Ph.D. Dissertation of Medical Sciences

Peptidyl-prolyl *cis-trans* isomerase NIMA interacting 1 (Pin1) stabilizes HIF-2α in breast cancer

유방암에서 Peptidyl-prolyl cis-trans isomerase NIMA- interacting 1에 의한 HIF-2α의 안정화

August 2023

Graduate School of Convergence Science and Technology

Seoul National University
Department of Molecular Medicine and Biopharmaceutical Sciences

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Abstract

Peptidyl-prolyl isomerase (Pin1) is overexpressed in the majority of cancers and binds to target proteins containing phosphorylated serine or threonine residues followed by proline (S/T-P) that can be isomerized thereby altering stability, subcellular localization and function of target proteins. The oncogenic transcription factor HIF-2α harbors the pSer/Thr-Pro motif. This prompted us to investigate whether Pin1 could bind to HIF-2α and influence its stability and function in the context of implications in breast cancer development and progression. The correlation between Pin1 and HIF-2α in the triple negative breast cancer cells was found positive. Interaction between Pin1 and HIF-2α was assessed by co-immunoprecipitation and an *in situ* proximity ligation assay. I found that inhibition of Pin1 enhanced the ubiquitination and degradation of HIF-2α. In contrast to the protein expression of HIF-2α., its mRNA levels were not altered by Pin1. Notably, the phosphorylation of HIF-2α at Ser672, Ser696 and Ser790 is essential for its interaction with Pin1. I identified phosphorylated Ser790 as an important site for the stability of HIF-2α. I also found PHD2 could be potential binding partner of Pin1. Further, phosphorylated Ser125, Thr168 and Ser174 residues in PHD2 were found to be essential for Pin1 binding. In conclusion Pin1 plays a role in breast cancer progression through stabilization of HIF-2α through direct interaction or indirectly by binding to PHD2.

Key words:

Pin1, HIF-2α, Protein-Protein interaction, Stabilization, Breast cancer, Hypoxia, PHD2

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Table of Contents

List of Figures

Chapter 1

Chapter 2

Chapter 3

List of Tables

Chapter 1.

List of Abbreviations

Abbreviations	Explanation
Pin1	Peptidyl-prolyl cis-trans isomerase NIMA-interacting 1
HIF-2α	Hypoxia inducible factor -2 alpha
PHD	Prolyl hydroxylase domain containing protein
HRE	Hypoxia-response element
ODD	Oxygen dependent degradation domain
pVHL	Von Hippel Lindau Protein
RT-qPCR	Reverse transcription-quantitative polymerase chain reaction
siRNA	Small interfering RNA
MAPK	Mitogen-activated protein kinase
ERK	Extracellular regulated protein kinase
WT	Wild-type
KO	Knockout
PTMs	Post-translational modifications
BC	Breast cancer
PBS	Phosphate-buffered saline
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase

IHC	Immunohistochemistry
IF	Immunofluorescence
VEGF	Vascular endothelial growth factor
PKA	Protein kinase A
PPIase	Peptidyl-prolyl isomerase
BRCA1	Breast cancer type 1
PI3K	phosphoinositide 3-kinase
HER2	human epidermal growth factor receptor 2
TNBC	Triple-negative breast cancer
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
CHX	Cycloheximide
FBS	Fetal bovine serum
EDTA	Ethylenediaminetetraacetic acid
SDS	Sodium dodecyl sulfate
PAGE	Polyacrylamide gel electrophoresis
PBST	Phosphate-buffered saline containing 0.1% tween 20
DMEM/F12	Dulbecco’s modified Eagle’s medium/F12
SD	Standard deviation

DMEM	Dulbecco's modified Eagle's medium

Chapter 1.

General Overview

Chapter 1. General Overview

1. Breast cancer

Breast cancer is the most common malignancy among women worldwide. In 2020, there were 2.3 million women diagnosed with breast cancer and 685 000 deaths globally. As of the end of 2020, there were 7.8 million women alive who were diagnosed with breast cancer in the past 5 years, making it the world's most prevalent cancer. Thus, breast cancer accounts for approximately 12% of all new cancer cases and 25% of all cancers in women [1]. It is a heterogeneous disease categorized into three main intrinsic subtypes based on expression of hormone receptors (HRs) including estrogen receptor (ER) and progesterone receptor (PgR) and human epidermal growth factor receptor 2 (HER2): HR-positive/HER2-negative (luminal-type: >70%), HER2- positive (15–20%) and HR- and HER2-negative (triple-negative BC; TNBC: 15%) [2,3]. Triple negative breast cancer (TNBC) is a subtype of breast malignancy that lacks HR expression and HER2 gene amplification, and it accounts for about quarter of newly diagnosed breast cancers. Due to the lack of targetable hormone receptors and HER2 expression, TNBC is associated with a poor prognosis and a limited range of therapeutic options, making it a challenging subject for breast cancer research [3].

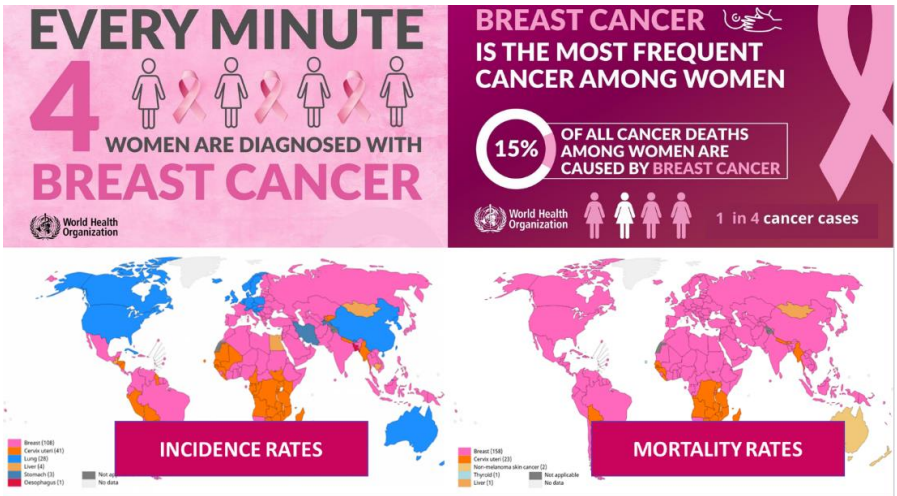

Figure 1. Overview of breast cancer worldwide. Adopted from: World Health Organization 2021

2. HIF-2α

2.1. Hypoxia and cancer

Hypoxia triggers important cellular stress responses allowing tumor cells to survive under extreme conditions, including the stabilization of the hypoxia-inducible factor (HIF) proteins [4-10]. Hypoxia is a common feature of most of solid tumors resulting from an imbalance between oxygen supply and consumption by tumor cells. Hypoxic tumor areas are characterized by a disrupted vasculature causing inefficient oxygen and nutrient supply to neighboring cells [11-13]. Hypoxia is one of the key factors in inducing the development of resistant cells with an aggressive phenotype [12,13], which leads to poor prognosis in patients due to the decreased efficacy of chemo- and radiotherapy [14,15]. Accurate measurement of tumor hypoxia in patients together with the design of novel anti-hypoxia treatments has largely been a major goal in cancer research [15-20].

Under normoxic conditions, prolyl-hydroxylation promotes HIF-α degradation via the von Hippel–Lindau (VHL) ubiquitin/proteasome pathway. Under hypoxia, this regulation is suppressed, leading to the stabilization of three independent. HIF-α subunits (HIF-1α, HIF-2α, and HIF-3α) that dimerize with the constitutively expressed HIF-β and activate the transcription of genes via hypoxia responsive elements in their promoter region. Hypoxia has negative impacts on prolyl hydroxylation of HIF-α proteins [21-24].

2.2. Rol of HIF-2α in cancer

The HIF-2α protein, also named endothelial PAS domain protein-1, is a transcriptional activator and a key mediator of the cellular adaptation to oxygen deprivation (hypoxia), HIF-2α is present over 50% similarity with HIF-1α in their amino acid sequence identity [21, 22]. In physiologic conditions HIF-2α is more restricted to specific cell types, e.g., kidney, lung, and heart [25]. Specific activity of HIF-2α differently contributes to total HIF target gene expression among many types of cancers, which may influence the characteristics of these tumors and the outcome of patients [26]. HIF-2α is a crucial transcription factor that plays important roles in physiological processes such as erythropoiesis and vascularization [25, 27]. It is also involved in the tumor progression and metastasis of many types of cancer in pathological conditions [28,29] including cell proliferation, resistance to apoptosis, epithelial-to-mesenchymal transition, cell metabolism, angiogenesis, and resistance to therapy [28-30]. Hypoxia-induced HIF-2α expression and its subsequent chain of events make this protein a relevant marker of tumor hypoxia and a promising target for anticancer therapies with novel inhibitors.

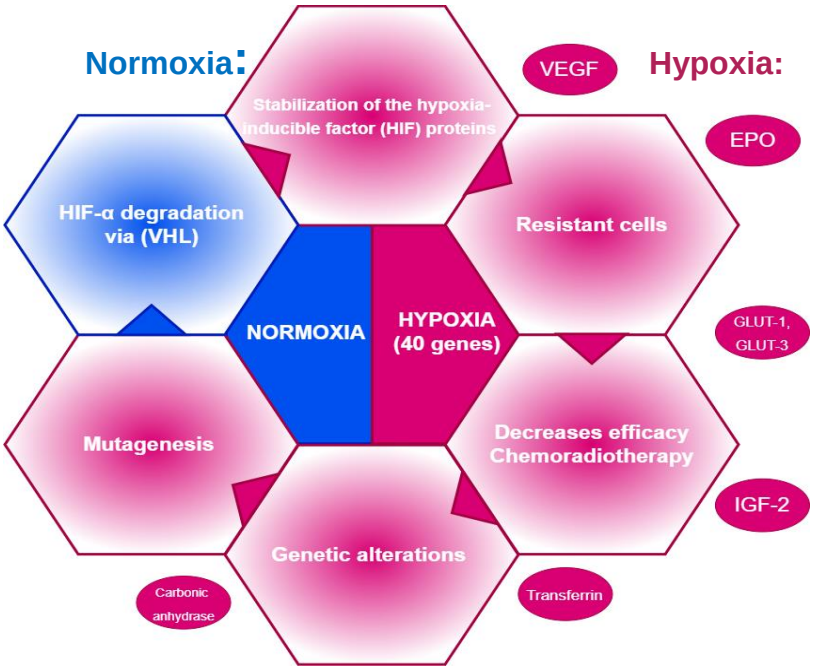

Figure 2. Negative impacts of hypoxia

2.3. Regulation of HIF-2α under normoxia and hypoxic conditions

HIF-2, thus, constitutes a validated target of anti-cancer therapy [29, 30]. HIF-2α is regulated by oxygen in a similar fashion to HIF-1α. In the presence of oxygen, HIF-2α is modified by HIF-specific prolyl 4-hydroxylases (PHDs), leading to proteasomal degradation mediated in part by the Von Hippel Lindau tumor suppressor protein (VHL) [31]. HIF-2α is also hydroxylated at an asparagine residue by factor inhibiting HIF (FIH, also known as HIF1AN), which inhibits HIF-2α interaction with the transcriptional co-activators CREB-binding protein (CBP, also known as CREBBP) and p300 (EP300) (collectively termed CBP/p300) [31-33]. Under hypoxia, PHDs and FIH, which use oxygen as a substrate, become inactive. As a result, HIF-2α hydroxylation is inhibited, leading to HIF-2α stabilization, transport into the nucleus, dimerization with ARNT,

DNA binding, co-activator recruitment and formation of an active transcriptional complex.

Growth factors, hormones or elevated oncogenic signaling in cancer cells can induce HIF-α independently of oxygen levels, both in terms of expression levels and activity [34,35]. Those oxygen-independent mechanisms involve the extensive post-translational modification of HIF-α subunits and their interaction with different proteins. 'Pseudohypoxia' is a condition in which cells exhibit signs of hypoxia (oxygen deficiency) despite adequate oxygen levels in the surrounding environment. This phenomenon is often associated with dysregulation of the cellular response to hypoxia, specifically involving the HIF proteins [36, 37].

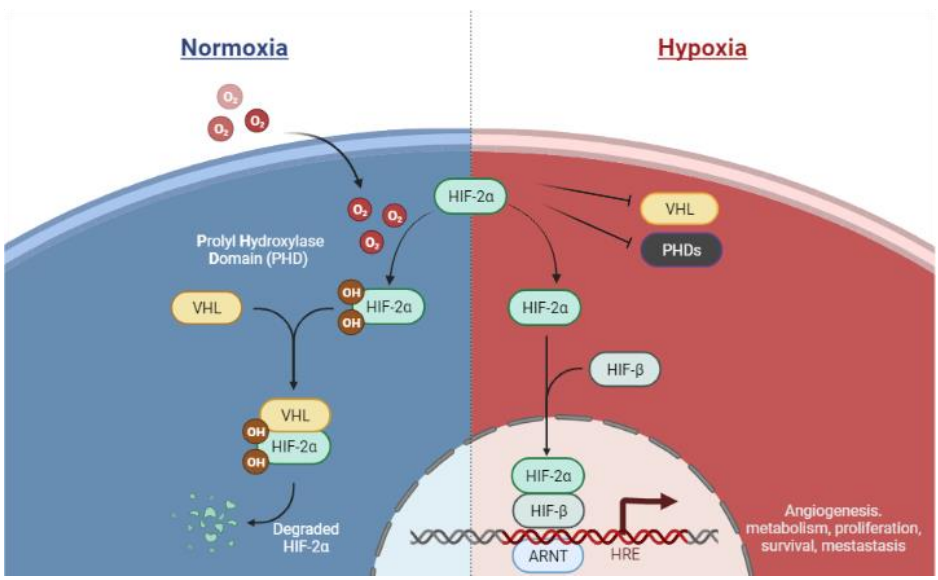

Figure 3. HIF-2α under normoxia and hypoxic conditions

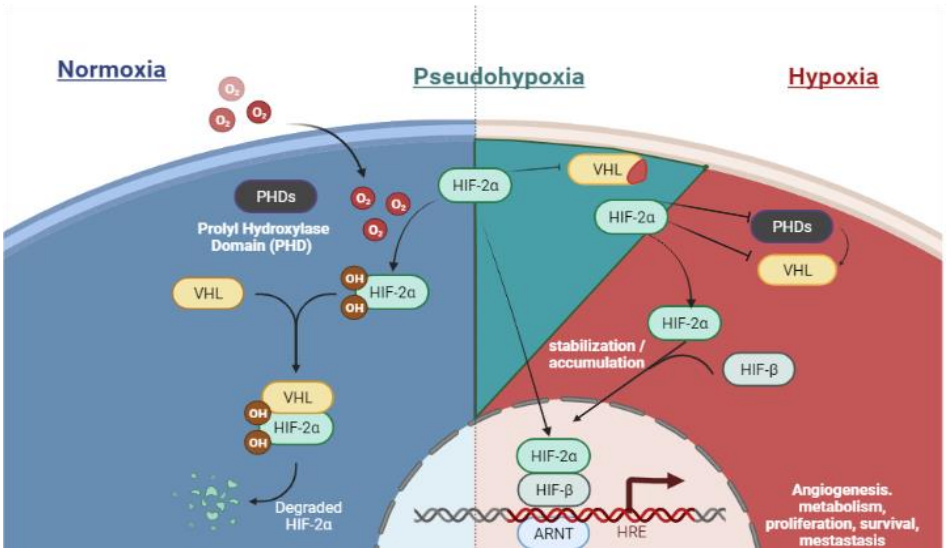

Figure 4. HIF-2α under pseudohypoxia

2.4. Pseudohypoxia or oxygen-independent stabilization of HIF-2α

In certain conditions, HIF-2α can be stabilized and activated, independently of oxygen levels, leading to increased HIF-2α activity even in normoxic conditions. This phenomenon is referred to as "oxygen-independent stabilization of HIF-2α " or "pseudohypoxia." [36, 37]. Pseudohypoxia is a condition characterized by the erroneous activation of HIF-2α and its downstream signaling pathways, despite adequate oxygen availability [36].

2.5. Mechanisms responsibles for oxygen-independent HIF-2α stabilization

Pseudohypoxia can result from mutations in HIF signaling pathway genes or alterations in factors that regulate HIF stability, such as HIF prolyl hydroxylase enzymes. This condition may have significant implications in various diseases, including cancer [36-41].

2.5.1. Genetic mutations

Mutations in genes encoding HIF-α subunits, particularly HIF-2α, can disrupt the prolyl hydroxylation sites or prevent the binding of prolyl hydroxylases (PHDs), leading to reduced HIF degradation.

2.5.2. Loss of PHD activity

Dysfunction or inactivation of PHDs can impair their ability to hydroxylate HIF, resulting in its stabilization.

2.5.3. Activation of signaling pathways

Certain oncogenic signaling pathways, such as the PI3K/AKT and RAS/ERK pathways, can promote HIF stabilization by inhibiting PHD activity or enhancing HIF translation.

2.5.4. Loss of von Hippel-Lindau (VHL) protein

VHL is part of an E3 ubiquitin ligase complex responsible for targeting hydroxylated HIF for proteasomal degradation. Mutations or inactivation of VHL can prevent HIF degradation, leading to its stabilization. The implications of oxygen-independent stabilization of HIF in cancer, result in the activation of HIF target genes, which are involved in promoting cell survival, angiogenesis, metabolism, and other processes associated with tumor growth and progression.

2.6. Post-translational modifications of HIF-2α: Phosphorylation

The most well-studied post-translational modification of HIF-α subunits, which affects their function other than hydroxylation, is phosphorylation. Modification of HIF-α subunits by phosphorylation can affect their stability, subcellular localization, and transactivation potential. There are numerous kinases known to modify HIF-1α, and the effect of phosphorylation on HIF-1α function has been extensively studied [40-42]. However, our knowledge concerning HIF-2α regulation by phosphorylation is more limited. Open

questions include whether the phosphorylation-dependent mechanisms that regulate HIF-1α activity also apply to HIF-2α or whether there are isoform-specific modifications mediated by different protein kinases

2.6. Phosphorylation of HIF-2α

It has been reported that HIF-2α is phosphorylated at T324 by protein kinase D1 (PKD1, also known as PRKD1) and, as a result, its interaction with the transcription factor specificity protein 1 (SP1) is inhibited, thus promoting the expression of nijmegen breakage [42,43]. HIF-2α is also phosphorylated by casein kinase 2 (CK2) at T844, which results in increased HIF-2α transcriptional activity, possibly by lowering the affinity of HIF-2α for FIH [44]. Two residues of HIF-2α, S383 and T528 are targeted by casein kinase 1δ (CK1δ, also known as CSNK1D). These modifications lead to efficient HIF-2α nuclear accumulation and full HIF-2 transcriptional activity [43,44].

Table 1. Panels of target genes regulated by HIF-1α and HIF-2α

HIFs	Target Genes	Function
HIF-1	HK	Glucose Metabolism
	LDH	
	PDHK	
	CAIX	pH Regulation
	SDF-1	Cell Migration
	CXCR4	
HIF-1/HIF-2	VEGF	Angiogenesis
	PDGF	
	Ang4	
HIF-2	ADRP	Nutrient Uptake
	Glut-1	
	EPO	Polycythema
	Oct4	Dedifferentiation

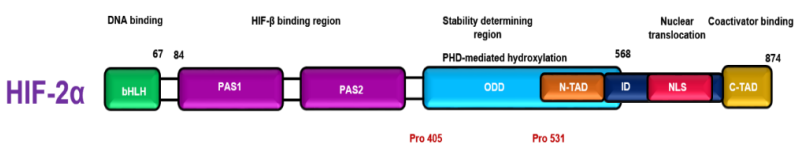

Figure 5. HIF-2α structure

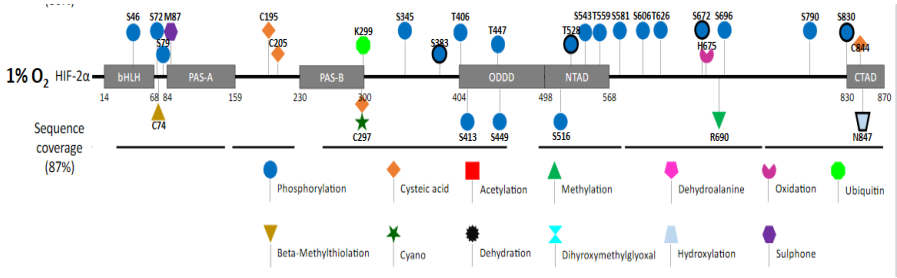

Figure 6. Post-translational modification of HIF-2α

Table 2. List of HIF-2α phosphorylation sites and their effects

HIF-2α /Sites	Kinase	Results	References
Serine 383	Casein kinase 1 delta (CK1σ)	Nuclear accumulation of HIF-2α and full HIF-2 transcriptional activity	Pangou E. et al, 2016
Threonine 528	Casein kinase 1 delta (CK1σ)	Nuclear accumulation of HIF-2α and full HIF-2 transcriptional activity	Pangou E. et al, 2016
Serine 672	ERK ½ (MAPK3 y MAPK1)	HIF-2α Transcriptional activity / nucleocytoplasmic shutting	Gkotinakou l. et al, 2019
Threonine 324	kinase D1 (PKD1, also known as PRKD1)	protein 1 (SP1) is inhibited, syndrome protein 1 (NBS1)	(To et al., 2006).
Threonine 844	casein kinase 2 (CK2)	Increased HIF-2 transcriptional activity	Gradin et al., 2002)

3. Pin1

3.1. Critical role of Pin1 in cancer

Pin1, also known as peptidyl-prolyl cis-trans isomerase NIMA-interacting 1 (PPIase), comprises 163 amino acid residues and has an 18 kDa relative molecular mass. It possesses one nuclear localization signal and two functional domains. The tryptophan-tryptophan central domain (WW domain) is located at the amino terminus, responsible for recognizing and binding to the pSer/Thr-Pro motif of substrate proteins. In contrast, the PPIase catalytic domain is present at the C-terminus, responsible for *cis-trans* isomerization [45-48].

Pin1 plays a crucial role in the post-translational regulation of target protein functions by isomerizing specific phospho-serine/threonine-proline motifs present in its substrate proteins [47]. It is engaged in various cellular activities such as cell cycle, cell proliferation, cell motility, and apoptosis [48]. The cellular function of Pin1 involves isomerizing phosphorylated substrates and regulating downstream signaling pathways. In signal transmission, cells respond to extracellular and intracellular inputs through diverse regulatory mechanisms like epigenetic modulation, allosteric regulation, and post-translational modifications (PTMs). PTMs modify the structures of corresponding proteins, influencing their interaction with other proteins, compartmentalization, intracellular processing, stability, and functions. Dysregulation of Pin1 is hence associated with the onset of several illnesses [49,50].

Pin1, initially identified as a regulator of mitosis, is implicated in the pathogenesis of certain cancers. It has been shown to stabilize numerous oncogene regulators; in contrast, it also promotes the degradation of various proteins with tumor-suppressive and growth-inhibitory functions [47,48]. Subsequent studies revealed that Pin1 facilitates multiple signaling

pathways in cancer [48]. The deregulation of Pin1, particularly its aberrant overexpression, plays a significant role in cancer development. Cancer metastasis, which is the leading cause of death in cancer patients, is associated with higher Pin1 expression in metastatic cancer compared to primary tumors [49, 50]. Pin1 overexpression is linked to the promotion of epithelial-mesenchymal transition (EMT) through the downregulation of E-cadherin [51-53]. Overall, Pin1's multifaceted role in cancer makes it a crucial target for potential therapeutic interventions.

3.2. Pin1-induced promotion of tumor growth

Pin1 promotes tumor growth, and its overexpression has been associated with poor clinical outcomes in cancer patients [55, 56]. Pin1 has been shown to activate more than 50 oncogenic proteins and growth promoters and shut down at least 20 tumor suppressors and growth inhibitors through positive and negative feedback regulations. Pin1 is overexpressed and activated in many different types of cancer [54,55]. Likewise, Pin1 expression is considerably higher in tumor cell lines than in normal cells. Cancer cell growth is inhibited when the Pin1 gene is knocked down, resulting in cancer cell death.

3.3. The significance of Pin1 phosphorylation

Protein phosphorylation is a reversible PTM that influences the biological activities of proteins in eukaryotic cells. It is one of the most prevalent PTMs, particularly phosphorylation of serine or threonine residues preceding a proline (pSer/Thr-Pro), which occurs in a wide range of proteins involved in cell cycle progression. This modification is catalyzed by proline-directed kinases. Pin1, a unique PPIase among other PPIases, is central to cancer treatment and development due to its substrate specificity in recognizing phosphorylated Ser/Thr-Pro moieties (pSer/Thr-Pro). It

possesses a highly conserved two-domain structure, with an N-terminal WW domain that binds specific pSer/Thr-Pro modules and a C-terminal PPIase domain responsible for *cis-trans* isomerization [56-66]. Different PTMs, including phosphorylation, influence stability of Pin1, subcellular localization, substrate binding, and catalytic activity.

The phosphorylation status of Pin1 is highly variable, and it plays a critical role in dictating protein activity, subcellular localization, and the establishment of recruitment platforms for interacting proteins. Phosphorylation sites, especially serines or threonines preceding proline (Ser/Thr-Pro) targeted by proline-directed kinases such as cyclin-dependent kinases (CDKs), mitogen-activated protein kinases (MAPKs), and others, deserve particular attention. Frequently altered as a downstream consequence of oncogenic driver mutations, protein phosphorylation is significantly implicated in a variety of cellular processes [63-68]

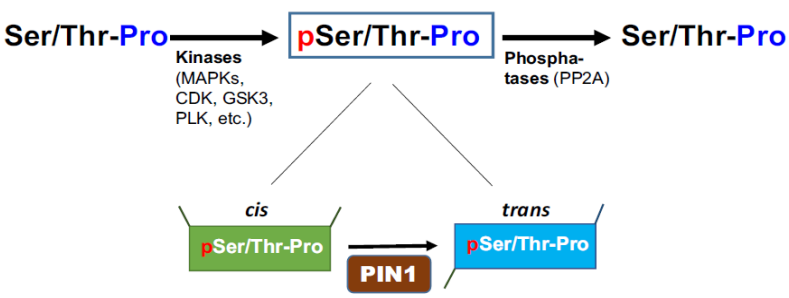

Figure 7. Pin1 as signal transduction modifier

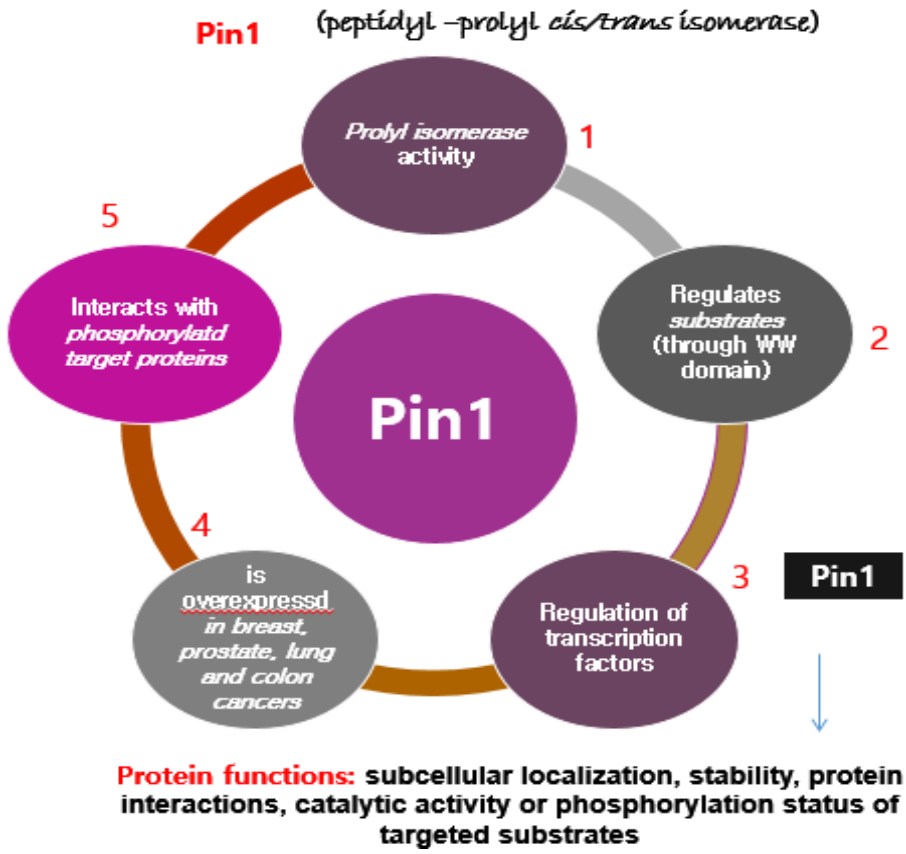

Figure 8. Cellular functions of Pin1

4. PHD2

4.1. PHDs as an oxygen sensor

HIF-1 and HIF-2 are the main executors of the cellular response to hypoxia. They are negatively regulated by the HIF PHD family members, PHD1, PHD2, and PHD3. When specific prolyl residues in the alpha subunits of HIF1 and HIF2 are hydroxylated, they become targeted for ubiquitination and proteasomal degradation [69,70]. Although hypoxia reduces the activity of PHDs, their catalytic activity is still observed even at 1% oxygen [70,71]. In fact, under nearly anaerobic conditions, HIFs are still hydroxylated [71,72]. PHD2 is considered the primary oxygen sensor among the PHD isoforms (PHD1, PHD2, and PHD3) [72].

