약학박사학위논문

Peptidyl-prolyl *cis-trans* isomerase NIMA-interacting 1 (Pin1) directly binds and stabilizes Nrf2 in breast cancer

인체 유방암에서 Peptidyl-prolyl *cis-trans* isomerase NIMA-interacting 1 (Pin1)과의 직접 결합을 통한 Nrf2의 안정화 연구

2020년 8월

서울대학교 대학원

분자의학 및 바이오제약학과

Soma Saeidi

Peptidyl-prolyl *cis-trans* isomerase NIMA-interacting 1 (Pin1) directly binds and stabilizes Nrf2 in breast cancer

by

Soma Saeidi

A thesis submitted in partial fulfillment of the requirements

for the degree of

DOCTOR OF PHILOSOPHY

(Molecular Medicine and Biopharmaceutical Sciences)

Under the supervision of Professor Young-Joon Surh

at the Department of

Molecular Medicine and Biopharmaceutical Sciences,

Seoul National University

August 2020

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지도교수 서 영 준

이 논문을 약학박사 학위논문으로 제출함

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Soma Saeidi

Soma Saeidi의 약학박사 학위논문을 인준함

2020 년 7 월

위 원 장 _____

부 위 원 장 _____

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Dedicated to my parents

ACKNOWLEDGMENTS

First and foremost, I would like to express my special and sincere gratitude and the deepest appreciation to my supervisor, Professor Young-Joon Surh, for the continuous support of my Ph.D. study and research whose patience, encouragement and immense knowledge have motivated me throughout my Ph.D. course during which his valuable guidance and advice utterly helped. Beside my advisor, I would like to admire the members of my dissertation committee, Prof. Seong-Jin Kim, Prof. Yu-Jin Lee, Prof. Marc Diederich and Prof. Hye-Kyung Na, for their contribution as my committee members and for their encouraging and insightful comments and suggestions. I appreciate all for letting my viva be an unforgettable moment.

My sincere recognition also goes to Dr. Su-Jung Kim for supporting and encouraging me at the first steps of my research. The authors are grateful to Professor Donna Zhang of University of Arizona for generous supply of Nrf2 serine mutant constructs. Also acknowledgement for the people (Drs. Yamamoto, Motohashi and Hiroshi Kitamura) in Sendai for supply of Keap1 MEFs. The authors also thank Prof. Joon Jeong (Yonsei University) for generous supply of patients' specimens.

I am very indebted to my friends and lab mates whom I had such productive and delightful times in the laboratory with. Many thanks to all those wonderful former members of the laboratory who have offered up some help or advice.

Lastly, I would like to thank my family for all their love and encouragement. Words cannot express how grateful I am to my father and my mother for all of the sacrifices that they have made for me over the years, to my sister and brothers who gave me a reason to carry on, even through the difficult times, which sustained me thus far, and above all, to my loving, supportive, inspiring and patient brother, Shahram, for his unconditional love, constant support and encouragement, to whom I owe the person I have become and the joy and meaning of life I have found.

Soma Saeidi,

August, 2020

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Under the supervision of Professor Young-Joon Surh

Department of Molecular Medicine and

Biopharmaceutical Sciences

Seoul National University

Peptidyl-prolyl *cis-trans* isomerase NIMA-interacting 1 (Pin1) specifically recognizes phosphorylated serine or threonine of a target protein and isomerizes the adjacent proline residue. Overexpression of Pin1 has been found in many types of malignancies, suggesting its oncogenic function. Recent studies have revealed constitutive activation of Nrf2, a transcription factor that regulates cellular redox

homeostasis, in some transformed or cancerous cells, conferring an advantage for their growth and survival. Silencing of *Pin1* by using siRNA or pharmacologic inhibition blocked the accumulation of Nrf2, thereby suppressing proliferation and clonogenicity of MDA-MB-231 human breast cancer cells and xenograft tumor growth in nude mice. Since Nrf2 harbours pSer/Thr-Pro motifs, I investigated whether Pin1 could directly interact with Nrf2 in the context of its implications in breast cancer development and progression. I found that Pin1 binds to Nrf2 which stabilizes this transcription factor by hampering proteasomal degradation. Notably, the interaction between Pin1 and Nrf2 was dependent on the phosphorylation of Nrf2 at Ser 215, 408 and 577. In another study, Keap1, the main inhibitor of Nrf2, was found to be phosphorylated at Ser 104 and Thr 277. These amino acids are preceded by proline and hence can be the putative binding sites for Pin1. I found the direct interaction between Keap1 and Pin1, and this was abolished upon substation of Ser 104 and Thr 277 with Alanine. The interaction of Nrf2 with Keap1 was markedly increased when Pin1 was downregulated. On the other hand, Keap1 knockout embryonic fibroblasts exhibited the enhanced interaction between Nrf2 and Pin1. Therefore, it is likely that Pin1 and Nrf2 may compete with each other for Keap1 binding. In conclusion, Pin1 plays a role in stabilization and constitutive activation of Nrf2 interfering the interaction between Nrf2 and Keap1.

Key words:

Pin1, Nrf2, Keap1, Protein-Protein interaction, Breast cancer

Student Number: 2015-30790

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LIST OF ABBREVIATIONS

Abbreviations	Explanation
Pin1	Peptidyl-prolyl cis-trans isomerase NIMA-interacting 1
Nrf2	Nuclear factor-erythroid2-related factor 2
Keap1	Kelch-like ECH-associated protein 1
WT	Wild-type
KO	Knockout
PBS	Phosphate-buffered saline
RT-qPCR	Reverse transcription-quantitative polymerase chain reaction
siRNA	Small interfering RNA
MAPK	Mitogen-activated protein kinase
ERK	Extracellular regulated protein kinase
JNK	c-Jun N-terminal kinase
MEF	Mouse embryonic fibroblast
PTMs	Post-translational modifications
BC	Breast cancer
H&E	Hematoxylin and eosin
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
IHC	Immunohistochemistry
IF	Immunofluorescence
AURKA	Aurora kinase A
C/EBPα	CCAAT/enhancer binding protein α

DAPK1	Death-associated protein kinase 1
DPP4	Dipeptidyl peptidase 4
IL-22	Interleukin-22
miR	microRNA
MLK3	Mixed-lineage kinase 3
PKA	Protein kinase A
PPIase	Peptidyl-prolyl isomerase
RSK2	Ribosomal S6 kinase 2
SENP1	SUMO Specific Peptidase 1
SUMO	Small ubiquitin-related modifier
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
NAC	N-acetylcysteine
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
DMEM	Dulbecco's modified Eagle's medium
SD	Standard deviation

Chapter I

General overview

1. Peptidyl-prolyl *cis-trans* isomerase NIMA-interacting 1 (Pin1) in cancer

Pin1, a peptidyl-prolyl *cis*/*trans* isomerase (PPIase), isomerizes specific phospho-serine/threonine-proline motifs present its substrate proteins, and hence plays a role in post-translational regulation of the target protein functions. Deregulation of Pin1 notably, the aberrant overexpression of Pin1 is implicated in the pathogenesis of certain cancers. Pin1 has been shown to stabilize numerous oncogene regulators. In contrast, Pin1 also promotes the degradation of various proteins that have tumor suppressive and growth inhibitory functions [1]. Pin1 was initially identified as a regulator of mitosis, but subsequent studies showed that it facilitates multiple signaling pathways in cancer [2]. Cancer metastasis is the leading cause of death in cancer patients. Research revealed that the expression of Pin1 is much higher in the metastatic cancer compared with primary tumor [3, 4]. Pin1 overexpression promotes epithelial-mesenchymal transition (EMT) through downregulation of E-cadherin [5- 7].

1.1. Pin1 has critical roles in breast cancer development and progression

Breast cancer (BC) is a heterogeneous disease consisting of distinct subtypes that are characterized by different histo-pathological features, specific genetic and epigenetic alterations, and diverse aggressive characters acquired during malignant progression [8]. Pin1 is involved in all main cellular processes of BC development and progression [5, 7, 9-16]. Pin1 is overexpressed in the majority of BCs and

correlates with worse clinical outcome, pointing to its essential role in phosphorylation-dependent oncogene events of breast carcinogenesis [5-7, 9, 10, 17].

1.2. Regulation of Pin1 gene expression in human breast cancer

The expression of Pin1 is immediately regulated by transcription factors E2F and NOTCH. Besides, the CCAAT/enhancer binding protein-α (C/EBPα)-p30 increases Pin1 expression by recruiting E2F to the promoter of Pin1. Neu/Ras signaling can upregulates expression of Pin1, and overexpression of Pin1 in Neu/Ras-expressing mammary epithelial cells accounts for their transformed phenotypes [6, 11, 16]. Oncogenic signaling molecules known to activate E2F transcription factor, such as Her2, H-Ras, PI3K and p38, induce *Pin1* mRNA expression [13, 16, 18, 19]. Given the presence of E2F consensus sequence in Pin1 promoter, the above finding implies the direct transcriptional activation of Pin1 by E2F. In BC cells, Pin1 mRNA levels were found to be reduced by the miR-200c small non-coding RNA, a well-known keeper of epithelial fate and repressor of metastasis [7].

1.3. Expression, post-translational modifications and subcellular localization of Pin1

In human BC, deregulation of Pin1 protein expression and activity are relevant to its development and progression. In fact, while normal breast epithelial cells express low levels of nuclear Pin1, BC cells exhibit elevated accumulation of Pin1 both in the nucleus and cytoplasm and display highly phosphorylated Pin1 expression [5]. Different phosphorylation and other post-translational modifications of Pin1 have been identified which influence its stability, subcellular localization, substrate binding and catalytic activity (**Fig. 1-1 and Table 1-1**). Interestingly, the phosphorylation status is highly variable whereas the levels of total Pin1 do not differ significantly during cell cycle progression. For instance, polo-like kinase 1, an early trigger for G2/M transition involved in centrosome maturation and mitotic spindle establishment, has been found to phosphorylate Ser65 in the catalytic domain of Pin1 [19]. This phosphorylation increases Pin1 stability, a relevant event during mitosis [19].

On the other hand, Ser16 phosphorylation by protein kinase A (PKA) and aurora kinase A (AURKA) abolishes Pin1 ability to bind to substrates and is required for exit from mitosis [20]. Phosphorylation by PKA has been shown to affect Pin1 localization in nuclear speckles, by facilitating its nuclear export [21]. Of note, Gonadotropin signaling regulates the activity of Pin1 through a nuclear-cytoplasmic shuttling mechanism based on Ser16 phosphorylation by PKA or protein kinase C (PKC) and its subsequent dephosphorylation by calcineurin [22]. The tumor suppressor, death-associated protein kinase 1 (DAPK1), phosphorylates

Pin1 at Ser71, fully inhibiting its isomerase activity, nuclear localization and cellular function [23]. DAPK1 expression correlates with phospho- Pin1 ^{Ser71} levels in human breast tumors. Accordingly, reduction of Pin1 expression or restoration of DAPK1 in cancer cells effectively suppresses the manifestation of tumorigenic phenotypes [23].

Mixed-lineage protein kinase 3 (MLK3) was shown to phosphorylate Pin1 on Ser138, fostering Pin1 catalytic activity and nuclear localization. A significant difference in the levels of phospho-Pin1^{Ser71} between normal and cancer tissue has been observed, although total protein levels were comparable, suggesting that MLK3-induced phosphorylation of Pin1 could be an early event in oncogenesis and a reliable marker for Pin1 activation in BC [24]. Sumoylation of Lys6 or Lys63 is another important post-translational modifications that inhibits Pin1 functions, and is reverted by de-sumoylation by SUMO Specific Peptidase 1 (SENP1) [25]. The aforementioned post-translational modifications might influence the interaction of the nuclear localization signal sequence, contained in the PPIase domain of Pin1, with proteins of the nuclear import machinery [26]. In the nucleus, Pin1 activity affects both specific transcription factors (e.g., p53, p63, p73, ERα, Notch1, NF-κB, c-Myc, MEF2C, etc. [27-30] and global transcriptional as well as post-transcriptional regulators (e.g., histones, RNApolII, RNA binding proteins, ADAR1, etc.) [27, 29, 31-34].

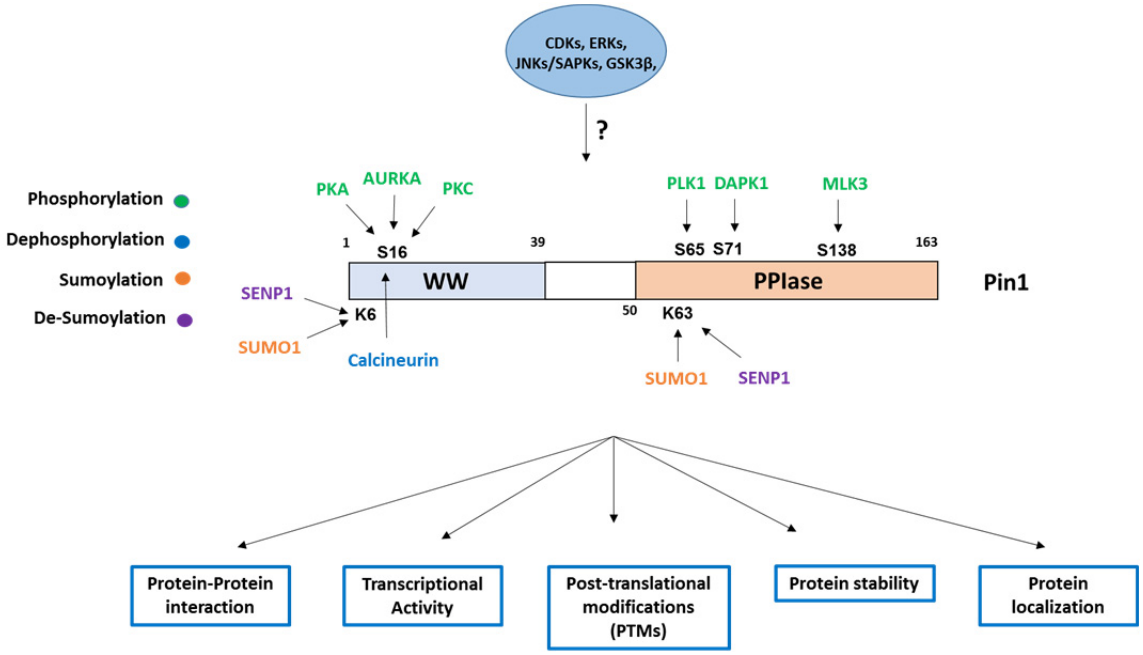


Figure 1-1. Impact of Pin1 modifications on biological functions of its substrates

Table 1-1. Modulation of Pin1 expression or activity

Pin1 regulation	Pin1 regulators	Pin1 binding site	Pin1 activity	REFs
Transcription	E2F1, HER2, H-RAS, PI3K	E2F site	Increase	35, 36
	IL-22, DPP4, C/EBPα-P30	E2F site	Increase	37
	NOTCH1 and NOTCH4	BS1 site	Decrease	38
	RB1	E2F site	Decrease	39, 40
	BRCA1	Unknown	Decrease	41
	MAPKs	E2F	Increase	42
Translation	miR200b, miR200c, miR296-5p	3′UTR	Decrease	43, 44, 45
Phosphorylation	PLK1	Ser65	Increase	46
	MLK3	Ser138	Increase	47
	DAPK1	Ser71	Decrease	48
	PKA, RSK2, AURKA	Ser16	Decrease	49, 50, 51
	COT	Ser16	Increase	52
Sumoylation	SUMO1 and SUMO2	Lys6, Lys63	Decrease	53
Desumoylation	SENP1	Lys6, Lys63	Increase	53
Deubiquitylation	PLK1	Unknown	Increase	46
Acetylation		Lys46	Unknown	54, 55
Oxidation		Met130 and Met146	Unknown	56
		Cys113	Decrease	57, 58
Foreign sources	Theileria annulata (Parasite)	Pin1 PPIase domain	Increase	59
	12-O-tetradecanoylphorbol-13-acetate	Ser16	Decrease	60

1.4. The significance of Pin1 phosphorylation

Protein phosphorylation is a reversible post-translational modification implicated in a variety of cellular processes that have impacts on protein activity, dictate subcellular localization and induce the establishment of recruitment platforms for interacting proteins. Frequently altered as a downstream consequence of oncogenic driver mutations, protein phosphorylation is central to cancer treatment as well as development [61].

Among the phosphorylation sites, serines or threonines preceding proline (Ser/Thr-Pro) that are targeted by proline-directed kinases, such as cyclin-dependent kinases (CDKs), mitogen-activated protein kinases (MAPKs), and glycogen synthase kinase-3β (GSK-3β), deserve particular attention. Compared with other PPIases, Pin1 is unique in the context that specifically recognizes phosphorylated Ser/Thr-Pro moieties (pSer/Thr-Pro). Such unique substrate specificity is conferred by its highly conserved two-domain structure consisting of an N-terminal WW domain binding specific pSer/Thr-Pro modules and a C-terminal PPIase domain catalyzing their *cis-trans* isomerization [27, 29].

1.5. Pin1 regulates signaling molecules associated with cancer and cancer stem cells

Pin1 prevents the protein degradation of numerous oncogenes/growth-promoting regulators, including AKT, β-catenin, c-Fos, c-Jun, cyclin D1, estrogen receptor

(ER), Hbx, HER2, HIF-1, Mcl-1, Nanog, NF-κB, NUR77, Oct4, PML-RARα, Stat3, and Tax [29, 36,63-66]. In contrast, Pin1 also promotes the degradation of various proteins involved in tumor suppression and growth inhibition, including Daxx, Fbw7, FoxO4, GRK2, KLF10, PML, RARα, RBBP8, RUNX3, Smad, SMRT, SUV39H1, and TRF1 [29, 67, 63, 66-69].

1.6. Pin1 mediates drug resistance of breast cancer

Pin1 might directly mediate chemoresistance by directly binding to PKB/AKT [62], MCL-1 [9] or Notch1, that promotes the cell survival (e.g., Survivin and Bcl-2) and drug efflux pump genes (e.g., ABCG2) [6]. Pin1 was found to be overexpressed in ER+ tumors and cell lines [5] and it is thought to confer treatment resistance through induction of EMT and angiogenesis [15, 70] as well as degradation of CDK10 [71. Pin1 functions as an essential catalyst of the ERα-HER2 crosstalk [67], supporting the idea that Pin1 inhibitors may re-sensitize tumors to endocrine therapies.

1.7. Pin1 inhibitors

Several modes of action of both the WW and PPIase domains have been proposed [72], and it appears that inhibition of either domain may have a therapeutic potential. In their therapeutic applications, difficulties have been encountered mainly due to low substrate affinity or specificity (e.g., EGCG and Juglone), poor solubility (e.g.,

PiB) or cell-permeability (e.g., peptide inhibitors) [72]. All-*trans* retinoic acid (ATRA) is a known therapeutic for the treatment of acute promyelocytic leukemia (APL) by targeting retinoic acid receptor a (RARα) and causing degradation of the oncoprotein PML-RAR. ATRA inhibits and degrades active Pin1 selectively in cancer cells by directly binding to the substrate phosphate- and proline-binding pockets in the Pin1 active site [73].

The Pin1-ATRA co-crystal structure revealed that the carboxyl group of ATRA formed salt bridges with K63 and R69 of Pin1, both of which are responsible for binding the phosphate of pS71 of Pin1 [74]. The possibility that S71 phosphorylation affects ATRA sensitivity has been examined. Indeed, the levels of S71 phosphorylation in different cell lines inversely correlated with ATRA sensitivity. Thus, ATRA selectively ablates active non-phosphorylated Pin1 and thereby inhibits multiple cancer-driving pathways in ER-positive, HER2-positie and triple negative human breast cancer (TNBC) cells (**Fig. 1-2**). ATRA also dose-dependently reduced expression of both endogenous and exogenous cyclin D1. ATRA (tretinoin) directly and selectively binds, inhibits and ultimately degrades active Pin1, thereby exerting potent anticancer activity against acute APL and TNBC by simultaneously blocking multiple Pin1-regulated cancer-driving pathways [73]. Therefore, ATRA has potent anti-tumor activity against TNBC through ablation of Pin1 [66].

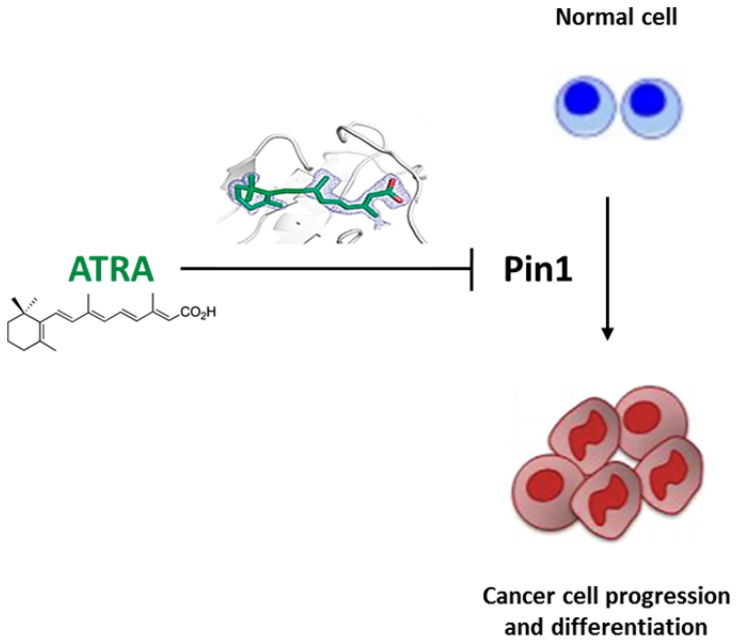


Figure. 1-2. ATRA inhibits the tumorigenic function of Pin1

2. Nrf2 in cancer

Nrf2 is a master regulator of numerous cytoprotective genes [75, 76]. Like other proteins, the Nrf2 protein is degraded by the ubiquitin-proteasome system in the cytoplasm [77]. Kelch-like ECH-associated protein 1 (Keap1) is a component of the Cullin 3 (CUL3)-based E3 ubiquitin ligase complex and controls the stability and accumulation of Nrf2 [78]. Inactivation of Keap1 strongly induces Nrf2 overexpression, and this phenomenon is often observed in cancer cells. Cancer cells can thus acquire malignancy by perverting Nrf2 activity [79]. At least four pathways have been reported to be involved in Nrf2 activation in cancer cells [80], including somatic mutations within the Nrf2, Keap1, or Cul3 genes [81-83], epigenetic silencing of the Keap1 gene [84], cysteine modification by oncometabolites such as fumarate [85, 86], and the accumulation of Keap1 interacting proteins, such as p62/Sqstm1 [87] and p21 [88]. All these molecular events result in disrupted binding of Keap1 to Nrf2, causing aberrant accumulation of Nrf2 in cancer cells.

2.1. Nrf2 promotes tissue invasion and metastasis

During EMT, epithelial cells lose expression of the adhesion protein E-cadherin in favor of N-cadherin. In cancer cells, Nrf2 promotes EMT by downregulation of E-cadherin expression through unknown mechanisms [89, 90]. Expression of Nrf2 is important for the migration of malignant cells [91, 92]. Cancer cells that have

constitutively high levels of Nrf2 can grow in an anchorage-independent manner and have a higher metastatic capacity [93].

2.2. Role of Nrf2 in resistance to chemotherapy

Nrf2 is also responsible for regulation of expression of efflux transporters, especially those of ATP-binding cassette (ABC) family which pump out xenobiotics from the cell against a concentration gradient. Nrf2 binds to the antioxidant responsive element (ARE)-like sequences in the promoters of multidrug resistance-associated proteins (MRP) genes like *Mrp1*, *Mrp2*, *Mrp3*, *Mrp4*, and *Abcg2* and enhances their expression, conferring chemoresistance in cancer cells [94]. Nrf2 plays key role in the development of drug resistance in patients undergoing chemotherapy. The activity of Nrf2 in cancer cells decreases their sensitivity to the common chemotherapeutic agents like doxorubicin, carboplatin, cisplatin, etc [95].

2.3. Keap1 as an inhibitor of Nrf2

Two Keap1 molecules are able to bind to one Nrf2 molecule [96], and the BTB domain is responsible for the homodimerization of Keap1 and the subsequent inhibition of Nrf2 [97]. When transfected into cells, Nrf2 would accumulate in the nucleus. When co-transfected with Keap1, however, the two would co-localize in the cytoplasm. Moreover, in the presence of both Keap1 and a panel of electrophiles, this co-localization is lost and Nrf2 again localizes in the nucleus [98]. Under basal

conditions, it is the interaction with Keap1 that facilitates the proteasomal degradation and high turnover of Nrf2 protein resulting in a half-life of approximately 10–20 min [99, 100].

In the absence of oxidative stress, Nrf2 is sequestered in the cytosol by the Keap1 homodimer which acts as a substrate adaptor for the ubiquitination of Nrf2 in a Cul3-dependent manner [101]. When cells are under oxidative stress or in the presence of electrophiles, subsets of the cysteine residues in Keap1 are modified. This renders Keap1 molecules saturated with Nrf2 that is no longer targeted for degradation and newly synthesized, and free Nrf2 accumulates in the cytosol. Consequently, Nrf2 translocates to the nucleus where it binds to the ARE, activating the transcription of defence genes [102, 103]. Thus, thiol modifications of Keap1 potentially result in its conformational change, which results in the release of Nrf2 from the low affinity binding site (latch), disturbing the transfer of ubiquitin.

2.4 Keap1-independent regulation of Nrf2

Nrf2 has been shown to be regulated by mechanisms independent of Keap1. These include regulation at the transcriptional level by Arylhydrocarbon receptor (AhR)-ARNT inducing Nrf2 expression and NF-κB which has been proposed to bind to an ARE within the Nrf2 promotor region. At the post-transcriptional level, components of the Nrf2 pathway are regulated by several micro-RNAs (miR-28, 34, 144, 200). Post translationally, Nrf2 is phosphorylated, ubiquitinated and acetylated by a distinct set of enzymes. Each post-translational modification affects Nrf2 differently

by altering its interaction with Keap1; Nrf2 localization; Nrf2 protein degradation and Nrf2 DNA binding. A number of proteins have been identified as Nrf2 binding partners, but their mechanisms of action are unknown [104, 105].

2.5. Post-translational modifications of Nrf2

There have been several studies suggesting that phosphorylation of Nrf2 may contribute to its nuclear exclusion and degradation [106, 107, 108]. Nrf2 contains many serine, threonine and tyrosine residues, which may provide sites for phosphorylation by different kinases [108]. These include MAPKs, PI3K/AKT, PKC, and GSK3β (**Fig. 1-3**). Additionally, several serine/threonine residues in Nrf2 have been identified to be phosphorylated by a panel of MAPKs [109]. Recent studies have highlighted the involvement of c-Jun N-terminal kinase (JNK) and extracellular signal-regulated kinase 1/2 (ERK1/2) in the activation of Nrf2. Butylated hydroxyanisole was shown to increase the phosphorylation of both ERK1/2 and JNK1/2 to activate Nrf2 which was released from Keap1 and translocated to the nucleus under the control of ERK and JNK signalling pathways [110] (**Fig. 1-3**). Besides phosphorylation and ubiquitinylation, there is paucity of data on the other types of post-translational modifications (e.g., acetylation) of Nrf2 (**Fig. 1-4**).

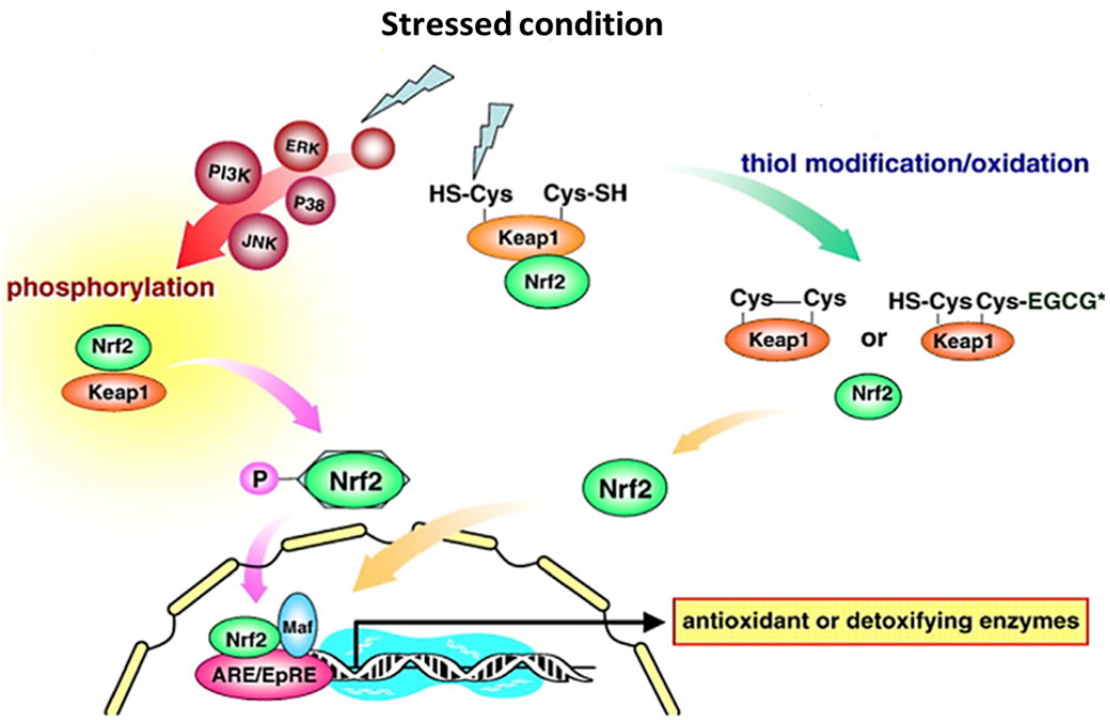
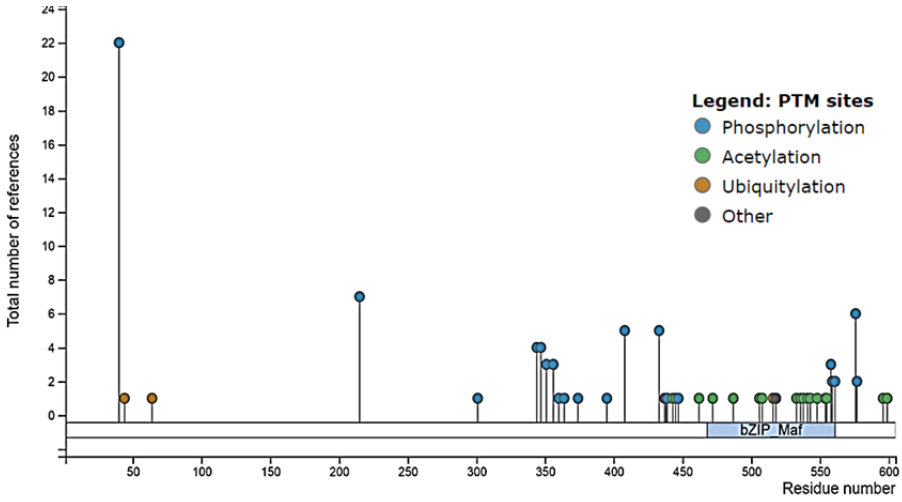


Fig 1-3. Phosphorylation-induced Nrf2 stabilization. Adopted from: Hanna Lewandowska et al., 2016



Effects on Modified Protein	Triggered By
activity, induced	S40-p
intracellular localization	S40-p , S215-p , T395-p , S408-p , S433-p , T439-p , S558-p , T559-p , Y576-p , S577-p
molecular association, regulation	S40-p , R437-me

Effects on Biological Processes	Triggered By
transcription, induced	S40-p , S215-p , S408-p , R437-me , S558-p , T559-p , S577-p

Fig 1-4. Post-translation modifications of Nrf2. Source: PhpsphositePlus (https://www.phosphosite.org/)

2.6. Protein stabilization of Nrf2

Despite evidence showing the effect of several Nrf2 activators on a wide variety of signalling pathways, little is known about the interplay among these pathways and how they coordinate to contribute to the turnover/fate of the Nrf2. The identification of GSK3β as a key regulator of Nrf2 stability has provided an insight into the activation of Nrf2 by phosphorylation and it may act as a common downstream effector for a number of Nrf2 inducers [111].

tert-Butylhydroquinone (tBHQ) stabilization of Nrf2 is dependent on the MAPK/ERK signalling cascade as Nrf2 induction by tBHQ is inhibited in the presence of MAPK/ERK inhibitors, suggesting that the MAPK/ERK signalling cascade drives this stability through phosphorylation [112]. p62, also known as sequestosome 1 (SQSTM1), is a ubiquitin-binding protein that targets protein aggregates for degradation via the autophagic pathway. p62 competes with Nrf2 for binding to Keap1, and binding of p62 to Keap1 leads to the degradation of Keap1 and the consequent Nrf2 stabilization [113, 114].

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STATEMENT OF PURPOSE

Nrf2 is a transcription factor that integrates cellular stress signals and rescues cells from a wide range of noxious stimuli. However, recent studies have revealed the constitutive overexpression of Nrf2 in transformed or cancerous cell lines and human tumor tissues, which may confer an advantage for cancer cell survival and growth. The peptidyl-prolyl cis-trans isomerase, Pin1 is overexpressed in many types of malignancies and promotes tumorigenesis through activation of distinct cancer-driving pathways. We speculate that Nrf2 harbouring the pSer/Thr-Pro motif provides a binding site for Pin1. We found that the expression of Pin1 and its mRNA transcript was highly increased in the MDA-MB-231 human breast cancer cell line, compared with the immortalized human benign breast epithelial MCF10A cells. Furthermore, genetic or pharmacologic inhibition of Pin1 blunted accumulation of the Pin1 substrate, Nrf2 and effectively suppressed proliferation and clonogenic activity of MDA-MB-231 cells and their growth in a xenograft mouse model. We found that Keap1 harbours putative binding sites for Pin1 binding. Occupying Keap1 pSer/Thr/Pro motifs by Pin1 may lead to the activation and nuclear translocation of Nrf2.

CHAPTER II

Pin1 stabilizes Nrf2 in a Keap1 independent manner in breast cancer

ABSTRACT

Peptidyl-prolyl *cis-trans* isomerase NIMA-interacting 1 (Pin1) has been frequently overexpressed in many types of malignancies, suggesting its oncogenic function. It recognizes phosphorylated serine or threonine of a target protein and isomerizes the adjacent proline residue, thereby altering folding, subcellular localization, stability, and function of target proteins. Recent studies have revealed constitutive overactivation of Nrf2, a redox-sensitive transcription factor that regulates cellular redox homeostasis, in certain transformed or cancerous cell lines and human tumor tissues. Aberrant activation of Nrf2 confers an advantage for cancer cell survival and growth. Since Nrf2 harbors the pSer/Thr-Pro motif, we investigated whether Pin1 could regulate the stability of Nrf2 in the context of its implications in breast cancer development and progression. This study indicates that mRNA and protein levels of Pin1 were highly increased in the human breast cancer MDA-MB-231 cell line compared with those in the non-tumorigenic MCF10A cells. Silencing of Pin1 by using siRNA or a pharmacologic inhibitor markedly increased the ubiquitination of Nrf2 and consequently reduced its stability, thereby suppressing proliferation and clonogenicity of MDA-MB-231 cells. In contrast, the overexpression of Pin1 resulted in accumulation of Nrf2 in the nucleus, without affecting its transcription. Notably, the phosphorylation of Nrf2 at serine 215, 408 and 577 is essential for its interaction with Pin1. Keap1, the negative regulator of Nrf2, was found to harbor Serine 104 and Threonine 277, which constitute the WW binding motif for interaction with Pin1. This interaction may lead to competition between Nrf2 and

Pin1 for Keap1 binding and consequently, Nrf2 can be stabilized. These findings, taken all together, suggest that Pin1 plays a role in breast cancer progression through stabilization and constitutive activation of Nrf2.

1. INTRODUCTION

Triple-negative breast cancer (TNBC) is an aggressive cancer subtype. It is the rarest form of breast cancer, yet still accounts for 15-20% of cases. It is unresponsive to anti-hormonal and Her2-targeted therapies, due to the absence of estrogen and progesterone receptors and excess Her2 receptor [1]. Consequently, TNBC patients are less likely to survive the first five years after diagnosis compared to those with other forms of breast cancer and are prone to relapse and death because of the higher tendency to metastasize [2]. With limited treatment options and a poorer prognosis, it is essential to continue further research on TNBC.

Peptidyl-prolyl *cis-trans* isomerase NIMA-interacting 1 (Pin1), consisting of an N-terminal WW and a C-terminal PPIase domains, is a member of the pervulin subfamily of peptidyl prolyl *cis*/*trans* isomerases (PPIases). It specifically isomerizes the proline residue of substrate proteins, preceded by phosphorylated Ser/Thr residues. By changing the conformation of the bound proteins, Pin1 modulates their subcellular localization, stability, interaction with other proteins, and biological activities [3, 4, 5]. Pin1 overexpression has frequently been observed in several types of malignancies including gastric, prostate and breast cancer [6, 7]. It regulates various cancer-related proteins such as β-catenin and Cyclin D1 via an isomerization-mediated conformational change [3, 4, 8].

Pin1 has been suggested to be a prognostic marker in several cancer types [9]. According to previous reports, Pin1 overexpression was associated with

transformation and uncontrolled growth of tumors [10, 11]. Ablation of Pin1 in HER2 or H-Ras transgenic mice or p53-knockout mice suppressed tumorigenesis [12-14]. The oncogenic activity of Pin1 is largely attributed to its ability to stabilize/activate oncoproteins and/or to destabilize/inactivate tumor suppressors [15, 16]. Many transcription factors and their regulators important for tumor development are known to be regulated by Pin1 [14, 16-20].

Nuclear factor E2-related factor 2 (Nrf2) is a leucine zipper transcription factor responsible for the cellular redox balance. Under basal conditions, Keap1 serves as a substrate scaffold protein for Cul3-containing E3 ubiquitin ligase, which can induce ubiquitin-proteasome degradation of Nrf2. Oxidants or electrophiles can modify the sensor cysteine residues of Keap1, which disrupts its interaction with Nrf2. As a result, Nrf2 is liberated from Keap1 and translocates to the nucleus where it binds to the antioxidant response element (ARE) or electrophile response elements (EpRE) present in the promoters of target genes [21-24].

In recent years, Nrf2 and its target proteins have been shown to play differential roles in cancer development and progression, acting as tumor suppressors or tumor promotors [22]. While transient induction of Nrf2 in normal cells activates cellular defense signalling against various oncogenic insults, constitutively elevated accumulation of Nrf2 in certain cancer cells can create a redox environment that favours tumor growth and provokes resistance to chemotherapy [25-29]. As such, high levels of Nrf2 in tumors are generally correlated with poor prognosis [27].

Nrf2 harbours multiple Ser/Thr-Pro motifs of which Serine 215 (S215), 408 (S408), and 577 (S577) residues were found to undergo phosphorylation [30], and can hence be a *bona fide* substrate of Pin1. This prompted us to explore the possibility that Pin1 binds and structurally modifies Nrf2, thereby hampering interaction with Keap1 for degradation. To test this possibility, we conducted a series of experiments to measure direct interaction between Pin1 and Nrf2 and to determine whether such interaction, if any, could be influenced by mutating aforementioned serine residues and inhibiting their phosphorylation/dephosphorylation. Additionally, we also examined whether the Pin1 could stabilize Nrf2 by binding to Keap1 and subsequently interrupting the sequestration of Nrf2 by Keap1.

2. MATERIALS AND METHODS

2.1. Reagents and antibodies

Dulbecco's modified Eagle's medium (DMEM), penicillin/streptomycin mixtures and fetal bovine serum (FBS) were obtained from Gibco BRL (Grand Island, NY, USA). TRIzol® and Stealth™ RNAi negative control duplexes were purchased from Invitrogen Life Technologies, Inc. (Carlsbad, CA, USA). Primary antibodies for goat monoclonal antibody against Pin1 (sc-46660), goat monoclonal (sc-81342) antibody against Nrf2, and goat monoclonal antibody against Keap1 (sc-365626) were supplied by Santa Cruz Biotechnology (Santa Cruz, CA, USA). Primary antibody for goat polyclonal antibody against Nrf2 (ab137550) was supplied by Abcam (Cambridge, UK). Polyclonal antibody against HA-tag (71-5500) was a product of Thermo Fisher Scientific (Thermo Fisher; MA, USA). Monoclonal antibody against Flag-tag (F1804-1MG) was a product of Sigma-Aldrich (St. Louis, MO, USA). Monoclonal antibody against Myc-tag (sc-9E10) was a product of Santa Cruz Biotechnology (Santa Cruz, CA, USA). Secondary antibodies were purchased from Zymed Laboratories Inc. (San Francisco, CA, USA). Dithiothreitol (DTT), and cycloheximide (CHX) were purchased from Sigma Aldrich (St. Louis, MO, USA). Western blot detection kit (Absignal) was obtained from Abclon (Seoul, South Korea). Control and *Pin1* targeting si-RNA were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All other chemicals used were in the purest form available commercially. Calf intestinal alkaline phosphatase (CIP CIP; cat.

number, P0114) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Human breast tumor specimens as well as adjacent normal tissues for Western blot analysis of Pin1, Nrf2 and Keap1 proteins were obtained from the biorepository of Lab of Breast Cancer Biology at the Cancer Research Institute, Seoul National University (IRB No., 1405-088-580). Human breast cancer tissue slides (including both adjacent and malignant tissues), obtained from the Yonsei University Hospital, were used for immunofluorescence staining.

2.2. Cell culture

Human breast cancer (MDA-MB-231) and human embryonal kidney (HEK293T) cell lines obtained from American type culture collection were maintained in DMEM containing 5% FBS at 37°C in a 5% CO_2/95% air incubator.

2.3. Anchorage-independent growth assay

To prepare the hard agar layer, 2.5 ml of the boiled agarose solution (3.3%) dissolved in phosphate-buffered saline (PBS) was added immediately to 60-mm dishes using a pre-warmed pipette and then kept in the 37 °C incubator to solidify. To prepare the soft agar layer containing the cells, MDA-MB-231 (1×10^5) cells were suspended in the 0.33% agarose solution with gentle mixing, and 2.5 ml of this solution was inoculated on top of the hard agar layer. After allowing the solution to harden as a soft agar for 4 h, 2.5 ml of the fresh medium was added to the top of the hardened soft agar layer followed by all-*trans* retinoic acid (ATRA) treatment.

After one week of incubation, anchorage-independent growth (spherical formation containing >10 cells) was scored using a light microscope. The total number of foci per 1×10^5 cells in a well was counted. For experiments with siPin1 MDA-MB-231 cells, cells were plated in 6-well plates at a density of 150 cells per well. The DMEM medium was changed every other day. After one week of incubation, the colonies were fixed in cold methanol and stained by 0.5% crystal violet for 4 h. The stained colonies were washed with PBS to remove the excess dye. Quantitative changes in clonogenicity were determined by extracting stained dye with 10% acetic acid, and the absorbance at 570 nm was measured.

2.4. Wound healing assay

MDA-MB-231 cells pre-treated with control or Pin1 siRNA (72 h incubation) were plated into the ibidi culture insert on 6 well dishes. After 5- or 7- h incubation for appropriate cell attachment, the culture-insert was gently removed by using sterile tweezers. Cell migration was observed under the microscope.

2.5. Preparation of cytosolic and nuclear extracts

Cells were pelleted by centrifugation at 1700 x *g* for 5 min after washing with cold PBS and suspended in ice-cold hypotonic buffer A [10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT and 0.2 mM phenylmethylsulfonyl fluoride (PMSF). Following incubation in an ice bath for 15 min, cells were centrifuged again at 6,000 x *g* for 5 min and the supernatant was collected as a cytosolic fraction.

The remaining cell pellets were washed by buffer A, twice and resuspended in ice-cold buffer C containing 20 mM HEPES (pH 7.9), 20% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, and 0.2 mM PMSF and were incubated at 0°C for 1 h. After vortex mixing, the resulting suspension was centrifuged at 18,000 x *g* for 15 min, and the supernatant was collected as a nuclear extract and stored at -70°C.

2.6. *In situ* proximity ligation assay (PLA)

PLA was carried out using the DUOLinkTM kit (OLINK; Uppsala, Sweden) according to the manufacturer's instructions. In brief, MDA-MB-231 cells transfected with Myc-Nrf2 and pcDNA-Pin1 or Flag-Keap1 and pcDNA-Pin1, on glass coverslips were fixed, permeabilized, and blocked with blocking solution (0.1% Triton in PBS containing 5% bovine serum albumin) and incubated with the antibodies against Pin1 monoclonal (1:100), Nrf2 polyclonal (1:200), Pin1 polyclonal (1:200), and Keap1 monoclonal (1:100) overnight at 4°C. PLA plus and minus affinity probes were then added and incubated for an additional 1 h at 37°C. The probes were hybridized using a ligase to be a closed circle. The DNA was then amplified (a rolling-circle amplification) and detected by fluorescence microscopy.

2.7. Xenograft assay

For the xenograft assay, 4-week-old male BALB/c nude mice were purchased from Central Lab Animal Inc. (Seoul, South Korea). After one week of the acclimation

period, 5×10^6 scrambled or Pin1 siRNA transfected MDA-MB-231 cells re-suspended in equal volumes of PBS and Matrigel (total volume of 200 μl were subcutaneously injected into the flanks of mice to generate breast cancer xenograft tumors (n=4 per group). The tumor size was regularly measured with digital calipers and calculated according to the formula, $V=0.5\ ab^2$, where 'a' is the longest and 'b' is the shortest perpendicular diameters. After mice were killed, tumors were excised and fixed in formalin for further analysis. All experimental protocols for animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of Seoul National University.

2.8. Tissue array

Human paraffin-embedded breast cancer tissue array with adjacent normal tissues (US Biomax, Inc., cat. no. BC08118a; Rockville, MD, USA) was subjected to deparaffinization with xylene. Following antigen retrieval by heated citrate buffer, sections were permeabilized and blocked according to the standard protocol. After overnight incubation at 4°C with anti-Pin1, and anti-Nrf2 antibodies, the tissue sections were washed with PBS and then labeled with secondary antibody conjugates of traditional fluorophores, such as fluorescein (FITC), tetramethyl-rhodamine (TRITC) for 1 h at room temperature. The slides were then analyzed under a fluorescent microscope.

Human breast cancer slides, obtained from the Breast Center of Servance Hospital, Younsei University, were used to detect Pin1, Nrf2 and Keap1 proteins by

immunofluorescence staining. Preparation of tissue was approved by the Institutional Review Board (IRB) and the use of these tissue array slides was exempt as anonymous, archived specimens. For antibody staining, tissue slides were deparaffinized, rehydrated through an alcohol series, and then boiled in Antigen Unmasking Solution. After incubation with a blocking solution containing 10% bovine serum albumin in PBS, the sections were stained overnight at 4°C with 1:100 dilution of a Pin1 and Keap1 primary antibodies and 1:200 dilution of Nrf2 antibody. After incubation with a secondary antibody (Alexa 488 for the green signal and Alexa 546 for red; Invitrogen), sections were mounted in PBS containing 4′, 6′-diamidino-2-phenylindole (DAPI; Invitrogen). The slides were then visualized under a fluorescent microscope.

2.9. QuantSeq 3'mRNA sequencing Library

2.9.1. RNA isolation

Total RNA was isolated using Trizol® reagent (Invitrogen). The RNA quality was assessed by Agilent 2100 bioanalyzer using the RNA 6000 Nano Chip (Agilent Technologies, Amstelveen, The Netherlands), and RNA quantification was performed using ND-2000 Spectrophotometer (Thermo Inc., DE, USA).

2.9.2. Library preparation and sequencing

For control and test RNAs, the construction of the library was performed using QuantSeq 3' mRNA-Seq Library Prep Kit (Lexogen, Inc.; Vienna, Austria) according to the manufacturer's instructions. In brief, each total RNA was prepared and an oligo-dT primer containing an Illumina-compatible sequence at its 5' end was hybridized to the RNA and reverse transcription was performed. After the degradation of the RNA template, second-strand synthesis was initiated by a random primer containing an Illumina-compatible linker sequence at its 5' end. The double-stranded library was purified by using magnetic beads to remove all reaction components. The library was amplified to add the complete adapter sequences required for cluster generation. The finished library is purified from PCR components. High-throughput sequencing was performed as single-end 75 sequencings using NextSeq 500 (Illumina, Inc.; San Diego, CA, USA).

2.9.3. Data analysis

QuantSeq 3' mRNA-Seq reads were aligned using Bowtie2. Bowtie2 indices were either generated from the genome assembly sequence or the representative transcript sequences for aligning to the genome and transcriptome. The alignment file was used for assembling transcripts, estimating their abundances and detecting differential expression of genes. Differentially expressed gene was determined based on counts from unique and multiple alignments using coverage in Bedtools. The RC (Read Count) data were processed based on the quantile normalization method using EdgeR within R using Bioconductor. Gene classification was based

on searches done by DAVID (http://david.abcc.ncifcrf.gov/) and Medline databases (http://www.ncbi.nlm.nih.gov/). The results were presented as mean ± SD. To determine the statistical significance, the Student's unpaired *t*-test was used, and *p*-value of less than 0.05 was considered significant.

2.10. Western blot analysis

MDA-MB-231 cells were lysed in lysis buffer [250 mM sucrose, 50 mM Tris-HCl (pH 8.0), 25 mM KCl, 5 mM $MgCl_2$, mM EDTA, 2 mM NaF, 2 mM sodium orthovanadate, and 1 mM PMSF for 1 h on ice followed by centrifugation at 18,000 x *g* for 20 min. The protein concentration of the supernatant was measured by using the BCA reagents (Pierce; Rockford, IL, USA). Protein (30 μg) was separated by running through 8% and 12% SDS-PAGE gel and transferred to the PVDF membrane (Gelman Laboratory; Ann Arbor, MI, USA). The blots were blocked with 5% non-fat dry milk PBST buffer for 1 h at room temperature. The membranes were incubated overnight at 4°C with 1:1,000 dilution of one of the antibodies of Pin1 (Santa Cruz Biotechnology; Santa Cruz, CA, USA), Nrf2 (Santa Cruz Biotechnology; Santa Cruz, CA, USA and Abcam; Cambridge, UK) or Keap1 (Santa Cruz Biotechnology; Santa Cruz, CA, USA). Equal lane loading was assured using β-actin (Sigma-Aldrich Co; St. Louis, MO, USA). The blots were rinsed three times with PBST buffer for 10 min each. Washed blots were treated with 1: 5,000 dilution of the horseradish peroxidase conjugated-secondary antibody (Pierce Biotechnology; Rockford, IL, USA) for 1 h and washed again three times with PBST buffer. The transferred proteins were visualized with an enhanced

chemiluminescence detection kit (Amersham Pharmacia Biotech; Buckinghamshire, UK). The blots were quantified by using Gel-Pro Analyzer to calculate the mass/IOD of each lane. Each protein of 3 independent analyses was normalized with β-actin by using an excel program. We used Prism and Sigma Plot for quantification.

Human breast cancer tissues (including both adjacent and malignant tissues), obtained from the archives of the Breast Care Center of Seoul National University Hospital (SNUH), were also used to detect Pin1, Nrf2 and Keap1 proteins by Western blot analysis.