4.2. Localization of PHD2

The expression patterns, as well as the subcellular localization of PHDs, have been associated with tumorigenesis. Different studies the promoting role of PHD2 nuclear localization in carcinoma cell growth. It has been shown that increased levels and nuclear translocation of PHD2 are associated with tumor aggressiveness [73]. High nuclear expression of PHD2 increases anchorage-independent carcinoma cell growth [73,74]. Similarly, increased PHD expression and nuclear PHD translocation have been linked to poor survival in pancreatic endocrine tumors [75,76]. However, the impacts of PHD intracellular localization on tumor development, the precise localization of cellular oxygen sensing, and the molecular mechanisms underlying nuclear import and export of the three PHDs are still not well understood.

4.3. The non-canonical function of PHD2 in breast cancer

PHD2 is best known as an oxygen sensor that hydroxylates HIF-α subunits under normoxic conditions, leading to their proteasomal

degradation. However, recent research has uncovered additional functions of PHD2 in breast cancer. In breast cancer cells, PHD2 has been shown to have diverse functions that contribute to tumorigenesis and cancer progression. Some of the non-canonical functions of PHD2 in breast cancer cells have been reported elsewhere [77-82].

4.3.1. Regulation of metastasis

PHD2 has been implicated in promoting breast cancer metastasis by regulating the epithelial-mesenchymal transition (EMT), a process by which cancer cells acquire invasive and migratory properties

4.3.2. Angiogenesis promotion

PHD2 can influence angiogenesis, the formation of new blood vessels, which is crucial for tumor growth and metastasis

4.3.3. Regulation of cancer stem cells

PHD2 has been linked to the maintenance and expansion of breast cancer stem cells, which are a subpopulation of cells responsible for tumor initiation and therapy resistance

4.3.4. Control of cancer cell metabolism

PHD2 can impact cellular metabolism in breast cancer cells, influencing how cancer cells generate energy and building blocks necessary for rapid proliferation

4.3.5. Interaction with other signaling pathways

PHD2 can interact with various signaling pathways and transcription factors, contributing to altered gene expression and cellular responses in breast cancer

4.3.6. Modulation of the tumor microenvironment

PHD2 can influence the tumor microenvironment, affecting the interactions between cancer cells and surrounding stromal cells. Understanding the non-canonical functions of PHD2 in breast cancer cells is essential for developing targeted therapies that can effectively inhibit its oncogenic activities. The complexity of PHD2's roles in breast cancer requires further research to uncover specific mechanisms and potential therapeutic targets.

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Statement of Purpose

Pin1 interacts with diverse protein substrates, thereby causing their conformational changes through cis/trans-isomerization of peptide bonds preceding a specific proline residue adjacent to phosphorylated serine or threonine residue (pS/T-P). The Pin1-mediated peptidyl-prolyl isomerization consequently influences the function of its substrate proteins through multiple mechanisms. Pin1 acts as an oncoprotein by regulating several kinases and phosphatases involved in cell proliferation, cell cycle progression, cell metabolism, apoptosis, etc. Pin1 is frequently overexpressed in diverse human cancers, but its expression in normal tissues is relatively low.

Pin1 binds to the substrates phosphorylated at a specific serine or threonine residue and catalyzes cis/trans isomerization of the peptide bond prior to adjacent proline. HIF-2α harbors 11 such motifs. To the best of my knowledge, the present study explores for the first time that Pin1 interacts via its WW and PPIase domains with HIF-2α phosphorylated on specific serine/threonine residues.

My study facilitates designing the small molecule pharmacological inhibitors of Pin1 that can block the proliferative and metastatic ability of breast cancer. It also paves the way to elucidation of the novel signaling mechanism underlying breast cancer progression.

Chapter 2.

Oxygen-independent stabilization of HIF-2α in breast cancer through direct interaction with peptidyl-prolyl *cis-trans* isomerase NIMA-interacting 1

Abstract

Peptidyl-prolyl *cis-trans* isomerase NIMA-interacting 1 (Pin1) isomerizes the nearby proline (Pro) residue when it detects phosphorylated serine (Ser) or threonine (Thr) of target proteins, altering their structure, stability, function, and interaction with other proteins. Hypoxia-inducible factor 2α (HIF-2α), a transcription factor that transactivates many oncogenic genes under hypoxic conditions, harbours the pSer/Thr-Pro motif. We found for the first time that Pin1 binds to HIF-2α physically in normoxic as well as hypoxic conditions in triple-negative breast cancer cells. The level of ubiquitinated HIF-2α was significantly raised by Pin1 knockdown, while its mRNA transcript expression was unaffected. In agreement with this observation, the cycloheximide chase assay demonstrated that Pin1 prolonged the stability of HIF-2α. Serine 672, 696, and 790 of HIF-2α were found to undergo phosphorylation. Of these, the main amino acid involved in the Pin1 binding and HIF-2α stabilization was identified as serine 790, located in the nuclear export signal region of HIF-2α. The tissue array with human breast cancer tissues showed elevated expression of HIF-2α as well as Pin1 compared to adjacent normal tissues. Knockdown of Pin1 or HIF-2α diminished breast cancer cell migration and colony formation. In conclusion, Pin1 stabilizes HIF-2α through direct interaction, which aids in the growth of breast cancer.

Keywords: Breast cancer, HIF-2α, Pin1, Protein-protein interaction, Pseuodohypoxia

1. Introduction

Breast cancer constitutes one of the most prevalent malignancies in women worldwide. In 2020, there were over 2.3 million new cases and 685,000 deaths globally from breast cancer [1]. Triple-negative breast cancer (TNBC), a severe form of breast malignancy, has a higher propensity to metastasize and is less susceptible to Her2-targeted therapy and anti-hormonal treatment [2,3]. As a result, it is important to find trustworthy new treatment targets by elucidating the molecular mechanisms underlying TNBC pathophysiology.

Peptidyl–prolyl cis-trans isomerase NIMA-interacting 1 (Pin1), often overexpressed in human cancers, mediates several oncogenic signaling pathways involved in drug resistance and tumor progression, which accounts for poor clinical outcomes in human cancer patients [4-7]. It has been proposed that Pin1 is a prognostic indicator [8], as its aberrant overexpression contributes to the uncontrolled growth of tumors [9,10]. Phosphorylation-dependent prolyl isomerization mediated by Pin1 represents an important post-translational regulation mechanism in intracellular signaling [11-13]. Pin1 is made up of a PPIase (peptidyl-prolyl cis/trans-isomerase) domain and an N-terminal WW region. Pin1 interacts with specific phosphorylated Ser/Thr-Pro sites present in a subset of proteins [14-16]. This causes conformational changes in the target proteins, influencing their function, subcellular localization, and stability. Pin1's ability to destabilize/inactivate tumor suppressors and/or stabilize/activate oncoproteins is largely responsible for its role in cancer development and progression [17-18].

By means of stabilization of hypoxia inducible factor (HIF-α) proteins, hypoxia induces upregulation of various types of oncoproteins and important cellular stress responses [19-22]. One of the main causes of the growth of

cancerous cells with an aggressive phenotype is hypoxia, which diminishes the effectiveness of chemotherapy and radiation therapy [23-25], leading to a poor prognosis for patients [26,27]. There are three isoforms of HIF-α found in mammalian species: HIF-1α and HIF-2α, the most prevalent forms, and less defined HIF-3α. Despite sharing similarities in the structure and being controlled by prolyl hydroxylase domains (PHDs), HIF-1α and HIF-2α have different transcriptional targets and physiological functions [28-30]. Both HIF-1α and HIF-2α are necessary for cancer cell viability in an oxygen-deprived/hypoxic tumor microenvironment.

HIF-2α is an activator of transcription and an essential modulator of the biological response to hypoxia.-It is generated by the *EPAS1* gene, an analog of *HIF1A*, with functions in pathological conditions as well as physiological processes like erythropoiesis and vascularization [31-33]. Like HIF-1α, PHD-mediated destabilization of HIF-2α can be hampered in hypoxia, but stabilization of HIF-2α under non-hypoxic conditions remains largely unresolved.

Phosphorylation of HIF-α subunits after translation can change their transactivation potential, subcellular localization, and stability [31]. Ser 383, Ser 672, Thr 324, Thr 528, and Thr 844 residues present in the pSer/Thr-Pro motifs of HIF-2α may undergo phosphorylation [32]. However, there is paucity of data on the phosphorylation of HIF-2α. HIF-2α has the pSer/Thr-Pro motif [34], which prompted us to look into whether Pin1 could bind and stabilize HIF-2α in breast cancer. We ran a series of tests to verify the physical interaction between Pin1 and HIF-2α and to assess its impact on the stability and function of HIF-2α in relation to implications for the progression of breast cancer.

2. Materials and methods

2.1. Reagents and antibodies

We used different media such as Dulbecco's modified Eagle's medium (DMEM) and RPMI 1640 medium, supplemented with fetal bovine serum (FBS) and penicillin/streptomycin combinations, or DMEM/F12 medium, which were purchased from Gibco BRL (Grand Island, NY, USA).

Stealth™ RNAi-negative control duplexes and Trizol® were obtained from Invitrogen Life Technologies Corporation (Carlsbad, CA, USA). US Biomax (Rockville, MD, USA) supplied the human breast cancer tissue microarray (LVI5050). Pin1 and β-actin antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). HIF-1α and HIF-2α antibodies were obtained from Novus Biologicals (Centennial, CO, USA). Additionally, HA-tag polyclonal antibody, ubiquitin antibody and secondary antibodies were purchased from Thermo Fisher Scientific, Inc. (Waltham, MA, USA), Cell Signaling Technology (Bervely, MA, USA), and Zymed Laboratories (San Francisco, CA, USA), respectively. Cycloheximide (CHX), MG-132, and dithiothreitol (DTT) were supplied from Sigma-Aldrich (St. Louis, MO, USA). A hypoxia chamber was the product of Forma Scientific (*Marietta*, OH, USA). We purchased a Western blot kit (Absignal) from Abclon (Seoul, South Korea).

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2.2. Cell culture and treatments

The American Type Culture Collection provided the human breast cancer cell lines (MDA-MB-231, invasive MCF7 and MDA-MB-468 cells), and non-oncogenic MCF10A and HEK293T cell lines. MCF7 human breast cancer cells were maintained in RPMI cell culture media, while the other

cell lines were grown in DMEM. All of these media contained 100 ng/mL antibiotic combination and 5% FBS, and they were all grown at 37℃ in an incubator with 5% CO_2 and 95% air. MCF10A cells were cultured as described previously [35]. Normoxic conditions for experiments in cell cultures were defined elsewhere [36]. When required for hypoxia conditions, cells were maintained at 37℃ with 1% O_2.

2.3. DNA plasmid transient transfection and small interfering RNA (siRNA)

For siRNA transfection (20 nM), oligonucleotides of siRNA targeting Pin1 #1 were 5'-GCU CAGG CCGA GUG UACUA-3' (sense) and 5'-UAG UAC ACU CGG CCU GAGC-3' (antisense); for HIF-2α siRNA #1, 5'-CCC GGA UAG ACU UAU UGCCAA-3' (sense) and 5’-UUG GCA AUA AGU CUA UCCGGG-3’ (*antisense)*. siRNA oligonucleotides were obtained from Bioneer, Inc. (Seoul, South Korea). *Santa Cruz Biotechnology, Inc.* (Dallas, TX, USA) supplied the control siRNA and Pin1 siRNA #2 (sc-36230). These siRNAs were transfected for 48 h into breast cancer cells with lipofectamine RNAiMAX, provided by Life Technologies Corporation (Carlsbad, CA, USA). Cosmo Genetech Company (Seoul, South Korea) developed full-length HIF-2*α* and all HIF-2*α* mutant constructs. HEK293T cells in an 100-mm dish at a density of 5×10^4 cells/cm^2 were grown in a complete growth medium to 90% confluence. Lipofectamine 2000 obtained by Thermo Fisher Scientific, Inc. (Waltham, MA, USA) was transfected to cells with WT-HA-HIF-2*α* and pcDNA-Pin1 constructs. After 24 h of transfection, lysed cells were prepared for immunoprecipitation or Western blot analyses.

2.4. Western blot analysis

Cells were lysed in lysis buffer [50 mM Tris-HCl (pH 8.0), 250 mM sucrose, 25 mM KCl, 5 mM $MgCl_2$, 1 mM ethylenediaminetetraacetic acid (EDTA), 2 mM, 2 mM NaF, and 1 mM phenylmethylsulfonyl fluoride (PMSF)], on ice for 1 h, and then centrifuged 20 minutes at 18,000 x *g*. Using the BCA protein kit (Pierce; Rockford, IL, USA), the protein concentrations in the supernatant were determined. Protein (40 μg) was separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The protein blots were blocked at 37℃ for 1 h with either 5% skim dry milk in PBST or TBST (phosphate or Tris-buffered saline buffer containing 0.1% Tween-20) buffer. The blocked membranes were exposed to primary antibodies for Pin1, HIF-2α, actin, and HA for an entire night at 4℃. On the next day, the membranes were incubated with a secondary antibody from Pierce Biotechnology (Rockford, IL, USA) during 1 h and then washed with PBST/TBST solution for 10 min.

2.5. Preparation of nuclear and cytoplasmic extracts

The cells were washed with cold PBS and subsequently suspended in hypotonic buffer A [10 mM HEPES (pH 7.9),10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM DTT, and 0.2 mM PMSF] on ice. Then, cells were centrifuged at 1700 x *g* for 5 min. The cytosolic fractions were obtained by collecting the supernatant after the centrifugation. The remaining cells were washed with buffer A twice before resuspended in buffer C, and ice-cold buffer constituted with 420 mM NaCl, 20 mM HEPES (pH 7.9), 20% glycerol, 1.5 mM $MgCl_2$, 0.5 mM DTT, 0.2 mM PMSF, and 0.2 mM EDTA. The cell suspension was kept in ice for one hour followed by centrifugation at 18,000 x *g* for 15 minutes. The supernatant containing the nuclear extracts was stored at -80℃ until use.

2.6. Real-time polymerase chain reaction (RT-qPCR) analysis

Total RNA was extracted from MDA-MB-231 cell lines using the Trizol® reagent from Invitrogen (Carlsbad, CA, USA). The RNA quality was determined using the RNA 600 Nano chip provided by Agilent Technologies (Amstelveen, Netherlands), followed by quantification using the ND-2000 spectrophotometer from Thermo Inc., (Wilmington, DE, USA). The gene expression of Pin1, HIF-1α, and HIF-2α was determined by real-time PCR in accordance with an established protocol, which used the RealHelix™ SYBR Green I qPCR kit from NanoHelix Co., Ltd. (Seoul, South Korea). The fluorescent signals from the PCR products were measured using the 7500 Fast Real-time PCR system (Waltham, MA, USA) and quantified by employing the comparative cycle threshold method. The data were analyzed based on the mean of a minimum of three independent experiments. The PCR primer sequences used were as follows: *Pin1*, 5′-TGA TCA ACG GCT ACA TCC AG-3′ (F) and 5′-CAA ACG AGG CGT CTT CAA AT-3’ (R); *HIF-1α*, 5’-GAA CGT CGA AAA AGT CTC-3’ (F) and 5’ -CCT TAT CAA GAT GCG AAC TCA CA-3’ (R); *HIF-2α,* 5’-CGG AGG TGT TCT ATG AGC TGG-3’ (F) and 5’ -AGC TTG TGT GTT CGC AGG AA-3’ (R); *GAPDH*, 5’- CAT GAG AAG TAT GAC AAC AGC CT-3’ (F) and 5’ -AGT CCT TCC ACG ATA CCA AAG T-3’ (R) [37].

2.7. QuantSeq 3’mRNA sequencing library

The libraries for the control and test RNAs were generated using the QuantSeq 3' mRNA-Seq Library Prep Kit by Lexogen, Inc. (Vienna, Austria) following the manufacturer's protocol. Briefly, a total of 500 ng RNA was produced, and an oligo-dT primer, including an illumine-compatible sequence at its 5’ end was annealed to the RNA for reverse transcription. A random primer initiates the second strand synthesis, after the degradation of the RNA template. Magnetic beads were employed to

remove the reaction by products from the double-stranded library. Adaptor sequences essential for cluster creation were added to the library via amplification, and the final library was purified from any PCR by products. High-throughput sequencing was carried out with the NextSeq 500 (Illumina, Inc.), with single-end 75 sequencings [35].

The mRNA-Seq reads from QuantSeq 3” were aligned using the Bowtie software for data analysis [38]. Bowtie2 indices were created either using the representative transcript sequences or the genome assembly sequence for aligning to the genome and transcriptome. The alignment file was utilized to assemble transcripts, calculate their abundances, and identify genes that expressed differently. Bedtools coverage [39] was used to count single and multiple alignments identifying differentially expressed genes. RC (Read Count) data were processed using EdgeR in R and Bioconductor, with the quantile normalization approach [40,41]. Medline (http://www.ncbi.nlm.nih.gov/) and DAVID (http://david.abcc.ncifcrf.gov/) database searches were used for gene classification. QuantaSeq 3’mRNA sequencing was carried out using eBiogen (Seoul, South Korea), and quality control, reads calling and aligment of the raw QuantSeq 3′ mRNA-Seq reads were done using fastqc, Bowtie2, and bedtools. EdgeR was used to determine the gene fold changes, using read counts, with modifications [34]. Any genes with fewer than 5 read counts per sample on average were excluded. Pathway changes following Pin1 perturbation were identified using Gene set enrichment analysis (GSEA) employing the clusterProfiler v, and the pathway annotations were obtained using the g:profiler (https://biit.cs.ut.ee/gprofiler/gost) and MSigDB (https://www.gsea-msigdb.org/gsea/msigdb/) websites. Plots were generated in R with ggplot2.

2.8. Immunoprecipitation

For immunoprecipitation of total proteins (100 μg), HEK 293T and MDA-MB-231 cells were lysed, and incubated with primary antibodies against IgG, HIF-2α, HA, or Pin1 overnight at 4°C, followed by precipitation using Protein A/G-PLUS Agarose bead suspension from Santa Cruz Biotechnology (*Dallas, TX, USA).* The mixture was then centrifuged at 1,000 x *g* for 1 min, supernatant was discarded, and precipitated beads were washed in cell lysis buffer. The immunoprecipitated beads were then prepared for Western blotting by resuspending in 24 μl of lysis buffer and 6 μl of 5X dye and heated at 95°C for 5 minutes before centrifuging to collect the supernatant.

2.9. The in situ proximity ligation assay (PLA)

The PLA was carried out using the DuoLink^{TM} kit from Sigma-Aldrich (St. Louis, MO, USA). HEK293T cells were transfected with HA-HIF-2α/pcDNA-Pin1 and control siRNA or Pin1 siRNA for 48 h. After fixing, permeabilization, and blocking with 0.1% Triton in PBS containing 5% bovine serum albumin (BSA), the cells were incubated with Pin1 monoclonal (1:100) and HIF-2α polyclonal (1:200) antibodies overnight at 4℃. The cells were then treated with two PLA affinity probes (PLUS and MINUS) and incubated at 37℃ for 1 h. The probes were hybridized to form a closed circle using a ligase enzyme, which were then amplified and detected using fluorescence microscopy from Nikon (Tokyo, Japan) [30, 37].

2.10. Tissue array analysis

For tissue array analysis, human paraffin-embedded breast cancer tissue array Cat. No. BC08118a provided by US Biomax, Inc. (Rockville, MD, USA) with surrounding normal tissues was deparaffinized with xylene,

followed by rehydration in series of (100%, 90%, 80%, and 70%) ethanol baths. Antigen retrieval was performed by boiling the sections in hot citrate buffer for 30 min, followed by permeabilization and blocking using a standard protocol. The tissue sections were washed in PBS and then incubated with antibodies against Pin1 and HIF-2α overnight at 4°C, followed by incubation with fluorescent-conjugated secondary antibodies (FITC-conjugated for HIF-2α, green signal; TRITC-conjugated for Pin1, red signal), for 1 h at room temperature. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI), and slides were imaged using a fluorescent microscope provided by Nikon (Tokyo, Japan).

2.11. Immunofluorescence staining

For immunocytochemistry staining, MDA-MB-231 cells were seeded in an 8-chambered plate at a density of 1 x 10^4 cells per well. Once the cells reached the 80% confluency, the cells were fixed, permeabilized and blocked before incubation with anti-Pin1 and anti-HIF-2α antibodies overnight. The cells were then labeled with fluorophore attached secondary antibodies (FITC and TRITC conjugated). DAPI staining was used for detecting nuclei. The slides were then scanned and imaged using a Nikon fluorescent microscope (Tokyo, Japan).

2.12. Clonogenic assay

For the clonogenic assay, MDA-MB-231 and MCF7 cells were seeded in 6-well plates at a density of 150–200 cells per well and cultured for 14 days before being transfected with control siRNA, Pin1 siRNA, or HIF-2α siRNA for 48 h. Media were changed every other day. After the 14-day incubation period, colonies were fixed with methanol at 4℃ for 1 h and then stained with 0.05% crystal violet (Sigma-Aldrich; St. Louis, MO, USA)

for 4 h. The excess dye was washed off with PBS, and colonies were visualized and counted manually using LAS-4000 image reader (Nikon; Tokyo, Japan) [37].

2.13. Migration assay

To evaluate the cell migration, MDA-MB-231 and MCF7 were pretreated with control, Pin1 or HIF-2α siRNAs and then plated into Culture-Inserts® (ibid; Regensburg, Germany). Cells were allowed to adhere well to the inserts for 24 h, after which they were gently removed using sterile tweezers. The cells were then monitored for their ability to migrate at different time points under a microscope (Nikon; Tokyo, Japan).

2.14. Evaluation of protein stability by using the CHX chase assay

MDA-MB-231 cells were transfected with Pin1 siRNA or control siRNA for 48 h. Protein translation was inhibited by treating the cells with 10 μM CHX after a 4 h exposure to hypoxia or MG132. The half-life of the HIF-2α protein was determined by collecting cells at different time points and analyzing the protein expression levels.

2.15. Identification of the phosphorylation sites of HIF-2α

For identification of the plausible phosphorylation sites, MDA-MB-231 cells were transfected with HA-HIF-2α, and immunoprecipitated samples were analyzed using SDS-PAGE. The bands containing the HA-HIF-2α were excised, and protein was eluted by the trypsin digestion procedure [42]. Phosphorylation of HA-HIF-2α was analyzed in peptides using a hybrid dual-cell quadrupole linear ion trap-orbitrap mass spectrometer (LTQ Orbitrap Velos, Thermo Fisher). MS/MS spectra were searched against a composite database of all translated human open reading frames and their

reversed complements using the SEQUEST algorithm. MS data were automatically captured using the Proteome Discoverer 2.5 software (Thermo Fisher Scientific Inc.; Waltham, MA, USA) [43].

2.16. Network analysis of protein-protein interaction

Protein-protein interaction was analyzed using the STRING tool (Search Tool for the Retrieval of Interacting Genes/Proteins, https://string-db.org).

2.17. Structural Similarity Index Measure (SSIM) assessment

The SSIM index is a quantitative measure for assessing the image similarity which analyzes the local patterns of pixel intensities. We utilized immunofluorescence tissues images with HIF-2α and Pin1 protein expression that were numerically coded systematically. To estimate the difference in the expression of proteins in normal and tumor tissue array, we employed a computational vision algorithm of structure similarity detection based on an established protocol [44,45].

2.18. Statistical analysis methods

All data are represented as mean ± standard deviation (SD) for a minimum of three independent experiments. Statistical analysis was conducted using either one-way ANOVA or two-tailed unpaired Student's *t*-tests, with $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$ indicating statistical significance, while ns, stood for not significant. The data were analyzed using the GraphPad Prism 8.0 software (GraphPad Software; San Diego, CA, USA).

3. Results

3.1. Correlation of overexpression of Pin1 with HIF-2α in breast cancer

Pin1 and HIF-2α expression was examined on human 90 breast cancer tissues and 10 nearby normal tissue microarrays by immunofluorescence staining as an initial step in exploring their potential involvement in the breast cancer progression. The invasive ductal carcinomas displayed highly elevated expression of Pin1 and HIF-2α compared with normal tissues (**Fig. 1A** and **Fig. 1B**). This was also confirmed by using structural similarity index measure where Pin1 and HIF-2α had a high-value algorithm in breast cancer tissues than normal counterparts (**Fig. S1**). In invasive ductal carcinoma tissues, there was a significant correlation between Pin1 and HIF-2α (**Fig. 1C**). Furthermore, both proteins displayed different levels of expression depending on the disease stages (**Fig. 1D**). Additionally, we compared expression levels of Pin1 and HIF-2α in MDA-MB-231 human breast cancer cells and non-oncogenic MCF10A cells using immunofluorescence staining. The data show that Pin1 and HIF-2α are co-localize in the MDA-MB-231 cancer cells (**Fig. 1E**).

Next, we used MCF10A-Ras cells to knockdown Pin1 using siRNA and performed RNA-Seq to see the connection between Pin1 and HIF-2α. Silencing of Pin1 suppressed transcription of some HIF-2α target genes, *Oct-4* and *VEGFA* encoding octamer binding transcription factor 4 (Oct-4) and vascular endothelial growth factor, respectively. Overall, there are increased inflammatory responses and reduced expression of genes involved in growth and proliferation (**Fig. 1F**). We also saw a significant reduction in glycolysis-related genes (**Fig. 1G**). These findings suggest an important interplay between Pin1 and HIF-2α in breast cancer to drive the expression of hypoxia response genes.

3.2. Pin1 and HIF-2α interact directly with each other in breast cancer

For more systematic analysis of Pin1 and HIF-2α interaction, we utilized the STRING database and found that Pin1 is closely associated with HIF-2α (**Fig. 2A**). We also looked into the possibility of physical interaction between the two proteins. MDA-MB-231 breast cancer cells exhibited the direct association between Pin1 and HIF-2α which was demonstrated by co-immunoprecipitation with an antibody against either HIF-2α (**Fig. 2B**) or Pin1 (**Fig. 2C**). Such interaction was also observed in two other human breast cancer cell lines, MDA-MB-468 (**Fig. S2A**) and MCF-7 (**Fig. S2B**).

Next, we overexpressed Pin1 and HIF-2α in MDA-MB-231 breast cancer cells with plasmids, HA-Pin1 and X-press HIF-2α, and verified the strong exogenous interaction between these two proteins (**Fig. 2D**). We also detected Pin1 and HIF-2α by the PLA, which generates a stronger fluorescence signal when two proteins are nearby (**Fig. 2E**). In MDA-MB-231 breast cancer cells transfected with the pcDNA-Pin1 plasmid, the cytosolic and nuclear levels of the Pin1 and HIF-2α complex were investigated. The interaction between the two proteins mostly took place in the nucleus, as depicted in (**Fig. 2F**). In MDA-MB-231 cells, Pin1 and HIF-2α may interact under hypoxic and normoxic conditions (**Fig. S2C**).

3.3. Pin1 or HIF-2α knockdown attenuates oncogenicity of breast cancer cells

We studied the functional significance of Pin1 and HIF-2α. Pin1 or HIF-2α knockdown diminished the clonogenicity of MCF-7 cells (**Fig. 3A**) and MDA-MB-231 cells (**Fig. 3B**) and their migrative capability (**Fig. 3C** and **Fig. 3D**).