2.11. Immunoprecipitation

MDA-MB-231 cells, human tissues and xenograft tissues were lysed in 250 mM sucrose, 50 mM Tris-HCl (pH 8.0), 25 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA, 2 mM NaF, 2 mM sodium orthovanadate, and 1 mM PMSF. Total protein (100 μg) was subjected to immunoprecipitation by rotation with Nrf2, Keap1, Myc, HA, or Flag primary antibodies at 4°C for overnight followed by the addition of protein A/G-agarose bead suspension (25% slurry, 40 μl). After centrifugation at 1,000 x *g* for 1 min, immunoprecipitated beads were collected by discarding the supernatant and washed with cell lysis buffer. The immunoprecipitate was then resuspended in 26 μL of lysis buffer and 4 μl of 5X dye and boiled for 5 minutes. The supernatant from each sample was collected by centrifugation and loaded on SDS-polyacrylamide gel.

2.12. Immunohistochemistry

For immunohistochemical analysis of the expression of Pin1, Nrf2, and Keap1, 4 mm sections of 10% formalin-fixed, paraffin embedded tissues from breast cancer patients were placed on glass slides and deparaffinized 3 times with xylene and rehydrated through graded alcohol bath. The deparaffinised sections were heated by using microwave and boiled twice for 10 min in (Target Retrieval sol. pH 9.0, DAKO, S2367). To diminish non-specific staining, each section was treated with 3% hydrogen peroxide blocking solution for 10 min. For the detection of respective protein expression, slides were incubated with Pin1 (1:100), Nrf2 (1:100), and Keap1 (1:50) antibodies at room temperature for 120 min in TBST followed by treatment with respective horseradish peroxidase-conjugated secondary antibody (rabbit/Mouse, DAKO, K5007). The peroxidase binding sites were detected by staining with 3,3'-diaminobenzidine tetrahydrochloride. Finally, counterstaining was performed using Mayer's hematoxylin. Stained tissues were visualized under a microscope and photographed.

2.13. Small interfering RNA (siRNA) and plasmid transient transfection

siRNA specifically targeting *Pin1* and non-specific siRNA were purchased from Santa Cruz Biotechnology (sc-36230). Full-length Nrf2 and all Nrf2 mutants were kindly provided by Professor Donna Zhang, University of Arizona. Full-length Keap1 and Keap1 mutants were generated by Cosmo Genetech Company.

HEK293T cells were seeded at a density of 2 x 10^5 cells/ml in 100-mm dish and grown to 90% confluence in complete growth media. Transient transfections with PCI-HA-Nrf2 as well as Flag-Keap1 derivatives were performed using the Lipofectamine 2000 transfection reagents according to the instructions supplied by the manufacturer (Invitrogen; Carlsbad, CA, USA). After 12 to 24 h transfection, cells were lysed for Western blot analysis.

2.14. Protein stability assay

The MDA-MB-231 cells after 72 h transfection with control or *Pin1* siRNA were treated with 10 μM CHX to block protein synthesis. The cells were collected at different time intervals for Western blot analysis.

2.15. Protein dephosphorylation assay

The dephosphorylation assay was conducted in accordance with the supplier's instructions. The HEK293T cells transfected with Myc-Nrf2 and pcDNA-Pin1 for 24 h. The lysate for the phosphatase treatment group was re-suspended in the CIP buffer. To the lysate (30 μg) was added CIP (1 unit of per μg protein), and the mixture was incubated for 60 min at 37°C before SDS-PAGE.

2.16. Statistical Analysis

All data are presented as the mean ± SD. Experiments were repeated at least three

times. Two-tailed unpaired Student's t tests or one-way ANOVA were used to evaluate the data. Statistical differences were considered significant at $*p < 0.05$; $**p < 0.01$, and $** p < 0.001$

3. RESULTS

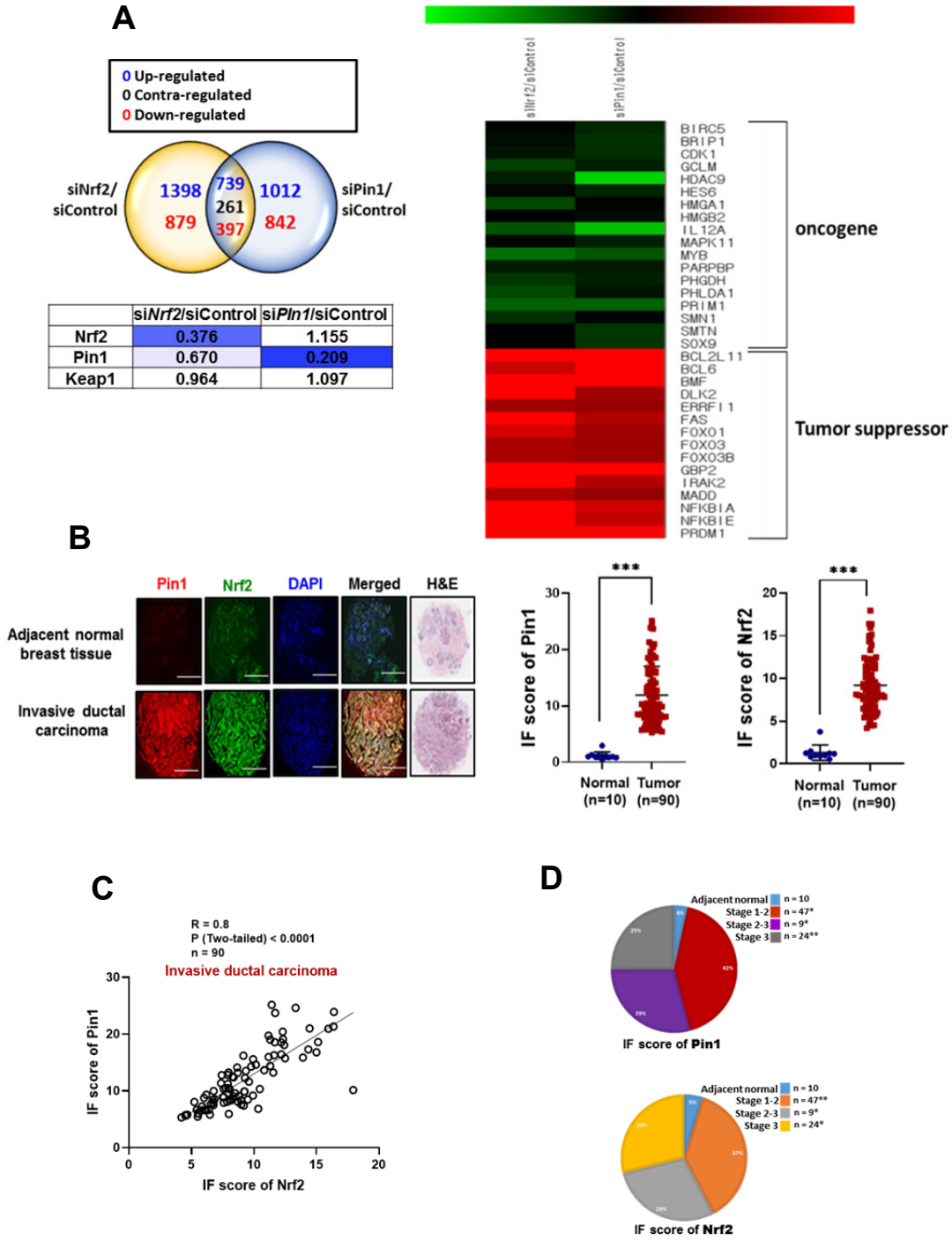
3.1. Pin1 and Nrf2 are overexpressed and correlated each other in human breast cancer

To investigate the correlation between Pin1 and Nrf2 in breast cancer, MDA-MB-231 cells were transfected with siRNA control, siNrf2 or siPin1 and subjected to the measurement of gene expression by RNASeq analysis. The in cells with a deficiency of Nrf2 or Pin1, as compared to control cells, were identified followed by a systemic analysis to narrow down the list of commonly expressed genes. There were 739 upregulated and 397 downregulated genes differentially expressed in cells knockdown for *Nrf2* and *Pin1*. Among these DEGs, 261 genes had identical trends (**Fig. 2-1A**).

Next, we investigated the clinical relevance of Nrf2 and Pin1 to breast cancer progression. For this purpose, we performed a microarray of 90 breast cancer tissues and 10 adjacent normal ones. While normal tissues exhibited relatively low immunofluorescence (IF) signals upon staining with antibodies recognizing Nrf2 and Pin1, the invasive ductal carcinomas showed highly enhanced intensities reflecting co-localization of both proteins (**Fig. 2-1B**). The positive correlation between Nrf2 and Pin1 was found in invasive ductal carcinoma tissues **(Fig. 2-1C)**. Moreover, IF scores of both proteins correlate with disease stages (**Fig. 2-1D**).

The overexpression of Nrf2 and Pin1 in breast cancer patients was further confirmed by Western blot analysis (**Fig. 2-2A**). Pin1 and Nrf2 were significantly

overexpressed in tumor tissues compared with those in adjacent normal tissues. We verified the co-localization of Nrf2 and Pin1 in the two molecular subtypes (luminal and TNBC) of cancerous tissues (140 each) compared with 50 normal tissues (**Fig. 2-2B**). The co-localization of Nrf2 and Pin1 was higher in both luminal and TNBC type tumors than normal tissues (**Fig. 2-2C** and **Fig. 2-2D**). Notably, a higher degree of co-localization was observed in the more aggressive TNBC than luminal breast cancer. IF staining of serial tissue microarray for Nrf2 and Pin1 indicated a positive correlation between these proteins in luminal and TNBC patient tissues, 140 each (**Fig. 2-2C** and **Fig. 2-2D**).



	si*Nrf2*/siControl	si*Pln1*/siControl
Nrf2	0.376	1.155
Pin1	0.670	0.209
Keap1	0.964	1.097

Figure 2-1. Overexpression of Pin1 and Nrf2 and their correlation in breast cancer

(A) MDA-MB-231 cells transfected with siRNA control, siNrf2 or siPin1 were subjected to the measurement of gene expression by RNASeq. The DEGs of cells with a deficiency of Nrf2 or Pin1, as compared to control cells, were identified followed by a systemic analysis to narrow down the list of commonly expressed genes. (B) Representative H&E and IF images of Pin1 and Nrf2 in breast cancer or adjacent normal tissues. Expression of Pin1 and Nrf2 was found to be higher in breast cancer tissues than in adjacent normal tissues, as determined by IF score (tissue microarray). The statistical significance was determined by two-tailed unpaired Student's *t*-test method. ***$p < 0.001$. (C) Spearman analysis of IF data showed that Pin1 was positively associated with Nrf2 ($r = 0.8$). (D) The percentage of breast cancer patients in different stages of the disease according to the levels of Pin1 and Nrf2 based on IF score.

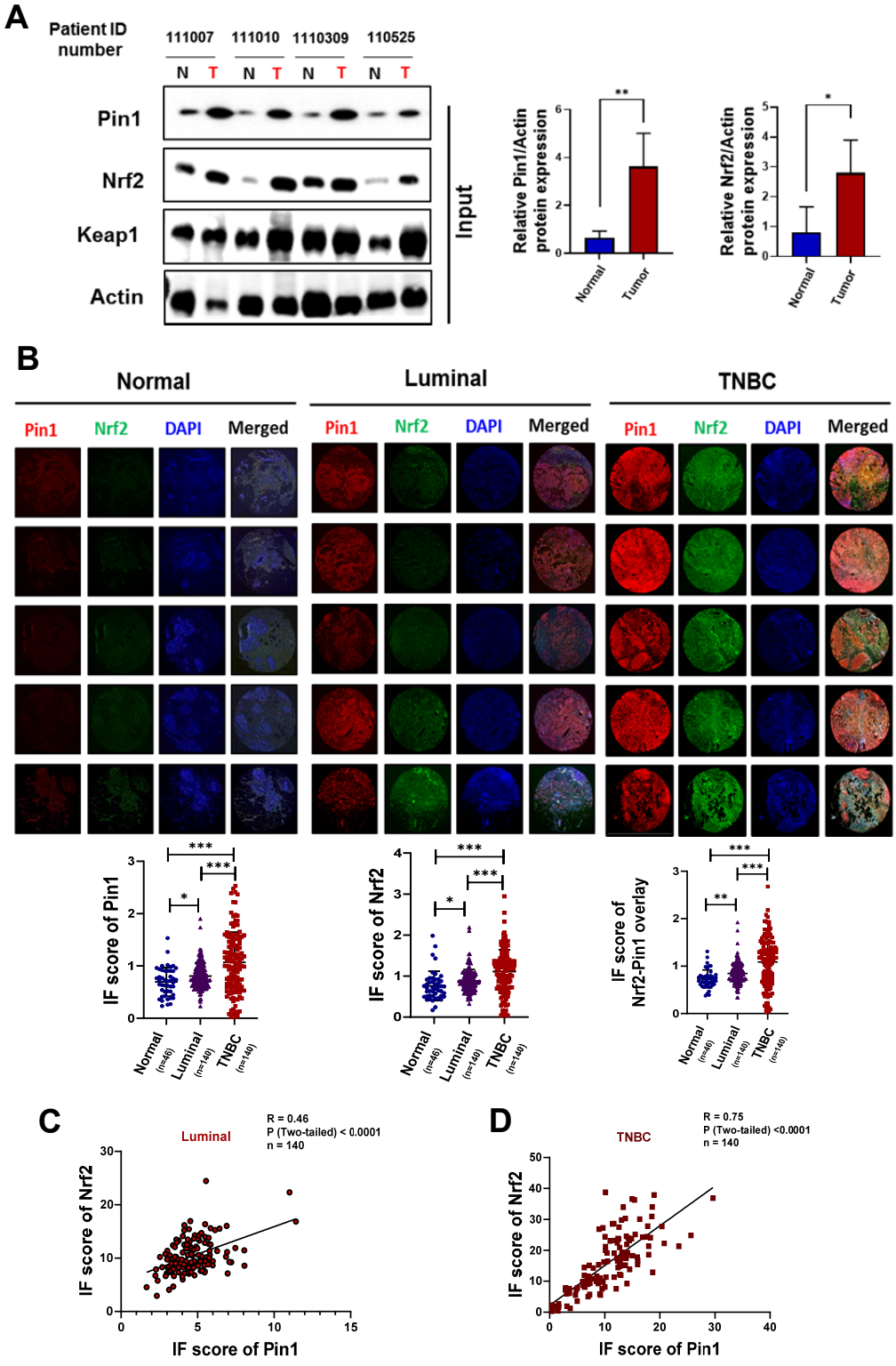

A
Patient ID number
111007
111010
1110309
110525
N T N T N T N T
Pin1
Nrf2
Keap1
Actin
Input
Relative Pin1/Actin protein expression
Relative Nrf2/Actin protein expression
Normal
Tumor
B
Normal
Luminal
TNBC
Pin1
Nrf2
DAPI
Merged
IF score of Pin1
IF score of Nrf2
IF score of Nrf2-Pin1 overlay
C
Luminal
R = 0.46
P (Two-tailed) < 0.0001
n = 140
IF score of Nrf2
IF score of Pin1
D
TNBC
R = 0.75
P (Two-tailed) <0.0001
n = 140
IF score of Nrf2
IF score of Pin1

Figure 2-2. Clinical relevance of Nrf2 and Pin1 determined by the tissue array

(A) The overexpression of Nrf2 and Pin1 in breast cancer patients was confirmed by Western blot analysis. Data are shown as the mean ± SD, and the statistical significance was determined by Student's *t*-test. *$p < 0.05$, and **$p < 0.01$. (B) Co-localization of Pin1 and Nrf2 in normal, luminal breast cancer and TNBC tissues was determined by IF analysis. Fifty normal and 140 each of luminal breast cancer and TNBC specimens were exposed to anti-Nrf2 and anti-Pin1 antibodies, and the IF scores of both proteins were measured for all samples and quantified. Representative images of 5 out of 140 stained specimens of each molecular sub-type are displayed, but the quantification was done with all samples. Data are shown as the mean ± SD (normal tissues; n=50, luminal and TNBC; n=140 each), and the statistical significance was determined by two-tailed unpaired Student's *t*-test method. (C and D) Spearman analysis of IF data showed that Pin1 was positively associated with Nrf2 in luminal (r = 0.46) and significantly in TNBC (r = 0.75) tissues compared with that in normal tissues ($r = - 0.2999$).

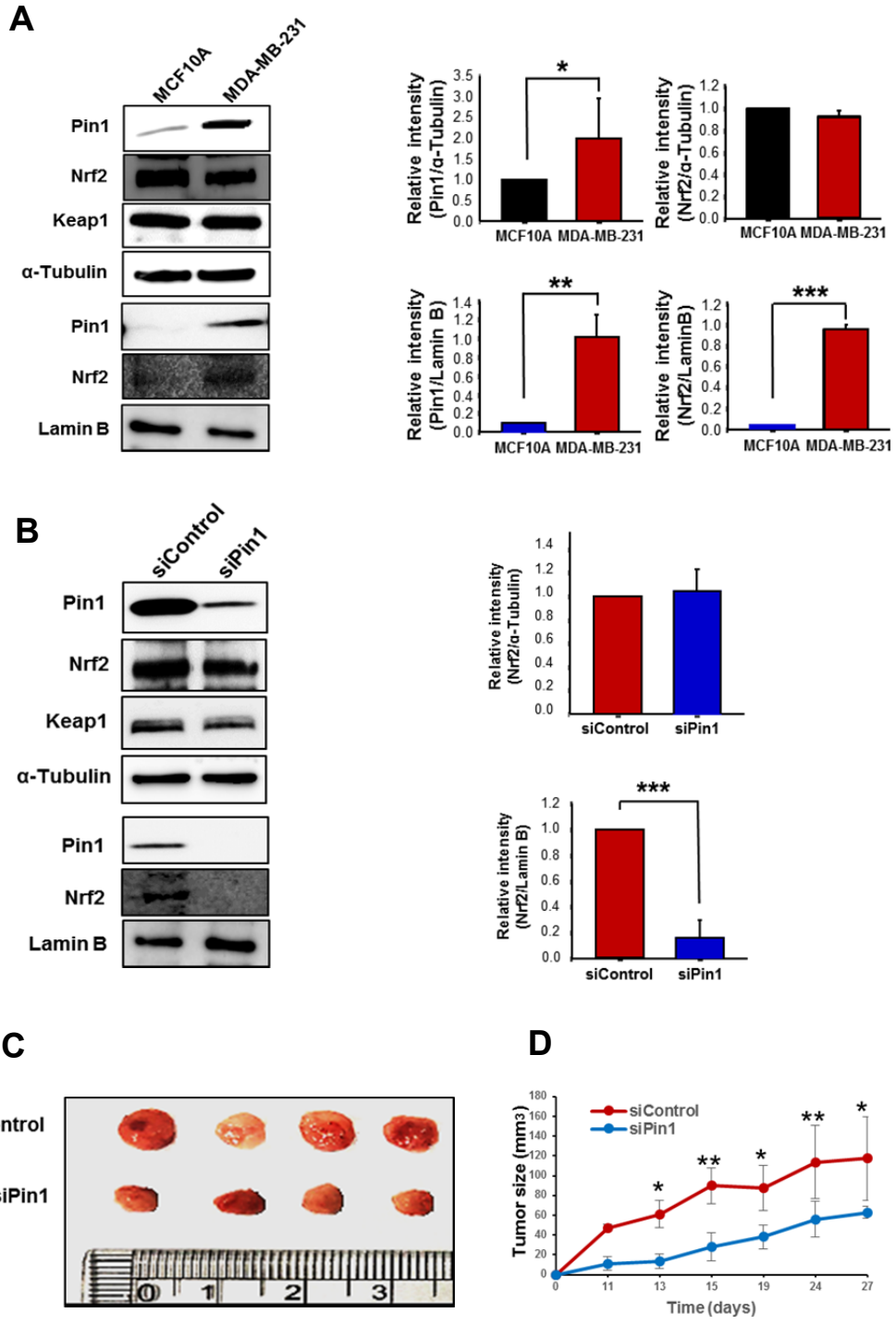
3.2. Pin1 upregulates Nrf2 expression in breast cancer cells and stimulates their growth in culture and in a xenograft mouse model

In order to explore the role of Nrf2 and Pin1 in breast cancer development and progression, we first compared their expression in MCF-10A cells, an immortalized non-oncogenic human breast epithelial cells, with that in malignant MDA-MB-231 human breast cancer cells. We found that the protein level of Pin1 was higher in both cytoplasmic and nuclear fractions of MDA-MB-231 cells compared with that in MCF-10A cells (**Fig. 2-3A).** Nrf2 protein was detected at a similar level in the cytoplasmic fraction of both cell lines, but there was markedly elevated nuclear accumulation in MDA-MB-231 cells (**Fig. 2-3A**). In another experiment, we treated MDA-MB-231 cells with either control siRNA or *Pin1* siRNA. The silencing of *Pin1* did not affect the expression of cytoplasmic Nrf2 protein (**Fig. 2-3B upper**). However, the nuclear accumulation of Nrf2 was abolished in the *Pin1* silenced cells (**Fig. 2-3B lower**).

To investigate the significance of the regulation of Nrf2 by Pin1 *in vivo*, we inoculated MDA-MB-231 cells treated with control or *Pin1* siRNA into nude mice. Mice treated with *Pin1* silenced cells developed tumors with a significantly reduced size compared with those injected with control cells (**Fig. 3C** and **Fig. 3D**). Both Pin1 and Nrf2 were overexpressed in the xenograft tumors derived from breast cancer cells transfected with control siRNA, but suppressed by siRNA knockdown of *Pin1* (**Fig. 3E**). The tumors were then collected and subjected to Western blot analysis. Tumors with silenced *Pin1* exhibited obvious depletion of Nrf2 and reduced expression of PCNA and VEGF which are representative proliferation and

pro-angiogenic markers, respectively. Acquisition of epithelial-mesenchymal transition (EMT) features is critical for cancer cell invasion and metastasis during tumor progression. *Pin1* knockdown also altered the expression of EMT markers, with N-cadherin and vimentin down-regulated and E-cadherin upregulated (**Fig. 2-3F**).

The regulation of breast cancer cell growth by Pin1 was further evidenced by marked reduction in colony formation when the MDA-MB-231 cells were treated with *Pin1* siRNA (**Fig. 2-4A**). Moreover, *Pin1* knockdown significantly attenuated the invasiveness (**Fig. 2-4B**) and migrative capability (**Fig. 2-4C**) of MDA-MB-231 cells, which were assessed by the trans-well migration assay and the wound healing assay, respectively. Similarly, pharmacologic inhibition of *Pin1* activity with ATRA abrogated the colony formation (**Fig. 2-4D**) as well as nuclear translocation of Nrf2 **(Fig. 2-4E)** in MDA-MB-231 cells.


A
MCF10A
MDA-MB-231
Pin1
Nrf2
Keap1
α-Tubulin
Pin1
Nrf2
Lamin B
Relative intensity (Pin1/α-Tubulin)
Relative intensity (Nrf2/α-Tubulin)
Relative intensity (Pin1/Lamin B)
Relative intensity (Nrf2/LaminB)
MCF10A MDA-MB-231
B
siControl
siPin1
Pin1
Nrf2
Keap1
α-Tubulin
Pin1
Nrf2
Lamin B
Relative intensity (Nrf2/α-Tubulin)
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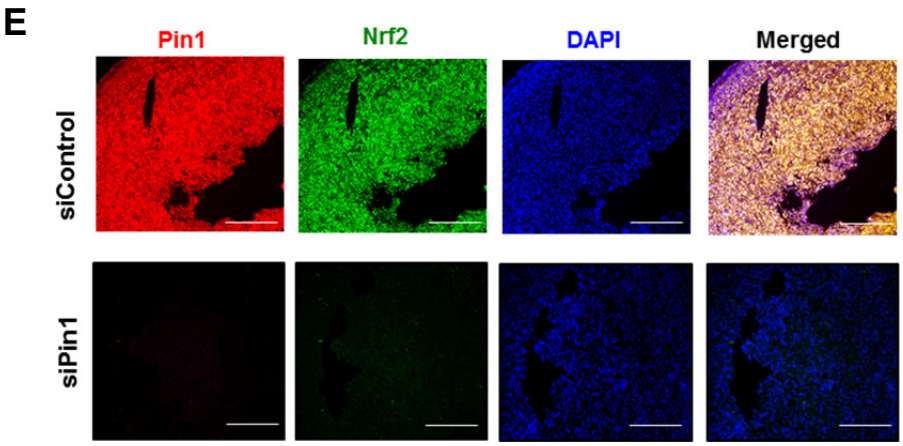
siPin1
D
siControl
siPin1
Tumor size (mm3)
Time (days)


E
Pin1
Nrf2
DAPI
Merged
siControl
siPin1

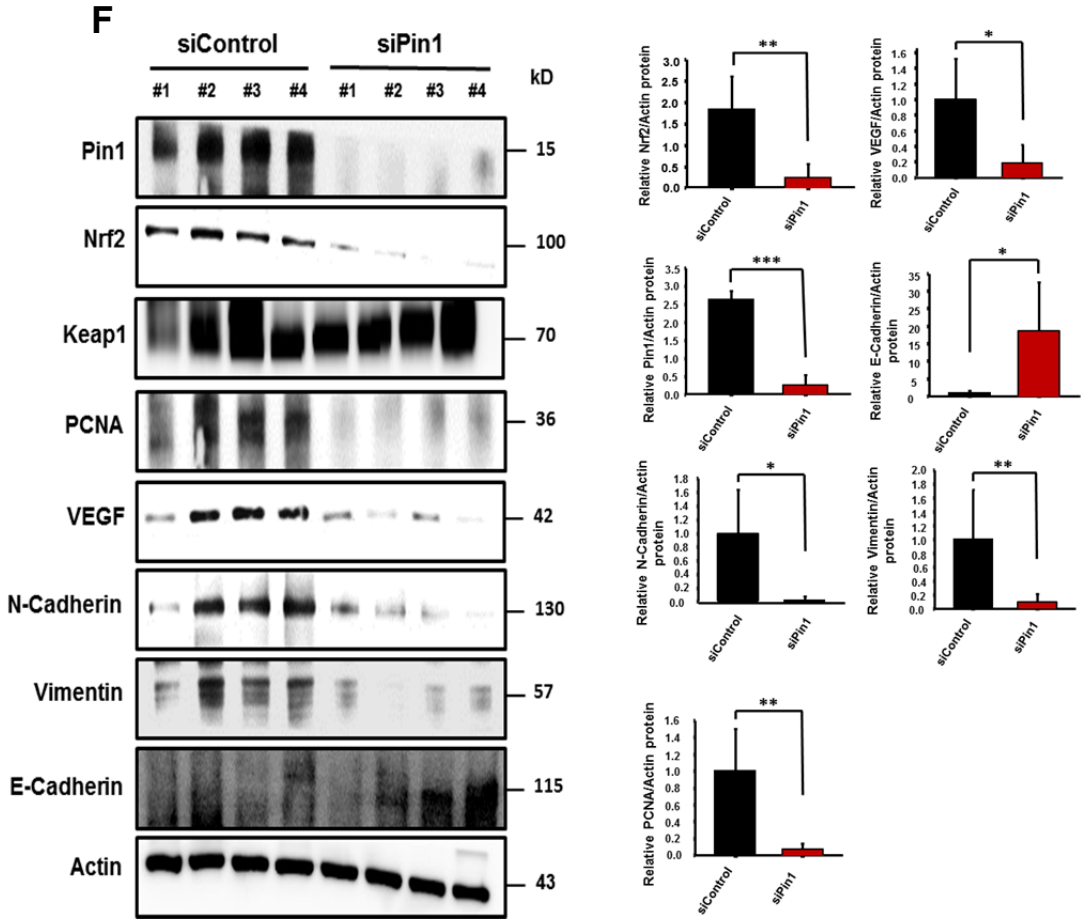
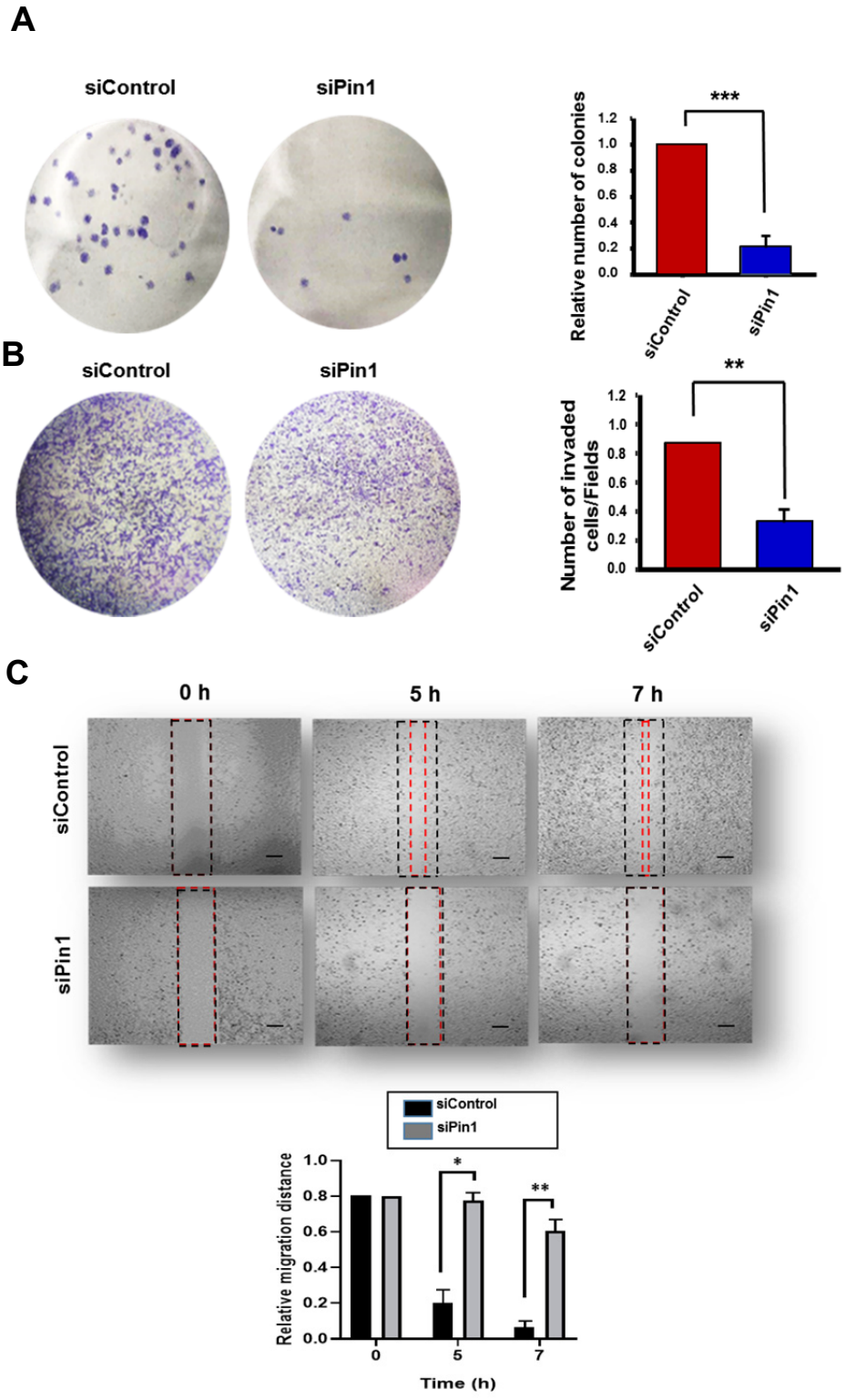
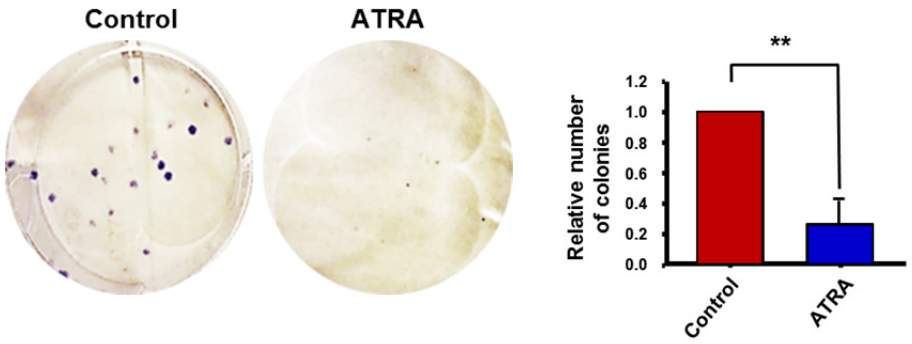

F
siControl
siPin1
#1 #2 #3 #4 #1 #2 #3 #4
kD
Pin1
15
Nrf2
100
Keap1
70
PCNA
36
VEGF
42
N-Cadherin
130
Vimentin
57
E-Cadherin
115
Actin
43
Relative Nrf2/Actin protein
Relative VEGF/Actin protein
Relative Pin1/Actin protein
Relative E-Cadherin/Actin protein
Relative N-Cadherin/Actin protein
Relative Vimentin/Actin protein
Relative PCNA/Actin protein
siControl
siPin1

Figure 2-3. Pin1-mediated upregulation of Nrf2 in breast cancer cells and stimulation of their growth in a xenograft model

(A) Comparative expression of Pin1 and Nrf2 proteins in the cytoplasmic and nuclear fractions of MCF10A and MDA-MB-231 cells. Data are shown as the mean ± SD of three independent experiments, and the statistical significance was determined by Student's *t*-test. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$. (B) Effects of *Pin1* silencing on Nrf2 protein expression in the cytoplasmic and nuclear fractions of MDA-MB-231 cells. Data are shown as the mean ± SD of three independent experiments, and the statistical significance was determined by Student's *t*-test. ***$p < 0.001$. (C, D) Effects of *Pin1* silencing on the growth of xenograft tumor growth. MDA-MB-231 cells transfected with control or *Pin1* siRNA treated to nude mice, and the tumor size was measured at the indicated time intervals (mean ± SEM; n=4). *$p < 0.05$ and **$p < 0.01$. (E) Overexpression and co-localization of Pin and Nrf2 in xenograft tumor tissues. (F) Effects of Pin1 silencing on expression of Nrf2 and, proliferative and EMT marker proteins in the xenograft tumor tissues. Data are shown as the mean ± SD (n=4), and the statistical significance was determined by Student's *t*-test. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.


A
siControl
siPin1
Relative number of colonies
***
1.2
1.0
0.8
0.6
0.4
0.2
0.0
siControl
siPin1
B
siControl
siPin1
Number of invaded cells/Fields
**
1.2
1.0
0.8
0.6
0.4
0.2
0.0
siControl
siPin1
C
0 h
5 h
7 h
siControl
siPin1
siControl
siPin1
Relative migration distance
*
**
1.0
0.8
0.6
0.4
0.2
0.0
0
5
7
Time (h)

D


Control
ATRA
**
Relative number of colonies
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Control
ATRA

E

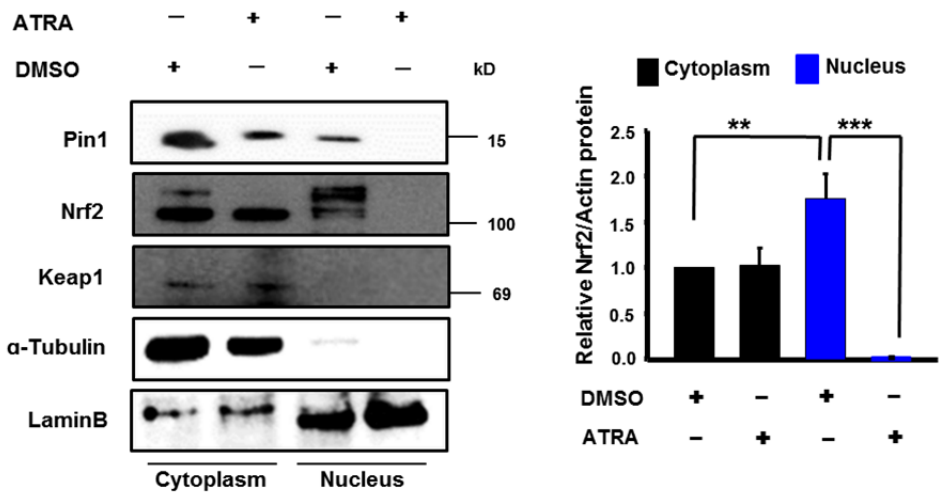

ATRA
DMSO
kD
Pin1
Nrf2
Keap1
α-Tubulin
LaminB
15
100
69
Cytoplasm
Nucleus
Cytoplasm
Nucleus
Relative Nrf2/Actin protein
**
***
2.5
2.0
1.5
1.0
0.5
0.0
DMSO
ATRA

Figure 2-4. Involvement of Pin1 in proliferation, migration and invasiveness of human breast cancer cells and nuclear accumulation of Nrf2

(A) MDA-MB-231 cells seeded in 6-well plates were treated with control or *Pin1* siRNA as described in Materials and Methods. Attached cells were photographed after crystal violet staining, and the proportion of attached cells was quantified by counting the number of colonies. A representative set of images from three independent experiments is shown. Data are shown as the mean ± SD, and the statistical significance was determined by Student's *t*-test. ***$p < 0.001$. (B) Invasiveness of MDA-MB-231 cells was measured using 24-well microchemotaxis chambers. The randomly chosen fields were photographed, and the number of cells migrated to the lower surface was counted. Data are shown as the mean ± SD (n=3), and the statistical significance was determined by Student's *t*-test. **$p < 0.01$. (C) MDA-MB-231 cells were transfected with control or *Pin1* siRNA and incubated for 72 h. Then, cell migration was visualized under a confocal microscope. Data are shown as the mean ± SD (n=3), and the statistical significance was determined by Student's *t*-test. *$p < 0.05$ and **$p< 0.01$. (D) The effects of the Pin1 inhibitor ATRA on growth of MDA-MB-231 cells. The cells were treated with ATRA (50 μM) for 48 h as described in Material and Methods, and the formation of colonies was measured. Data are shown as the mean ± SD (n=3), and the statistical significance was determined by Student's *t*-test. **$p < 0.01$. (E) The effects of ATRA on expression of Nrf2 in MDA-MB-231 cells. The cells were treated with ATRA (50 μM) for 48 h, and the expression of Nrf2 as well as Pin1 was measured

by Western blot analysis. Data are shown as the mean ± SD (n=3), and the statistical significance was determined by Student's *t*-test. $^{**}p < 0.01$ and $^{***}p < 0.001$.

3.3. Pin1 physically interacts with Nrf2

As Pin1 co-localizes with Nrf2 (**Fig. 2-1B** and **Fig. 2-2B**), we investigated whether both proteins could physically interact each other. The interaction was verified by PLA, which detects an enhanced fluorescent signal when two proteins are localized in proximity (**Fig. 2-5A**). The interaction between Nrf2 and Pin1 was further confirmed by a co-immunoprecipitation assay in MDA-MB-231 cells (**Fig. 2-5B**). In contrast to MDA-MB-231 cells, MCF-7 breast cancer cells have relatively low expression levels of Nrf2 and Pin1 (data not shown). Therefore, we overexpressed Nrf2 and Pin1 in MCF-7 cells to further verify the interaction between those two proteins. There was no pronounced interaction of HA-tagged Pin1 with Myc-tagged Nrf2 in the cytoplasm of MCF-7 cells, whereas interaction was detectable predominantly in the nucleus (**Fig. 2-5C**). Such interaction was also observed in xenograft tumors derived from MDA-MB-231 cells, but abolished in *Pin1* knockdown xenograft tumors (**Fig. 2-5D**). We also found that human breast cancer tissues exhibited the significantly elevated levels of the Pin1-Nrf2 complex, compared with normal tissues (**Fig. 2-5E**).

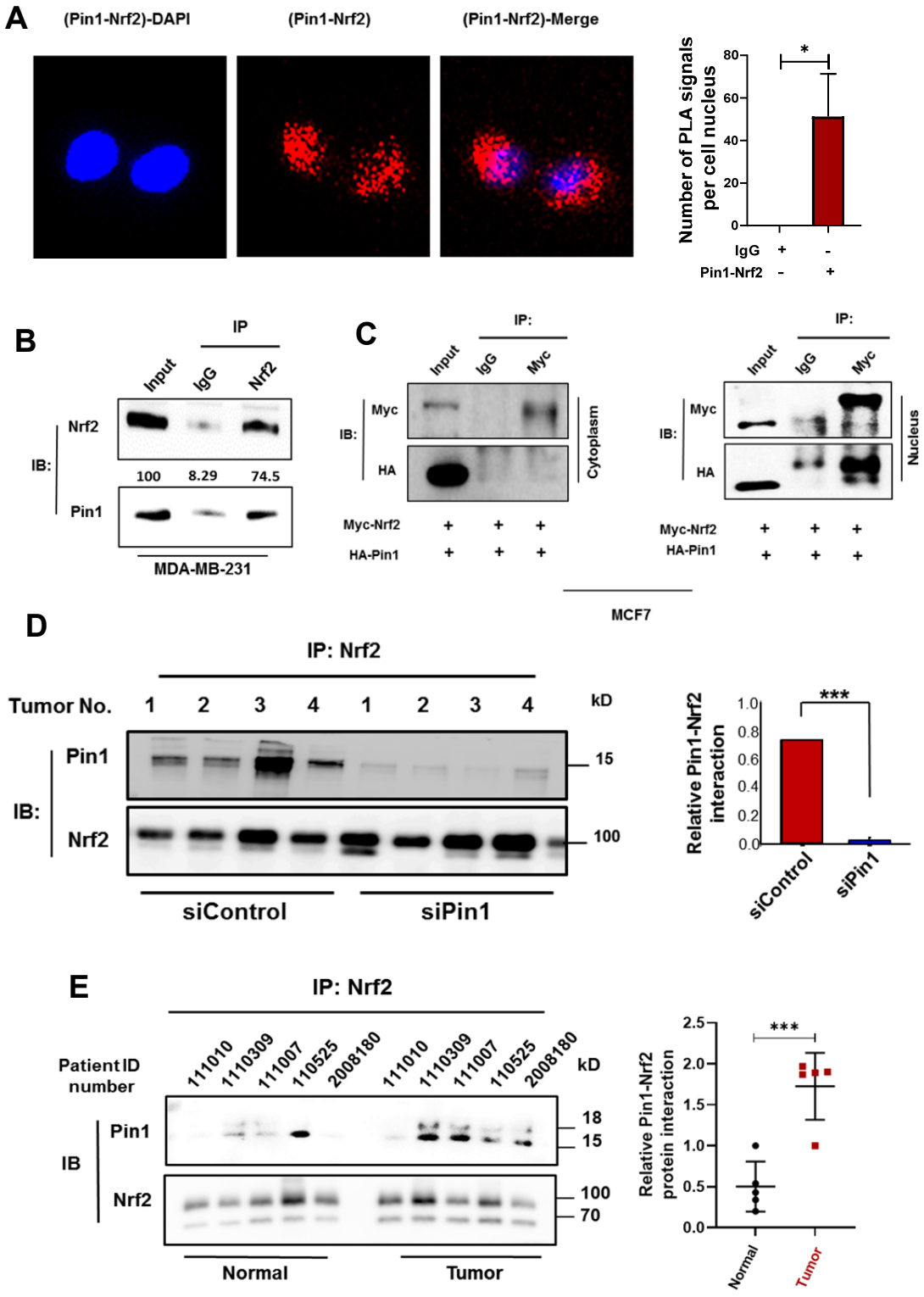

A
(Pin1-Nrf2)-DAPI
(Pin1-Nrf2)
(Pin1-Nrf2)-Merge
Number of PLA signals per cell nucleus
IgG
Pin1-Nrf2
B
IP
Input
IgG
Nrf2
IB:
Nrf2
Pin1
100
8.29
74.5
MDA-MB-231
C
IP:
Input
IgG
Myc
IB:
Myc
HA
Cytoplasm
Nucleus
Myc-Nrf2
HA-Pin1
MCF7
D
IP: Nrf2
Tumor No.
kD
Pin1
Nrf2
IB:
15
100
siControl
siPin1
Relative Pin1-Nrf2 interaction
E
IP: Nrf2
Patient ID number
111010
1110309
111007
110525
2008180
kD
IB
Pin1
Nrf2
18
15
100
70
Normal
Tumor
Relative Pin1-Nrf2 protein interaction

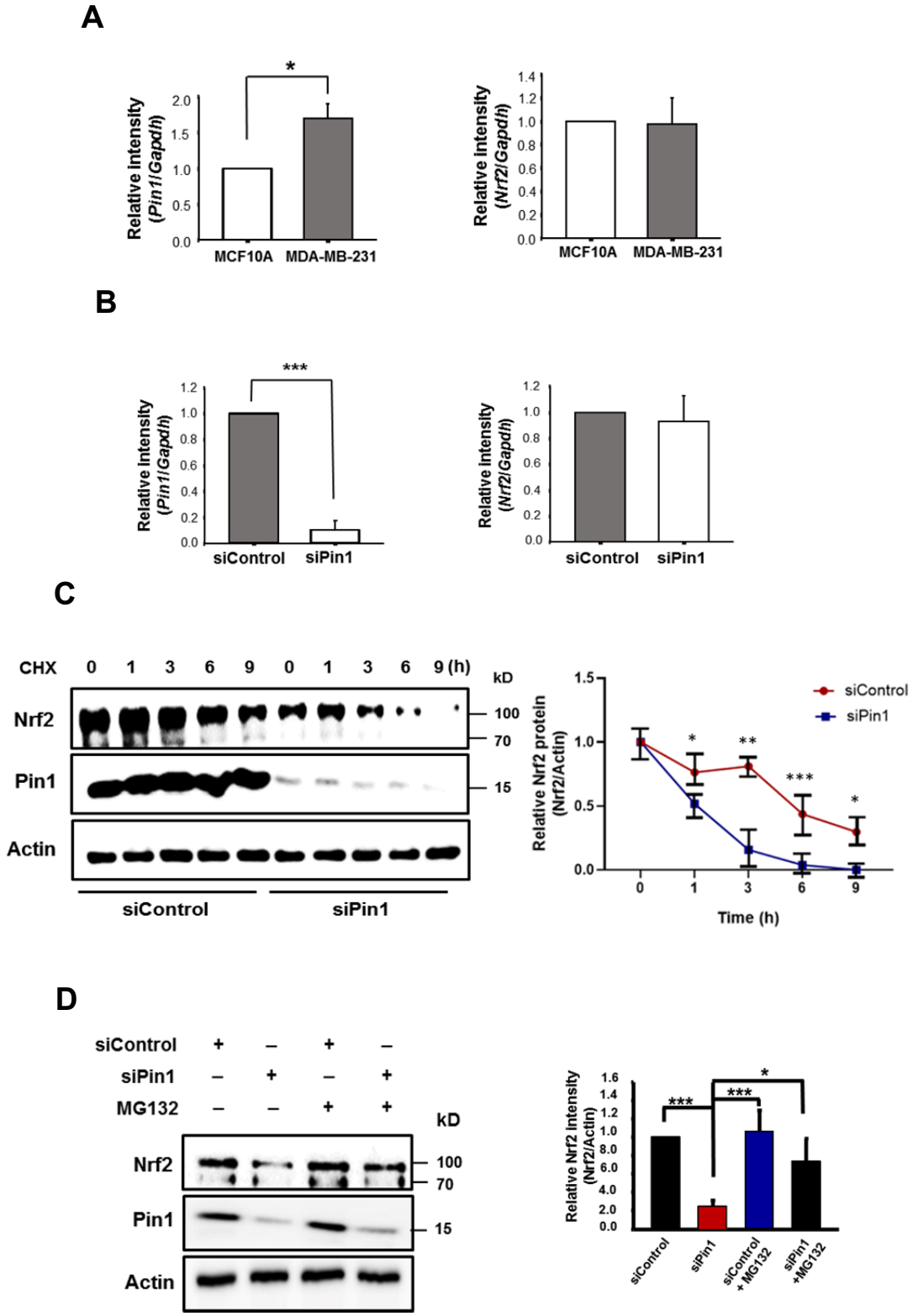
Figure 2-5. Physical interaction between Pin1 and Nrf2 in breast cancer cells and tissues

(A) Binding of Pin1 and Nrf2 *in situ*. The interaction of Pin1 with Nrf2 was visualized by Duolink analysis. Pin1 and Nrf2 were co-labeled with corresponding antibodies. Nuclei were counter-stained with DAPI (blue). Data are shown as the mean ± SD (n=3), and the statistical significance was determined by Student's *t*-test. $*p < 0.05$.

(B) Interaction between endogenous Pin1 and Nrf2 was examined in MDA-MB-231 cells. (C) Interaction between exogenous Pin1 and Nrf2 was examined upon overexpression of Myc-tagged Nrf2 and HA-tagged Pin1 in MCF-7 cells. Cell fractionation was conducted to ensure Pin1-Nrf2 interaction in the nucleus. (D) Effects of *Pin1* silencing on interaction between Pin1 and Nrf2 in the xenograft tumor lysates. Data are shown as the mean ± SD (n=4), and the statistical significance was determined by Student's *t*-test. $**p < 0.01$. (E) Comparison of Pin1-Nrf2 interaction in human breast cancer tissues with normal tissues. Data are shown as the mean ± SD (n=5), the statistical significance was determined by Student's *t*-test. $***p < 0.001$.

3.4. Pin1 stabilizes Nrf2 in human breast cancer cells

After confirmation of the direct interaction of Pin1 with Nrf2, we investigated whether this could affect the stability of Nrf2 in MDA-MB-231 cells. We found that MDA-MB-231 and MCF-10A cells express equivalent levels of *Nrf2* mRNA but a higher level of *Pin1* mRNA in the former cells (**Fig. 2-6A**). The silencing of *Pin1* had no effect on the expression of *Nrf2* mRNA (**Fig. 2-6B**), but markedly inhibited its protein expression (**Fig. 2-3B**). These findings suggest that Pin1-induced accumulation of Nrf2 is mediated through stabilization of the Nrf2 protein rather than promotion of gene transcription. In order to test this possibility, the cells were treated with control siRNA or *Pin1* siRNA prior to blockage of *de novo* protein synthesis with CHX. As shown in **Fig. 2-6C**, Pin1-silenced MDA-MB-231 cells exhibited a significantly higher degradation rate of pre-existing Nrf2 compared with cells transfected with negative control siRNA. Treatment with a proteasome inhibitor, MG-132 abolished the degradation of Nrf2 in the cells silenced for *Pin1* (**Fig. 2-6D**). Consistently, the knockdown of *Pin1* substantially increased the level of ubiquitinated Nrf2 (**Fig. 2-6E**), corroborating the stabilization of Nrf2 by Pin1.