3.4. Pin1 knockdown reduced HIF-2α protein expression in breast cancer cells.

HIF-2α protein levels in MDA-MB-231 breast cancer cells were significantly affected by Pin1 knockdown, but expression of its isoform, HIF-1α was not significantly changed (**Fig. 4A**). Immunofluorescence staining (**Fig. 4B**) confirmed that Pin1 knockdown resulted in the reduced HIF-2α protein expression. The expression of HIF-1α and HIF-2α mRNA transcripts was unaffected by Pin1 silencing (**Fig. 4C**), indicating that Pin1-induced accumulation of HIF-2α is accomplished by protein stabilization rather than *de novo* synthesis. PLA **(Fig. 4D)** and Western blot analysis (**Fig. S2D**) both showed that Pin1 knockdown reduced the direct interaction between Pin1 and HIF-2α. However, HIF-2α silencing had no effect on protein expression of Pin1 (**Fig. 5A**).

3.5. Pin1 stabilizes the HIF-2α protein in breast cancer

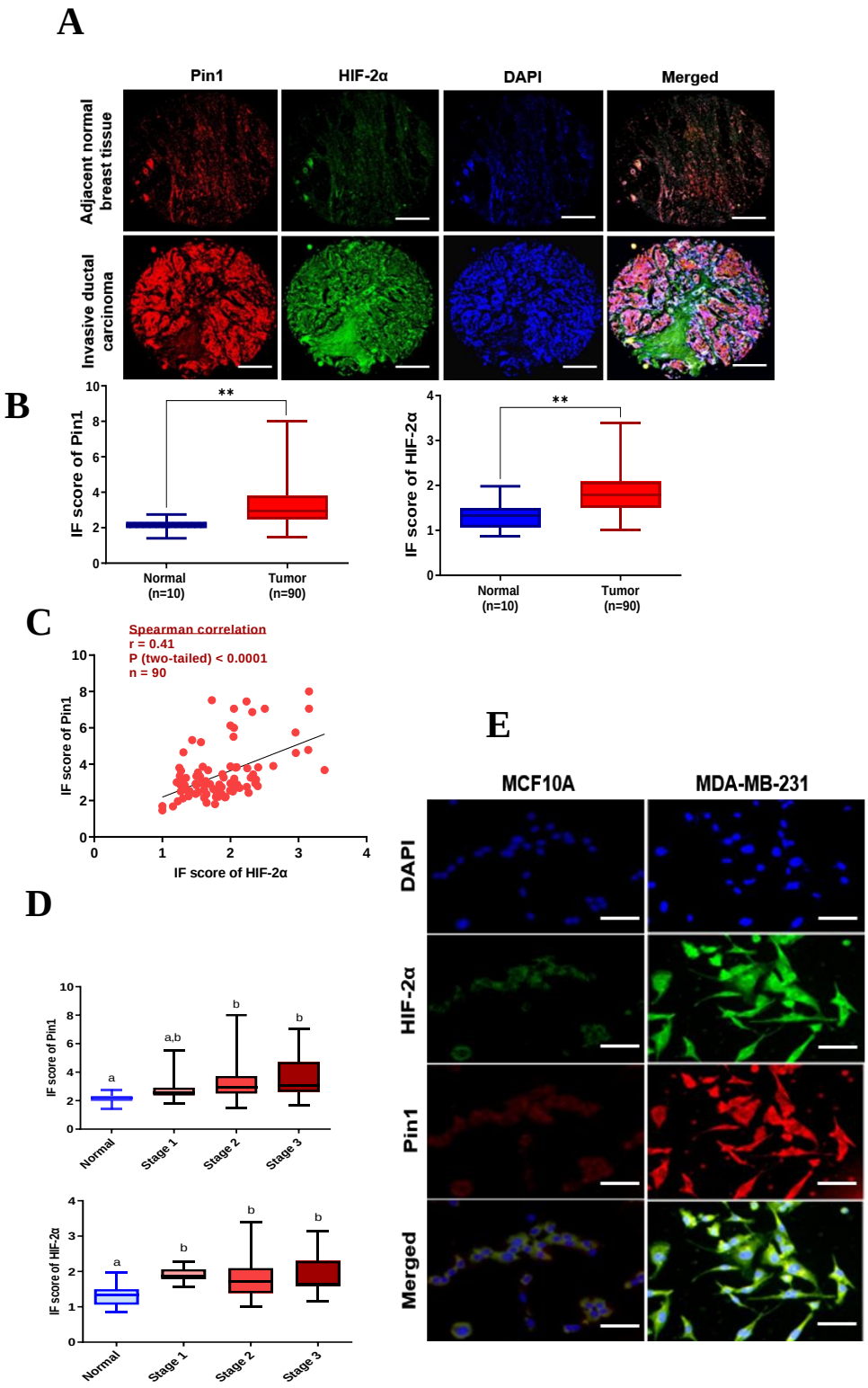
Pin1-silenced MDA-MB-231 breast cancer cells were treated with or without a proteasome inhibitor MG-132, the cell-membrane permeable proteasome inhibitor, in order to more precisely assess the stabilization of HIF-2α by Pin1. CHX was used to stop the synthesis of new proteins in the Pin1-silenced as well as siControl MDA-MB-231 breast cancer cells. The half-life of HIF-2α was significantly reduced in Pin1 silenced MDA-MB-231 cells (**Fig. 5B**). These results were supported by the fact that the knockdown of Pin1 significantly raised the level of ubiquitinated HIF-2α (**Fig. 5C**), supporting the Pin1's role in stabilizing HIF-2α. These findings suggest that Pin1 may inhibit the degradation of HIF-2α by the proteasomes.

3.6. HIF-2α is phosphorylated on certain serine residues (S672, S696, and S790) by Pin1.

In exerting its regulatory function, Pin1 binds to the target protein in a phosphorylation-dependent manner [46,47]. The peptide link between phosphorylated serine or threonine and proline (pSer/Thr-Pro) undergoes *cis* and *trans* isomerization by Pin1 when it interacts with a partner protein [10-13]. HIF-2α is phosphorylated by some kinases, such as PKD1, CK1, and CK2 [48-50]. To figure out which Ser/Thr residue(s) could be involved in Pin1-HIF-2α interaction, we analyzed the HIF-2α structure (**Fig. 6A**) and recognized 11 such motifs harboring Ser/Thr with an adjacent Pro residue (**Fig. 6B**). These motifs are ubiquitously present in different species (**Fig. 6C**). LC-MS/MS analysis identified three Ser residues (S672, S696, and S790) as prime sites for phosphorylation (**Fig. 6D**). We transfected the HEK293T cells with expression plasmids for pcDNA-Pin1 and HA-tagged wild-type (WT) HIF-2α or mutant constructs in order to identify which of the three Ser residues is/are crucial for Pin1 binding to HIF-2α. Pin1 and HIF-2α expression was unaffected by the serine substitution for any of the aforementioned serine residues (**Fig. 7A**). In the mutant cells expressing HIF-2α-S672A and HIF-2α-S696A, the interaction between HIF-2α and Pin1 was still discernible; however, S790A mutation abolished the Pin1 binding to HIF-2α (**Fig. 7B**). Consistent with this observation, more ubiquitinated HIF-2α was found in the mutant cells containing the HIF-2α-S790A (**Fig. 7C**). In line with this notion, S790A mutation rendered HIF-2α degraded faster (**Fig. 7D**). These results imply that the stabilization of HIF-2α depends on Pin1 binding to HIF-2α phosphorylated on S790. We also investigated the functional significance of S790 phosphorylation of HIF-2α. Cells expressing WT HIF-2α and Pin1 exhibited the relatively high clonogenic (**Fig. 7E**) and migrative (**Fig. 7F**) capabilities which were attenuated by S790A mutation.

3.7. Pin1 controls the stability of HIF-2α phosphorylated at S790.

Pin1 recognizes the proline residue adjacent to phosphorylated serine/threonine of the target protein. To further verify that the Ser790-Pro791 motif of HIF-2α is essential for its Pin1 binding, we utilized the mutant HIF-2α in which Pro791 is replaced by Ala (HIF-2α-P791A). The interaction of HIF-2α with both endogenous (**Fig. 8A**) and exogenous (**Fig. 8B**) Pin1 and HIF-2α was abolished by mutation at Pro791 as well as Ser790 in MDA-MB-231 cells, and this was confirmed by immunoprecipitation assay (**Fig. 8C**). However, ubiquitination of HIF-2α in the mutant cells in which proline 791 is replaced by alanine (HIF-2α-P791A) with or without MG-132 was not prominent in HEK293T cells (**Fig. 8D**). This suggests that Pro 791 adjacent to the phosphorylated Ser 790 is necessary for Pin1-HIF-2α binding, but not does not appear to influence the stabilization of the latter protein.


A
Pin1
HIF-2α
DAPI
Merged
Adjacent normal breast tissue
Invasive ductal carcinoma
B
IF score of Pin1
IF score of HIF-2α
**
Normal (n=10)
Tumor (n=90)
C
Spearman correlation
r = 0.41
P (two-tailed) < 0.0001
n = 90
IF score of Pin1
IF score of HIF-2α
D
IF score of Pin1
IF score of HIF-2α
a
a,b
b
Normal
Stage 1
Stage 2
Stage 3
E
MCF10A
MDA-MB-231
DAPI
HIF-2α
Pin1
Merged

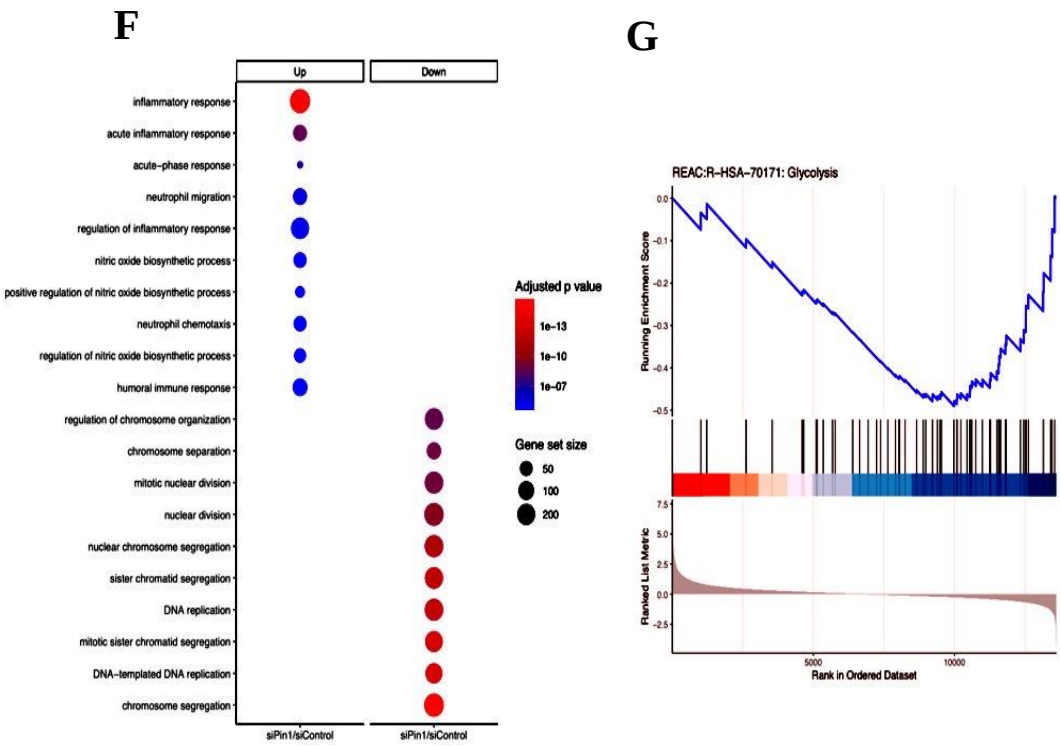


Figure 1. Overexpression of Pin1 and HIF-2α in breast cancer and their relationship

(A) Representative immunofluorescence (IF) images of and Pin1 and HIF-2α in breast tumor and surrounding normal tissue arrays. Scale bar, 200 μm. (B) Measurement of Pin1 and HIF-2α expression levels based on the tissue microarray IF score. The two-tailed unpaired Student's *t*-test $^{**}p < 0.01$ was used to establish the statistical significance. (C) Spearman analysis of IF data demonstrating a positive correlation between Pin1 and HIF-2α (n = 90), $r = 0.4$. (D) The relative IF scores of Pin1 and HIF-2α in different stages of breast cancer. (E) Pin1 and HIF-2α immunofluorescence staining in non-oncogenic MCF10A and MDA-MB-231 breast cancer cells. Scale bar, 200 µm. (F). RNA-Seq analysis of the Pin1-silencing-induced transcriptional response in MCF10A-ras cells. Gene set enrichment analysis (GSEA)

identified pathways in Gene Ontology: Biological Process (GO: BP) that were significantly up- and down-regulated. (G) GSEA plots show the downregulation of genes involved in the glycolysis pathway (REAC: R−HSA−70171). NES:-1.82, adjusted *p*-value: 0.006.

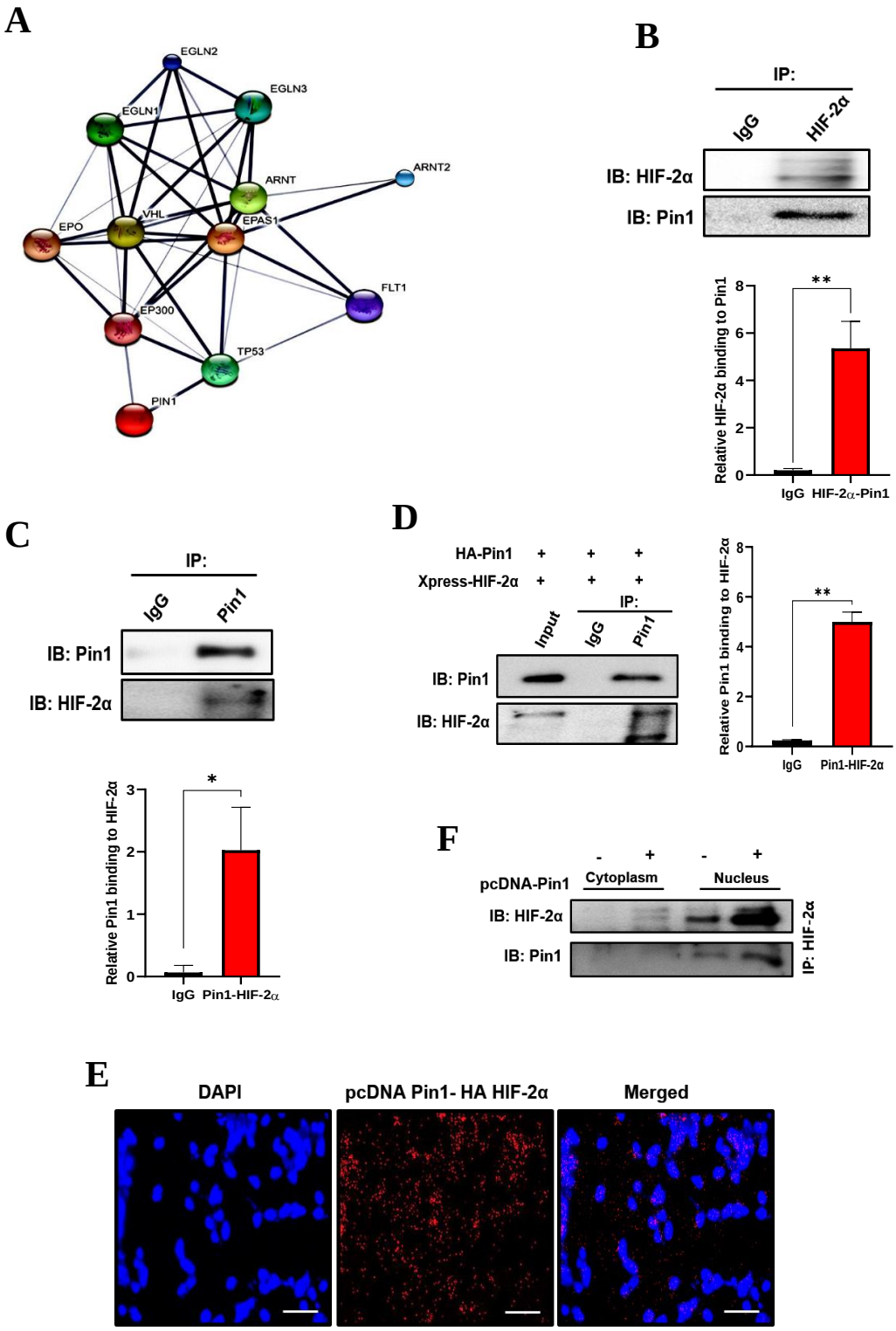

A
EGLN2
EGLN3
EGLN1
ARNT2
ARNT
VHL
EPO
EPAS1
FLT1
EP300
TP53
PIN1
B
IP:
IgG
HIF-2α
IB: HIF-2α
IB: Pin1
Relative HIF-2α binding to Pin1
**
IgG
HIF-2α-Pin1
C
IP:
IgG
Pin1
IB: Pin1
IB: HIF-2α
Relative Pin1 binding to HIF-2α
*
IgG
Pin1-HIF-2α
D
HA-Pin1
Xpress-HIF-2α
IP:
Input
IgG
Pin1
IB: Pin1
IB: HIF-2α
Relative Pin1 binding to HIF-2α
**
IgG
Pin1-HIF-2α
F
pcDNA-Pin1
Cytoplasm
Nucleus
IB: HIF-2α
IB: Pin1
IP: HIF-2α
E
DAPI
pcDNA Pin1- HA HIF-2α
Merged

Figure 2. Pin1 and HIF-2α Interaction in breast cancer cells

(A) Pin1 and HIF-2α interaction predicted by the STRING database. EPAS1 is the name of the gene encoding HIF-2α. (B, C) The immunoprecipitation technique was used to evaluate how endogenous Pin1 and HIF-2 interacts in MDA-MB-231 cells. The proteasome inhibitor MG132 (10 μM) was applied to MDA-MB-231 cells for 2 h. The protein lysates were immunoprecipitated for HIF-2α (B) or Pin1 (C) followed by immunoblot analysis. (D) Immunoprecipitation measurement of the exogenous interaction between Pin1 and HIF-2α overexpressed in MDA-MB-231 cells. (E) Pin1 and HIF-2α interaction was visualized by the PLA in MDA-MB-231 cells. Corresponding antibodies were used to co-label Pin1 and HIF-2α. DAPI was used to stain the nuclei. Red pots represent the Pin1 and HIF-2α complex. Scale bar, 200 μm. (F) The pcDNA-Pin1 plasmid was transfected into MDA-MB-231 cells, and Pin1 interaction with HIF-2α in the cytoplasm and nucleus fractions was measured as in (B). The statistical significance was established by the Student's *t*-test $*p < 0.05$ and $**p < 0.01$ values. Data are presented as the mean ± SD (n=3).

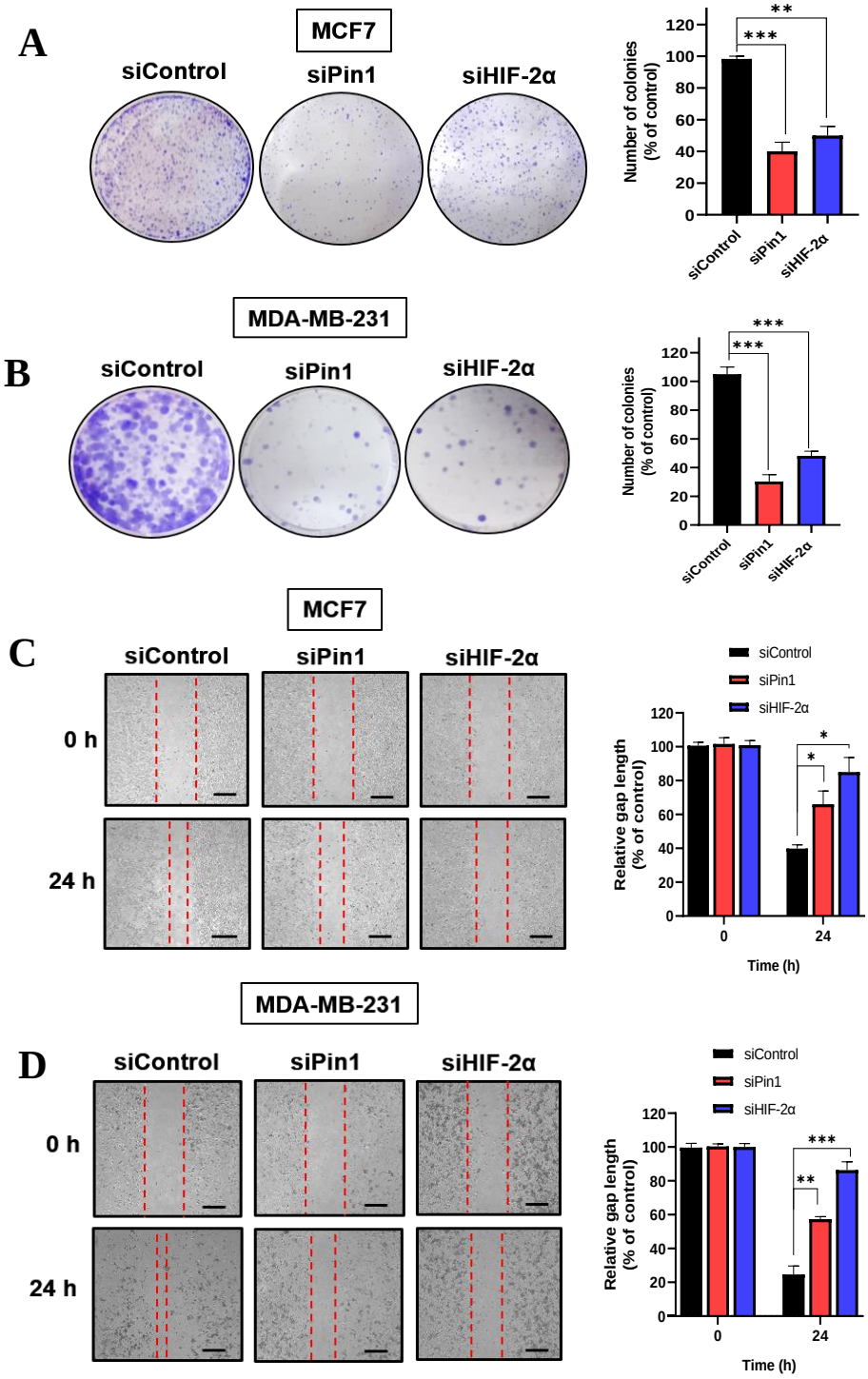
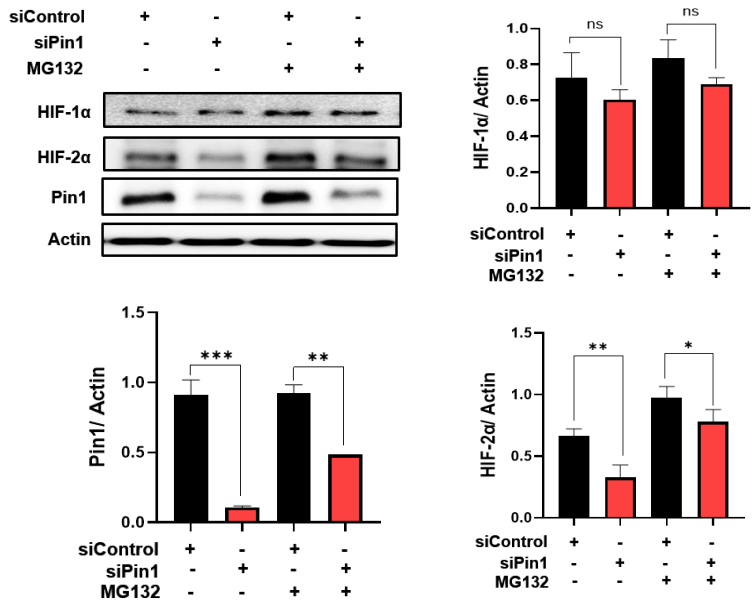

A
MCF7
siControl
siPin1
siHIF-2α
Number of colonies (% of control)
B
MDA-MB-231
C
0 h
24 h
Relative gap length (% of control)
Time (h)
D

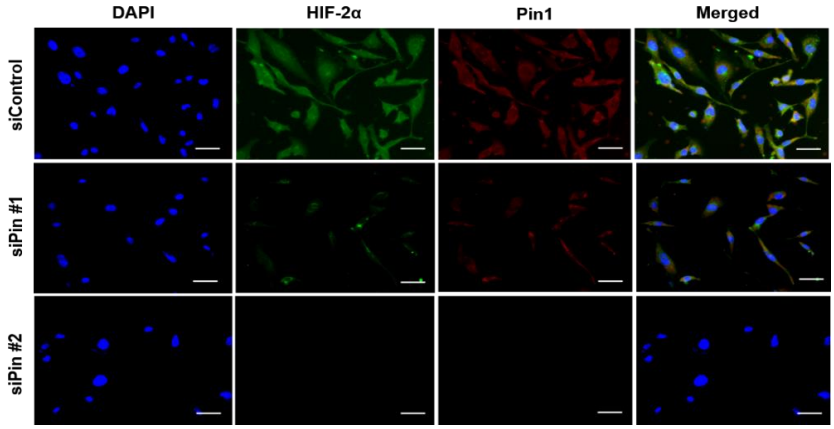
Figure 3. Oncogenic activity of Pin1 and HIF-2α in breast cancer cells

(A, B) Control, Pin1, or HIF-2α siRNA was transfected into MCF7 (A) and MDA-MB-231 (B) cells planted in 6-well plates according to the Materials and Methods section. Following crystal violet staining, attached cells were captured on camera, and the percentage of attached cells was determined by counting the number of colonies. Representative sets of photos from three separate experiments are displayed. The Student's *t*-test was used to establish the statistical significance of the data, which are presented as the mean ± SD, $^{**}p < 0.01$ and $^{***}p < 0.001$. (C, D) After being transfected with control, Pin1, or HIF-2α siRNA, MCF7 (C) and MDA-MB-231 (D) cells were incubated for 48 h. Then, using a confocal microscope, cell migration was viewed. The Student's *t*-test was used to establish the statistical significance of the data, which are presented as the mean ± SD (n=3). $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$, respectively.