A
*
Relative Intensity (Pin1/Gapdh)
2.0
1.5
1.0
0.5
0.0
MCF10A
MDA-MB-231
Relative Intensity (Nrf2/Gapdh)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
MCF10A
MDA-MB-231
B
***
Relative Intensity (Pin1/Gapdh)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
siControl
siPin1
Relative Intensity (Nrf2/Gapdh)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
siControl
siPin1
C
CHX 0 1 3 6 9 0 1 3 6 9 (h)
kD
Nrf2
100
70
Pin1
15
Actin
siControl
siPin1
Relative Nrf2 protein (Nrf2/Actin)
1.5
1.0
0.5
0.0
siControl
siPin1
*
**
***
*
0
1
3
6
9
Time (h)
D
siControl + – + –
siPin1 – + – +
MG132 – – + +
kD
Nrf2
100
70
Pin1
15
Actin
Relative Nrf2 intensity (Nrf2/Actin)
1.6
1.4
1.2
1.0
8.0
6.0
4.0
2.0
0.0
***
***
*
siControl
siPin1
siControl + MG132
siPin1 +MG132

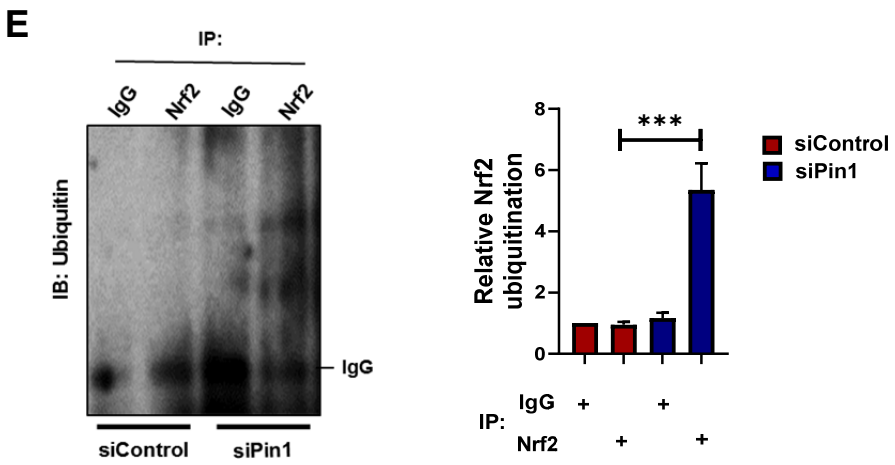


Figure 2-6. Regulation of Nrf2 stability by Pin1

(A) Comparison of mRNA expression of Pin1 and Nrf2 in non-oncogenic MCF10A and malignant MDA-MB-231 cells. Data are shown as the mean ± SD (n=3), and the statistical significance was determined by Student's *t*-test. **p* < 0.05. (B) The effect of *Pin1* silencing on Nrf2 gene expression. Data are shown as the mean ± SD (n=3), and the statistical significance was determined by Student's *t*-test. ****p* < 0.001. (C) The reduced stability of Nrf2 by *Pin1* silencing. MDA-MB-231 cells were transfected with control or *Pin1* siRNA expression vector for 72 h, followed by exposure to CHX (10 μM) for the indicated time periods. Cell lysates were subjected to Western blot analysis with anti-Pin1 and anti-Nrf2 antibodies. Data are shown as the mean ± SD (n=3), and the statistical significance was determined by Student's *t*-test. **p* < 0.05, ***p* < 0.01, and ****p* < 0.001. (D) Nrf2 protein levels were determined by Western blot analysis in control or *Pin1* knockdown MDA-

MB-231 cells with or without exposure to the proteasome inhibitor MG-132 (20 μM). Data are shown as the mean ± SD (n=3), and the statistical significance was determined by Student's *t*-test. **p* < 0.05, and ****p* < 0.001. (E) Effects of *Pin1* silencing on ubiquitination of Nrf2 in control and *Pin1* knockdown MDA-MB-231 cells. Nrf2 ubiquitination was determined by immunoprecipitation of Nrf2 with anti-ubiquitin antibody. Data are shown as the mean ± SD (n=3), and the statistical significance was determined by Student's *t*-test. ****p* < 0.001.

3.5. Pin1 binds to Nrf2 phosphorylated at specific serine (S215, S408 and S577) residues

It has been reported that Pin1 exerts its regulatory function in a phosphorylation-dependent manner [31, 32]. Nrf2 has been shown to be phosphorylated by mitogen-activated protein kinases (MAPKs) [30]. We examined whether the phosphorylation status of Nrf2 could affect its interaction with Pin1. The treatment of MDA-MB-231 cells with the pharmacologic MAPK inhibitors SP600125, SB203580, and U0126 effectively inhibited the activation of JNK, p38 MAPK, and ERK, respectively (**Fig. 2-7A**). As shown in **Fig. 2-7B**, the interaction between Nrf2 and Pin1 was substantially reduced by inhibitors of ERK and JNK, but not by the p38 inhibitor.

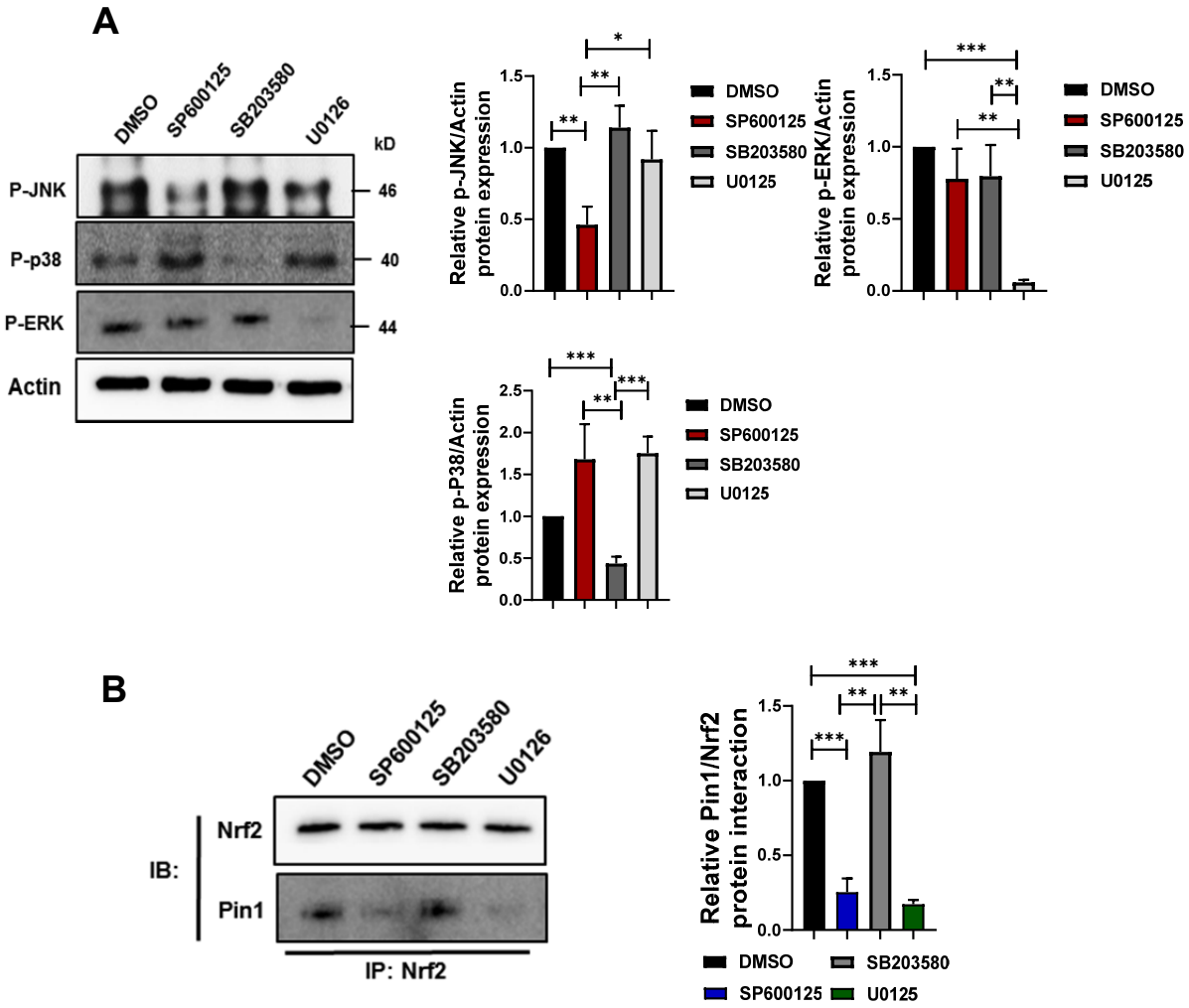
Pin1 binds to a partner protein and catalyzes *cis*/*trans* isomerization of the peptide bond between phosphorylated serine or threonine and proline (pSer/Thr-Pro). Nrf2 harbours 13 such motifs (**Fig. 2-7C**). Four serine and one threonine (S215, S408, S558, T559 and S577) residues were identified as prime sites on Nrf2 phosphorylated by endogenous kinases [30]. Three of them (S215, S408, S577) fit consensus sites for MAPKs, while the other two (S558 and S559) do not. To investigate the involvement of these 3 serine residues in Pin1-Nrf2 interaction, HEK293T cells were co-transfected with expression vectors for GST-Pin1 and HA-tagged wild-type (WT) Nrf2 or indicated mutant constructs. The alanine substitution for each of aforementioned serine residues did not affect the expression of both Pin1 and Nrf2 as resultant mutant cells express both Pin1 and Nrf2 at the levels equivalent to those in WT cells (**Fig. 2-7D** upper). Notably, interaction between Nrf2 and Pin1 was barely detectable in the mutant cells expressing $Nrf2^{S215A}$,

$Nrf2^{S408A}$ or $Nrf2^{S577A}$ (**Fig. 2-7D** bottom and quantification in **Fig. 2-7E**). Consistent with this observation, more ubiquitinated Nrf2 was detected in the cells harbouring the mutated Nrf2 that cannot be phosphorylated at the corresponding serine residues (**Fig. 2-7F**).

To further verify that the interaction between Pin1 and Nrf2 is phosphorylation-dependent, we overexpressed Pin1 and Nrf2 in HEK 293T cells and then treated the cell lysates with the alkaline phosphatase, CIP prior to the immunoprecipitation assay. Treatment of cell lysates with CIP repressed the interaction between Pin1 and Nrf2 (**Fig. 2-8A**). In addition, the degradation of Nrf2 was dramatically enhanced after treating cells with CIP, indicative of a phosphorylation-dependent interaction between two proteins (**Fig 2-8B**).

Pin1 specifically binds target proteins harbouring the pSer/Thr-Pro consensus motifs through its N-terminal WW domain and catalyzes the *cis*/*trans* isomerization with its C-terminal PPIase domain [5, 9, 33]. The WW domain spans the first 39 amino acid residues of the Pin1 protein, while the PPIase domain contains amino acids 50 to163 in the C-terminal region (**Fig. 2-8C**) [33]. –You may add, in Fig. 8C below the linear structure, the image of the 3-dimensional structure of Pin1 with WW and PPIase indicated It has been reported that a point mutation of tryptophan at position 34 in the WW domain of Pin1 to alanine (Pin1-W34A) [34] and a point mutation of lysine at position 63 in the PPIase domain (Pin1-K63A) abolish the interaction of Pin1 with its substrates [35] and the isomerase activity, respectively. Overexpression of Pin1 mutant constructs (Pin1-W34 or Pin1-K63) did not affect the intracellular expression of Pin1 protein (**Fig. 2-8D** left). However, Pin1

mutation with a disrupted WW domain (W34A) or a catalytically inactive PPIase domain (K63A) abolished its interaction with Nrf2 (**Fig.** 2-**8D** right).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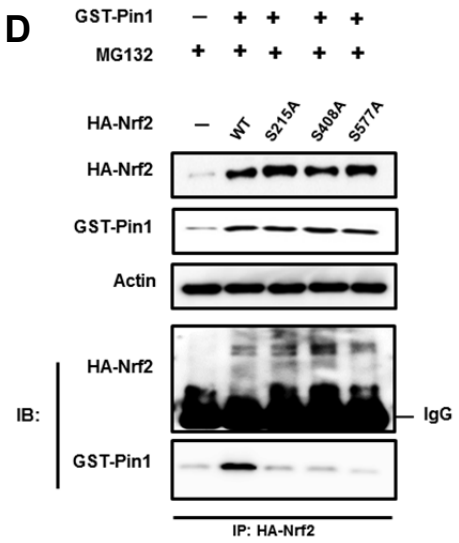
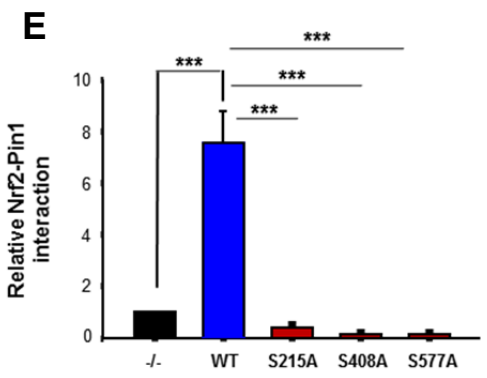
DMSO
SP600125
SB203580
U0126
kD
P-JNK
P-p38
P-ERK
Actin
46
40
44
Relative p-JNK/Actin protein expression
Relative p-ERK/Actin protein expression
Relative p-P38/Actin protein expression
DMSO
SP600125
SB203580
U0125
B
IB:
Nrf2
Pin1
IP: Nrf2
Relative Pin1/Nrf2 protein interaction
C
MMDLELPPPGLPSQQDMDLIDILWRQDIDLGVSREVFDFSQRRKEYELEKQKKLEKERQEQLQKEQEKAFFAQLQL
DEETGEFLPIQPAQHIQSETSGSANYSQVAHIPKSDALYFDDCMQLLAQTFPFVDDNEVSSATFQSLVPDIPGHIE
SPVFIATNQAQSPETSVAQVAPVDLDGMQQDIEQVWEELLSIPELQCLNIENDKLVETTMVPSPEAKLTEVDNYH
FYSSIPSMEKEVGNCSPHFLNAFEDSFSSILSTEDPNQLTVNSLNSDATVNTDFGDEFYSAFIAEPSISNSMPSPA
TLSHSLSELLNGPIDVSDLSLCKAFNQNHPESTAEFNDSDSGISLNTSPSVASPEHSVESSSYGDTLLGLSDSEVE
ELDSAPGSVKQNGPKTPVHSSGDMVQPLSPSQGQSTHVHDAQCENTPEKELPVSPGHRKTPFTKDKHSSRLEAHL
TRDELRAKALHIPFPVEKIINLPVVDFNEMMSKEQFNEAQLALIRDIRRRGKNKVAAQNCRKRKLENIVELEQDLD
HLKDEKEKLLKEKGENDKSLHLLKKQLSTLYLEVFSMLRDEDGKPYSPSEYSLQQTRDGNVFLVPKSKKPDVKKN

	Position in query protein	Sequence in query protein	Corresponding motif (phosphorylated residues in red)	Features of motif
1	153-154	SP	[pS/pT]P	WW domain binding motif
2	164-165	SP	[pS/pT]P	WW domain binding motif
3	215-216	SP	[pS/pT]P	WW domain binding motif
4	243-244	SP	[pS/pT]P	WW domain binding motif
5	301-302	SP	[pS/pT]P	WW domain binding motif
6	351-352	SP	[pS/pT]P	WW domain binding motif
7	356-357	SP	[pS/pT]P	WW domain binding motif
8	395-396	TP	[pS/pT]P	WW domain binding motif
9	408-409	SP	[pS/pT]P	WW domain binding motif
10	425-426	TP	[pS/pT]P	WW domain binding motif
11	433-434	SP	[pS/pT]P	WW domain binding motif
12	439-440	TP	[pS/pT]P	WW domain binding motif
13	577-578	SP	[pS/pT]P	WW domain binding motif





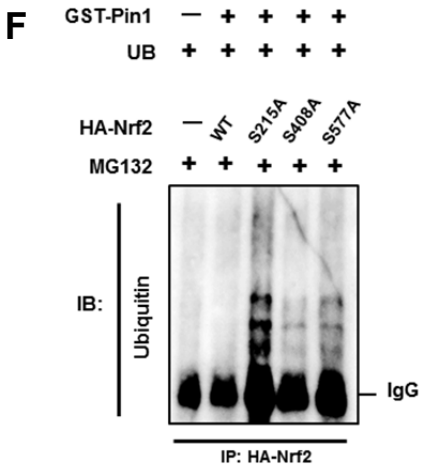


Figure 2-7. The importance of Nrf2 phosphorylation for its interaction with Pin1

(A, B) Effects of pharmacologic inhibition of MAPKs on interaction between Nrf2 and Pin1. MDA-MB-231 cells were pre-treated with a JNK inhibitor (SP00125; 20 μM), a p38 MAPK inhibitor (SB208530; 20 μM) or an ERK inhibitor (U0126; 20 μM), and phosphorylation of each MAPK (A) and interaction between Pin1 and Nrf2 (B) were measured by Western blot and co-immunoprecipitation assays, respectively. Data are shown as the mean ± SD (n=3), and the statistical significance was determined by Student's *t*-test. $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$. (C) Presence of the WW domain binding motifs in Nrf2. Nrf2 protein contains 13 WW binding motifs with pSer/Thr-Pro sequence. Source: Human Protein Reference Data base (http://www.hprd.org/). Among these, three serine residues (S215, S408 and S577) are known to be phosphorylated [30]. (D, E) Comparison of Pin1-Nrf2 interaction in WT and mutant cells. HEK293T cells were co-transfected with GST-Pin1 and HA-tagged WT Nrf2 or indicated mutant constructs in which specific serine residues are replaced by alanine, and then subjected to Western blot analysis (upper) or immunoprecipitation (lower) with anti-HA, followed by immunoblotting with anti-HA and anti-GST antibodies (D). All groups were treated with a proteasome inhibitor MG-132 (20 μM). Data are shown as the mean ± SD (n = 3), and the statistical significance was determined by Student's *t*-test. $***p < 0.001$ (E). (F) HEK293T cells were transfected with indicated plasmids expressing HA-Nrf2, GST-Pin1, HA ubiquitin, HA-Nrf2-S215A, HA-Nrf2-S408A, and HA-Nrf2-S577A. All groups were treated with a proteasome

inhibitor MG-132. Nrf2 immunoprecipitates were immunoblotted with anti-ubiquitin antibody.

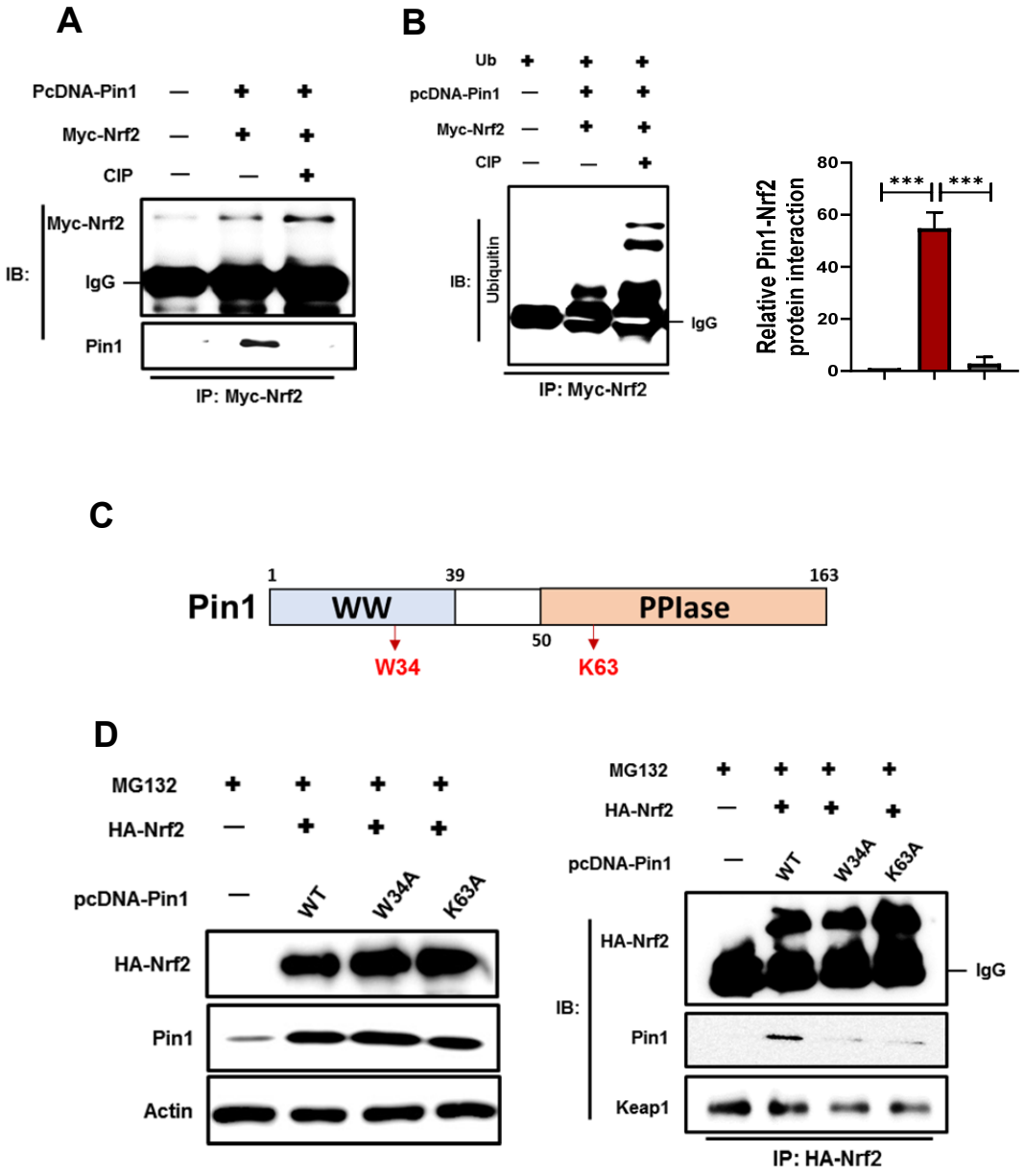


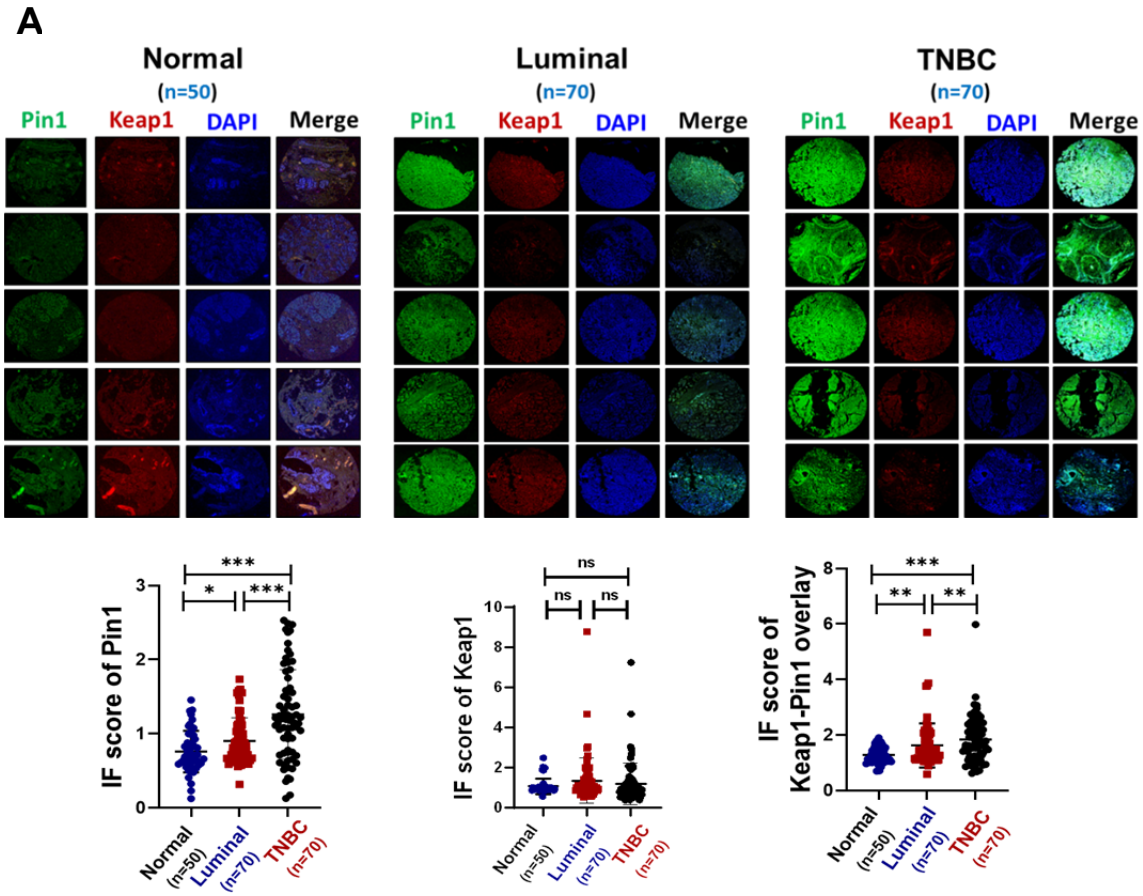
Figure 2-8. Involvement of WW and PPIase domains of Pin1 in its interaction with phosphorylated Nrf2

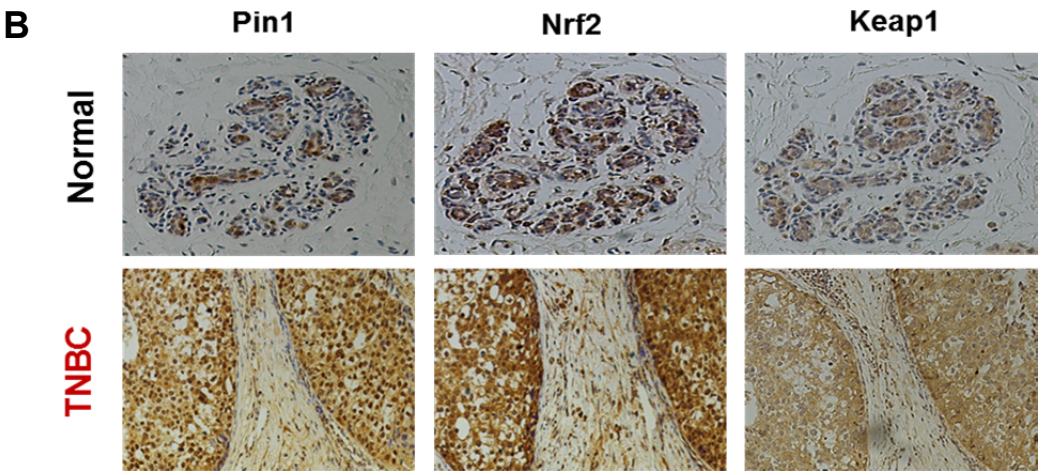
(A) HEK 293T cells were transfected with the Myc-Nrf2 and pcDNA-Pin1 expression plasmids. For those protein samples subjected to dephosphorylation, the lysate was re-suspend in the CIP buffer. CIP (30 U) was added, and the mixture was incubated at 37°C for 60 min. Nrf2 pulled-down complexes were subjected to SDS-PAGE and then immunoblotted with an anti-Myc and Pin1 antibodies. Data are shown as the mean ± SD (n=3), and the statistical significance was determined by Student's *t*-test. ***$p < 0.001$. (B) HEK293T cells were transfected with indicated plasmids expressing Myc-Nrf2, pcDNA-Pin1, and HA ubiquitin. Dephosphorylation of protein samples with CIP was conducted as described in Materials and Methods. Myc-Nrf2 immunoprecipitates were immunoblotted with anti-ubiquitin antibody**.**

(C) Pin1 consists of an N-terminal WW domain which recognizes the pSer/Thr-Pro motif of the binding partner and a C-terminal PPIase domain which has prolyl isomerase activity. (D) HEK293T cells were co-transfected with HA-Nrf2 and WT Pin1 or each of indicated Pin1 mutants (W34A and K63A) and then subjected to Western blot analysis (left) or immunoprecipitation (right) with anti-HA, followed by immunoblotting with anti-HA and anti-Pin1 antibodies. All groups were treated with a proteasome inhibitor MG-132.

3.6. Pin1, but not Keap1, is overexpressed in human breast cancer

As Nrf2 stability is mainly regulated by Keap1, we attempted to determine whether Pin1 can also interact with Keap1, thereby indirectly modulating Nrf2 stability. As shown in **Fig. 2-3A**, Pin1 was overexpressed in MDA-MB-231 breast cancer cells compared with non-oncogenic MCF10A cells. However, Keap1 expression was similar in both cell lines. Next, MDA-MB-231 cells were transfected with siRNA control, or siPin1 and subjected to Western blot analysis. The protein level of Pin1 protein was downregulated, but the expression of Keap1 was not changed (**Fig. 2-3B**). Then, we investigated the clinical relevance of Keap1 and Pin1 to breast cancer progression. For this purpose, we performed a tissue microarray of 70 breast cancer tissues and 50 normal ones. While normal tissues exhibited relatively low IF signals after staining with antibodies recognizing Pin1 and Keap1, the luminal and TNBC breast cancer tissues showed highly enhanced intensity of Pin1. However, Keap1 intensity was similar in normal, luminal, and TNBC tissues (**Fig. 2-9A**). The overexpression and co-localization of Nrf2 and Pin1 in breast cancer patients were further confirmed by Immunohistochemistry staining (**Fig. 2-9B**).


A
Normal
(n=50)
Luminal
(n=70)
TNBC
(n=70)
Pin1
Keap1
DAPI
Merge
IF score of Pin1
IF score of Keap1
IF score of Keap1-Pin1 overlay
ns
*
**
***
Normal (n=50)
Luminal (n=70)
TNBC (n=70)


B
Pin1
Nrf2
Keap1
Normal
TNBC

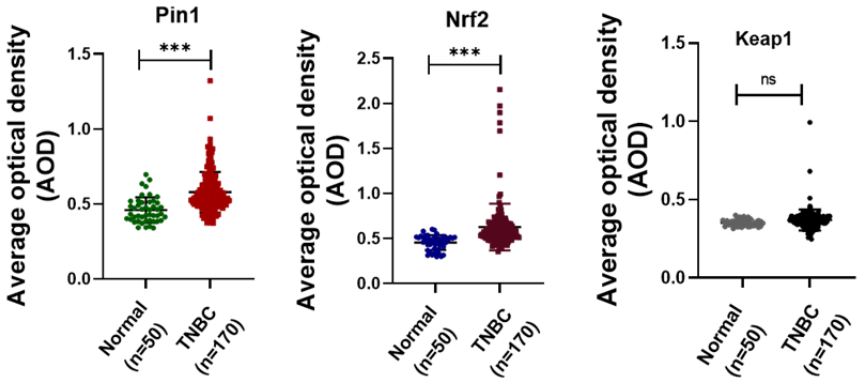
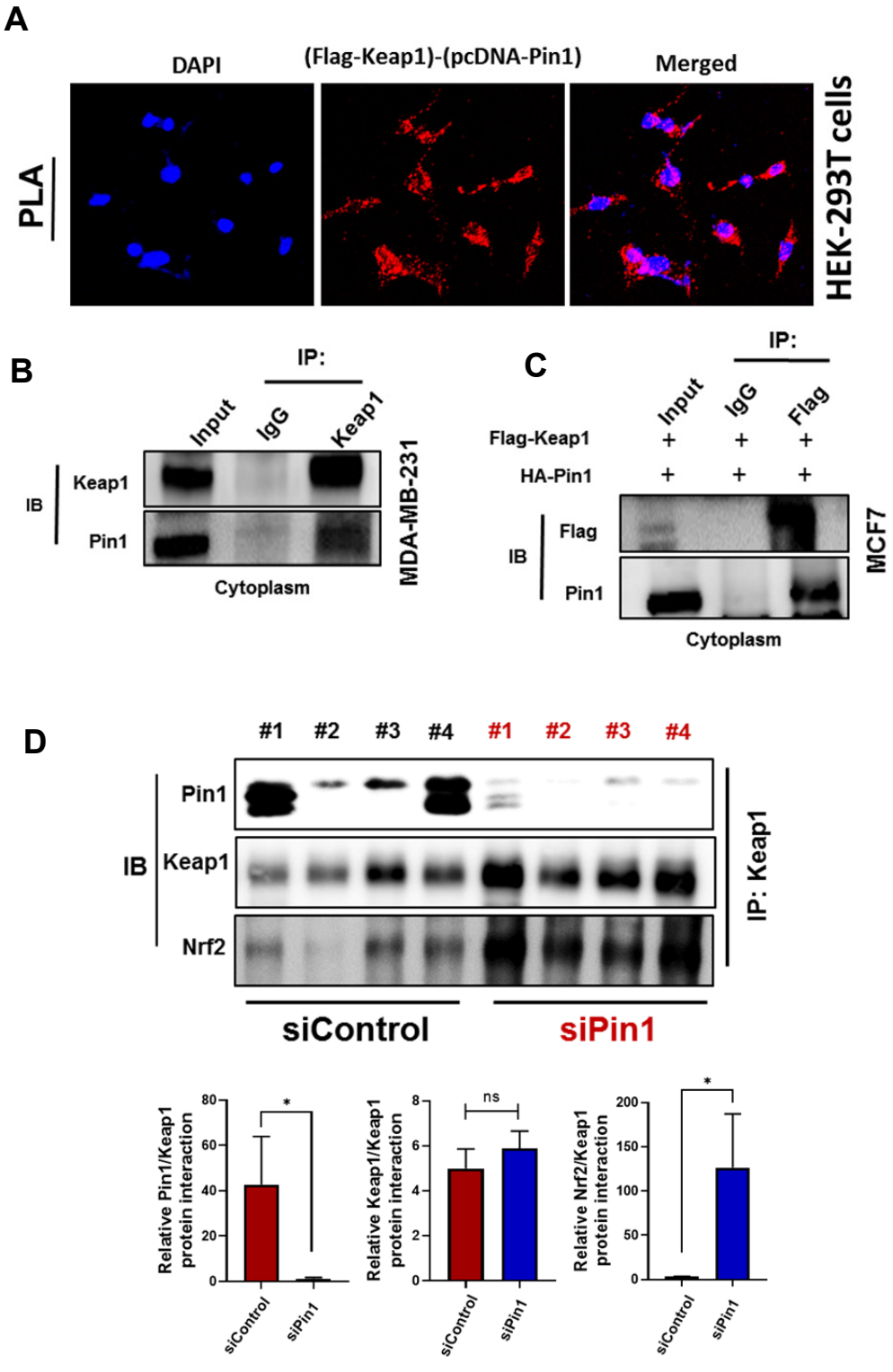


Figure 2-9. Clinical relevance of Pin1 and Keap1 determined by the tissue array

(A) Expression of Pin1 and Keap1 in normal, luminal breast cancer and TNBC tissues was determined by IF analysis. Fifty normal and 70 each of luminal breast cancer and TNBC specimens were exposed to anti-Pin1 and anti-Keap1 antibodies, and the IF scores of both proteins were measured. Representative images of 5 out of 70 stained specimens of each molecular sub-type are displayed, but the quantification was done with all samples. Data are shown as the mean ± SD (normal tissues; n=50, luminal and TNBC; n=70 each), and the statistical significance was determined by two-tailed unpaired Student's *t*-test method. (B) IHC data showed that Keap1 expression was not significantly different from the normal tissues.

3.7. Pin1 physically interacts with Keap1

We investigated whether Pin1 and Keap1 proteins could physically interact each other. The interaction was verified by PLA, which detects an enhanced fluorescent signal when two proteins are localized in proximity (**Fig. 2-10A**). The interaction of Pin1 and Keap1 in the cytoplasm of MDA-MB-231 cells was further confirmed by a co-immunoprecipitation assay (**Fig. 2-10B**). In contrast to MDA-MB-231 cells, MCF-7 breast cancer cells have relatively low expression levels of Pin1 and Keap1 (data not shown). Therefore, we overexpressed Keap1 and Pin1 in MCF-7 cells to further verify the interaction between those two proteins. There was a pronounced interaction of HA-tagged Pin1 with Flag-tagged Keap1 in the cytoplasm of MCF-7 cells, (**Fig. 2-10C**). Such interaction was also evident in xenograft tumors derived from MDA-MB-231 cells, but not in Pin1 silenced tumors (**Fig. 2-10D**). Under normal physiologic conditions, Nrf2 is sequestered in the cytoplasm as an inactive complex with Keap1 [22-24]. Notably, there was a concomitant increase in the interaction between Nrf2 and Keap1 in the *Pin1* silenced xenograft tumors which indicates that Pin1 and Nrf2 may compete with each other for keap1 binding (**Fig. 2-10D**). We found that human breast cancer tissues exhibited the significantly elevated levels of the Pin1-Keap1 complex, compared with normal tissues (**Fig. 2-10E**).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PLA
HEK-293T cells
B
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Input
IgG
Keap1
IB
Keap1
Pin1
Cytoplasm
MDA-MB-231
C
IP:
Input
IgG
Flag
Flag-Keap1
HA-Pin1
IB
Flag
Pin1
Cytoplasm
MCF7
D
#1
#2
#3
#4
#1
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*
ns
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siControl
siPin1

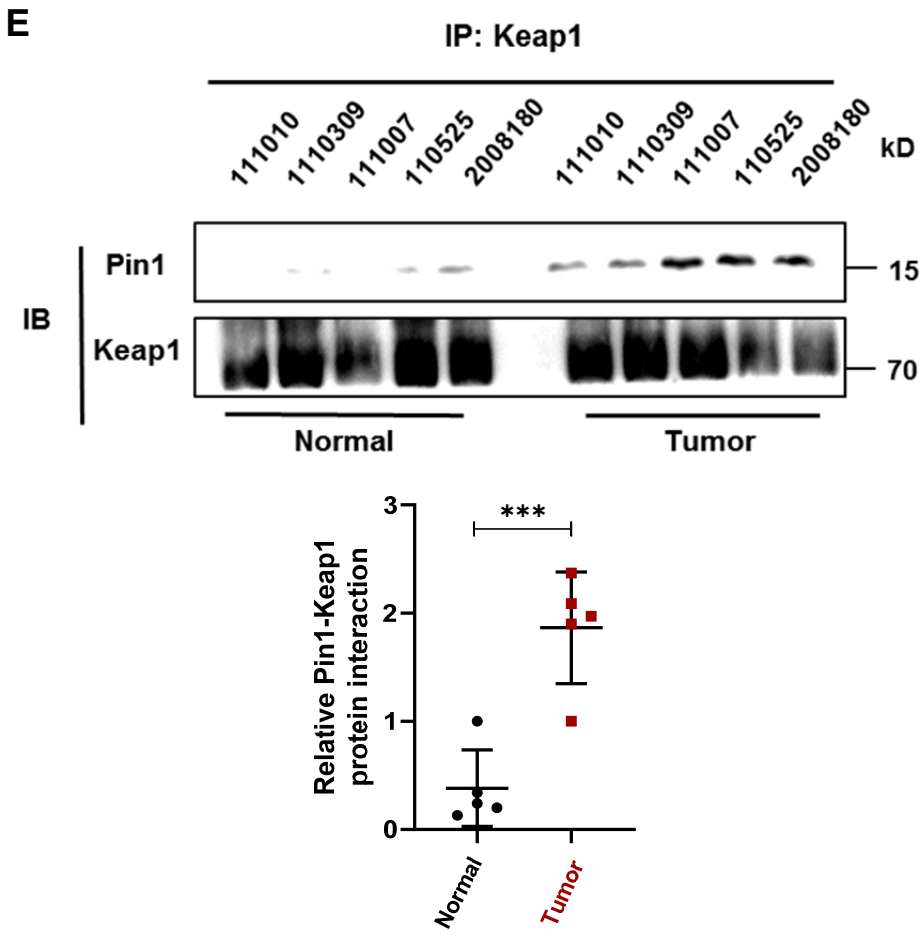


Figure 2-10. Physical interaction between Pin1 and Keap1 in breast cancer cells and tissues

(A) Binding of Pin1 and Keap1 *in situ*. The interaction of Pin1 with Keap1 was visualized by Duolink analysis. Pin1 and Keap1 were co-labeled with corresponding antibodies. Nuclei were counter-stained with DAPI (blue). (B) Interaction between endogenous Pin1 and Keap1 was examined in MDA-MB-231 cells. (C) Interaction between exogenous Pin1 and Keap1 was examined upon overexpression of HA-

tagged Pin1 and Flag-tagged Keap1 in MCF-7 cells. (D) Effects of *Pin1* silencing on interaction between Pin1 and Keap1 in the xenograft tumor lysates. Data are shown as the mean ± SD (n=4), and the statistical significance was determined by Student's *t*-test. $*p < 0.05$. (E) Comparison of Pin1-Keap1 interaction in human breast cancer tissues with normal tissues. Data are shown as the mean ± SD (n=5), the statistical significance was determined by Student's *t*-test. $***p < 0.001$.

3.8. Pin1 binds to Keap1 phosphorylated at specific serine (S104 and T277) residues

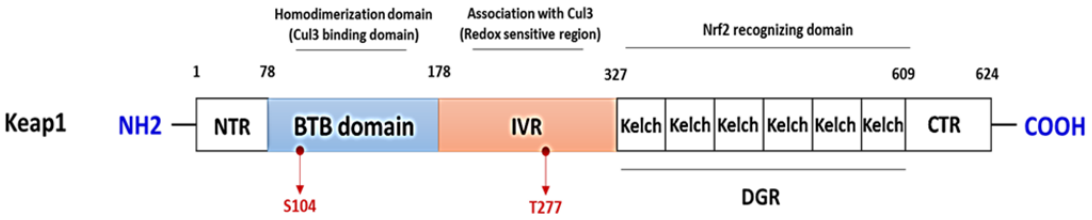
Pin1 binds to pSer/Thr-Pro motifs of the substrates and catalyzes *cis*/*trans* isomerization. Keap1 harbours 6 such motifs (**Fig. 2-11A**). Serine 104 and threonine 277 residues (S104 and T277) were identified as prime sites on Keap1 for Pin1 binding by the LC-MS/MS analysis (**Fig. 2-11B**). To investigate the involvement of these two residues in Pin1-Keap1 interaction, HEK293T cells were co-transfected with expression vectors for pcDNA-Pin1 and Flag-tagged WT Keap1 or indicated mutant constructs. The alanine substitution for each of aforementioned serine or threonine residues did not affect the expression of both Pin1 and Keap1 as resultant mutant cells express both proteins at the levels equivalent to those in WT cells (**Fig. 2-11C** upper). Notably, interaction between Pin1 and Keap1 was barely detectable in the mutant cells expressing Keap1^{S104A} and Keap1^{T277A}. However, the alanine substitution for adjacent prolines, Keap1^{P105A} and Keap1^{P278A}, did not affect the interaction of Pin1 and Keap1 (**Fig. 2-11C** bottom).

A

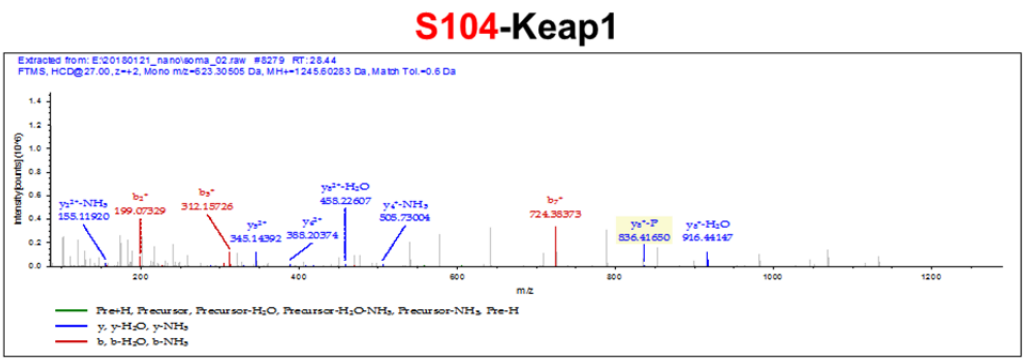
Protein name : Keap1

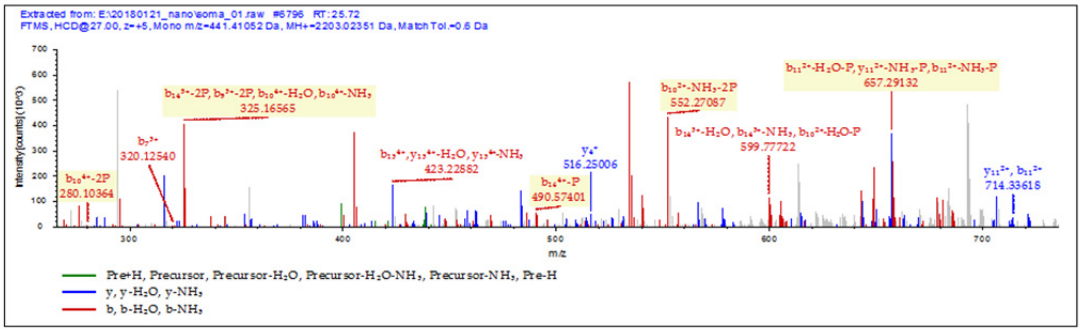
MQPEPRPSGAGARTRFLPLRSQRPEGAGDTVMYASTECKAEV**TP**SQHGNRTFSYTLEDHTKQAFGIMNELRLS
QQLCDVTLQVKYEDAPAAQFMAHKVVLASS**SP**VFKAMFTNGLREQGMEVVSIEGIHPKVMERLIEFAYTASIS
MGEKCVLHVMNGAVMYQIDSVVRACSDFLVQQLDPSNAIGIANFAEQIGCAELHQRAREYIYMHFGEVAKQEE
FFNLSHCQLVTLISRDDLNVRCESEVFHACINWVKYDCEQRRFYVQALLRAVRCHSL**TP**HFLQMQLQKCEILQ
SDSRCKDYLVKIFQELTLHKPTQVMPCRAPKVGRLIYTAGGYFRQSLSYLEAYNPSDGTWLRLADLQVPRSGL
AGCVVGGLLYAVGGRNN**SP**DGNTDSSALDCYNPMTNQW**SP**CAPMSVPRNRIGVGVIDGHIYAVGGSHGCIHHN
SVERYEPERDEWHLVAPMLTRRIGVGVAVLNRLLYAVGGFDGTNRLNSAECYYPERNEWRMI**TP**MNSIRSGAG

	Position in query protein	Sequence in query protein	Corresponding motif (phosphorylated residues in red)	Features of motif
1	43 - 44	TP	[pS/pT]P	WW domain binding motif
2	104 - 105	SP	[pS/pT]P	WW domain binding motif
3	277 - 278	TP	[pS/pT]P	WW domain binding motif
4	383 - 384	SP	[pS/pT]P	WW domain binding motif
5	404 - 405	SP	[pS/pT]P	WW domain binding motif
6	501 - 502	TP	[pS/pT]P	WW domain binding motif



B




T277-Keap1
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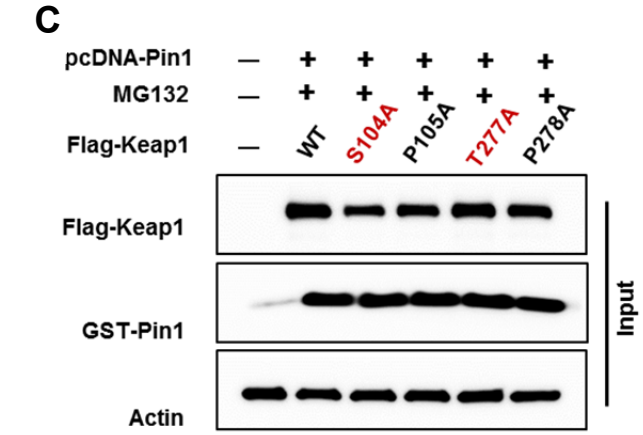
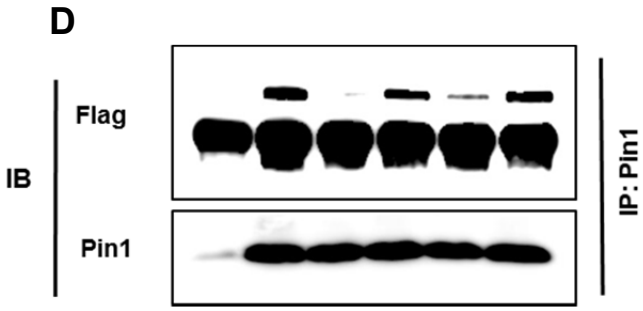
FTMS, HCD@27.00, z=+5, Mono m/z=441.41052 Da, MH+=2203.02351 Da, Match Tol.=0.6 Da
Intensity(counts)(10³)
m/z
b14 2+-2P, b9 2+-2P, b16 4+-H2O, b16 4+-NH3
325.16565
b7 2+
320.12540
b16 4+-2P
280.10364
b13 4+, y13 4+-H2O, y13 4+-NH3
423.22882
y4+
516.25006
b14 4+-P
490.57401
b16 2+-NH3-2P
552.27087
b14 2+-H2O, b14 2+-NH3, b16 2+-H2O-P
599.77722
b11 2+-H2O-P, y11 2+-NH3-P, b11 2+-NH3-P
657.29132
y11 2+, b11 2+
714.33618
Pre-H, Precursor, Precursor-H2O, Precursor-H2O-NH3, Precursor-NH3, Pre-H
y, y-H2O, y-NH3
b, b-H2O, b-NH3


C
pcDNA-Pin1
MG132
Flag-Keap1
WT
S104A
P105A
T277A
P278A
Flag-Keap1
GST-Pin1
Actin
Input


D
IB
Flag
Pin1
IP: Pin1

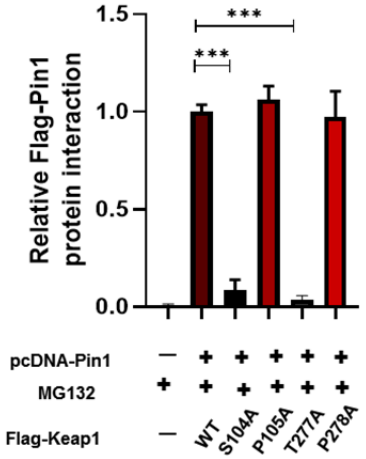

Relative Flag-Pin1 protein interaction
1.5
1.0
0.5
0.0
***
***
pcDNA-Pin1
MG132
Flag-Keap1
WT
S104A
P105A
T277A
P278A

Figure 2-11. The precise sites of Keap1 for its interaction with Pin1

(A) Presence of the WW domain binding motifs in Keap1. Keap1 protein contains 6 WW binding motifs with pSer/Thr-Pro sequence. Source: Human Protein Reference Data base (http://www.hprd.org/). (B) The peptide spectrum of Keap1 obtained by the LC-MS/MS analysis. Among WW domain binding motifs, two residues (S104 and T277) are identified as consensus sites for binding to Pin1. (C) Comparison of Pin1-Keap1 interaction in WT cells and mutant cells in which specific serine and threonine residues are replaced by alanine. HEK293T cells were co-transfected with pcDNA-Pin1 and Flag-tagged WT Keap1 or indicated mutant constructs and then subjected to Western blot analysis (upper) or immunoprecipitation (lower) with anti-Pin1, followed by immunoblotting with anti-Flag and anti-Pin1 antibodies (D). All groups were treated with a proteasome inhibitor MG-132 (20 μM). Data are shown as the mean ± SD (n = 3), and the statistical significance was determined by Student's *t*-test. ***$p < 0.001$ (E).