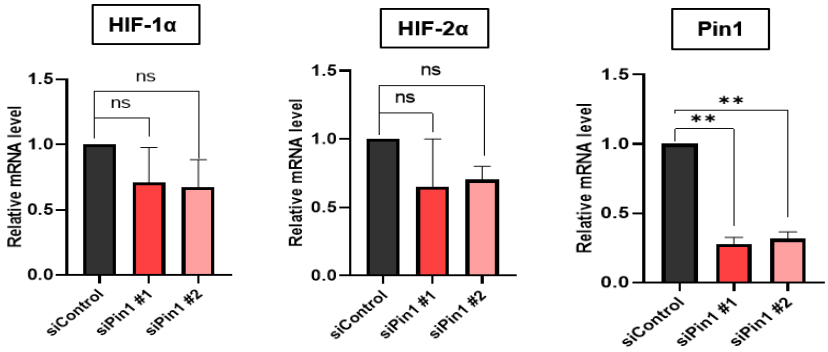
A


siControl + - + -
siPin1 - + - +
MG132 - - + +
HIF-1α
HIF-2α
Pin1
Actin
HIF-1α/ Actin
ns
ns
Pin1/ Actin
***
**
HIF-2α/ Actin
**
*

B


DAPI
HIF-2α
Pin1
Merged
siControl
siPin #1
siPin #2

C


HIF-1α
HIF-2α
Pin1
Relative mRNA level
ns
**
siControl
siPin1 #1
siPin1 #2

D

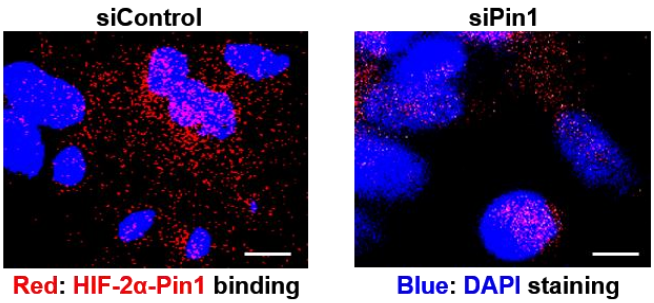


Figure 4. Pin1-mediated upregulation of HIF-2α in breast cancer cells

(A) MDA-MB-231 cells were treated with either control siRNA or Pin1 siRNA and exposed to the proteasome inhibitor MG132 (10 μM). Western blot analysis using anti-Pin1, anti-HIF-1α, and anti-HIF-2α antibodies was performed on cell lysates. (B) After Pin1 siRNA #1 or #2 was transfected into MDA-MB-231 cells, immunofluorescence (IF) staining was used to evaluate the expression of both Pin1 and HIF-2α. Scale bar, 200 μm. (C) Real-time PCR was used to assess the relative mRNA levels of Pin1, HIF-1α, and HIF-2α in MDA-MB-231 cells that had either received control siRNA or Pin1 siRNA treatment. (D) Interaction of Pin1 with HIF-2α in Pin1 knockdown MDA-MB-231cells confirmed by the PLA. Scale bar, 200 μm. The statistical significance was established using the Student's *t*-test, and the data are shown as the mean ± SD (n=3). *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$

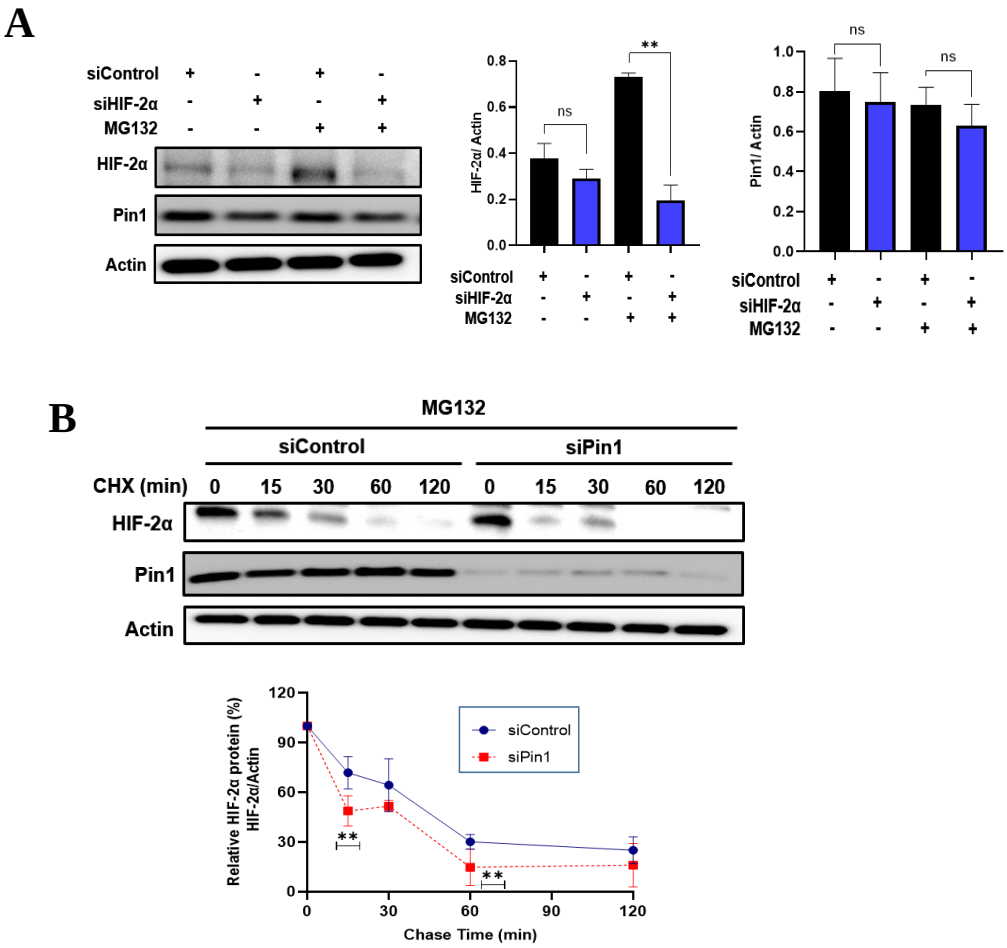
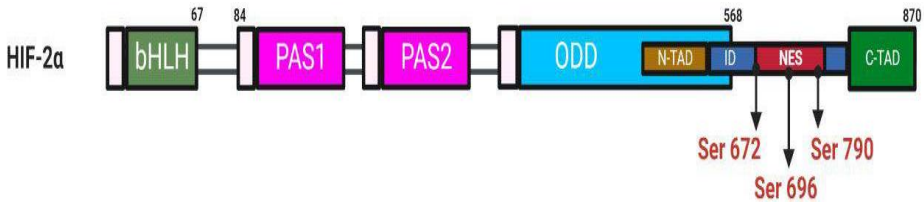

A
siControl
siHIF-2α
MG132
HIF-2α
Pin1
Actin
HIF-2α/ Actin
Pin1/ Actin
ns
**
B
MG132
siControl
siPin1
CHX (min)
0 15 30 60 120 0 15 30 60 120
HIF-2α
Pin1
Actin
Relative HIF-2α protein (%)
HIF-2α/Actin
Chase Time (min)
C
IP:
IgG
HIF-2α
MG132
IB: Ubiquitin
Actin
Relative HIF-2α Ubiquitanation
*
IgG
HIF-2α
HIF-2α + MG132

Figure 5. The functions of Pin1 in controlling HIF-2α stability

(A) MDA-MB-231 cells were exposed to the proteasome inhibitor MG132 (10 μM) after transfection with either control siRNA or HIF-2α siRNA. (B) The reduced HIF-2α stability as a result of Pin1 silencing. Following 48 h of control or Pin1 siRNA transfection, MDA-MB-231 cells were exposed to CHX (10 μM) for the indicated times. Western blot analysis using anti-Pin1 and anti- HIF-2α antibodies was performed on cell lysates. Student's *t*-test was used to establish the statistical significance of the data, which are presented as the mean ± SD (n=3) $^{**}p < 0.01$**.** (C) Effects of Pin1 silencing on ubiquitination of HIF-2α in Pin1 knockdown and control MDA-MB-231 cells. By applying an anti-ubiquitin antibody to immunoprecipitated HIF-2α, the ubiquitination of HIF-2α was identified. Pin1 knockdown MDA-MB-231 cells were exposed for 2 h to either MG132 (10 μM) or vehicle. The Student's *t*-test was used to establish the statistical significance of the data, which are presented as the mean ± SD (n=3). $^{*}p < 0.05$, and $^{**}p < 0.01$.

A



B

	Position in query protein	Sequence in query protein	Corresponding motif	Features of motif described in the literature
1	406 - 407	TP	[pS/pT]P	WW domain binding motif
2	469 - 470	TP	[pS/pT]P	WW domain binding motif
3	481 - 482	TP	[pS/pT]P	WW domain binding motif
4	484 - 485	SP	[pS/pT]P	WW domain binding motif
5	543 - 544	SP	[pS/pT]P	WW domain binding motif
6	559 - 560	TP	[pS/pT]P	WW domain binding motif
7	581 - 582	SP	[pS/pT]P	WW domain binding motif
8	626 - 627	TP	[pS/pT]P	WW domain binding motif
9	672 - 673	SP	[pS/pT]P	WW domain binding motif
10	696 - 697	SP	[pS/pT]P	WW domain binding motif
11	790 - 791	SP	[pS/pT]P	WW domain binding motif

C

```
Human        664 APLGPPVSPPHVSTFKTRSAKGFGARGPDVLSPAMVALSNKLKLKR---- LPLPQPPSAISPGENSKSRFPP
Chimpanzee       APLGPPVSPPHVSTFKTRSAKGFGARGPDVLSPAMVALSNKLKLKR---- LPLPQPPSAISPGENSKSRFPP
Rat              SQLEPPSTPPHVSMFKMRSAKDFGARGPYMMSPAMIALSNKLKLKR---- LPPSQPPSTRSPGENAKSGFPP
Mice             SQLEPPSAPPHVSMFKMRSAKDFGARGPYMMSPAMIALSNKLKLKR---- LPPPQPPSTRSSGENAKTGFPP
Canis            SPLGPPITSPHLSMFKKRSAKAFGPQGPDVMSPAMVALSNKLKLKR---- LP--PPPSVMSPGENTKSGFPP

                 : * ** :.**:* ** **** **.:** ::****:**********     **     ***. *.***:*: ***
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D

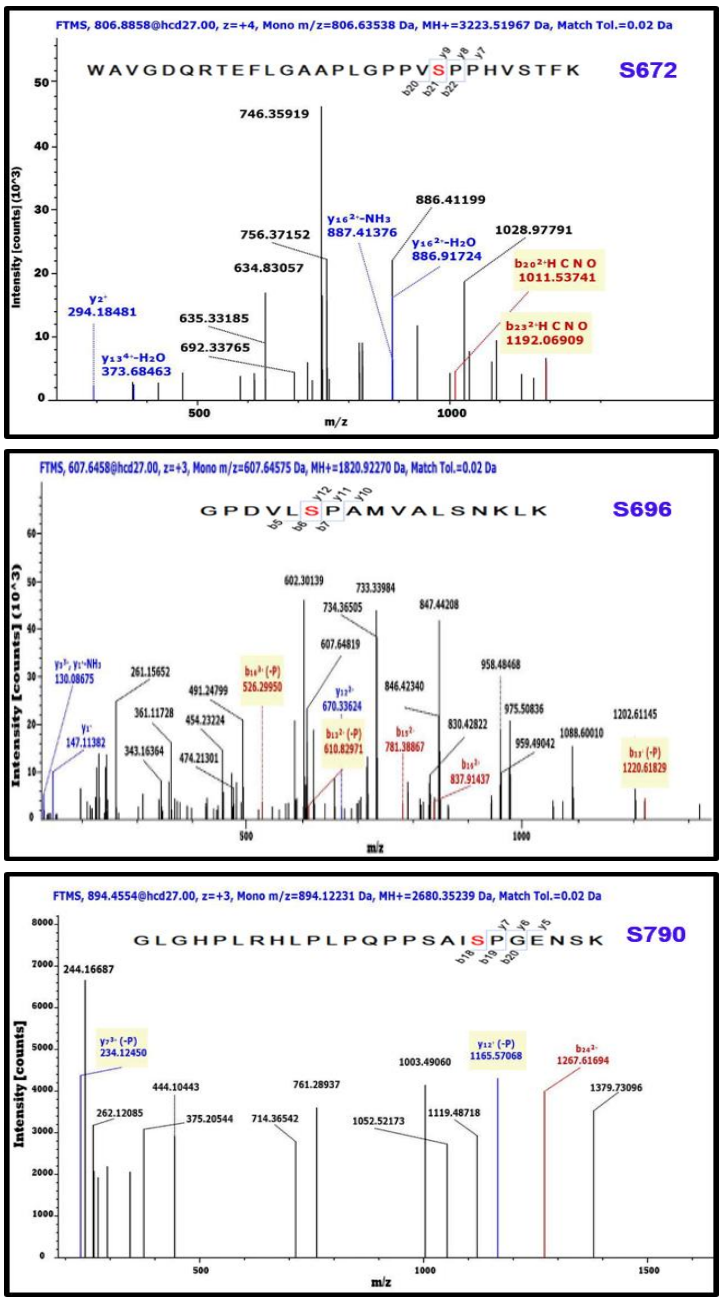
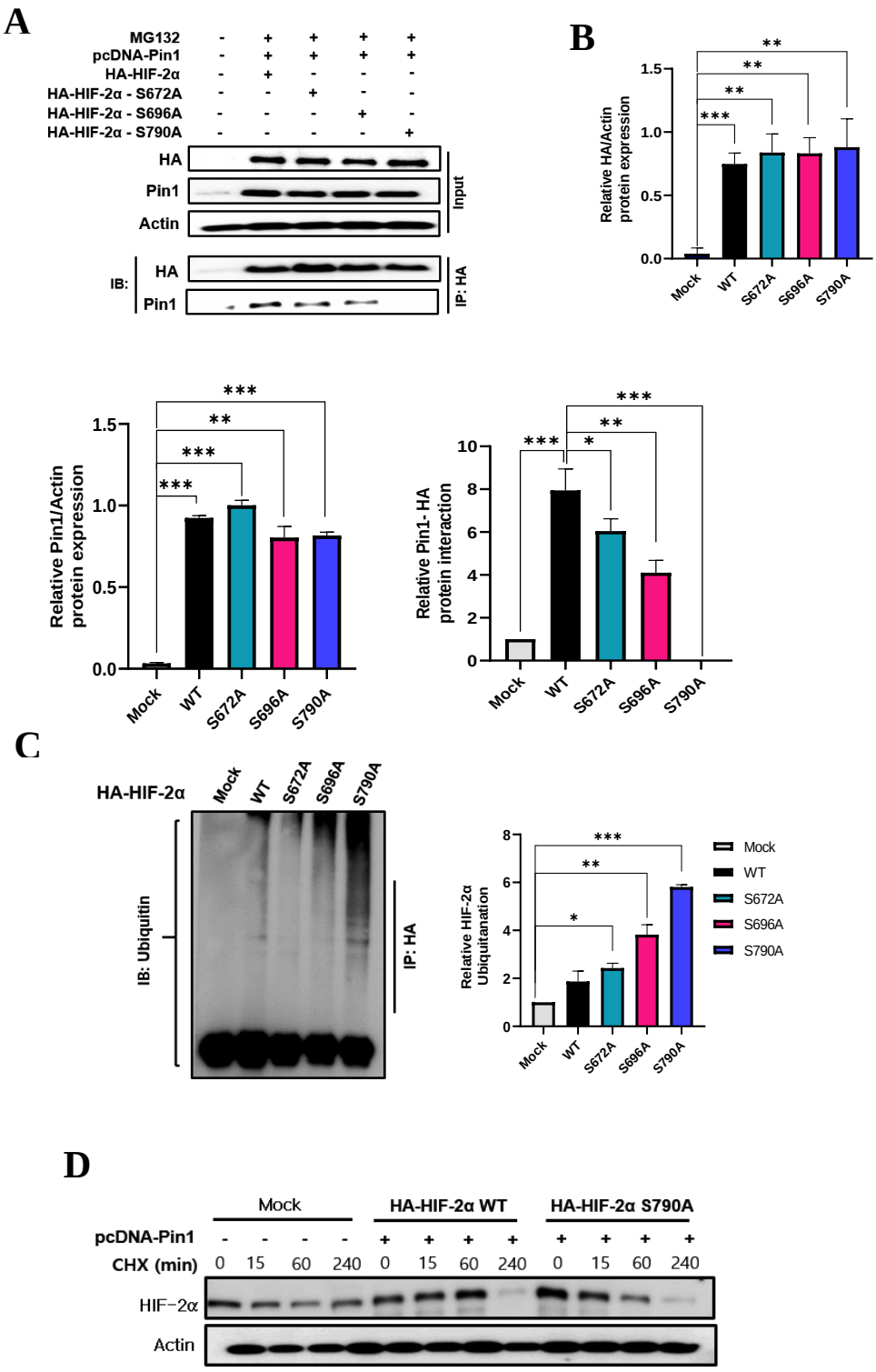


Figure 6. Identification of phosphorylation sites of HIF-2α

(A) The location of the phosphorylatable serines in the NES of full-length HIF-2α fragment is shown schematically. (B) HIF-2α has the WW domain

binding motifs. Eleven WW binding motifs with the pSer/Thr-Pro sequence are present in the HIF-2α protein. Human Protein Reference Database, available at http://www.hprd.org/ (C) Ubiquitous presence of S672, S696, and S790 in different species. (D) The LC-MS/MS analysis was used to get the peptide spectra of HIF-2α. Three residues (S672, S696, S790) of HIF-2α were found to be phosphorylated and recognized as the consensus binding locations for Pin1.


A
MG132
pcDNA-Pin1
HA-HIF-2α
HA-HIF-2α - S672A
HA-HIF-2α - S696A
HA-HIF-2α - S790A
HA
Pin1
Actin
Input
IB:
IP: HA
B
Relative HA/Actin protein expression
Mock
WT
S672A
S696A
S790A
Relative Pin1/Actin protein expression
Relative Pin1- HA protein interaction
C
HA-HIF-2α
IB: Ubiquitin
IP: HA
Relative HIF-2α Ubiquitanation
D
Mock
HA-HIF-2α WT
HA-HIF-2α S790A
pcDNA-Pin1
CHX (min)
HIF-2α
Actin

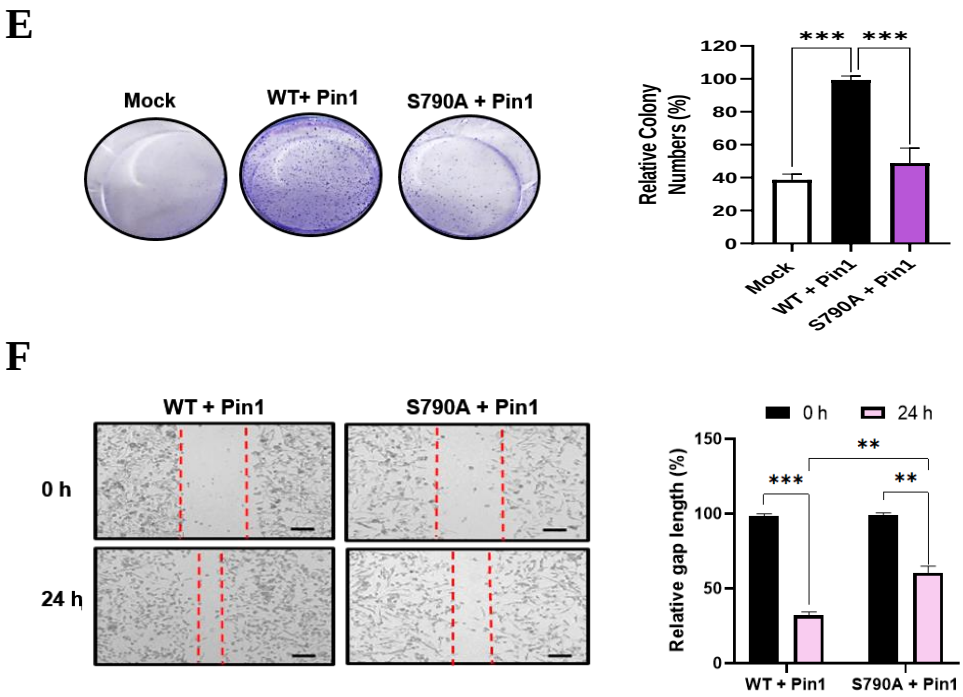


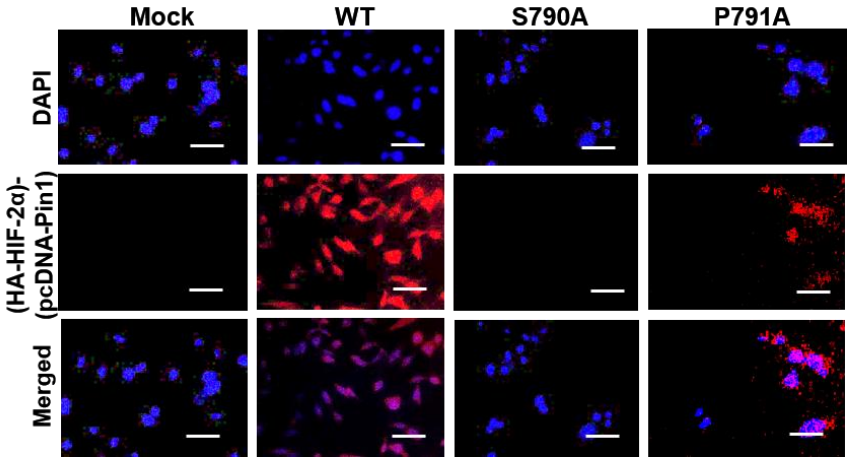
Figure 7. The precise sites of HIF-2α (S672, S696, and S790) involved in its binding to Pin1

(A) In the presence or absence of the proteasome inhibitor MG132, a comparison of the HIF-2α-Pin1 interaction was performed in wild-type (WT) and mutant cells in which a particular serine was changed into an alanine. pcDNA-Pin1 and HA-tagged WT HIF-2α or the corresponding mutant constructs were co-transfected into HEK293T cells, and the cell lysates were then analyzed by Western blot analysis or immunoprecipitation (IP). (B) Quantitative analysis of the interaction between Pin1 with wild-type or non-phosphorylatable mutants (HIF-2α S672, S696, and S790). (C) Ubiquitinylated HIF-2α (Ub-HIF-α) was measured by immunoprecipitation of HA, and then HEK293T cells were subjected to a Western blot experiment using an anti-ubiquitin antibody. (D) Comparative stability of HIF-2α-S790A in HEK293T cells transfected with WT or non-

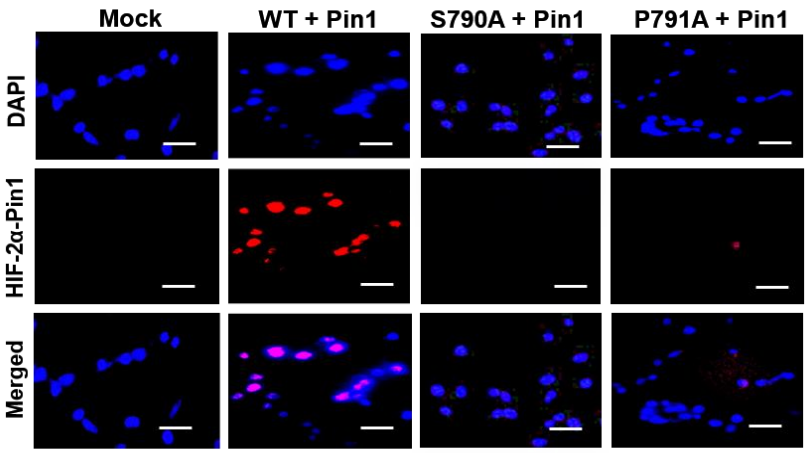
phosphorylatable mutant forms (S790A), assessed by the CHX chase assay. (E) MCF7 cells planted in 3-well plates were either co-transfected with pcDNA-Pin1 and HA-tagged WT HIF-2α or mutant forms (S790A). The cells were stained with crystal violet and then were photographed. (F) MDA-MB-231 cells planted in 2-well plates were either co-transfected with pcDNA-Pin1 and HA-tagged WT HIF-2α or mutant forms (S790A) for the cell migration assay.

The Student's *t*-test was used to establish the statistical significance of the data, which are presented as the mean ± SD (n = 3). $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$, respectively.

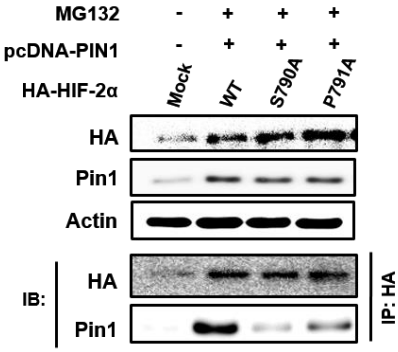
A

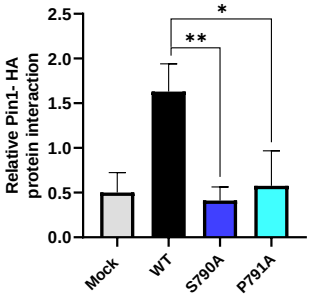

Mock
WT
S790A
P791A
DAPI
(HA-HIF-2α)-(pcDNA-Pin1)
Merged

B


Mock
WT + Pin1
S790A + Pin1
P791A + Pin1
DAPI
HIF-2α-Pin1
Merged

C


MG132 - + + +
pcDNA-PIN1 - + + +
HA-HIF-2α
Mock
WT
S790A
P791A
HA
Pin1
Actin
IB:
HA
Pin1
IP: HA


Relative Pin1- HA protein interaction
2.5
2.0
1.5
1.0
0.5
0.0
*
**
Mock
WT
S790A
P791A

D

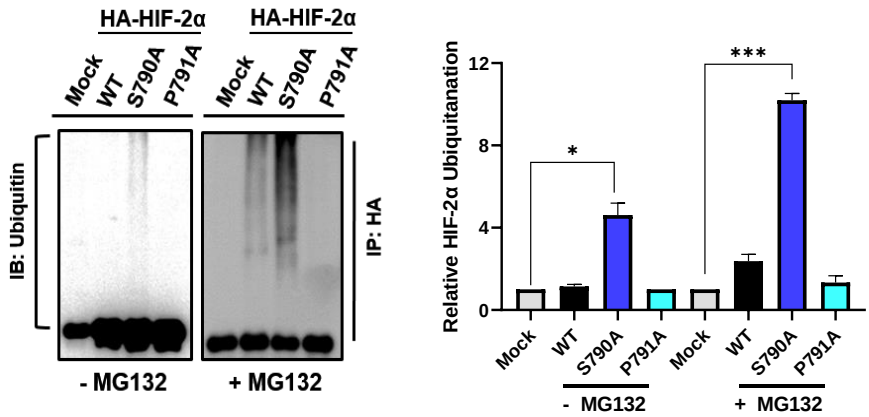


Figure 8. Verification of HIF-2α serine 790 as an essential site for its stabilization and Pin1 binding

(A, B) Binding of HIF-2α-S790A and P791A with Pin1 was assessed by the PLA in MDA-MB-231 cells without (A) or with (B) transfection with pcDNA-Pin1. Scale bar, 200 μm. (C) Comparison of Pin1 interaction with WT HIF-2α, HIF-2α-S790A, or HIF-2α-P791A. pcDNA-Pin1 and HA-tagged WT HIF-2α or the corresponding mutant constructs were co-transfected into HEK293T cells prior to Western blot and immunoprecipitation analyses. (D) Ubiquitin-HIF-2α was determined by immunoprecipitation of HA with a subsequent Western blot assay in HEK293T cells. Student's *t*-test. was used to establish the statistical significance of the data, which are shown as the mean± SD (n = 3). *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

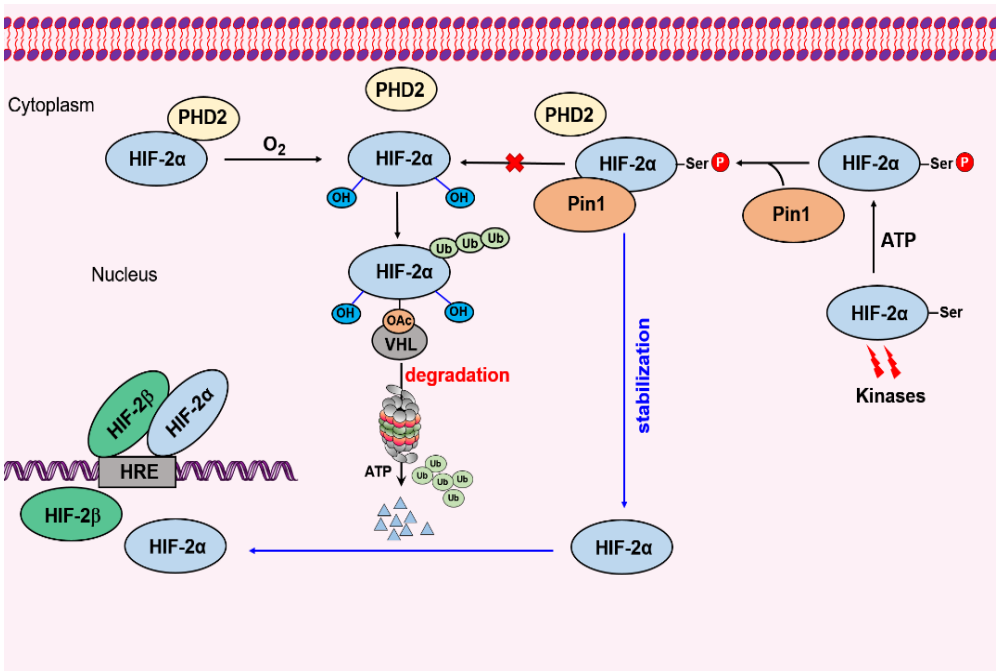
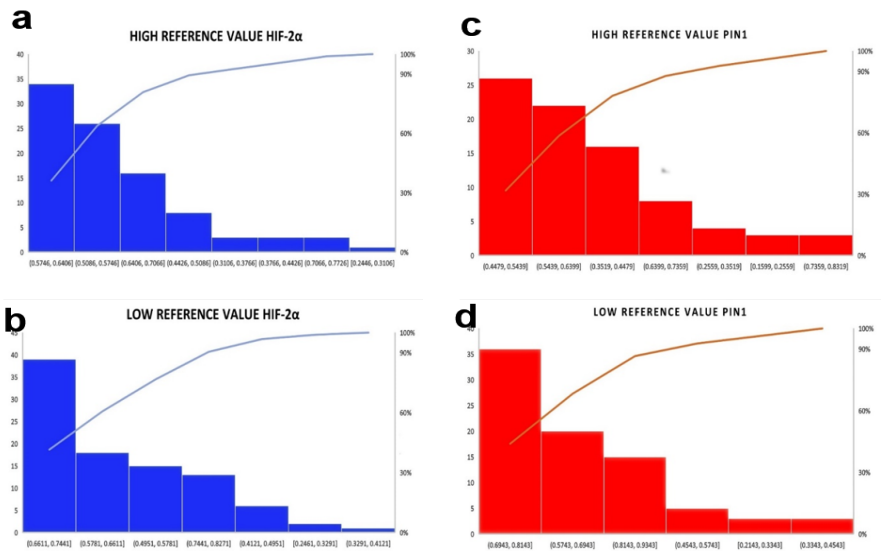


Figure 9. A proposed model for the regulation of HIF-2α by Pin1 in breast cancer

HIF-2α is subjected to proteasomal degradation in normoxia. HIF-2α undergoes phosphorylation at a specific serine residue (e.g., S790), which facilitates its interaction with Pin1. As a result, HIF-2α can avoid PHD2-mediated prolyl hydroxylation, ubiquitination and subsequent proteasomal degradation. The stabilized HIF-2α translocates to the nucleus where it upregulates the expression of genes including those involved in aerobic glycolysis (Warburg effect)

1A



1B

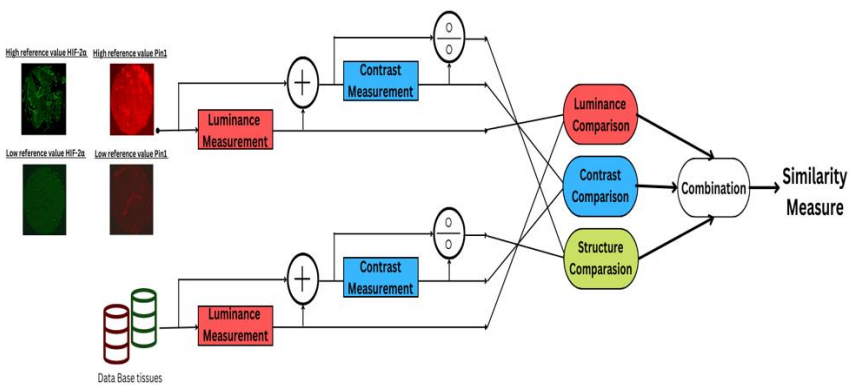


Figure S1. Structural Similarity Index Measure (SSIM) of breast tissue

(1A) Pareto plots of the amount of database breast tissue analyzed versus protein expression of Pin1 and HIF-2α measured by using SSIM (structural similarity index measure). (a,b) SSIM value algorithm of HIF-2α with high and low values, respectively. (c,d) SSIM value algorithm of Pin1 with high and low values, respectively. (1B) Adopted Diagram of the SSIM (structural similarity index measure) database breast Tissue [70].

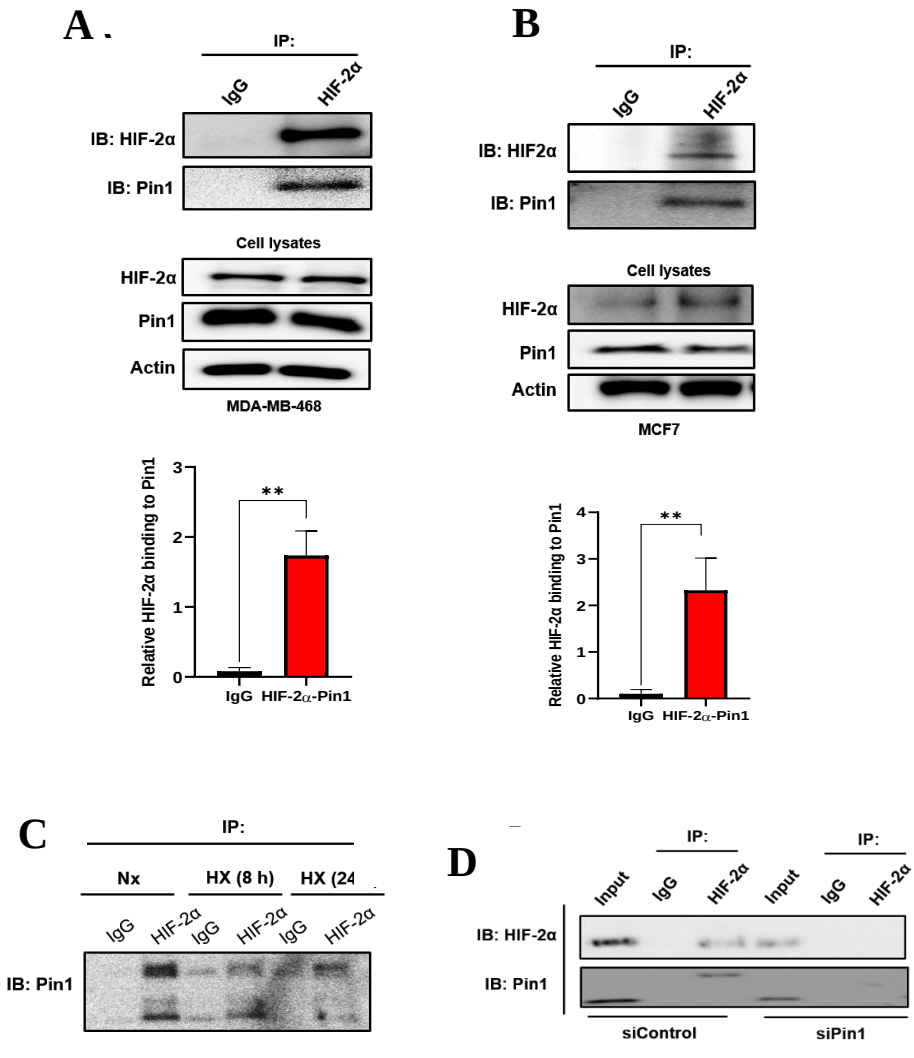


Figure S2. Interaction of Pin1 and HIF-2α in different breast cancer cell lines

(A, B) Using an immunoprecipitation technique, the interaction between endogenous Pin1 and HIF-2α was investigated in MDA-MB-468 cells and MCF7 cells. (C) Pin1 and HIF-2α interaction in normoxia and hypoxia was measured at 8 and 24 h in MDA-MB-231 cells. (D) Interaction of Pin1 with HIF-2α in Pin1 knockdown MDA-MB-231cells confirmed by immunoprecipitation.

4. Discussion

There is 48% amino acid sequence homology between HIF-2α and HIF-1α, particularly in the structural and functional motifs. Despite this analogy, HIF-2α differs significantly from HIF-1α in terms of its expression patterns, physiological functions, regulatory mechanisms, and gene specificity in oxygen homeostasis [51,52]. While both HIF-1α and HIF-2α can bind to the same consensus sequence, their target genes can vary depending on the context. HIF-2α regulates 1454 genes while HIF-1α controls 701 genes, with 303 of their targets being identical [53].

In general, HIF-1α preferentially induces transcription of genes that encode glycolytic enzymes, such as hexokinase 1 and lactate dehydrogenase A [54]. By contrast, HIF-2α upregulates genes involved in migration, including one encoding erythropoietin (EPO) [55], and the stem cell factor Oct-4 [56]. Erythropoiesis is a dynamic process regulated by oxygen in vertebrates. EPO is the key hormone responsible for effective erythropoiesis. In an animal model of breast cancer, EPO has been shown to promote lymph node tumor metastasis [57]. It also has a pro-angiogenic activity that may be disadvantageous in breast cancer [58]. Notably, EPO can partially mimic the effects of hypoxia under normoxic conditions [59], which may be regulated by HIF-2α.

Pin1 functions as an oncoprotein by controlling a number of signaling molecules involved in cell metabolism, cell cycle progression, and other processes [60]. Despite being constantly overexpressed in a variety of human malignancies, Pin1 expression is comparatively low in normal tissues [9,60]. Notably, Pin1 promotes the induction and maintenance of pluripotency through its regulation of Oct-4 [61]. Our RNAseq data suggest that Pin1 may activate Oct-4 via HIF-2 .

A study has examined the relationship between HIF-2 overexpression and overlapping clinical outcomes in patients with 18 different solid tumor

locations [25]. According to this study, there was a negative correlation between HIF-2 levels and poor overall, progression-free and metastasis-free survival. Moreover, the hypoxic tumor microenvironment influences metastasis, angiogenesis, and stemness by overexpressing HIF-2α. HIF-2α overexpression has been found in, various types of tumor, such as breast, colon, liver, and lung cancer [62-64]. In this investigation, we have demonstrated a positive correlation between Pin1 and HIF-2α expression in human breast carcinoma tissues.

Pin1 and HIF-2α have been shown to interact in colon cancer, and the resultant complex co-localizes in the nucleus under hypoxia [30]. Although the previous study suggested involvement of WW and PPIase domains of Pin1 in its binding to HIF-2α, phosphorylation sites of HIF-2α in the context of Pin1 binding were not identified [30]. In our present study, in contrast to hypoxia, aggressive TNBC cells exhibit a more significant interaction between Pin1 and HIF-2α in normoxia. In line with our findings, HIF-2α is often stabilized at even relatively high O_2 concentrations [53,65].

The Pin1 N-terminal WW domain identifies the pSer/Thr-Pro motif of a substrate protein, and the PPIase activity kept in the C-terminal domain isomerizes the proline residue found in that motif [34]. Both oxygen-dependent and oxygen-independent processes, including phosphorylation, are likely to modulate HIF-2α expression and transcriptional activity. Most notably, some serine residues of HIF-2α appear before proline residues, making HIF-2α a candidate substrate for Pin1. We hypothesize that Pin1 interacts with HIF-2α, which alters the structure and subsequently stability of HIF-2α. Of note, inhibition of Pin1 significantly decreased HIF-2α protein levels but not its mRNA levels. This finding suggests that Pin1 might control HIF-2α post-translationally. Our discovery lends supports to the hypothesis that Pin1 stabilizes HIF-2α by preventing its ubiquitination and proteasomal degradation.

In the current investigation, we have identified a unique O_2-independent regulatory mechanism underlying stabilization of HIF-2α by interaction with Pin1, which involves three different serine residues at positions 672, 696, and of this transcription factor. The inhibitory domain (ID) of HIF-2α, is located between the N-terminal activation (N-TAD) and the C-terminal transactivation (C-TAD) domains, where these serine residues, as well as the nuclear export signal (NES), are located. The function of ID is not fully understood, but it has been suggested to participates in the transactivation of HIFs [32,50].

The binding of Pin1 to HIF-2α was inhibited to different extents by a site-directed mutation of HIF-2α in which each of the aforementioned three serine residues was substituted with non-phosphorylatable alanine (S672A, S696A, and S790A. It has been reported that HIF-2α phosphorylated on serine 672 occurs in the majority of cells in both nucleus and cytoplasm [32]. Furthermore, extracellular signal-regulated protein kinases 1 and 2 (ERK1/2) have been shown to phosphorylate HIF-2α at serine 672 in hypoxia (1% O_2) [32,66]. Moreover, HIF-2α transcriptional activity is reduced when this location is changed to an alanine residue or the ERK1/2 pathway is inhibited, and this results in HIF-2α mislocalization to the cytoplasm without altering the protein expression levels [32]. HIF-2α phosphorylation on serine 696 was found for the first time in our study. There is no experimental data about this serine, but it is localized in NES, and hence could impact the transcriptional activity of HIF-2α.

According to our research, S790 close to NES is most crucial for HIF-2α interaction with Pin1. As a result, the physical contact between HIF-2α and Pin1 was abolished by its non-phosphorylatable mutation. The amount of HIF-2α in the nucleus determines its transcriptional activity; the S790 mutation may disrupt the NES sequence. This shows that Pin1 might be able to bind to HIF-2α via NES. Notably, a prior study revealed that CRM1-

dependent nuclear export of HIF-2α is inhibited by CK1-mediated phosphorylation of HIF-2α at S383 and T528 [50]. This has implications for how HIF-2α is distributed in the nucleocytoplasm. It is speculated that Pin1 promotes nuclear retention of HIF-2α by facilitating its binding to stationary nuclear or chromatin components [32,50].

Cancer cells are largely dependent on glycolysis in acquiring ATP despite the presence of oxygen, a phenomenon defined as Warburg effect. The majority of glycolytic enzymes as well as glucose transporter are known to be upregulated by HIF-1α. It has been mysterious how HIF-1α, prone to degradation in the presence of oxygen, can stimulate the aerobic glycolysis. Very recently, Yao and colleagues have reported the enhanced aerobic glycolysis in MDA-MB-231 breast cancer cells which was mediated by signal-induced proliferation-associated 1 (SIPA1) [67]. It is noticeable that SIPA1 binds to the promoter region of *EPAS1*, the gene encoding HIF-2α. The resulting HIF-2α up-regulation stimulated the expression of multiple glycolysis-related genes to increase aerobic glycolysis. Silencing of SIPA1 expression blocked the aerobic glycolysis and suppressed the growth and metastasis of breast cancer cells.

One of the salient features of our present work is oxygen-independent stabilization of the HIF-2α protein which can explain how HIF-2α can stimulate the aerobic glycolysis or Warburg effect in breast cancer cells observed by others [67]. The activities of HIF-2α are regulated by many additional signals, and the abundance of these signals is often associated with actual deprivation of oxygen [68]. The balance of these signals under different conditions gives Pin1 different opportunities to act. We speculate that cancer cells that are not deprived of oxygen may take advantage of Pin1, which mimics the activated hypoxia pathway, a phenomenon called, ‘pseudo hypoxia”.

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Chapter 3.

Role of Pin1 in non-canonical function of HIF-prolyl hydroxylase 2 in breast cancer cells

Abstract

Prolyl hydroxylase domain 2 (PHD2) is the main hypoxia-inducible factor (HIF)-prolyl hydroxylase. PHD2 in normoxia hydroxylates specific proline residues in HIF-1α and HIF-2α, which facilitates their ubiquitination and proteasomal degradation. Although the activity of PHD2 is reduced in hypoxia, significant levels of residual activity of this monooxygenase are still detected under hypoxic conditions. However, its role under hypoxia is poorly understood. Peptidyl-prolyl isomerase (Pin1) binds to target proteins containing phosphorylated serine or threonine residues followed by proline (pS/T-P). As PHD2 harbors several pS/T-P motifs, it may be a potential substrate of Pin1. We found for the first time interaction between Pin1 and PHD2 in human breast cancer MDA-MB-231 cells in normoxic and hypoxic conditions. Additionally, the breast cancer tissue array showed elevated expression of PHD2 as well as Pin1 in tumors compared to adjacent normal tissues. LC-MS/MS spectrometry identified three amino acid residues (S125, T168, and S174) of PHD2 undergoing phosphorylation. Among these, serine 125 was found to be the principal site required for Pin1 binding. As a novel binding partner of Pin1, oncogenic PHD2 can be explored as a therapeutic target for the treatment of breast cancer

Keywords: Breast cancer, hypoxia-inducible factor, PHD2, Pin1, Protein-protein interaction

1. Introduction

Prolyl hydroxylases (PHDs) are well known oxygen sensors that hydroxylate hypoxia inducible factors (HIFs) using molecular oxygen, thereby targeting them towards ubiquitin-mediated proteasomal degradation under normoxic conditions [1-3]. PHD2, one of the three PHD isoforms, is the primary hydroxylase for HIF proteins [1,4]. Regulation of oxygen homeostasis is critical in normal physiology; however, tissue oxygen tension is compromised in some disease conditions including cancer [5].

The differential regulation of PHD2 activity in tumor progression, tumor vasculature and metastasis versus in normal physiology has been investigated [6,7,8]. Under hypoxic conditions, PHD2 activity is repressed, elevating intracellular HIF levels. However, recent studies have demonstrated that substantial levels of residual PHD2 activity are still detected in hypoxia [9]. Notably, PHD2 haplodeficiency has been shown to impair metastasis in spontaneous cancer models [7]. In tumor endothelial cells, PHD2 can also induce metastasis [10], suggesting PHD2 as a new target for cancer therapy. In addition to its canonical function as an oxygen sensor, PHD2 can mediate several signaling pathways, independently of its hydroxylase function [11]. In breast cancer cells, PHD2 acts as a direct binding partner of epidermal growth factor receptor (EGFR), thereby maintaining the EGFR stability and activity [12].

Peptidyl-prolyl *cis-trans* isomerase NIMA-interacting 1 (Pin1) is an exclusive *cis-trans* isomerase that specifically binds to phospho-serine/threonine-proline (pSer/Thr-Pro) sequences of its substrate proteins, thereby altering their conformation, stability and functions [13,14]. Originally recognized as a cell cycle protein, Pin1 is often overexpressed in the tumor microenvironment, which facilitates cancer progression by

modulating numerous prooncogenic and tumor suppressive signaling pathways [15,16]. This drives cancer progression and is correlated with an unfavorable clinical prognosis [17-19]. Pin1 lies downstream of several well-established oncoproteins including RAS, NOTCH, E2F, PI3K, etc., and has been shown to regulate pathways governing hallmarks of cancer [20-23]. Consequently, targeting Pin1 is emerging as an appealing strategy to cancer therapy [24-27].

Both PHD2 and Pin1 have previously been established as crucial regulators of HIF proteins. While PHD2 prevents HIF accumulation in normoxic conditions, Pin1 directly binds to HIF-1α and stabilizes it in a phosphorylation-dependent manner [28]. We have previously shown that Pin1 inhibition impedes tumor progression, angiogenesis and hypoxia-induced upregulation of HIF-1α [28]. Very recently, we have reported that Pin1 is also involved in an oxygen-independent stabilization of HIF-2α in human breast cancer cells and patients' specimens [29]. Given the close involvement of PHD2 and Pin1 in HIF regulation and their overexpression in breast cancer, our investigation aimed to explore the association between PHD2 and Pin1 in the context of breast cancer progression.

2. Materials and methods

2.1. Reagents and antibodies

Dulbecco's modified Eagle's medium (DMEM), RPMI 1640 medium, fetal bovine serum (FBS), and penicillin/streptomycin were obtained from Gibco BRL (Grand Island, NY, USA). *Stealth™ RNAi-negative control duplexes and Trizol® were purchased from Invitrogen Life Technologies Corporation (Carlsbad, CA, USA).* The human breast cancer tissue microarray (LVI5050) was supplied by US Biomax (Rockville, MD, USA). Rabbit polyclonal antibodies against HIF-1α, HIF-2α, PHD2, and PHD3 were obtained from

Novus Biologicals (Centennial, CO, USA), and that for PHD1 was purchased from abcam (Cambridge, UK). Antibodies for Pin1, actin, lamin B_1, and α-tubulin were provided by Santa Cruz Biotechnology, Inc. (*Dallas, TX, USA*). The ubiquitin antibody was purchased from Cell Signaling Technology (Bervely, MA, USA). The secondary antibodies were acquired from Zymed Laboratories (San Francisco, CA, USA).

2.2. Cell culture

Human breast cancer (MDA-MB-231 and MCF-7) and HEK293T cell lines were obtained from the American Type Culture Collection. MDA-MB-231 and HEK293T cells were maintained in DMEM while MCF-7 human breast cancer cells were grown in RPMI cell culture media. They were grown with 5% FBS media mixed with 100 ng/mL antibiotic at 37℃ in an incubator with 5% CO_2 and 95% air. Cells in hypoxic conditions were grown at an atmosphere of 1% O_2.

2.3. Small interfering RNA (siRNA) and DNA plasmid transfection

Pin1 siRNA #1 was obtained from Bioneer, Inc. (Seoul, South Korea). The Control siRNA, Pin1 siRNA #2 (sc-36230), and HIF PHD2 siRNA (sc-45537) were supplied by *Santa Cruz Biotechnology, Inc.* (Dallas, TX, USA). Lipofectamine RNAiMAX was acquired from Life Technologies Corporation (Carlsbad, CA, USA) and transfected for 48 h into breast cancer cells. Full-length and mutants PHD2 were made by Cosmo Genetech Company (Seoul, South Korea). HEK293T cells were grown until 90% confluence, and Lipofectamine 2000 obtained by Thermo Fisher Scientific, Inc. (Waltham, MA, USA) was transfected into these cells with wild type (WT) HA-PHD2 and pcDNA-Pin1 constructs following the manufacture's

procedure. Western blot and immunoprecipitation assays were performed after 24 h of transfection.

2.4. Quantitative real-time polymerase chain reaction (RT-qPCR) analysis

Total RNA from MDA-MB-231 cells was extracted using the Trizol® reagent provided by Invitrogen *(Carlsbad, CA, USA).* The RNA 600 Nano chip by Agilent Technologies (Amstelveen, Netherlands) was used to determinate the RNA quality, followed by quantification using the ND-2000 spectrophotometer from Thermo Inc. (Wilmington, DE, USA). The expression of genes encoding Pin1, PHD1, PHD2, and PHD3 was determined by RT-qPCR in accordance with a standardized protocol, which used the RealHelixTM SYBR Green I qPCR kit from NanoHelix Co., Ltd. (Seoul, South Korea). The 7500 Fast Real-time PCR system (Waltham, MA, USA) was used for the fluorescent signals, and the products were measured and quantified by employing the comparative cycle threshold method. The PCR primer sequences for the qPCR were: *Pin1*, 5′-TGA TCA ACG GCT ACA TCC AG-3′ (F) and 5′-CAA ACG AGG CGT CTT CAA AT-3’ (R); *PHD1*, 5′--GGC AACT ACG TCA TCA ATG GG-3’ (F) and 3’-TGG GGA TTG TCA ACA TGC CTC-5′ (R); *PHD2*, 5′-TTG TTA CCC AGG CAA CGG AAC-3’ (F) and 3’-CCT TGG CGT CCC AGT CTTT-5′ (R); *PHD3*, 5′-GGC TGG GCA AAT ACT ATG TCAA-3’ (F) and 3’-GGT TGT CCA CAT GGC GAACA-5′ (R); *GAPDH*, 5’- CAT GAG AAG TAT GAC AAC AGC CT-3’ (F) and 5’ -AGT CCT TCC ACG ATA CCA AAG T-3’ (R).

2.5. Western blot analysis

Cells were lysed and centrifuged 18,000 x *g* for 20 min. The protein concentration was calculated using the BCA protein kit (Pierce; Rockford, IL, USA. Protein (30 μg) was separated using sodium dodecyl sulfate-

polyacrylamide gel electrophoresis (SDS-PAGE). The protein blots were blocked at 37℃ for 1 h with 5% skim dry milk in PBST (phosphate-buffered saline buffer containing 0.1% Tween-20) buffer. Primary antibodies against Pin1 and PHD2 were used for the blocked membranes overnight at 4℃.

2.6. Preparation of nuclear and cytoplasmic extracts

Cold PBS was used for washing the cells which were subsequently suspended in hypotonic buffer A on ice according to the previously reported procedure [29]. The supernatant containing the cytosolic fractions was collected after the centrifugation. The remaining cells were washed with buffer A twice and resuspended in buffer C. The cell suspension was kept in ice for 1 h followed by centrifugation at 18,000 x *g* for 15 min. The obtained nuclear extracts were kept at -70℃ until use.

2.7 Immunoprecipitation

HEK293T and MDA-MB-231 cells were lysed, and total proteins (80 μg) were incubated with primary antibodies overnight at 4℃, followed by precipitation using Protein A/G-PLUS Agarose bead suspension obtained from Santa Cruz Biotechnology (*Dallas, TX, USA).* After the mixture was centrifuged at 1,000 x *g* for 1 min, supernatant was discarded, and precipitated beads were washed in cell lysis buffer. The immunoprecipitated beads were then prepared as described previously [30].

2.8 In situ proximity ligation assay (PLA)

The DuoLink^{TM} kit from Sigma-Aldrich (St. Louis, MO, USA) was used to carry out the PLA. HEK293T cells were transfected with HA-

PHD2/pcDNA-Pin1, control siRNA or Pin1 siRNA for 48 h. The cells were fixed, permeabilized, blocked with blocking solution (0.1% Triton in PBS containing 5% bovine serum albumin) and incubated with Pin1 monoclonal (1:100) and PHD2 polyclonal (1:200) antibodies overnight at 4℃. PLA affinity probes (PLUS and MINUS) were then added and incubated at 37℃ for 1 h. Then, probes were detected using fluorescence microscopy from Nikon (Tokyo, Japan) [29, 30]

2.9. Tissue array analysis

Paraffin-embedded human breast cancer tissue arrays [Cat. No. BC08118a provided by US Biomax, Inc. (Rockville, MD, USA)] with surrounding normal tissues were deparaffinized with xylene, followed by rehydration in a series of (100%, 90%, 80%, and 70%) ethanol baths. Antigen retrieval was performed by boiling the sections in the hot citrate buffer for 30 min, followed by permeabilization and blocking using a standard protocol. The tissue sections were washed in PBS and then incubated with antibodies against Pin1 and PHD2 overnight at 4℃, followed by incubation with fluorescent-conjugated secondary antibodies (FITC-conjugated for PHD2, green signal; TRITC-conjugated for Pin1, red signal) for 1 h at room temperature. Nuclei were stained with 4’,6-diamidino-2-phenylindole (DAPI), and slides were imaged using a fluorescent microscope provided by Nikon (Tokyo, Japan).

2.10. Immunofluorescence microscopy

For immunocytochemistry staining, MDA-MB-231 cells were seeded in an 8-chambered plate at a density of 1 x 10^4 cells per well. Once the cells reached the 80% confluency, they were fixed, permeabilized and blocked before incubation with anti-Pin1 and anti-PHD2 antibodies overnight. The

cells were then labeled with fluorophore attached secondary antibodies (FITC and TRITC conjugated). DAPI staining was used for detecting nuclei. The slides were then scanned, and stained cells were visualized under a Nikon fluorescent microscope (Tokyo, Japan).

2.11. Clonogenic assay

For the clonogenic assay, MDA-MB-231 and MCF7 cells were seeded in 6-well plates at a density of 150–200 cells per well and cultured for 14 days before being transfected with control siRNA, Pin1 siRNA, or PHD2 siRNA for 48 h. Media were changed every other day. After the 14-day incubation period, colonies were fixed with methanol at 4℃ for 1 h and then stained with 0.05% crystal violet (Sigma-Aldrich; St. Louis, MO, USA) for additional 4 h. The excess dye was washed off with PBS, and colonies were visualized and counted using LAS-4000 image reader (Nikon; Tokyo, Japan) [29].

2.12. Migration assay

To evaluate the cell migration, MCF7 cells were pretreated with control, Pin1 or PHD2 siRNAs and then plated into Culture-Inserts® (ibid; Regensbur, Germany). Cells were allowed for 24 h to adhere well to the inserts, after which they were gently removed using sterile tweezers. The cells were then monitored for their ability to migrate at different time points under a microscope (Nikon; Tokyo, Japan).

2.13. Identification of phosphorylation sites on PHD2

For identification of the plausible phosphorylation sites, MDA-MB-231 cells were transfected with HA-PHD2, and immunoprecipitated samples

were analyzed using SDS-PAGE. The bands containing the HA-PHD2 were excised, and protein was eluted by the trypsin digestion procedure [31]. Phosphorylation of HA-PHD2 was analyzed in peptides using a hybrid dual-cell quadrupole linear ion trap-orbitrap mass spectrometer (LTQ Orbitrap Velos, Thermo Fisher). MS/MS spectra were searched against a composite database of all translated human open reading frames and their reversed complements using the SEQUEST algorithm. MS data were automatically captured using the Proteome Discoverer 2.5 software (Thermo Fisher Scientific Inc.; Waltham, MA, USA)

2.14. Survival analysis

Gene expression quantification data from all TCGA-BRCA cohort (in tpm) and associated clinical data were obtained from UCSC Xena Browser. Patients were sub-grouped into PHD2 (Egln1) high and low groups based on log2 (tpm+0.001) values with the cut-off of 10% and 90% percentiles. Survival analysis was conducted using overall survival time in R with survival and survminer and plotted with ggsurvplots. Additionally, survival analysis based on the PHD2 mRNA expression level was evaluated using METABRIC data.

2.15. Transcriptomic analysis of TCGA-BRCA cohort

Whole transcriptome raw read count data and associated clinical data were obtained from GDC portal (https://portal.gdc.cancer.gov/). Patients were stratified into PHD2 (Egln1) expression high and low groups based on normalized readcount values produced in DESeq2, with the cut-off of 25% and 75% percentile. Differential expression analysis and Gene Set Enrichment Analysis (GSEA) were performed using DESeq2 and clusterProfiler, respectively. Plots were generated with ggplot2 and pheatmap.

2.16. Network analysis of protein-protein interaction

Protein-protein interaction was analyzed using the STRING tool (Search Tool for the Retrieval of Interacting Genes/Proteins, https://string-db.org).

2.17. Statistical analysis

All data are presented as mean ± standard deviation (SD) for a minimum of three independent experiments. Statistical analysis was conducted using either one-way ANOVA or two-tailed unpaired Student's *t*-tests, with $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$ indicating statistical significance, while ns stands for 'not significant'. The data were analyzed using the GraphPad Prism 8.0 software (GraphPad Software; San Diego, CA, USA).

3. Results

3.1. Overexpression of PHD2 and Pin1 and their functional relationship in breast cancer

Both Pin1 and PHD2 are overexpressed in breast tumor as measured in breast cancer tissue microarrays (**Fig. 1A** and **Fig. 1B**), and there is a strong positive correlation between Pin1 and PHD2 expression (**Fig. 1C**). However, there was no significant correlation of Pin1 or PHD2 expression with patient characteristics like tumor stage or patient age (**Fig. S1A and B**). Survival analysis evaluated using the TCGA-BRCA cohort revealed reduction of survival time in patients with high PHD2 (**Fig. 1D**). Likewise, METABRIC data analysis showed association of high PHD2 mRNA expression with poor prognosis in breast cancer patients (**Fig. 1E**). Functional studies revealed that knockdown of Pin1 or PHD2 diminished the clonogenicity

(**Fig. 1F**) and migrative capability (**Fig. 1G**) of MCF7 human breast cancer cells. Additionally, GSEA analysis showed that those tumors expressing high levels of *Egln1* encoding PHD2 have an increased epithelial to mesenchymal transition signature which potentially augments its aggressiveness (**Fig. S1C**).

3.2. Transcriptomic profile of PHD2 from TCGA-BRCA

Transcriptomic profile of high and low PHD2 from the TCGA-BRCA cohort showed in the heatmap the regulation of top 20 up- and down-regulated genes such as *PKD1* and *RPL7P9* (**Fig. 2A**) and top 15 up-regulated and down-regulated pathways in breast cancers with high PHD2 (**Fig. 2B** and **Fig. 2C**). The data show that PHD2 high cancers have upregulated inflammatory signaling, switched cell surface adhesion molecules, and reduced respiration as a result of hypoxia response.

3.3. Identification of Pin1 binding sites on PHD2

Pin1 is known to specifically bind to pSer/Thr-Pro residues on substrate proteins to induce their conformational changes, function and stability. PHD2 has serine and threonine residues with adjacent proline in its structure (**Fig. 3A**) and 4 candidate sites (pSer/Thr-Pro) for Pin1 binding have been identified, three of which have the serine residues (S12, S125 and S174) and the other one has a threonine (T168) as depicted in **Fig. 3B**. Of these, S125, T168, and S174 were found to be phosphorylated as determined by LC-MS/MS analysis (**Fig. 3C**). Analysis of the PHD2 amino acid sequences revealed the conserved pSer-Pro (pS125-P126 or pS174-P175) and p-Thr/Pro (pT168-P169) in 3 species; only pSer-Pro (pS125-P126) in 4 species and only pThr-Pro (pT168-P) in 7 species (**Fig. 4D**).

After confirmation of the existence of the pSer/Thr-Pro consensus sequences in PHD2, we explored the possibility of its interaction with Pin1 first by the string bioinformatics tool from curated and experimentally database (**Fig. 4A**). This was verified by immunoprecipitation; Pin1 and PHD2 interact in both normoxic and hypoxic conditions in MDA-MB-231 cells (**Fig. 4B**) and also in another human breast cancer cell line MCF-7 (**Fig. S1D**). Next, we overexpressed Pin1 and PHD2 in MDA-MB-231 breast cancer cells with plasmids, PCDNA-Pin1 and HA-PHD2, and the florescence signal was detected by PLA that demonstrated interactions between the two proteins (**Fig. 4C**).

Nuclear translocation of PHD2 has been shown to be associated with cancer cell growth and tumor-aggressiveness [32-34], We investigated the intracellular localization of PHD2 and Pin1 in MDA-MB 231 and MCF-7 breast cancer cells. Our data show that the majority of PHD2 is localized in the nucleus while Pin1 is detected mainly in the cytoplasm in both cell lines (**Fig. 4D and 4E**). Western blot analysis of total lysates from both cells revealed sustained PHD2 protein expression even under hypoxia (**Fig. S1E and F) (Fig. 4E**). Of note, hypoxia-induced enhancement of PHD2-Pin1interaction mainly occurred in the cytoplasm (**Fig. 4F**).

3.4. PHD2 expression regulated by Pin1

In a subsequent experiment, siRNA-mediated Pin1 knockdown inhibited PHD2 expression in normoxic (**Fig. 5A**) and hypoxic conditions (**Fig. S1G**). However, expression of other isoforms of PHDs, such as PHD1 and PHD3, was not affected (**Fig. S1H**). PHD2 expression was affected after Pin1 knockdown (**Fig. 5B**). Immunofluorescence staining confirmed that Pin1 silencing resulted in the down-regulation of PHD2 (**Fig. 5C**). Under the same experimental conditions, the relative interaction between Pin1 and PHD2 was also dampened (**Fig. 5D**). On the other hand, PHD2 silencing did

not alter the Pin1 protein expression (**Fig. 5E**). Given that the major function of PHD2 is HIF hydroxylation, we checked PHD2-HIF-2α interaction after Pin1 knockdown. siRNA-mediated Pin1 inhibition abrogated the PHD2-HIF-2α interaction in breast cancer cells (**Fig. S2A**).

3.5. Serine 125 of PHD2 as a critical site for Pin1 binding and oncogenicity

Site-directed mutagenesis studies in which aforementioned serine (S125 and S174) and threonine (T168) residues with adjacent proline were substituted by non-phosphorylatable alanine demonstrated that Ser125 is most essential for Pin1 binding (**Fig. 6A**). Further, more ubiquitinated PHD2 was found in the S125A mutant cells (**Fig. 6B**). These findings suggest that the stabilization of PHD2 depends on its phosphorylated Ser125 residue (pS125) that is recognized by Pin1.

We then investigated the functional significance of S125A phosphorylation of PHD2 in MDA-MB-231 and MCF-7 cells. Cells expressing WT PHD2 and Pin1 exhibited the relatively high clonogenicity in both cell lines (**Fig. 6C**) and migrative capability in MDA-MB-231 cells (**Fig. 6D**) capabilities which were attenuated by S125A mutation.

5. Discussion

Cancer cells are often exposed to low oxygen concentrations (hypoxia). In order to grow and survive in the hypoxic environment, they upregulate erythropoietin, which stimulates the production of red blood cells (erythrocytes). HIF-2 is a key transcription factor involved in erythropoiesis, thereby increasing the total capacity of erythrocytes to carry oxygen. In hypoxic tumor microenvironment, cancer cells can shift their

primary metabolic strategy from predominantly mitochondrial respiration towards increased glycolysis to maintain ATP levels [35]. This metabolic switch has been known to be mainly regulated by HIF-1α which induces an enhanced expression of glycolytic enzymes. However, recent studies have shown that HIF-2α activated as a consequence of aberrant expression of signal-induced proliferation-associated 1 (SIPA1) enhances expression of multiple glycolysis-related genes in breast cancer cells [36].

In order to adapt to different oxygen concentrations in tumor microenvironment, cancer cells must be able to sense changes in the oxygen gradient and respond accordingly. There have been great advances in our understanding how cellular signal transduction pathways are regulated by hypoxia. The potential candidates for oxygen sensors include the PHD family of enzymes that require molecular oxygen for their catalytic activity [37]. PHD family proteins consist of three proteins known as PHD1, PHD2 and PHD3. Although they share the same structure, these proteins exhibit different catalytic activities and specializations [38-40]. Despite prominent involvement of PHDs in negatively regulating the stability of HIF-2α as well as HIF-1α in normoxic conditions, their roles in hypoxic tumor microenvironment remain elusive. The PHD proteins require oxygen and 2-oxyglutarate as substrates, along with Fe (II) and ascorbate as cofactors. They catalyze the oxidation of highly conserved proline residues present in HIF proteins and function as the actual oxygen sensors within cells. [41,42].

Data regarding functions of PHD2 in various types of cancer are conflicting; while some studies suggest tumor promoting effects while others indicating tumor suppressive effects exerted by this monooxygenase [43]. In addition to its direct role in the regulation of cancer cell proliferation and growth, PHD2 has been demonstrated to play a vital role in tumor vascularization [10] and the tumor microenvironment [7],

contributing to cancer progression. The expression patterns of PHD isoforms exhibit notable differences between cancerous and corresponding normal tissues. A substantial overexpression of PHD2 is observed in several types of human malignancies including, lung, liver, kidney, and breast cancers [7], similar to our recent findings on a pro-tumorigenic function and overexpression of PHD2 in breast cancer. In contrast, low levels of PHD2 expression are associated with a worse prognosis in colorectal cancer [45].

So far, the majority of PHD2 involvement in tumor microenvironment is attributed to its role in regulating the HIF protein levels in hypoxic cancer cells versus surrounding normal cells that are oxygen abundant. However, several non-canonical functions of PHD2 which are independent of HIF hydroxylation are beginning to be uncovered. For example, PHD2 negatively regulates NF-κB regardless of its catalytic activity to hydroxylate HIF [8]. In addition, PHD2 binding to EGFR is imperative for EGFR stability and activate ERK and PI3K signaling pathways involved in cancer cell growth and survival [12,46]. Although PHDs require oxygen for an optimal function, it does not necessarily mean that oxygen concentrations directly influence their catalytic activity; PHD may even work in the hypoxic tumor microenvironment in which ambient oxygen at a substantial concentration is still present.

Phosphorylation is the principal mechanism that control the activities of many enzyme systems. But, phosphorylation alone is not sufficient, though necessary, for regulation of PHD function. It has been speculated that hypoxia increases the ROS production, which could activate a series of kinases. Several proteins that regulate PHD2 expression via phosphorylation have been identified: these include, hypoxia regulated proteins mTOR, p70S6K, and GSK3β which phosphorylate PHD2 [47,48]. PHD2 is composed of 426 residues and has a distinct structural arrangement. It

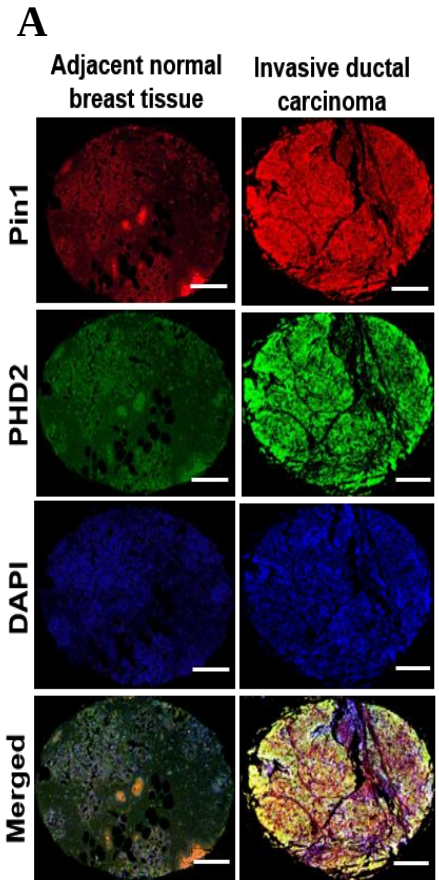
consists of a long intrinsically disordered N-terminal region spanning residues 1–187, followed by a well-structured oxygenase domain that serves as the catalytic center (residues 188–418). [48-50]. The phosphorylation of Ser/Thr residues preceding the proline moiety and subsequent Pin1-mediated proline isomerization serve as a regulatory mechanism for numerous oncogenic and tumor suppressor proteins [51-53].

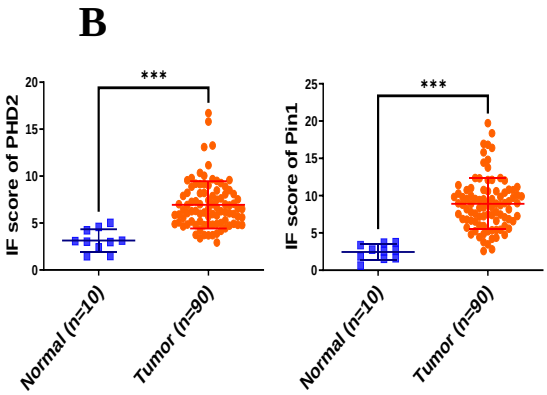
It has been reported that PHD2 localizes mainly in the cytoplasm [43]. However, PHD2 can shuttle between the cytoplasm and nucleus. Notably, PHD2 protein expression occurs predominantly in the nucleus of tumor tissues [38], and the nuclear localization is greater than the cytosolic localization in hypoxic conditions [9]**.** Consistent with these findings, our current study shows that in MDA-MB-231 and MCF7 human breast cells, the PHD2 expression is higher in nucleus than in cytoplasm, which is further increased in hypoxia. Similar results were observed in U-2OS cells: strong expression in nucleus in both normal and hypoxia conditions and NO treatment [54].

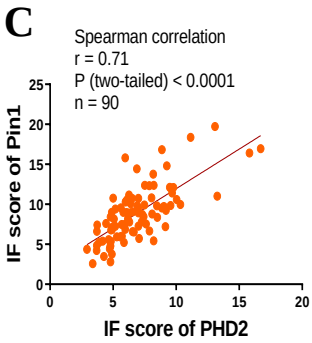
In the current investigation, we have identified specific serine and threonine residues of PHD2 required for its interaction with Pin1. A study with colon cancer showed functions of PHD2 exerted in an oxygen-independent manner through post-translational modification [9]. While PHD2 is the main regulator of HIF-1α stability, its phosphorylation status influences HIF-1α hydroxylation [42]. Ser125 in PHD2 was already reported to be phosphorylated by some kinases [9]. Ser125 phosphorylation can modulate PHD2 activity but does not influence the PHD2 interaction with HIF-1α [9]. PHD2 can also be activated through S125 phosphorylation which is catalyzed by the mTOR pathway, especially P70S6K [9]. In our study Ser125 of PHD2 is important for its stabilization through interaction with Pin1. Other serines, such as S12 and S14, were found to undergo

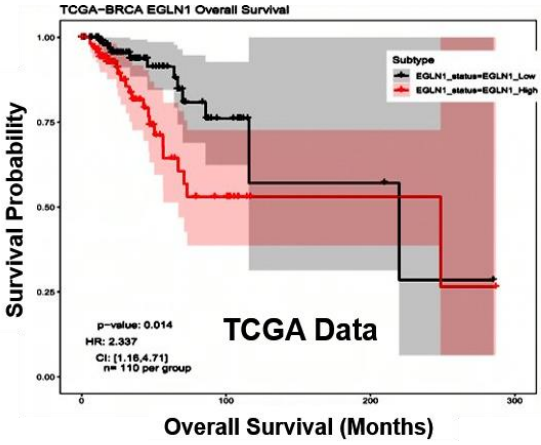
phosphorylation, but their role in PHD2 activity is not clarified yet [9]

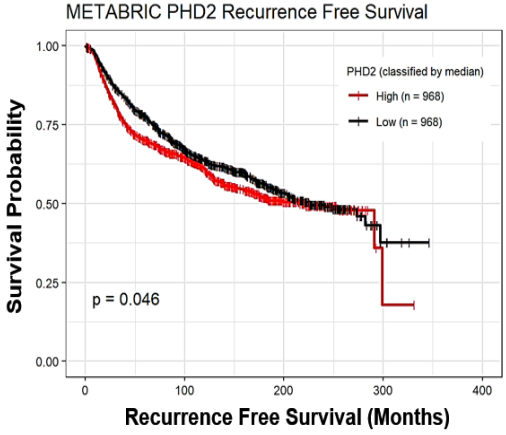
PHD2 phosphorylation on threonine 168 and serine 174 were found for the first time in our study. One of the salient features of our present work is the role for nuclear-oncogenic PHD2 that could influence the progression of breast cancer. The activities of PHD2 are regulated by many additional signals, and the abundance of these signals is often associated with actual deprivation of oxygen. We observed a strong correlation between PHD2 and Pin1 expression in breast cancer tissues. It is noticeable that hypoxia upregulates the PHD2-Pin1 interaction. Aberrant PHD2 overexpression is associated with adverse outcomes in breast cancer which could potentially increase its aggressiveness and mesenchymal transition. Therefore, pro-oncogenic PHD2 can be explored as a therapeutic target for the treatment of breast cancer.


A
Adjacent normal breast tissue
Invasive ductal carcinoma
Pin1
PHD2
DAPI
Merged

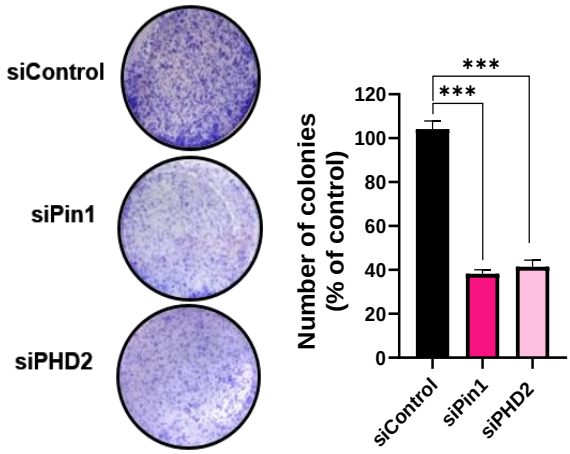

B
***
IF score of PHD2
IF score of Pin1
Normal (n=10)
Tumor (n=90)


C
Spearman correlation
r = 0.71
P (two-tailed) < 0.0001
n = 90
IF score of Pin1
IF score of PHD2


D
TCGA-BRCA EGLN1 Overall Survival
Subtype
EGLN1_status=EGLN1_Low
EGLN1_status=EGLN1_High
p-value: 0.014
HR: 2.337
CI: [1.16,4.71]
n= 110 per group
TCGA Data
Survival Probability
Overall Survival (Months)


E
METABRIC PHD2 Recurrence Free Survival
PHD2 (classified by median)
High (n = 968)
Low (n = 968)
p = 0.046
Survival Probability
Recurrence Free Survival (Months)

F



G

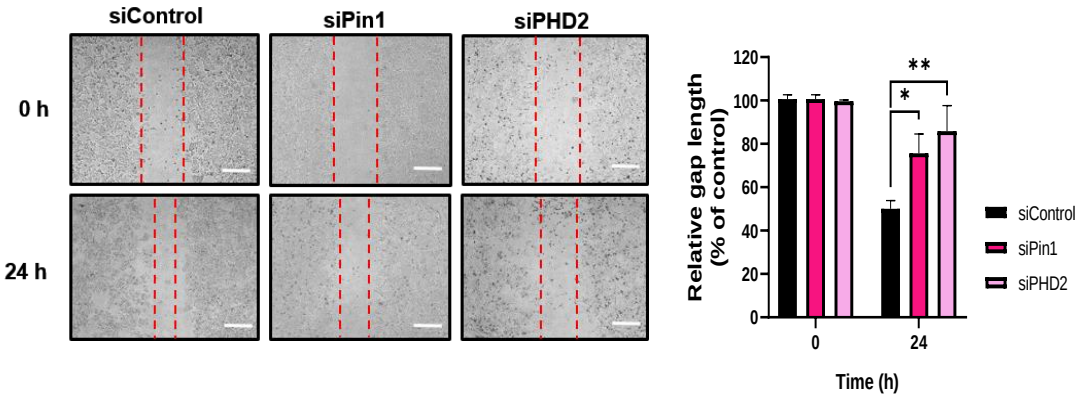
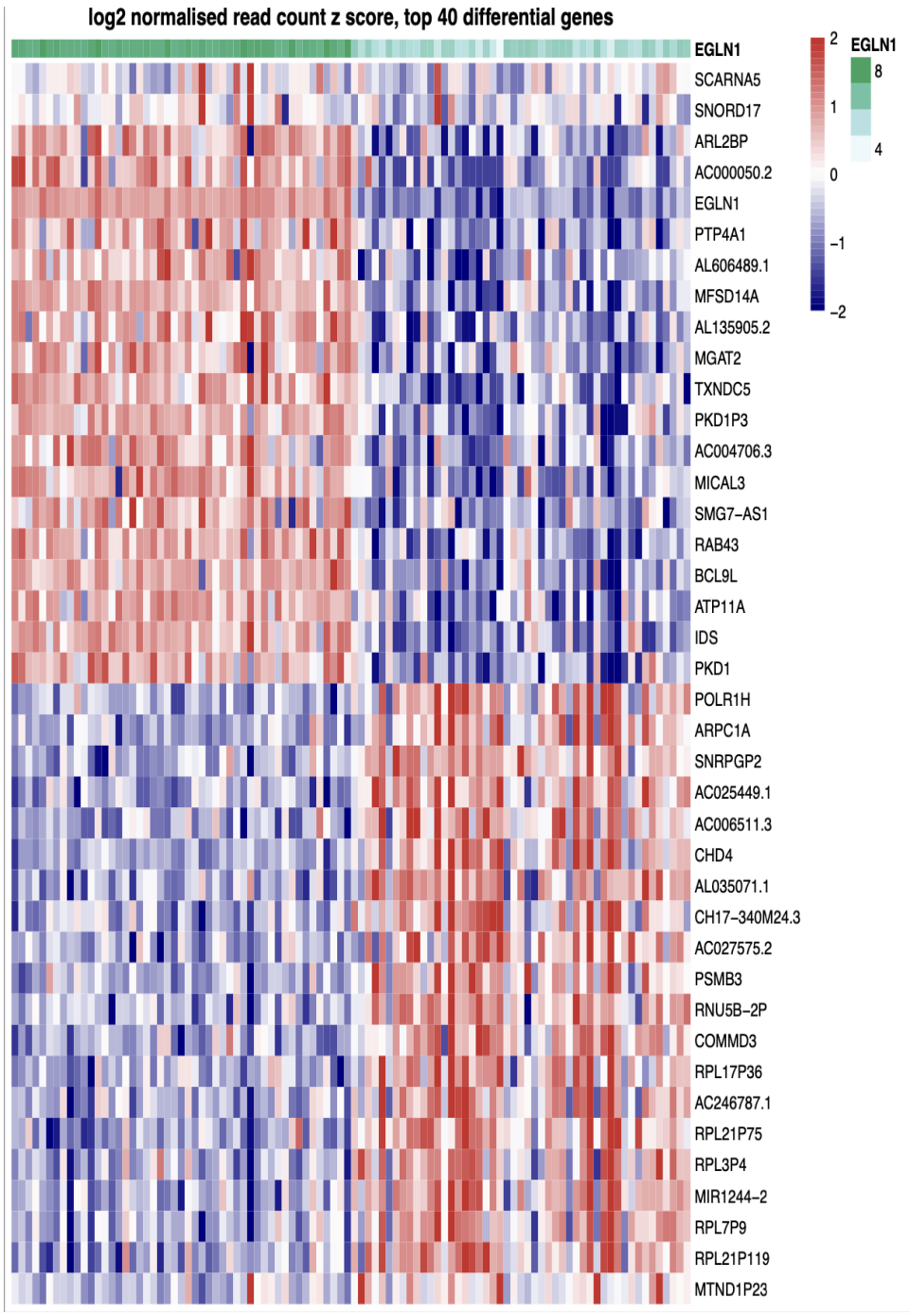


Figure 1. Overexpression of PHD2 and Pin1 and their functional relationship in breast cancer

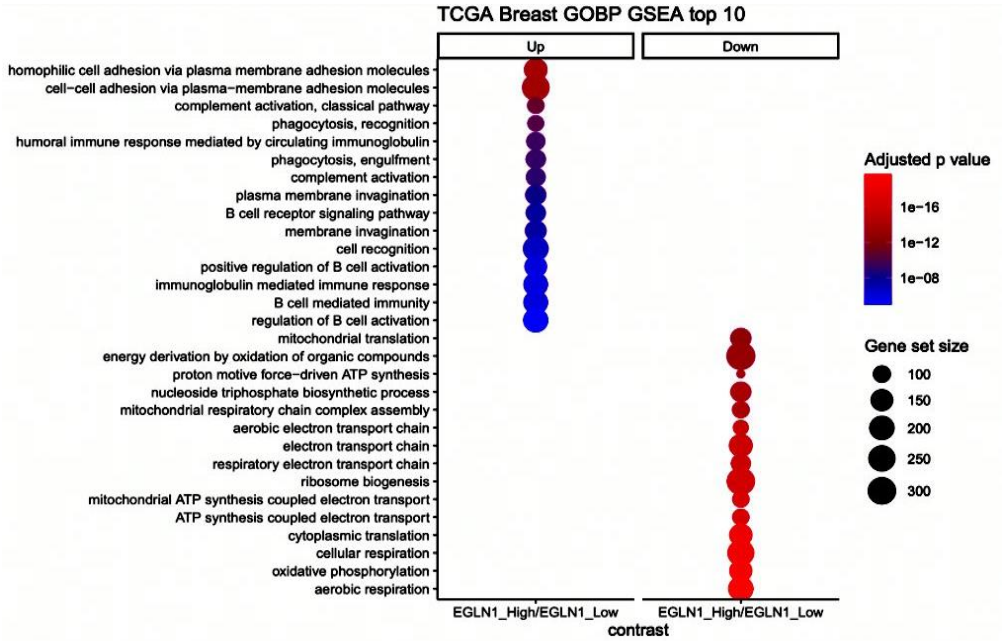
(A) Representative immunofluorescence (IF) images of and Pin1 and PHD2 in breast tumor and surrounding normal tissue arrays. Scale bar, 200 μm. (B) Measurement of Pin1 and PHD2 expression levels based on the tissue microarray IF score. The two-tailed unpaired Student's *t*-test (***$p < 0.001$). (C) Spearman analysis of IF data demonstrating a strong, positive correlation between Pin1 and PHD2 (n = 90), $r = 0.71$. (D) Survival analysis

of PHD2- (Egln1) high expression vs. -low expression breast cancer patients in TCGA-BRCA cohort. The grouping was based on log2 (tpm+0.001) values with the cutoff of 10% and 90% percentiles. The RNA expression and phenotype data was obtained from UCSC Xena Browser. (E) METABRIC Data-PHD2 Survival analysis by mRNA expression level in breast cancer patients (p = 0.046). (F-G) Oncogenic activity of Pin1 and PHD2 in breast cancer cells. (F) Control, Pin1, or PHD2 siRNA was transfected into MCF7 cells in 6-well plates according to the Materials and methods section. Following crystal violet staining, attached cells were captured on camera, and the percentage of attached cells was determined by counting the number of colonies. Representative sets of photos from three separate experiments are displayed. The Student's *t*-test was used to establish the statistical significance of the data, which are presented as the mean ± SD. ***p < 0.001. (G) After being transfected with siRNA for control, Pin1, or PHD2, MCF7 cells were incubated for 24 h. Then, using a confocal microscope, cell migration was viewed. The Student's *t*-test was used to establish the statistical significance of the data, which are presented as the mean ± SD (n=3). *p < 0.05 and **p < 0.01.

A


log2 normalised read count z score, top 40 differential genes
EGLN1
SCARNA5
SNORD17
ARL2BP
AC000050.2
EGLN1
PTP4A1
AL606489.1
MFSD14A
AL135905.2
MGAT2
TXNDC5
PKD1P3
AC004706.3
MICAL3
SMG7−AS1
RAB43
BCL9L
ATP11A
IDS
PKD1
POLR1H
ARPC1A
SNRPGP2
AC025449.1
AC006511.3
CHD4
AL035071.1
CH17−340M24.3
AC027575.2
PSMB3
RNU5B−2P
COMMD3
RPL17P36
AC246787.1
RPL21P75
RPL3P4
MIR1244−2
RPL7P9
RPL21P119
MTND1P23
2
1
0
−1
−2
EGLN1
8
4

B


TCGA Breast GOBP GSEA top 10
Up
Down
homophilic cell adhesion via plasma membrane adhesion molecules
cell−cell adhesion via plasma−membrane adhesion molecules
complement activation, classical pathway
phagocytosis, recognition
humoral immune response mediated by circulating immunoglobulin
phagocytosis, engulfment
complement activation
plasma membrane invagination
B cell receptor signaling pathway
membrane invagination
cell recognition
positive regulation of B cell activation
immunoglobulin mediated immune response
B cell mediated immunity
regulation of B cell activation
mitochondrial translation
energy derivation by oxidation of organic compounds
proton motive force−driven ATP synthesis
nucleoside triphosphate biosynthetic process
mitochondrial respiratory chain complex assembly
aerobic electron transport chain
electron transport chain
respiratory electron transport chain
ribosome biogenesis
mitochondrial ATP synthesis coupled electron transport
ATP synthesis coupled electron transport
cytoplasmic translation
cellular respiration
oxidative phosphorylation
aerobic respiration
EGLN1_High/EGLN1_Low
EGLN1_High/EGLN1_Low
contrast
Adjusted p value
1e−16
1e−12
1e−08
Gene set size
100
150
200
250
300

C

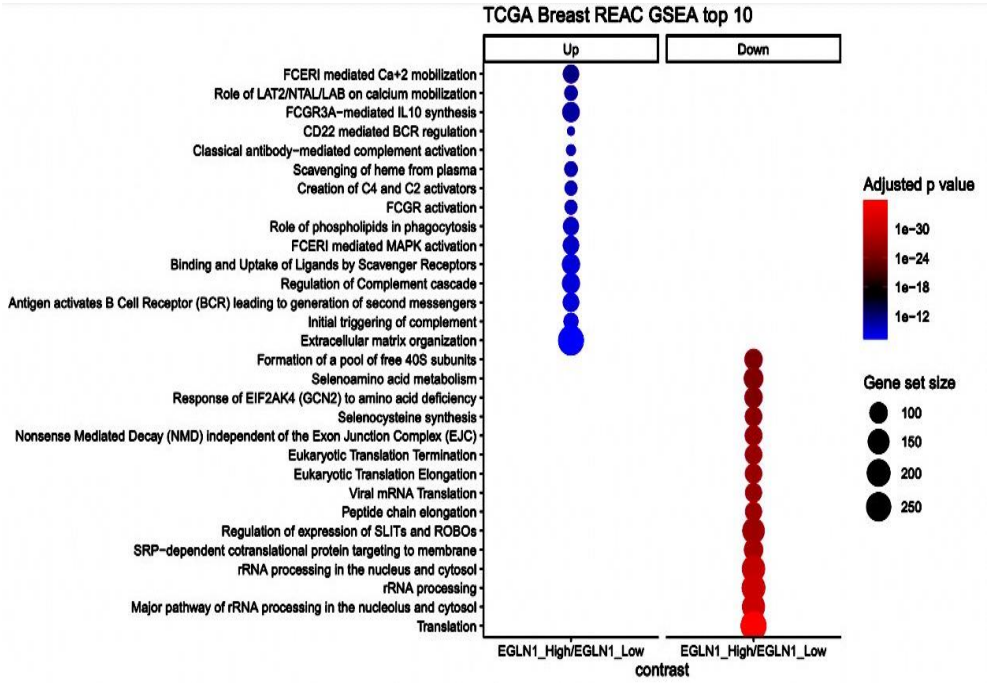

TCGA Breast REAC GSEA top 10
Up
Down
FCERI mediated Ca+2 mobilization
Role of LAT2/NTAL/LAB on calcium mobilization
FCGR3A−mediated IL10 synthesis
CD22 mediated BCR regulation
Classical antibody−mediated complement activation
Scavenging of heme from plasma
Creation of C4 and C2 activators
FCGR activation
Role of phospholipids in phagocytosis
FCERI mediated MAPK activation
Binding and Uptake of Ligands by Scavenger Receptors
Regulation of Complement cascade
Antigen activates B Cell Receptor (BCR) leading to generation of second messengers
Initial triggering of complement
Extracellular matrix organization
Formation of a pool of free 40S subunits
Selenoamino acid metabolism
Response of EIF2AK4 (GCN2) to amino acid deficiency
Selenocysteine synthesis
Nonsense Mediated Decay (NMD) independent of the Exon Junction Complex (EJC)
Eukaryotic Translation Termination
Eukaryotic Translation Elongation
Viral mRNA Translation
Peptide chain elongation
Regulation of expression of SLITs and ROBOs
SRP−dependent cotranslational protein targeting to membrane
rRNA processing in the nucleus and cytosol
rRNA processing
Major pathway of rRNA processing in the nucleolus and cytosol
Translation
EGLN1_High/EGLN1_Low
EGLN1_High/EGLN1_Low
contrast
Adjusted p value
1e−30
1e−24
1e−18
1e−12
Gene set size
100
150
200
250

Figure 2. Transcriptomic profile of PHD2 from TCGA-BRCA

(A) Transcriptomic profile of PHD2-high (top 25% patients with highest normalized counts of *EGLN1*) vs *EGLN1*-low (lower 25% percent) breast cancers from the TCGA-BRCA cohort. Heatmap shows top 20 up- and down-regulated genes. (B-C) Top up-regulated and down-regulated pathways in cancers with high *EGLN1* (B) GO: BP (C) Reactome.

A