3.9. Pin1 competes with Nrf2 for interaction with Keap1

Previously, we showed that silencing Pin1 enhanced the interaction of Nrf2 with Keap1 in xenograft mice tumors. We speculated that Pin1 may compete with Nrf2 for binding to Keap1. To test this supposition, the subcellular fractionation of Keap1 WT and knockout mouse embryonic fibroblast (MEF) cells was examined and the interaction of Pin1 with Nrf2 in the presence and absence of Keap1 was checked. The cytoplasmic protein expression of Pin1 and Nrf2 did not change in Keap1 WT and knockout cells. However, the nuclear expression of both Pin1 and Nrf2 was upregulated in Keap1 knockout cells (**Fig. 2-12A** upper**).** The interaction between Pin1 and Nrf2 was dramatically higher in Keap1 knockout cells compared with that in WT cells in both the cytoplasmic and nuclear fractions (**Fig. 2-12A** lower). The overexpression and co-localization of Nrf2 and Pin1 in MEFs cells were further confirmed by IF staining (**Fig. 2-12B**). Pin1 and Nrf2 were significantly overexpressed and co-localized into the nucleus in MEFs Keap1 knockout cells compared with those in WT cells **(Fig. 2-12B)**.

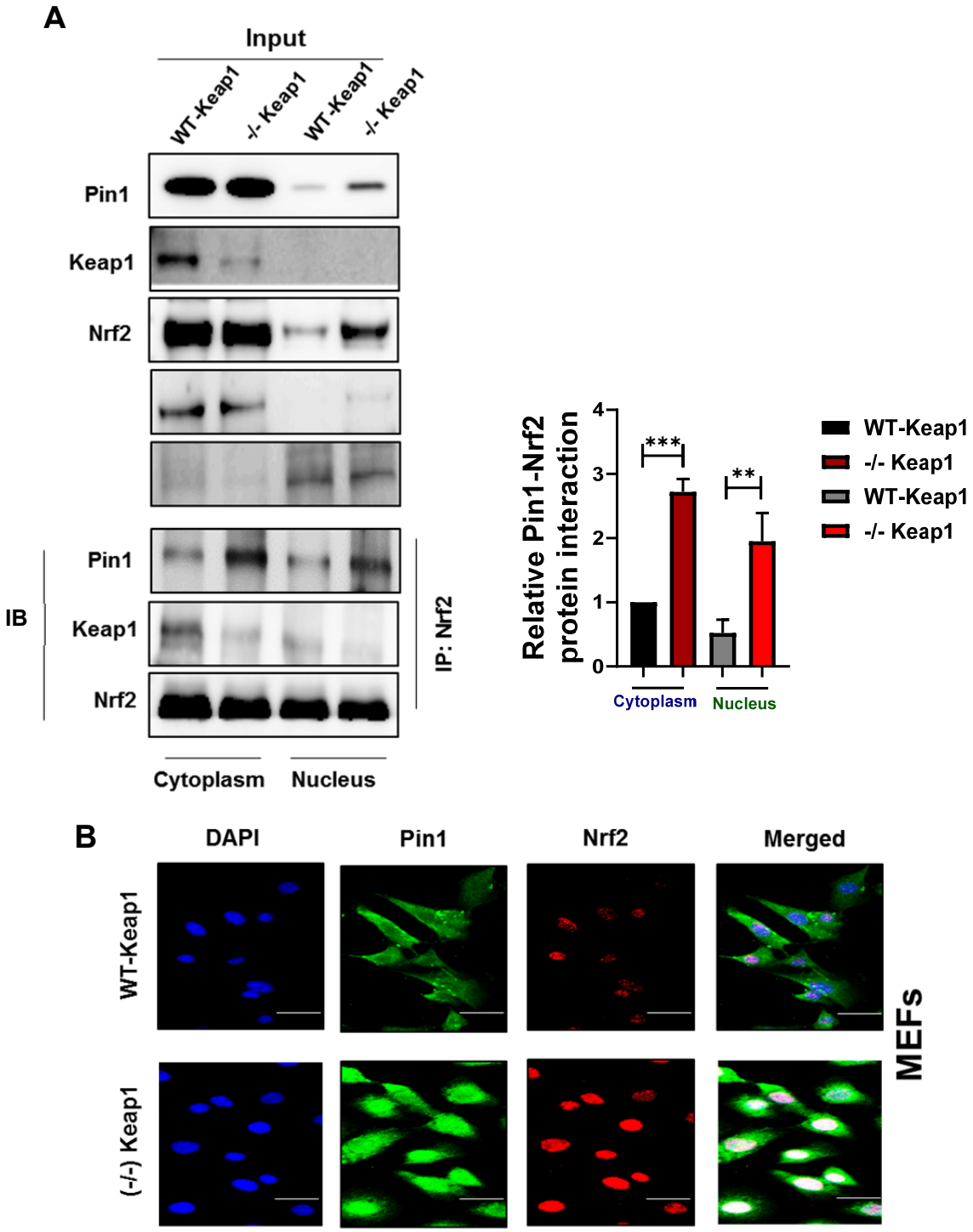

A
Input
WT-Keap1
-/- Keap1
WT-Keap1
-/- Keap1
Pin1
Keap1
Nrf2
IB
Pin1
Keap1
Nrf2
IP: Nrf2
Cytoplasm
Nucleus
Relative Pin1-Nrf2 protein interaction
***
**
WT-Keap1
-/- Keap1
WT-Keap1
-/- Keap1
Cytoplasm
Nucleus
B
DAPI
Pin1
Nrf2
Merged
WT-Keap1
(-/-) Keap1
MEFs

Figure 2-12. Competition of Pin1 and Nrf2 for binding to Keap1 and their nuclear translocation in mouse embryonic fibroblast

(A) Keap1 WT and knockout MEFs were subjected to cellular fractionation and the expression of Pin1, Nrf2 and Keap1 was examined by Western blot analysis. The interaction between Pin1 and Nrf2 was assessed in both cytoplasm and nucleus in WT and Keap1 knockout cells. Data are shown as the mean ± SD (n=3), and the statistical significance was determined by Student's *t*-test. $^{**}p < 0.01$ and $^{***}p < 0.001$. (B) The expression and nuclear co-localization of both Pin1 and Nrf2 were determined by IF staining.

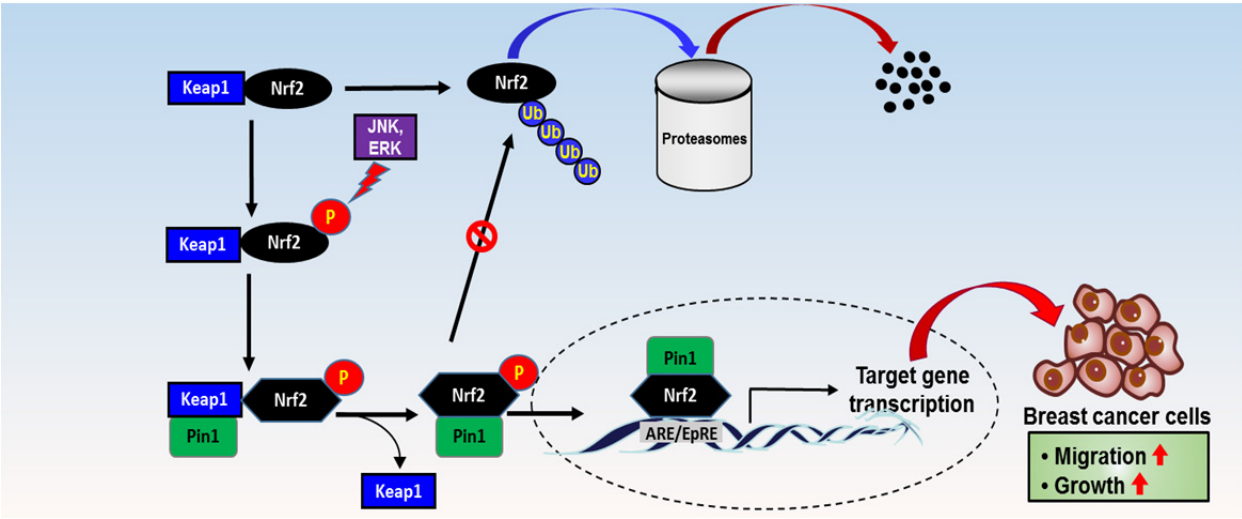


Fig 2-13. A proposed model for the regulation of Nrf2/Keap1 axis by Pin1 in breast cancer

Nrf2 is sequestered in the cytoplasm by Keap1 and subjected to proteasomal degradation. Nrf2 and Keap1 undergo phosphorylation at specific serine residues (e.g., S215, 408 and 577 of Nrf2 and S104 and T277 of Keap1), which facilitates their interaction with Pin1. This may cause a conformational change of Nrf2 and Keap1, with concomitant dissociation of Nrf2 from Keap1. As a result, ubiquitination and proteasomal degradation of Nrf2 can be prevented.

4. DISCUSSION

Although Nrf2 is known to upregulate expression of antioxidant/phase II carcinogen detoxifying enzymes and other cytoprotective proteins, accumulating evidence reveals that Nrf2 may accelerate cancer progression [22, 25, 30, 36-38]. Thus, it has been reported that deregulated overactivation of Nrf2 is consistently involved in tumor growth and survival [25-28, 33, 34]. Mutation in Nrf2 or its inhibitory protein Keap1 has been attributed to constitutive overexpression/overactivation of Nrf2 in cancer cells [22, 26, 38, 39]. However, post-translational modification of Nrf2 and interaction with other proteins that compete with Keap1 can alter its stability and subcellular localization [19, 28, 29, 40].

Pin1 interacts with diverse protein substrates, thereby causing their conformational changes through *cis/trans*-isomerization of a specific proline residue [16, 20, 41]. Pin1-mediated prolyl isomerization consequently influences the function of its substrates 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. [41]. Although Pin1 is frequently overexpressed in diverse human cancers, its expression in normal tissues is relatively low [8].

Pin1 has been shown to interact with Nrf2, and the resulting complex co-localizes in the nucleus in pancreatic cancer cells [19]. However, the above study did not provide the mechanistic details of Pin1-Nrf2 interaction and its functional

implications. In this study, we have shown that the expression of Pin1 is positively correlated with the accumulation of Nrf2 in human breast cancer tissues as well as breast cancer cell lines. Notably, such interaction is more pronounced in highly aggressive TNBC cells. The genetic or pharmacologic inhibition of Pin1 markedly reduced the levels of Nrf2 in the nucleus. However, silencing Pin1 had no significant effect on the mRNA expression of Nrf2, indicating that Pin1 may regulate Nrf2 in a post-translational way. Our data indicate that Pin1 stabilizes Nrf2 by inhibiting its ubiquitination and proteasomal degradation.

Pin1 recognizes the pSer/Thr-Pro motif of a substrate protein via its N-terminal WW domain and isomerizes the proline residue present in that motif by the PPIase activity retained in the C-terminal domain. Nrf2 is known to be phosphorylated at S215, S408 and S577 by MAPKs [30]. We speculate that such phosphorylation may dictate Pin1 to interact with Nrf2 and subsequently alters the conformation and stability of this transcription factor. Most notably, each of these serine residues precedes a proline residue (**Fig. 7C**), rendering Nrf2 suitable as a substrate of Pin1. Although two other amino acids (S559 and T559) are also known to undergo phosphorylation, they do not constitute the WW domain binding motif. Our present study demonstrates that the site-directed mutation of Nrf2, replacing each of the aforementioned 3 serine residues by alanine (S215A, S408A and, S577A), abolishes the binding of Pin1 to Nrf2, indicating that phosphorylation of Nrf2 in these specific serine residues is essential for its interaction with Pin1. Furthermore, treatment with a phosphatase CIP as well as pharmacologic inhibition of Nrf2 phosphorylation blunted the interaction of Nrf2 and Pin1.

Of interest, the binding of the substrate protein to the WW domain of Pin1 could result in conformational changes in the PPIase domain, enhancing its binding capacity to the *cis* configured pS/T-P motif and subsequently its catalytic activity. In addition to phosphorylation of Nrf2 at specific serine residues, both WW and PPIase domains of Pin1 are most likely to be required for its binding to Nrf2 as site-directed mutation of each domain negated their interaction. These findings suggest that Pin1 interaction with Nrf2 is dependent on phosphorylation.

ERK and JNK are proline-directed protein kinases, and proline exhibits two conformations, *cis* and *trans* [42-46]. Pin1 catalyzes the proline isomerization of substrates phosphorylated by ERK or JNK [46]. Pin1 overexpression correlates with cyclin D1 levels in human breast cancer tissues, and Pin1 can cooperate with either JNK or activated Ras to increase transcriptional activity of c-Jun towards the cyclin D1 promoter [7]. According to our present study, ERK and JNK are potential kinases responsible for Pin1 interaction with Nrf2 in MDA-MB-231 breast cancer cells. Under certain circumstances, Pin1 binds to pSer/Thr-Pro motifs of target proteins through its WW domain, independently of its catalytic (isomerase) activities that reside in the PPIase domain [9]. Further studies should follow to determine whether Pin1 does isomerize Nrf2 at a proline residue in the WW domain binding motif, thereby provoking conformational changes of Nrf2.

Under basal conditions, Nrf2 is sequestered in the cytoplasm by an inhibitory protein, Keap1. Keap1 functions as a substrate adaptor protein for the Cullin3-Rbx1 E3 ubiquitin ligase complex. Keap1 binding to Cullin3 enables the complex to degrade Nrf2 through ubiquitination. We speculate that Pin1 binds to the

phosphorylated Nrf2 which may change the conformation of Nrf2 (**Fig. 2-13**). This would hamper its sequestration by Keap1 and subsequent ubiquitin-proteasomal degradation. We also found that Pin1 directly interacts with Keap1 in breast cancer tissues as well as breast cancer cells compared with adjacent normal tissues. It has been reported that mutation of a conserved serine (S104A) residue within the Keap1 BTB/POZ domain disrupts dimerization of Keap1 and eliminates its ability to sequester Nrf2 in the cytoplasm and repress Nrf2 transactivation [47]. In addition, we identified the two specific amino acids in Keap1 (S104 and T277) critical for Pin1 binding by using LC-MS/MS analysis. Substitution of these amino acids with alanine dramatically reduced the interaction of Pin1 with Keap1. Thus, Pin1 may compete with Keap1 for Nrf2 binding. In line with this notion, we found a robust increase in the level of the Nrf2-Keap1 complex when Pin1 was deficient. On the other hand, the interaction between Pin1 with Nrf2 was increased in the absence of Keap1 deficient MEFs. The stabilized Nrf2 translocates to the nucleus together with Pin1, thereby regulating transcription of its target genes (**Fig. 2-13**). In addition, Pin1 dissociated from Nrf2 may translocate back to the cytoplasm to capture phosphorylated Nrf2 for stabilization and activation. In this context, it is interesting to note that Pin1 shuttles between cytoplasm and nucleus [31, 45].

Together, our current study reveals Nrf2 and Keap1 as novel binding partners of Pin1 and suggests that the Pin1-mediated stabilization of Nrf2 is attributable to its interaction with both Nrf2 and Keap1, which is implicated in breast cancer growth and progression. To the best of our knowledge, this is the first demonstration that Pin1 interacts via its WW domain with Nrf2 and its specific

inhibitor Keap1, harbouring proline residues preceded by specific serine residues. In this context, Nrf2 and Keap1 are a *bona fide* substrates of Pin1.

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Chapter III

H-*Ras* induces Nrf2-Pin1 interaction:

Implications for breast cancer progression

ABSTRACT

Increased expression and/or activation of H-*Ras* is often associated with tumor aggressiveness in breast cancer. Peptidyl-prolyl isomerase (Pin1) is a unique peptidyl-prolyl *cis*/*trans* isomerase that interacts with phosphorylated serine or threonine of a target protein and isomerizes the adjacent proline residue. Pin1 is prevalently overexpressed in human cancers, and its overexpression correlates with poor prognosis. Nuclear factor E2-related factor 2 (Nrf2) is a master regulator of cellular redox homeostasis. The sustained activation/accumulation of Nrf2 has been observed in many different types of human malignancies, conferring an advantage for growth and survival of cancer cells. The activated form of H-*Ras* (GTP-H-*Ras*) is highly overexpressed in human breast cancer tissues. In our present study, silencing of H-*Ras* decreased the invasiveness of MDA-MB-231 human breast cancer cells and abrogated the interaction between Pin1 and Nrf2 in these cells. *Pin1* knockdown blocked the accumulation of Nrf2, thereby suppressing proliferation and clonogenicity of MCF10A-*Ras* human mammary epithelial cells. We found that Pin1 binds to Nrf2 which stabilizes this transcription factor by hampering proteasomal degradation. In conclusion, H-*Ras* activation in cooperation with the Pin1-Nrf2 complex represents a novel mechanism underlying breast cancer progression and constitutive activation of Nrf2 and can be exploited as a therapeutic target.

Keywords:

H-Ras, Pin1, Nrf2, Protein-protein interaction, Breast cancer

1. INTRODUCTION

Activating mutations of Ras oncogene have been implicated in the development and progression of many different forms of human malignancies (Forbes et al., 2011; Prior et al., 2012). So far three Ras isoforms were identified. These include K-Ras, N-Ras and H-Ras. Ras belongs to small G protein family with intrinsic GTPase activity and is a major regulator of a plethora of pathophysiological events including growth, proliferation, cytoskeleton integrity, adhesion, migration, differentiation, and survival of cells (Khan et al., 2019).

A point mutation in codon 12 which substitutes Asp for Gly (G12D) has been found in H-Ras and N-Ras (Franks et al., 1997). Although both H-Ras and N-Ras can transform MCF10A cells, only H-Ras induces invasive and migratory phenotypes in these cells (Moon et al., 2000). Thus, aberrant activation of H-Ras signaling has been suggested as a prognostic marker of breast cancer (Clark et al., 1995; Geyer et al., 2018; Moon et al., 2008; Yong et al., 2011). Moreover, H-Ras and K-Ras oncogenes regulate different biological processes, which may differentially impact the overall process of carcinogenesis. While H-Ras is mostly involved with regulation of genes controlling cell morphology related to the epithelial-mesenchymal transition, K-Ras preferentially modulates gene expression responsible for cytokine signaling, cell adhesion, and colonic development. (Roberts et al., 2006).

Peptidyl-prolyl cis-trans isomerase NIMA-interacting 1 (Pin1), consisting of an N-terminal WW domain and a C-terminal peptidylprolyl isomerase (PPIase)

domain, interacts with a protein harbouring phosphorylated serine (Ser)/threonine (Thr) residues that precedes proline (Pro). As a consequence, the conformation of the bound proteins is altered, which influences their subcellular localization, stability, interaction with other proteins, and biological activities (Lu et al., 2002; Ryo et al., 2001; Ryo et al., 2002).

Pin1 has been shown to be upregulated in several different types of cancer tissues (Bao et al., 2004; Wulf et al., 2001). Pin1 overexpression is associated with neoplastic transformation and uncontrolled growth of tumors (Han et al., 2017; Xu et al., 2016). The oncogenic activity of Pin1 is largely attributed to its ability to stabilize/activate oncoproteins and/or to destabilize/inactivate tumor suppressors (Girardini et al., 2011; Takahashi et al., 2007). Ablation of Pin1 in H-Ras transgenic mice or p53-knockout mice suppressed tumorigenesis (Hu et al., 2017; Nicole Tsang et al., 2013; Yeh et al., 2004). It has been reported that H-Ras signaling cooperates with Pin1, which leads to enhanced transcriptional activity of c-Jun towards Cyclin D1 (Wulf et al., 2001).

Nuclear factor E2-related factor 2 (Nrf2) is a leucine zipper transcription factor that plays an essential role in maintaining the cellular redox balance against oxidative stress. In basal conditions, Nrf2 forms an inactive complex with the inhibitory protein Keap1. Keap1 facilitates degradation of Nrf2 through the ubiquitin-proteasome system. Some electrophilic molecules and reactive oxygen species (ROS) modify the critical sensor cysteine residues of Keap1, which can disrupt its sequestration of Nrf2 in the cytoplasm. As a result, Nrf2 is released from Keap1 and translocates to the nucleus where it binds to the antioxidant response

element (ARE) or electrophile response elements (EpRE) present in the promoter regions of target genes (Itoh et al., 1999; Motohashi et al., 2004; Taguchi et al., 2017; Yu et al., 2000).

In recent years, Nrf2 and some of its target proteins have been shown to play differential roles in multi-stage carcinogenesis, acting either as tumor suppressors or tumor promotors (Taguchi et al., 2017). While transient induction of Nrf2 in normal cells activates a broad spectrum of cellular defense signaling pathways against various carcinogenic insults, constitutively elevated accumulation of Nrf2 in transformed or malignant cells can create a redox environment that favours tumor growth and promotes resistance to anticancer therapy (Cheng et al., 2019; Ge et al., 2017; Lu et al., 2017; Rojo de la Vega et al., 2018; Wang et al., 2008). As such, persistent activation of Nrf2 in tumors is generally correlated with poor prognosis (Ge et al., 2017).

Nrf2 harbours multiple Ser/Thr-Pro motifs (Sun et al., 2009), and can hence be a putative substrate of Pin1. The expression of Nrf2 and Pin1 has been reported to be induced by H-Ras activation (Funes et al., 2014; Han et al., 2016; Kamimura et al., 2011; Kitamura et al., 2017; Lim et al., 2019; Liang et al., 2019; Ryo et al., 2002; Ryo et al., 2009). This prompted us to explore the possibility that Pin1 binds and structurally modify Nrf2 in H-Ras transformed mammary epithelial cells, thereby influencing the proliferation and survival of these cells.

2. MATERIALS AND METHODS

2.1. Reagents and antibodies

Dulbecco's Modified Eagle Medium (DMEM) Nutrient mixture F-12 (Ham), DMEM, penicillin/streptomycin mixtures and fetal bovine serum (FBS) were obtained from Gibco BRL (Grand Island, NY, USA). TRIzol® reagent and Stealth™ RNAi negative control duplexes were purchased from Invitrogen Life Technologies, Inc. (Carlsbad, CA, USA). Primary antibodies for Pin1 and vascular endothelial growth factor (VEGF) were supplied by Santa Cruz Biotechnology (Santa Cruz, CA, USA). Primary antibodies for Nrf2 and GTP-H-Ras were supplied by Abcam (Abcam; Cambridge, UK). Secondary antibodies were purchased from Zymed Laboratories Inc. (San Francisco, CA, USA). Dithiothreitol (DTT) and cycloheximide (CHX) were purchased from Sigma-Aldrich (St. Louis, MO, USA). A Western blot detection kit (Absignal) was obtained from Abclon (Seoul, South Korea). Control siRNA and Pin1 targeting siRNA were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All other chemicals used were in the purest form available commercially. Human breast cancer tissue slides (including both adjacent and malignant tissues), obtained from the biorepository of Lab of Breast Cancer Biology at the Cancer Research Institute, Seoul National University (IRB No., 1405-088-580), were used to detect GTP-H-Ras, Nrf2 and Pin1 proteins

2.2. Cell culture

MCF10A and MCF10A-*Ras* cells were cultured in DMEM/F12 supplemented with 5% horse serum, 0.5 μg/ml hydrocortisone, 10 μg/ml insulin, 20 ng/ml epidermal growth factor, 0.1 μg/ml cholera enterotoxin, 100 units/ml penicillin-streptomycin, 2 mM L-glutamine, and 0.5 μg/ml amphotericin. Cells were maintained in a humidified atmosphere with 95% air and 5% CO2 at 37°C. The human breast cancer (MDA-MB-231) cell line obtained from American type culture collection was maintained in DMEM containing 5% FBS at 37°C in a 5% CO2/95% air incubator.

2.3. Lentiviral production and infection

Lentiviruses were produced by transfecting HEK293T cells using lentiviral vectors. In brief, HEK293T cells transfected with PIN1 shRNA lentiviral vector were re-transfected with VSV-G-, pLP1- and pLP2-expressing plasmids, and lentiviral supernatants were collected at 48 h and 72 h post-transfection. MCF10A-*Ras* cells were infected with PIN1-shRNA or control virus with 5 μg/ml polybrene, and stable clones were selected using 1 μg/ml puromycin.

2.4. Anchorage-independent growth assay

To prepare the hard agar layer, 2.5 ml of the boiled agarose solution (3.3%) dissolved in phosphate-buffered saline (PBS) was added immediately to 60-mm

dishes using a pre-warmed pipette and then kept in the 37 °C incubator to solidify. To prepare the soft agar layer containing the cells, MCF10A-*Ras* (1 × 105) or the same number of MCF10A cells were suspended in the 0.33% agarose solution with gentle mixing, and 2.5 ml of this solution was inoculated on top of the hard agar layer. After allowing the solution to harden as a soft agar for 4 h, 2.5 ml of the fresh medium was added to the top of the hardened soft agar layer. After 3 to 4 weeks of incubation, anchorage-independent growth (spherical formation containing >10 cells) was scored using a light microscope. The total number of foci per 1 × 105 cells in a well was counted. For experiments with shPin1 stable MCF10A-*Ras* cells or siH-*Ras* MDA-MB-231 cells, cells were plated in 6-well plates at a density of 150 cells per well. The DMEM/F-12 medium was changed every other day. After one week of incubation, the colonies were fixed in cold methanol and stained by 0.5% crystal violet for 4 h. The stained colonies were washed with PBS to remove the excess dye. Quantitative changes in clonogenicity were determined by extracting stained dye with 10% acetic acid, and the absorbance at 570nm was measured.

2.5. Wound healing assay

Pre-treated MCF10A-*Ras* transfected with control or Pin1 shRNA Lentiviral vector were plated into the ibidi culture insert on 6 well dishes. After 5 h incubation for appropriate cell attachment, the culture-insert was gently removed by using sterile tweezers. Cell migration was observed under the microscope.