```
MANDSGGPGGPSPSERDRQYCELCGKMENLLRCSRCRSSFYCCKEHQRQDWKKHKLVCQ
GSEGALGHGVGPHQHSGPAPPAVPPPRAGAREPRKAAARRDNASGDAAKGKVKAKPPAD
PAAAASPCRAAAGGQGSAVAAEAEPGKEEPPARSSLFQEKANLYPPSNTPGDALSPGGG
LRPNGQTKPLPALKLALEYIVPCMNKHGICVVDDFLGKETGQQIGDEVRALHDTGKFTD
GQLVSQKSDSSKDIRGDKITWIEGKEPGCETIGLLMSSMDDLIRHCNGKLGSYKINGRT
KAMVACYPGNGTGYVRHVDNPNGDGRCVTCIYYLNKDWDAKVSGGILRIFPEGKAQFAD
IEPKFDRLLFFWSDRRNPHEVQPAYATRYAITVWYFDADERARAKVKYLTGEKGVRVEL
NKPSDSVGKDVF
```

B

	Position in query protein	Sequence in query protein	Corresponding motif	Corresponding
1	12 - 13	SP	[pS/pT]P	WW domain binding motif
2	125 - 126	SP	[pS/pT]P	WW domain binding motif
3	168 - 169	TP	[pS/pT]P	WW domain binding motif
4	174 - 175	SP	[pS/pT]P	WW domain binding motif

C

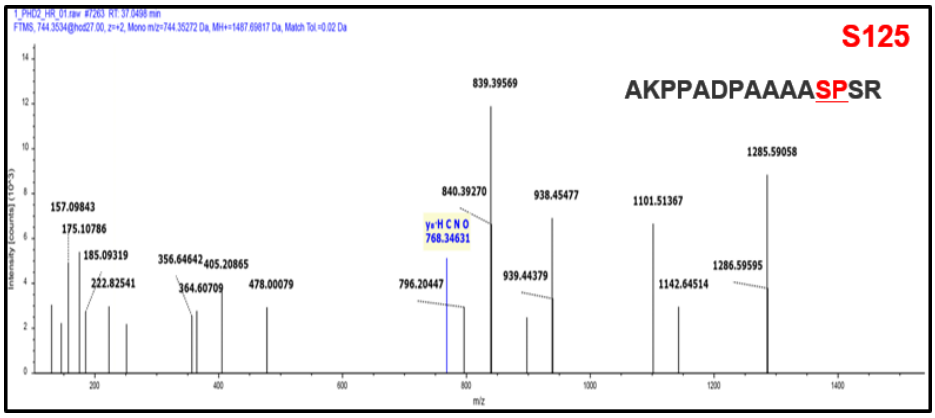
```
3 species

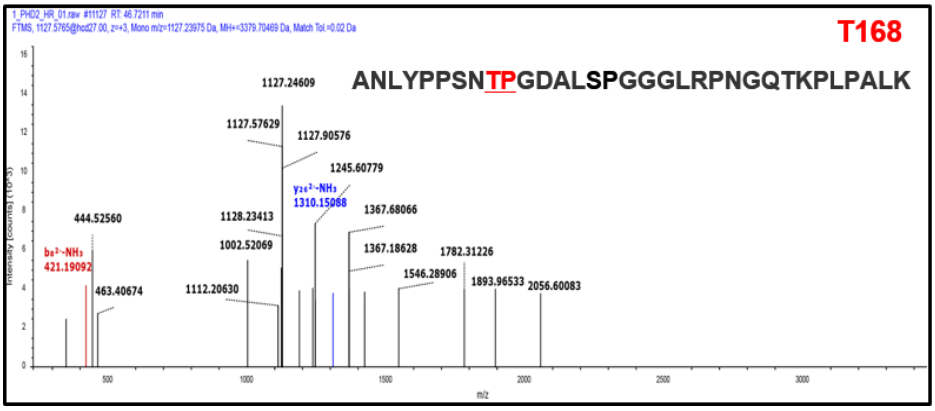
Human       121 AAAASPCRAAAGGQGSAVAAEAEPGKEEPPARSSLFQEKANLYPPSNTPGDALSPGGGLR 180
Chimpanzee      AAAASPGRAAAGGQGSAVAAEAEPGKEEPPARSSLFQEKANLYPPSNTPGDALSPGGGLR
Rhesus          AAAASPGRAAPGGQGSAVAAEAEPGKEEPPARSSLFQEKANLYPPSNTPGDALSPGGGLR
                ******.***.*****************************************.****

5 species
Human       121 AAAASPCRAAAGGQGS-AVAAEAEPGKEEPPARSSLFQEKANLYPPSNTPGDALSPGGGLR 180
Cow             AAAASPPRAAPGGQGRVAAAAEAEPAKEDLLSRSLL-----------NTPGDGLSHGGGLR
Horse           AAAASSPRAAPGGQGP------AEAGKEEALARSPPYQEKANLYPPSNTPGEGLSHGGGLR
Chimpanzee      AAAASPGRAAAGGQGS-AVAAEAEPGKEEPPARSSLFQEKANLYPPSNTPGDALSPGGGLR
Rhesus          AAAASPGRAAPGGQGS-AVAAEAEPGKEEPPARSSLFQEKANLYPPSNTPGDALSPGGGLR
                *****. ***.****       **..**:  :**             ****:.** .****

7 species
Human       121 AAAASPCRAAAGGQGS-AVAAEAEPGKEEPPARSSLFQEKANLYPPSNTPGDALSPGGGLR 180
Rat             TQAGS-------GPGP------AEPSSEDPPPSRSPGPERASLCPAGGGPGEALSPGGGLR
Mouse           AQARS-------GPGP------AEPGSEDPPLSRSPGPERASLCPAGGGPGEALSPGGGLR
Cow             AAAASPPRAAPGGQGRVAAAAEAEPAKEDLLSRSLL-----------NTPGDGLSHGGGLR
Horse           AAAASSPRAAPGGQGP------AEAGKEEALARSPPYQEKANLYPPSNTPGEGLSHGGGLR
Chimpanzee      AAAASPGRAAAGGQGS-AVAAEAEPGKEEPPARSSLFQEKANLYPPSNTPGDALSPGGGLR
Rhesus          AAAASPGRAAPGGQGS-AVAAEAEPGKEEPPARSSLFQEKANLYPPSNTPGDALSPGGGLR
                : * *       * *       **...*:                . **:.** .****
```

D


S125
AKPPADPAAAASPSR
839.39569
1285.59058
840.39270
938.45477
1101.51367
157.09843
175.10786
yₖHCNO
768.34631
185.09319
356.64642
405.20865
222.82541
364.60709
478.00079
796.20447
939.44379
1142.64514
1286.59595
m/z


T168
ANLYPPSNTPGDALSPGGGLRPNGQTKPLPALK
1127.24609
1127.57629
1127.90576
1245.60779
y₂₆²⁺-NH₃
1310.15068
1367.68066
444.52560
1128.23413
1002.52069
b₈²⁺-NH₃
421.19092
1367.18628
1782.31226
1546.28906
463.40674
1112.20630
1893.96533
2056.60083
m/z

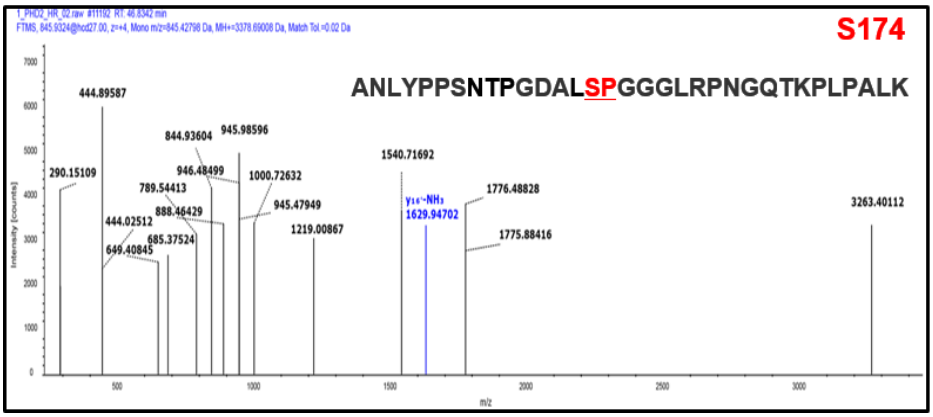
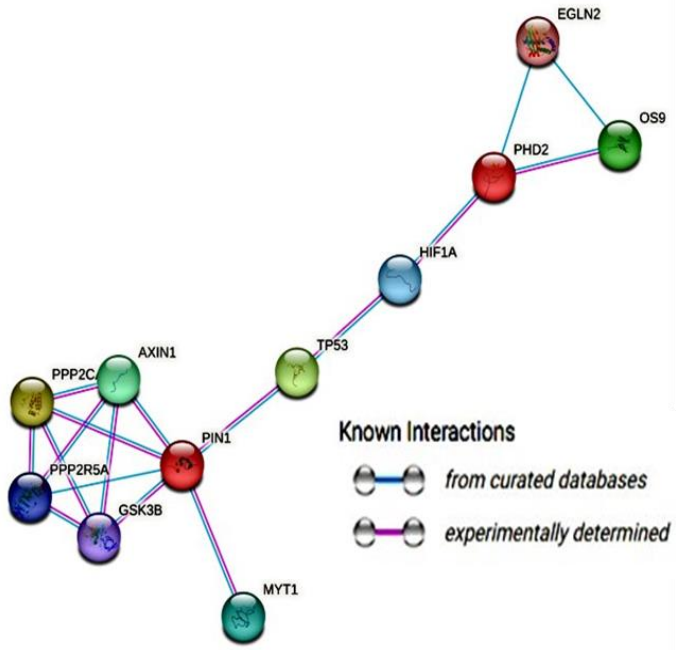

S174
ANLYPPSNTPGDALSPGGGLRPNGQTKPLPALK
444.89587
844.93604
945.98596
1540.71692
290.15109
946.48499
1000.72632
789.54413
1776.48828
y₁₆⁺-NH₃
1629.94702
3263.40112
888.46429
945.47949
444.02512
1219.00867
685.37524
1775.88416
649.40845
m/z

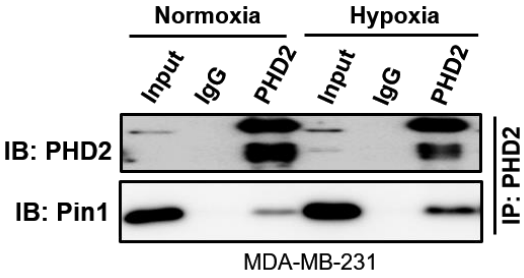
Figure 3. Identification of phosphorylation sites of PHD2

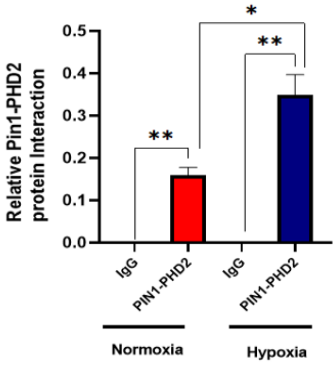
(A) The location of the phosphorylatable serines in PHD2 fragment is shown schematically. (B) PHD2 has the WW domain binding motifs. Four WW binding motifs with the pSer/Thr-Pro sequence are present in the PHD2 protein. Human Protein Reference Database, available at http://www.hprd.org/ (C) PHD2 sequences in S125, T168, and S174 in different species by multiple sequence alignment. (D) The LC-MS/MS analysis was conducted to get the peptide spectra of PHD2. Three residues (S125, T168, S174) of PHD2 were found to be phosphorylated and recognized as the consensus binding locations for Pin1

A

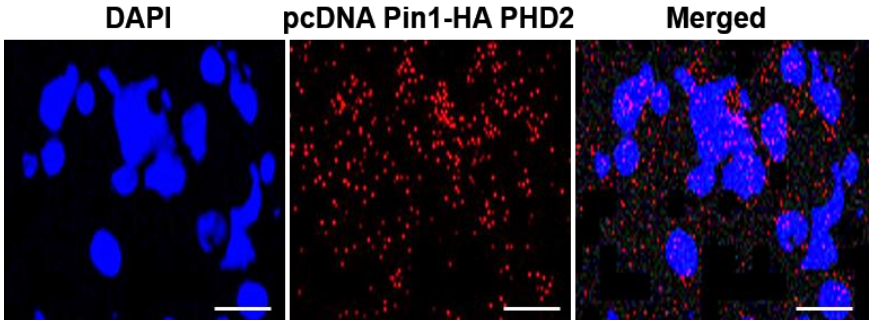

EGLN2
OS9
PHD2
HIF1A
TP53
AXIN1
PPP2C
PIN1
PPP2R5A
GSK3B
MYT1
Known Interactions
from curated databases
experimentally determined

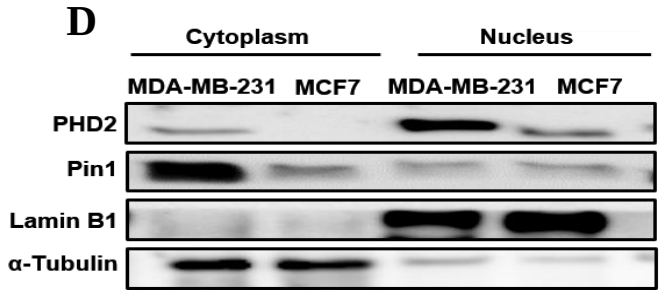
B

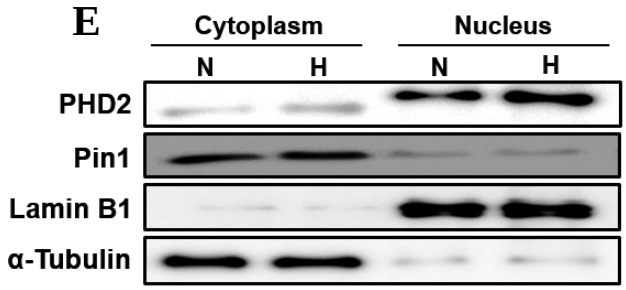

Normoxia
Hypoxia
Input
IgG
PHD2
Input
IgG
PHD2
IB: PHD2
IB: Pin1
IP: PHD2
MDA-MB-231

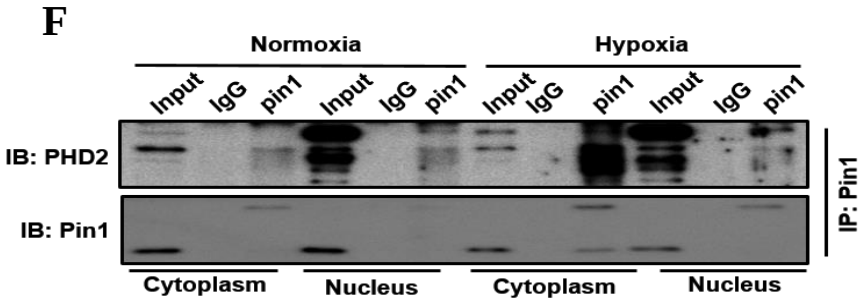

Relative Pin1-PHD2 protein Interaction
0.5
0.4
0.3
0.2
0.1
0.0
*
**
**
IgG
PIN1-PHD2
IgG
PIN1-PHD2
Normoxia
Hypoxia

C


DAPI
pcDNA Pin1-HA PHD2
Merged


D
Cytoplasm
Nucleus
MDA-MB-231
MCF7
MDA-MB-231
MCF7
PHD2
Pin1
Lamin B1
α-Tubulin


E
Cytoplasm
Nucleus
N
H
N
H
PHD2
Pin1
Lamin B1
α-Tubulin


F
Normoxia
Hypoxia
Input
IgG
pin1
Input
IgG
pin1
Input
IgG
pin1
Input
IgG
pin1
IB: PHD2
IB: Pin1
IP: Pin1
Cytoplasm
Nucleus
Cytoplasm
Nucleus

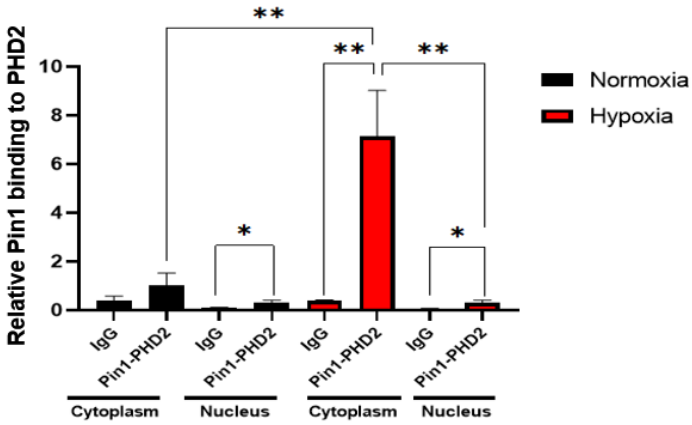
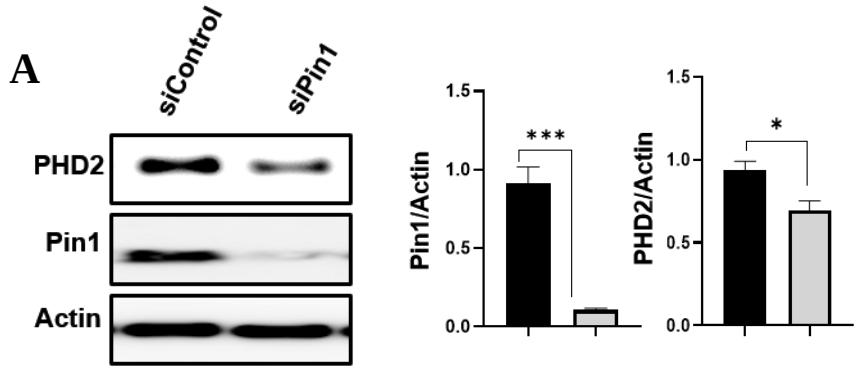
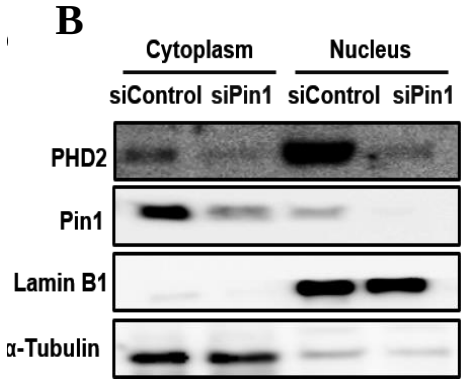
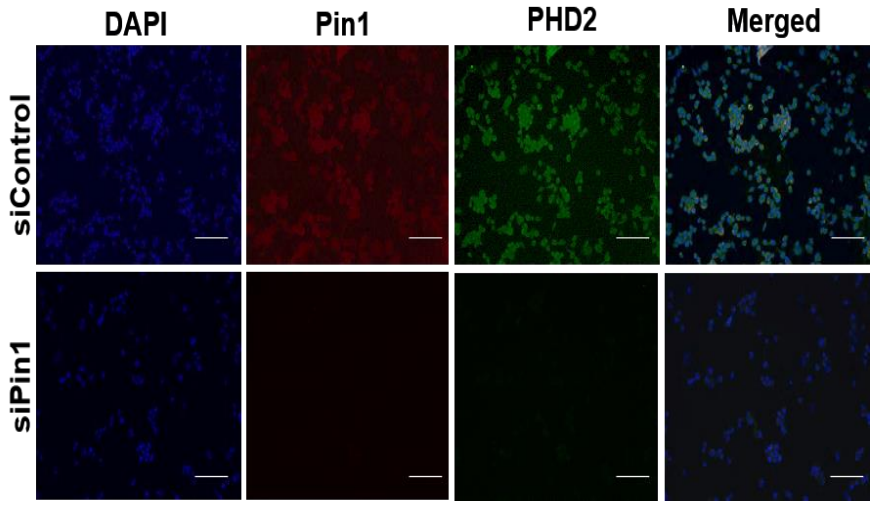

Relative Pin1 binding to PHD2
10
8
6
4
2
0
**
**
**
*
*
Normoxia
Hypoxia
IgG
Pin1-PHD2
IgG
Pin1-PHD2
IgG
Pin1-PHD2
IgG
Pin1-PHD2
Cytoplasm
Nucleus
Cytoplasm
Nucleus

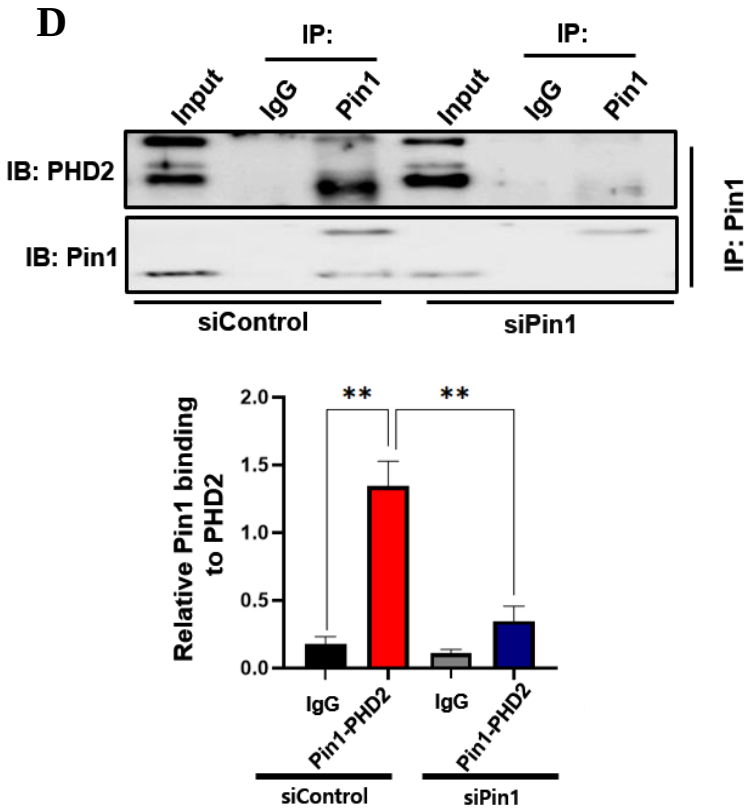
Figure 4. Pin1 and HIF-2α Interaction in breast cancer cells

(A) Pin1 and PHD2 interaction predicted by the STRING database. (B) The immunoprecipitation technique was used to evaluate how endogenous Pin1 and PHD2 interact in MDA-MB-231 cells in normoxic and hypoxic conditions. Results are expressed as means ± SD (n = 3). $*p < 0.05$ and $**p < 0.01$ (C) Interaction between ectopically expressed Pin1 and PHD2 was visualized by the PLA in MDA-MB-231 cells. Corresponding antibodies were used to co-label Pin1 and PHD2. DAPI was used to stain the nuclei. Red dots represent the Pin1 and PHD2 complex. Scale bar, 200 μm. (D) Comparison of Pin1 and PHD2 protein expression in cytoplasm and nucleus of MDA-MB-231 and MCF7 cells. (E) Localization of Pin1 and PHD2 protein expression in cytoplasm and nucleus of MDA-MB-231 in normoxic and hypoxic conditions. N, normoxia; H, hypoxia. (F) The endogenous interaction between Pin1 and PHD2 in cytoplasm and nucleus in normoxic and hypoxic conditions. Protein lysates of MDA-MB-231 cells were immunoprecipitated with Pin1 antibody and the proteins were detected with PHD2 and Pin1 antibodies. Results are expressed as means ± SD (n = 3). $*p < 0.05$ and $**p < 0.01$.


A
siControl
siPin1
PHD2
Pin1
Actin
Pin1/Actin
1.5
1.0
0.5
0.0
***
PHD2/Actin
1.5
1.0
0.5
0.0
*


B
Cytoplasm
Nucleus
siControl siPin1 siControl siPin1
PHD2
Pin1
Lamin B1
α-Tubulin


C
DAPI
Pin1
PHD2
Merged
siControl
siPin1


D
IP:
IP:
Input
IgG
Pin1
Input
IgG
Pin1
IB: PHD2
IB: Pin1
IP: Pin1
siControl
siPin1
Relative Pin1 binding to PHD2
2.0
1.5
1.0
0.5
0.0
**
**
IgG
Pin1-PHD2
IgG
Pin1-PHD2
siControl
siPin1

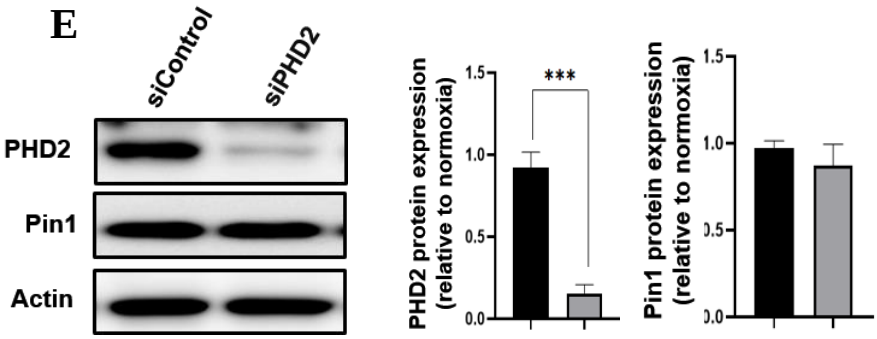
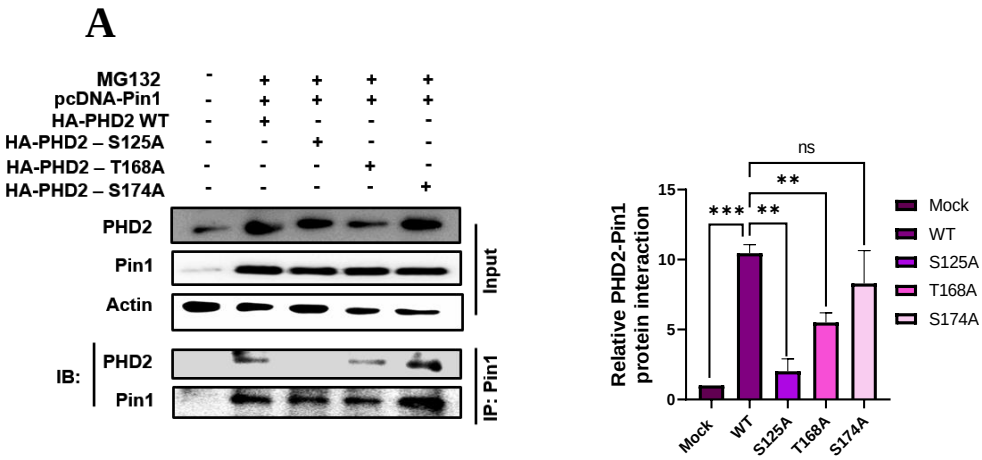
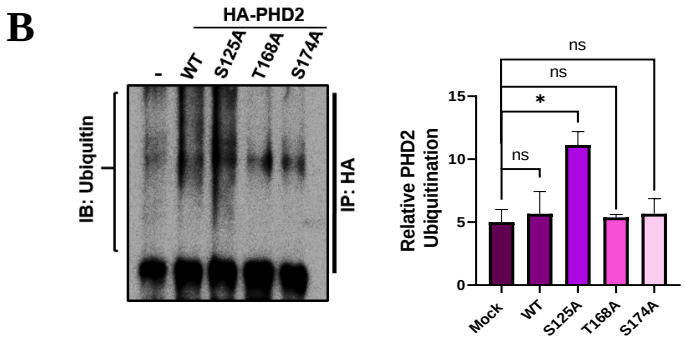
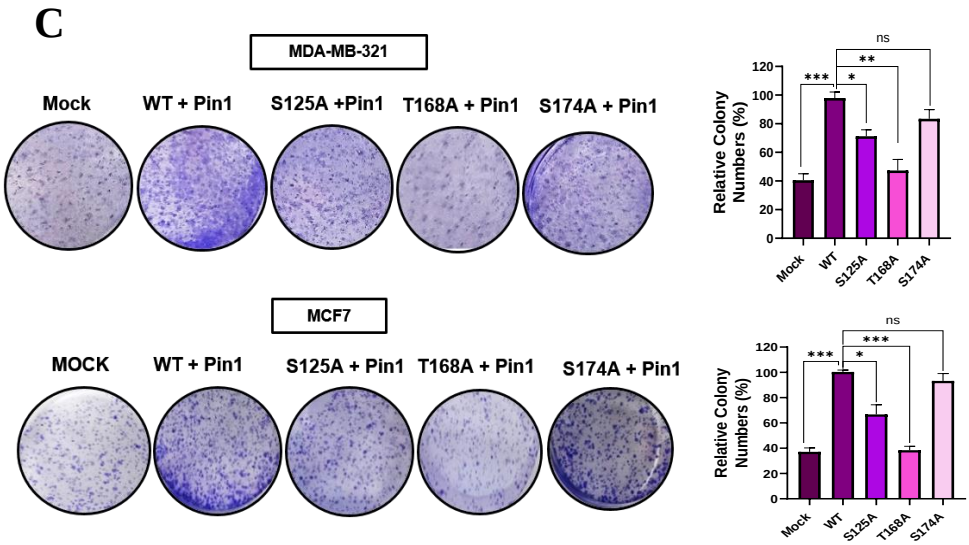

E
siControl
siPHD2
PHD2
Pin1
Actin
PHD2 protein expression (relative to normoxia)
1.5
1.0
0.5
0.0
***
Pin1 protein expression (relative to normoxia)
1.5
1.0
0.5
0.0

Figure 5. Effects of Pin1 knockdown on expression of PHD2 in breast cancer cells

(A) MDA-MB-231 cells were treated with either control siRNA or Pin1 siRNA. Western blot analysis using anti-Pin1 and anti-PHD2 antibodies was performed on cell lysates. *$p < 0.05$ and ***$p < 0.001$. (B) Comparison of Pin1 and PHD2 protein expression in cytoplasm and nucleus of MDA-MB-231 transiently transfected with control siRNA and Pin1 siRNA. (C) After Pin1 siRNA was transfected into MDA-MB-231 cells, immunofluorescence (IF) staining was used to evaluate the expression of both Pin1 and PHD2. Scale bar, 200 μm. (D) Interaction of Pin1 with PHD2 in Pin1 knockdown MDA-MB-231 cells confirmed by immunoprecipitation. **$p < 0.01$. (E) Protein expression of both Pin1 and PHD2 in MDA-MB-231 cells transfected with PHD2 siRNA. Results are expressed as means ± SD (n = 3). ***$p < 0.001$.


A
MG132
pcDNA-Pin1
HA-PHD2 WT
HA-PHD2 – S125A
HA-PHD2 – T168A
HA-PHD2 – S174A
PHD2
Pin1
Actin
Input
IB:
PHD2
Pin1
IP: Pin1
Relative PHD2-Pin1 protein interaction
Mock
WT
S125A
T168A
S174A
ns
**
***
**


B
HA-PHD2
-
WT
S125A
T168A
S174A
IB: Ubiquitin
IP: HA
Relative PHD2 Ubiquitination
ns
ns
*
ns
Mock
WT
S125A
T168A
S174A


C
MDA-MB-321
Mock
WT + Pin1
S125A +Pin1
T168A + Pin1
S174A + Pin1
Relative Colony Numbers (%)
ns
**
***
*
MCF7
MOCK
WT + Pin1
S125A + Pin1
T168A + Pin1
S174A + Pin1
Relative Colony Numbers (%)
ns
***
***
*

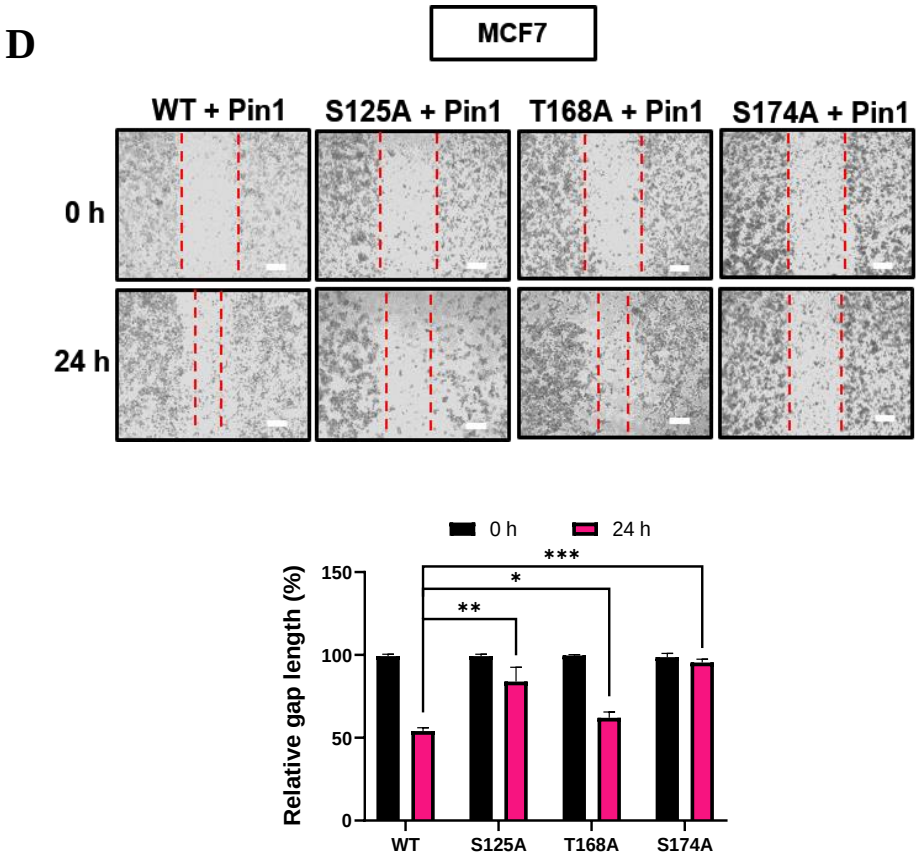


Figure 6. The precise sites of PHD2 (S125, T168, and S174) involved in Pin1 binding

(A) PHD2-Pin1 interaction was compared in wild type (WT) and mutant cells in which a particular serine was changed into an alanine. pcDNA-Pin1 and HA-tagged WT PHD2 or the corresponding mutant constructs were co-transfected into HEK293T cells, and the cell lysates were then subjected to immunoprecipitation (IP) analysis. Quantitative analysis of the interaction between Pin1 with WT or non-phosphorylatable mutants (PHD2 S125, T168, and S174). $^{**}p < 0.01$, and $^{***}p < 0.001$. ns, not significant. (B)

Ubiquitinylated PHD2 (Ub-PHD2) in HEK293T cells was measured by immunoprecipitation of HA, followed by a Western blot experiment using an anti-ubiquitin antibody. *$p < 0.05$. ns, not significant. (C) MDA-MB-231 and MCF7 cells were co-transfected with pcDNA-Pin1 and HA-tagged WT PHD2 or mutant forms (S125A, T168A, and S174A). The cells were stained with crystal violet and then photographed. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$. ns, not significant. (D) MCF7 cells in 4-well plates were co-transfected with pcDNA-Pin1 and HA-tagged WT PHD2 or mutant forms (S125A, T168A, and S174A) for the cell migration assay. The results are presented as the mean ± SD (n = 3). *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

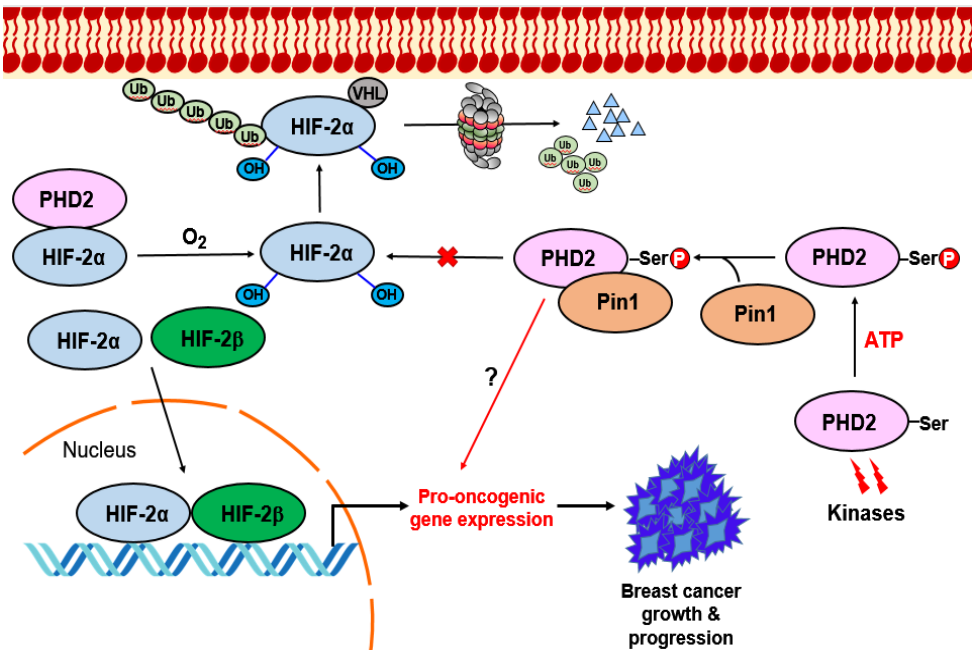
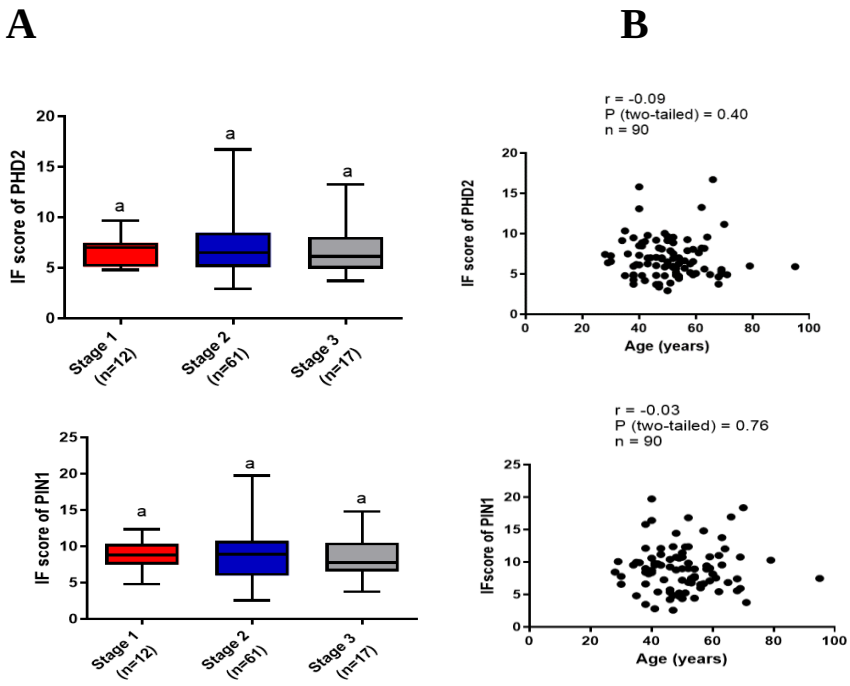
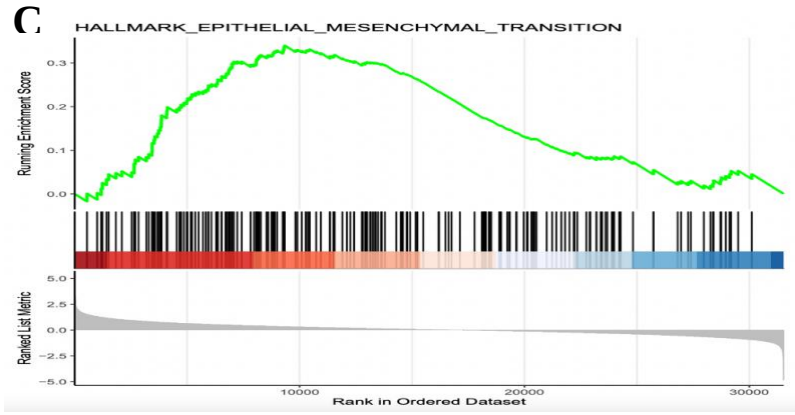
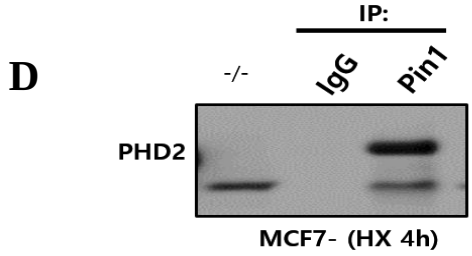


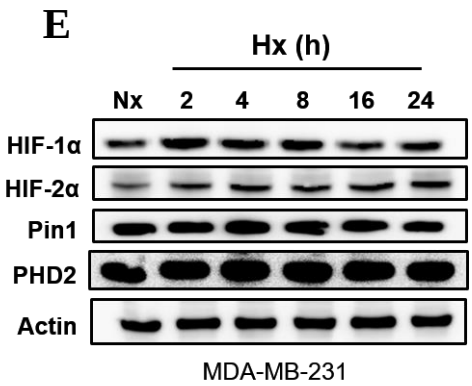
Figure 7. A proposed model for the regulation of PHD2 by Pin1 in breast cancer

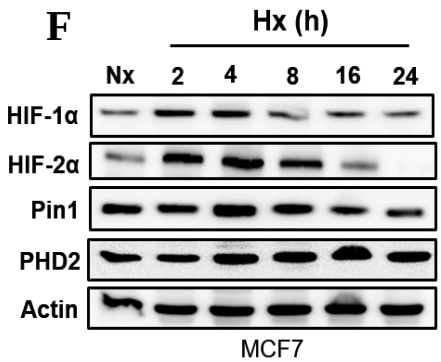
PHD2 is subjected can act in normoxia and hypoxia. PHD2 undergoes phosphorylation at a specific serine residue (e.g., S125), which facilitates its interaction with Pin1. As a result, PHD2 is unable to hydroxylate HIF-2α and translocates to the nucleus where it might be involved in the proliferation of breast cancer.

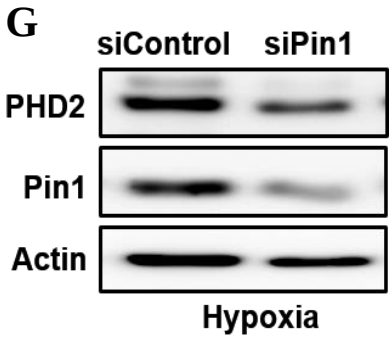

A
B
IF score of PHD2
Stage 1 (n=12)
Stage 2 (n=61)
Stage 3 (n=17)
r = -0.09
P (two-tailed) = 0.40
n = 90
Age (years)
IF score of PIN1
r = -0.03
P (two-tailed) = 0.76
n = 90
IFscore of PIN1

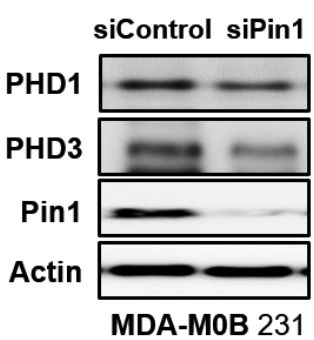

C
HALLMARK_EPITHELIAL_MESENCHYMAL_TRANSITION
Running Enrichment Score
Ranked List Metric
Rank in Ordered Dataset


D
IP:
-/-
IgG
Pin1
PHD2
MCF7- (HX 4h)


E
Hx (h)
Nx
2
4
8
16
24
HIF-1α
HIF-2α
Pin1
PHD2
Actin
MDA-MB-231


F
Hx (h)
Nx
2
4
8
16
24
HIF-1α
HIF-2α
Pin1
PHD2
Actin
MCF7


G
siControl
siPin1
PHD2
Pin1
Actin
Hypoxia


H
siControl
siPin1
PHD1
PHD3
Pin1
Actin
MDA-M0B 231

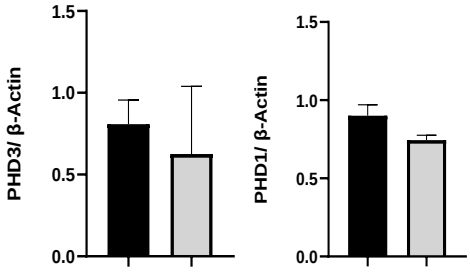

1.5
1.0
0.5
0.0
PHD3/ β-Actin
1.5
1.0
0.5
0.0
PHD1/ β-Actin

Figure S1. Association between PHD2 and Pin1 in breast cancer

(A) The relative IF scores of Pin1 and PHD2 in different stages and (B) ages of breast cancer patients. (C) GSEA analysis shows PHD2 (Egln1) high tumors have increased epithelial to mesenchymal transition signature which potentially increase its aggressiveness. adjusted p-value: 0.033, NES: -1.46. (D) Interaction of Pin1 and PHD2 in MCF7 breast cancer cell line in hypoxia 4 h. (E-F) Western blot analysis using anti-HIF-1α, HIF-2α, anti-Pin1 and anti-PHD2 antibodies was performed on cell lysates in normoxia and different times in hypoxia in (E) MDA-MB-231 and (F) MCF7 cells. (G) MDA-MB-231 cells were treated with either control siRNA or Pin1 siRNA in hypoxia for 4 h. (H) Western blot analysis using anti-Pin1, PHD1 and PHD3 antibodies was performed on cell lysate in Pin1 knockdown MDA-MB-231 cells. Data from three different experiments are presented as mean ± SD and the statistical significance was analyzed by the Student's *t*-test. Nx, normoxia; Hx, hypoxia.

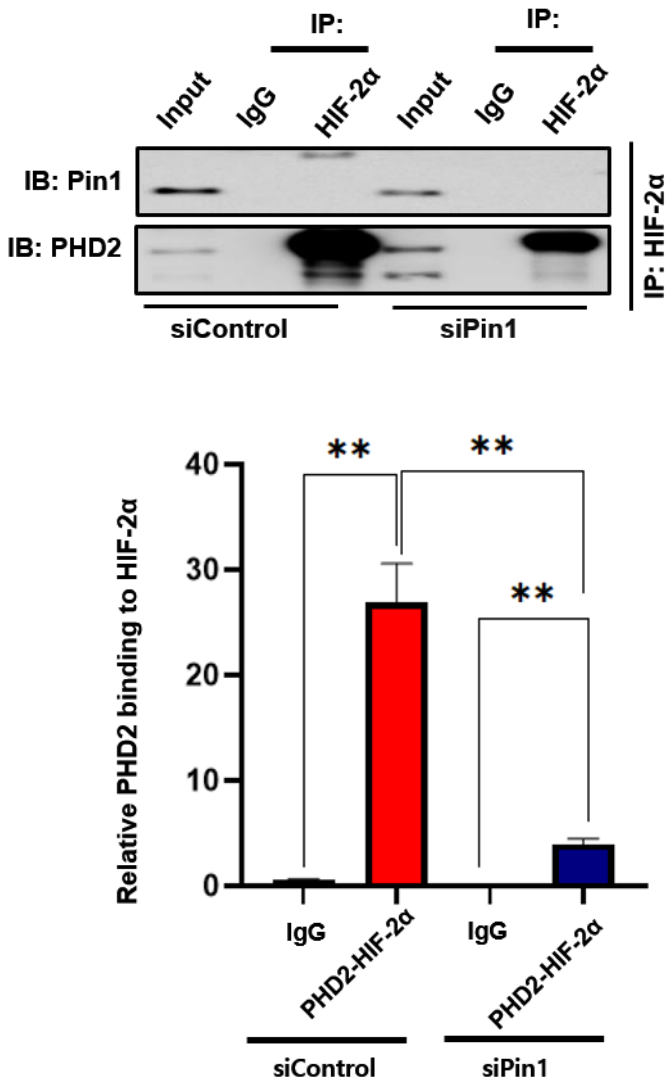


Figure S2. Interaction between Pin1 and PHD2 in Pin1 knockdown breast cancer cell line

Using an immunoprecipitation technique, the interaction between Pin1 and PHD2 was investigated in Pin1 knockdown MDA-MB-231 cells. Data from three different experiments are presented as mean ± SD and the statistical significance was analyzed by the Student's *t*-test $^{**}p < 0.01$

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Conclusion

In conclusion, we propose a novel mechanism responsible for controlling HIF-2α, with serine 790 as an essential phosphorylation site for both its stability and transcriptional activity. To the best of our knowledge, this is the first instance in which HIF-2α is stabilized under ambient oxygen through interaction of its NES domain with Pin1. Of note, the serine 790 residue located in the NES domain is critical in the stabilization of HIF-2α. HIF-2α's ability to interact with Pin1 was hindered by even a single serine-to-alanine alteration. According to our research, Pin1 binding is a key component of an oxygen-independent (pseudohypoxia) HIF-2α regulation mechanism.

Peptidyl-prolyl *cis-trans* isomerase NIMA interacting 1 stabilizes HIF2-α in breast cancer

ABSTRACT

요약(국문초록)

유방암에서 Peptidyl-prolyl cis-trans isomerase NIMA-interacting 1에 의한 HIF-2a 의 안정화

Peptidyl-prolyl cis-trans isomerase NIMA-interacting 1 (Pin1)은 대부분의 인체암에서 과발현되며 인산화된 serine 또는 threonine 다음에 오는 proline 잔기를 갖는(S/T-P) 단백질에 결합함으로써 그 안정성, 세포 내 분포 및 기능에 영향을 미친다. 대표적 종양성 전사인자인 HIF-2α 역시 이러한 S/T-P 구조를 가지므로 Pin1과 결합할 가능성이 높다. 이를 토대로 본연구에서는 Pin1과의 결합을 통한 HIF-2α 의 안정화 및 활성화가 유방암 진행에 미치는 영향을 살펴보았다. 삼중음성 인체유방암 세포주에서 Pin1과 HIF-2α 의 상관성을 확인하였다. Pin1과 HIF-2α 사이의 직접 결합은 co-immunoprecipitation and an in situ proximity ligation assays 방법으로 확인하였다. 또한 Pin1의 억제는 HIF-2α의 유비퀴틴화를 통한 분해를 증가시켰다. HIF-2α 단백질 발현과는 달리 그 mRNA 발현양은 Pin1에 의해 영향을 받지 않았다. HIF-2α의 Ser672, Ser696 and Ser790 잔기에서의 인산화는 이 전사인자가 Pin1과 결합하는데 있어서 매우 중요하다. 이중에서 Ser790의 인산화가 HIF-2α의 안정화에 중요한것으로 확인되었다.

HIF-2α의 proline 수산화에 중요한 효소인 prolyl hydroxylase domain 2 (PHD2) 또한 Pin1과 결합함을 알 수 있었다. PHD2의 Ser125, Thr168 그리고 Ser174가 Pin1과의 결합에 중요함을 확인하였다. 결론적으로 Pin1은 HIF-2α와의 결합을 통해 직접적으로 또한 PHD2와의 결합을 통해 간접적으로 HIF-2α 을 안정화시키고 이를 통해 유방암 증식과 진행에 역할을 하는것으로 사료된다.

keywords: Pin1, HIF-2α, Protein-Protein interaction, Breast cancer, Hypoxia, PHD2

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