2.6. Preparation of cytosolic and nuclear extracts

Cells were pelleted by centrifugation at 1,700 x *g* for 5 min after washing with cold PBS and suspended in ice-cold hypotonic buffer A [10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT and 0.2 mM phenylmethylsulfonyl fluoride (PMSF)]. Following incubation in an ice bath for 15 min, cells were centrifuged again at 6,000 x *g* for 5 min and the supernatant was collected as a cytosolic fraction. The remaining cell pellets were resuspended in ice-cold buffer C containing 20 mM HEPES (pH 7.9), 20% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, and 0.2 mM PMSF and were incubated at 0°C for 1 h. After vortex mixing, the resulting suspension was centrifuged at 18,000 x *g* for 15 min, and the supernatant was collected as a nuclear extract and stored at -70°C.

2.7. In situ proximity ligation assay (PLA)

PLA was carried out using the DUOLinkTM kit (OLINK; Uppsala, Sweden) according to the manufacturer's instructions. In brief, MCF10A-*Ras* cells on glass coverslips were fixed, permeabilized, and blocked with blocking solution (0.1% Triton in PBS containing 5% bovine serum albumin) and incubated with the antibodies against Pin1 (1:100) and Nrf2 (1:200) for 1 h at 37°C. PLA plus and minus affinity probes were then added and incubated for an additional 1 h at 37°C. The probes were hybridized using a ligase to be a closed circle. The DNA was then amplified (a rolling-circle amplification) and detected by fluorescence microscopy.

2.8. Western blot analysis

MCF10A, MCF10A-*Ras* and MDA-MB-231 cells were lysed in lysis buffer [250 mM sucrose, 50 mM Tris-HCl (pH 8.0), 25 mM KCl, 5 mM MgCl2, mM EDTA, 2 mM NaF, 2 mM sodium orthovanadate, and 1 mM PMSF for 1 h on ice followed by centrifugation at 18,000 xg for 20 min. The protein concentration of the supernatant was measured by using the BCA reagents (Pierce; Rockford, IL, USA). Protein (30 μg) was separated by running through 8% or 12% SDS-PAGE gel and transferred to the PVDF membrane (Gelman Laboratory; Ann Arbor, MI, USA). The blots were blocked with 5% non-fat dry milk PBST buffer for 1 h at room temperature. The membranes were incubated overnight at 4 °C with 1:1000 dilution of Pin1 antibody, 1:2000 dilution of Nrf2 antibody, 1:1000 dilution of VEGF antibody, or 1:5000 dilution of GTP-H-*Ras*. Equal lane loading was assured using β-actin (Sigma-Aldrich Co.; St. Louis, MO, USA). The blots were rinsed three times with PBST buffer for 10 min each. Washed blots were treated with 1:5000 dilution of the horseradish peroxidase (HRP)-conjugated secondary antibody (Pierce Biotechnology; Rockford, IL, USA) for 1 h and washed again three times with PBST buffer. The transferred proteins were visualized with an enhanced chemiluminescence detection kit (Amersham Pharmacia Biotech; Buckinghamshire, UK).

2.9. Immunoprecipitation

Cells were lysed in 250 mM sucrose, 50 mM Tris-HCl (pH 8.0), 25 mM KCl, 5

mM MgCl2, 1 mM EDTA, 2 mM NaF, 2 mM sodium orthovanadate, and 1 mM PMSF. Total protein (100 μg) was subjected to immunoprecipitation by using rotation with Nrf2 primary antibody and protein A/G-agarose bead suspension at 4°C, overnight. After centrifugation at 1,000 x g for 1 min, immunoprecipitated beads were collected by discarding the supernatant and washed with cell lysis buffer. The immunoprecipitate was then resuspended in 24 μl of 6X SDS electrophoresis sample buffer and 5X loading dye and boiled for 5 min. The supernatant from each sample was collected by centrifugation and loaded on SDS-polyacrylamide gel.

2.10. Immunohistochemistry and immunofluorescence Analysis

For immunohistochemical analysis of the expression of GTP-H-Ras, 4 mm sections of 10% formalin-fixed, paraffin embedded tissues from breast cancer patients were placed on glass slides and deparaffinized 3 times with xylene and rehydrated through graded alcohol bath. The deparaffinized sections were heated by using microwave and boiled twice for 6 min in 10 mM citrate buffer (pH 6.0) for antigen retrieval. To diminish non-specific staining, each section was treated with 3% hydrogen peroxide and 4% peptone casein blocking solution for 15 min. For the detection of respective protein expression, slides were incubated with GTP-H-Ras antibody (1:500) at room temperature for 40 min in TBST followed by treatment with respective HRP-conjugated secondary antibody (rabbit). The peroxidase binding sites were detected by staining with 3,3'-diaminobenzidine

tetrahydrochloride. Finally, counterstaining was performed using Mayer's hematoxylin. For immunofluorescence staining of human paraffin-embedded breast cancer tissues and matched adjacent normal breast tissues, a standard protocol for deparaffinization, antigen retrieval, and permeabilization was followed. After overnight incubation at 4 °C with anti-Nrf2 (1:200) and anti-Pin1 (1:100) antibodies, the tissue sections were washed with PBS and then labeled with TRITC or FITC-conjugated secondary antibody for 1 h at room temperature. The slides were then visualized under a fluorescent microscope. For immunofluorescence analysis of Nrf2 and Pin1 in shControl or shPin1 MCF10A-*Ras* stable cells, cells were plated on the 8-well chamber slide (105 cells/well). Cells were fixed in 95% methanol for 10 min at −20 °C. After rinse with 1× diluted PBS, cells were incubated in 0.2% Triton X-100 in PBS for 5 min. After three washing steps with 1× PBS, cells were blocked for 2 h in fresh blocking buffer [1× PBS, pH 7.4, containing 5% bovine serum albumin (BSA)] and incubated overnight at 4 °C with anti-Nrf2 or anti-Pin1 antibody. After three washing steps with 1× PBS, the cells were incubated with a diluted (1:1000) TRITC-conjugated anti-mouse or FITC-conjugated anti-rabbit IgG secondary antibody in 1× PBS with 1% BSA at room temperature for 1 h. Cells were also stained with 4′,6-diamidino-2-phenylindole (DAPI) and rinsed with 1× PBS. Stained cells were visualized under a microscope and photographed.

2.11. Small interfering RNA (siRNA) and plasmid transient transfection

siRNA specifically targeting *Pin1* and non-specific si-control were purchased from Santa Cruz Biotechnology (sc-36230). siRNA specifically targeting H-*Ras* was purchased from Thermo Fisher Scientific. Transient transfections with Pin1 siRNA or H-*Ras* siRNA were performed using the Lipofectamine RNAi-MAX transfection reagents according to the instructions supplied by the manufacturer (Invitrogen; Carlsbad, CA, USA). After 48- to 72-h transfection, cells were lysed for Western blot analysis.

2.12. Protein stability assay

The MCF10A-*Ras* cells transfected with control or Pin1 Lentiviral shRNA were treated with 10 μM CHX to block protein synthesis. The cells were collected at different time intervals for Western blot analysis.

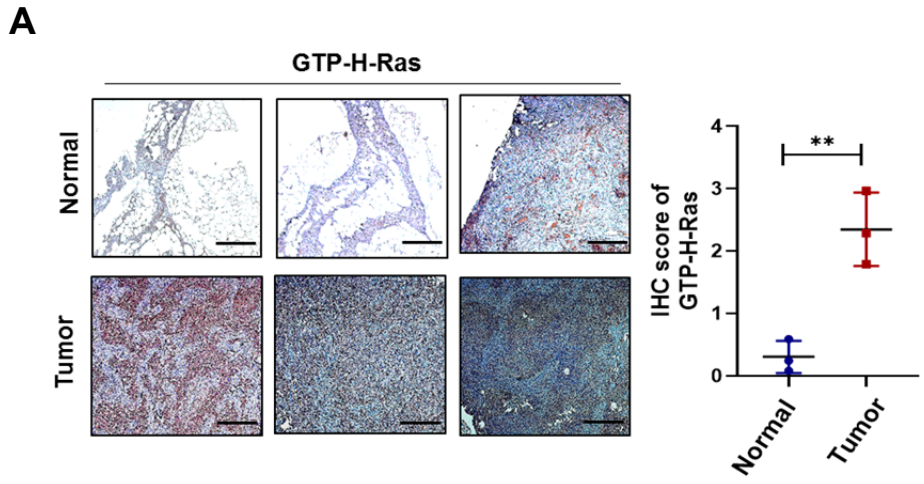
2.13. Statistical analysis

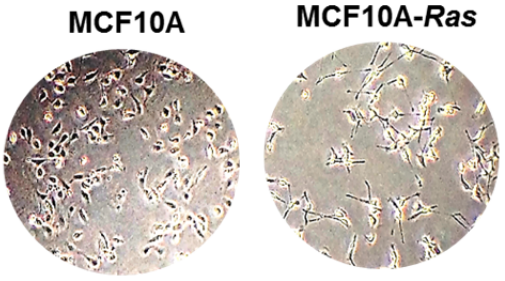
All data are presented as the mean ± SD. Experiments were repeated at least three times. Two-tailed unpaired Student's *t*-tests or one-way ANOVA were used to evaluate the data. Statistical differences were considered significant at $*p < 0.05$; $**p < 0.01$, and $** p < 0.001$.

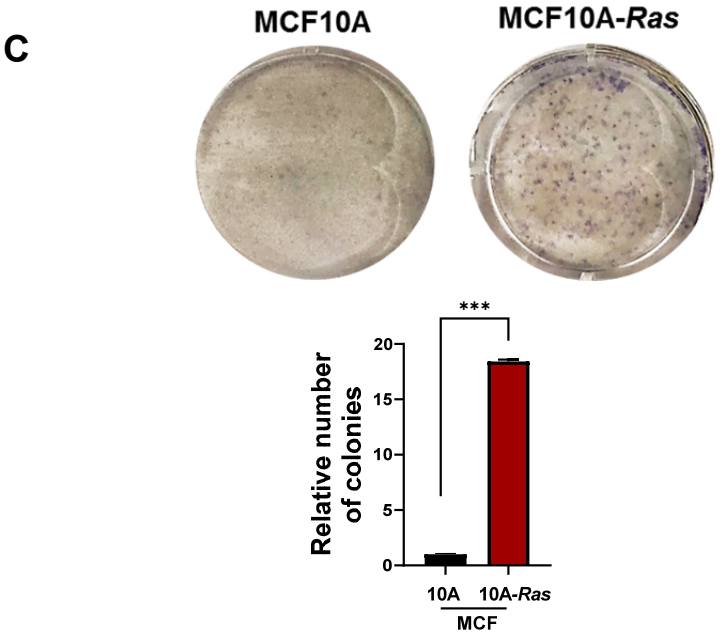
3. RESULTS

3.1. H-Ras is overexpressed and implicated in pathogenesis of human breast cancer

To investigate the correlation between H-*Ras* and breast cancer progression, we compared the expression of H-*Ras* in normal and tumor tissues from breast cancer patients. As illustrated in **Fig. 3-1A**, expression of the active form of H-*Ras* (GTP-bound) was upregulated in the tumor, compared with that in the normal tissues. We have demonstrated that H-*Ras* oncogene transforms non-oncogenic MCF10A human mammary epithelial cells and consequently changes their phenotypic characteristics. Thus, MCF10A-*Ras* cells are considerably elongated, whereas MCF10A cells have a round shape **(Fig. 3-1B)**. The number of colonies formed by anchorage-independent cell growth was dramatically increased in MCF10A-*Ras* cells compared to parental MCF10A cells **(Fig. 3-1C)**. Silencing of H-*Ras* resulted in marked reduction in migration (**Fig. 3-1D**) and clonogenicity (**Fig. 3-1E**) of MDA-MB-231 cells. Next, we investigated the expression of Nrf2 and Pin1 in human breast cancer tissues. As shown in **Fig. 3-1F**, the levels of both proteins were significantly higher in the tumor tissues than in the adjacent normal tissues, which correlated with activated H-*Ras* expression**.** Tumor tissues also exhibited significant upregulation of VEGF, a representative pro-angiogenic marker **(Fig. 3-1F)**.


A
GTP-H-Ras
Normal
Tumor
IHC score of GTP-H-Ras
**
Normal
Tumor


B
MCF10A
MCF10A-Ras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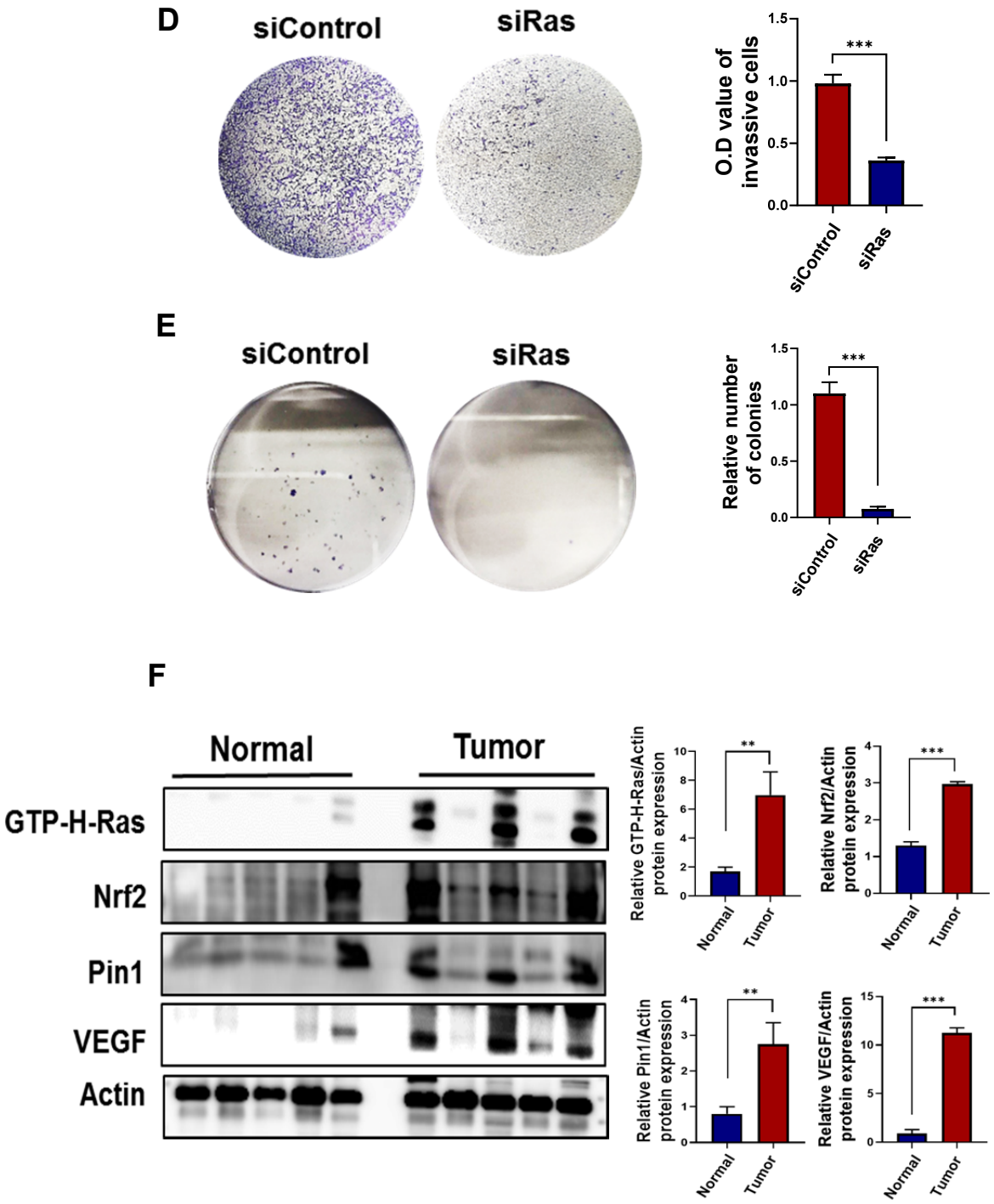
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siControl
siRas
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Normal
Tumor
GTP-H-Ras
Nrf2
Pin1
VEGF
Actin
Relative GTP-H-Ras/Actin protein expression
**
Normal
Tumor
Relative Nrf2/Actin protein expression
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Normal
Tumor
Relative Pin1/Actin protein expression
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Normal
Tumor
Relative VEGF/Actin protein expression
***
Normal
Tumor

Figure 3-1. Overexpression and functional role of H-*Ras* in breast cancer

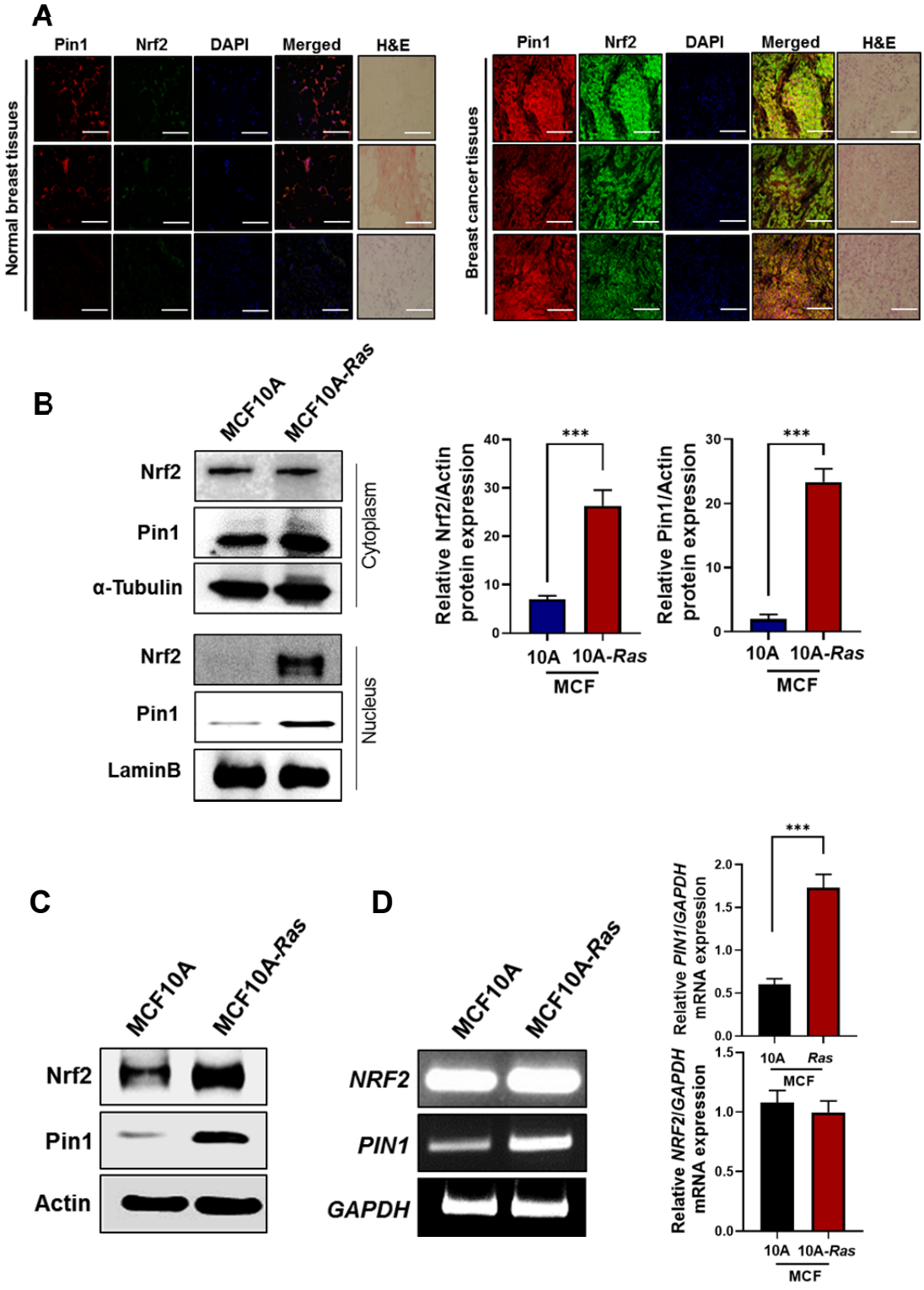
(A) Immunohistochemical analysis of GTP bound H-Ras in breast cancer specimens and adjacent normal tissues. **Significantly different from the control ($p < .01$). Scale bar: 200 μm. (B) The morphology of H-Ras transformed human mammary epithelial cells compared with the non-transformed MCF10A cells. (C) Comparison of anchorage-independent growth of MCF10A-*Ras* cells and MCF10A cells. Cells were treated as described in Materials and Methods. Attached cells were photographed, and the proportion of attached cells was quantified by counting the number of colonies. A representative set of images from three independent experiments is shown. Data are shown as the mean ± SD, and the statistical significance was determined by Student's t-test. ***$p < .001$. (D) Invasiveness of MDA-MB-231 cells was measured using 24-well microchemotaxis chambers. MDA-MB-231 cells were treated with control or H-Ras siRNA as described in Materials and Methods. The randomly chosen fields were photographed, and the number of cells migrated to the lower surface was counted. Data are shown as the mean ± SD of three independent experiments, and the statistical significance was determined by Student's *t*-test. ***$p < .001$. (E) MDA-MB-231 cells seeded in 6-well plates were treated with control or H-Ras siRNA as described in Materials and Methods. Attached cells were photographed after crystal violet staining, and the proportion of attached cells was quantified by counting the number of colonies. A representative set of images from three independent experiments is shown. Data are shown as the mean ± SD, and the statistical significance was determined by

Student's t-test. ***$p < .001$. (F) Comparative expression of GTP-H-Ras, Pin1, Nrf2 and VEGF in breast cancer tissues and adjacent normal tissues measured by Western blot analysis. **$p < .01$, and ***$p < .001$.

3.2. Pin1 and Nrf2 co-localize in nucleus of breast cancer cells

In order to explore the cooperative role of Nrf2 and Pin1 in breast cancer development and progression, we examined their co-localization in human breast cancer specimens. While normal tissues exhibited relatively low protein expression and immunofluorescence (IF) signals from antibodies recognizing Nrf2 and Pin1, the tumor tissues showed highly enhanced intensities and co-localization of both proteins (**Fig. 3-2A**). We also found that MCF10A-*Ras* cells express Nrf2 and Pin1 to a greater extent than the MCF10A parental cells (**Fig. 3-2B**). The protein level of Pin1 was higher in both cytoplasmic and nuclear fractions of MCF10A-*Ras* cells than that in MCF-10A cells (**Fig. 3-2C**). Nrf2 protein was found to be expressed at a similar level in the cytoplasmic fraction of both cell lines, but there was marked elevation in its nuclear accumulation in MCF10A-*Ras* cells (**Fig. 3-2C**). We found that both MCF10A-*Ras* and MCF-10A cells express equivalent levels of *Nrf2* mRNA, but a higher level of *Pin1* mRNA in the former cells (**Fig. 3-2D**).

In another experiment, the subcellular distribution of Nrf2 and Pin1 in both MCF10A and MCF10A-*Ras* cells was examined by immunofluorescence analysis. Consistent with the immunoblot data (**Fig. 3-2C**), Nrf2 and Pin1 were predominantly co-localized in the nucleus in MCF10A-*Ras* cells while they exist mainly in the cytoplasm in MCF10A cells **(Fig. 3-2E)**.


A
Pin1
Nrf2
DAPI
Merged
H&E
Normal breast tissues
Breast cancer tissues
B
MCF10A
MCF10A-Ras
Nrf2
Pin1
α-Tubulin
Cytoplasm
Nrf2
Pin1
LaminB
Nucleus
Relative Nrf2/Actin protein expression
Relative Pin1/Actin protein expression
***
10A
10A-Ras
MCF
C
MCF10A
MCF10A-Ras
Nrf2
Pin1
Actin
D
MCF10A
MCF10A-Ras
NRF2
PIN1
GAPDH
Relative PIN1/GAPDH mRNA expression
Relative NRF2/GAPDH mRNA expression
***
10A
Ras
MCF
10A
10A-Ras
MCF

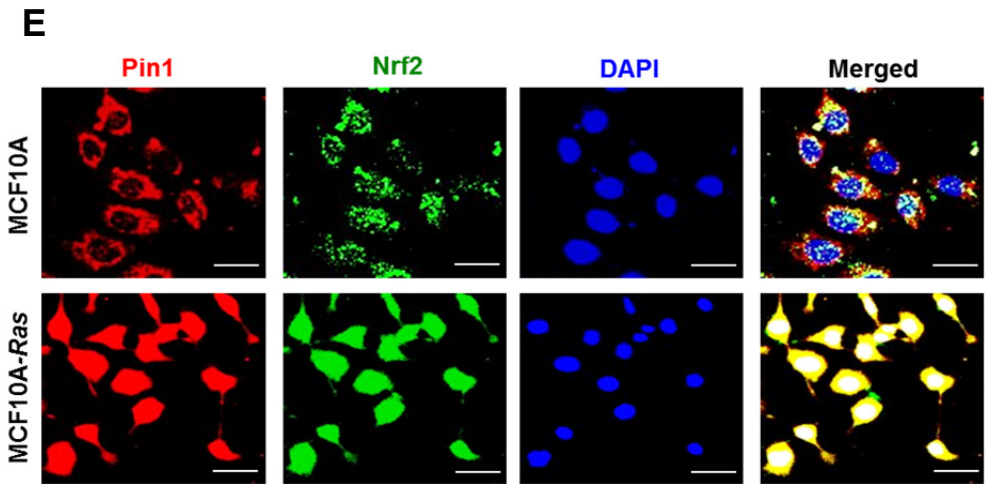
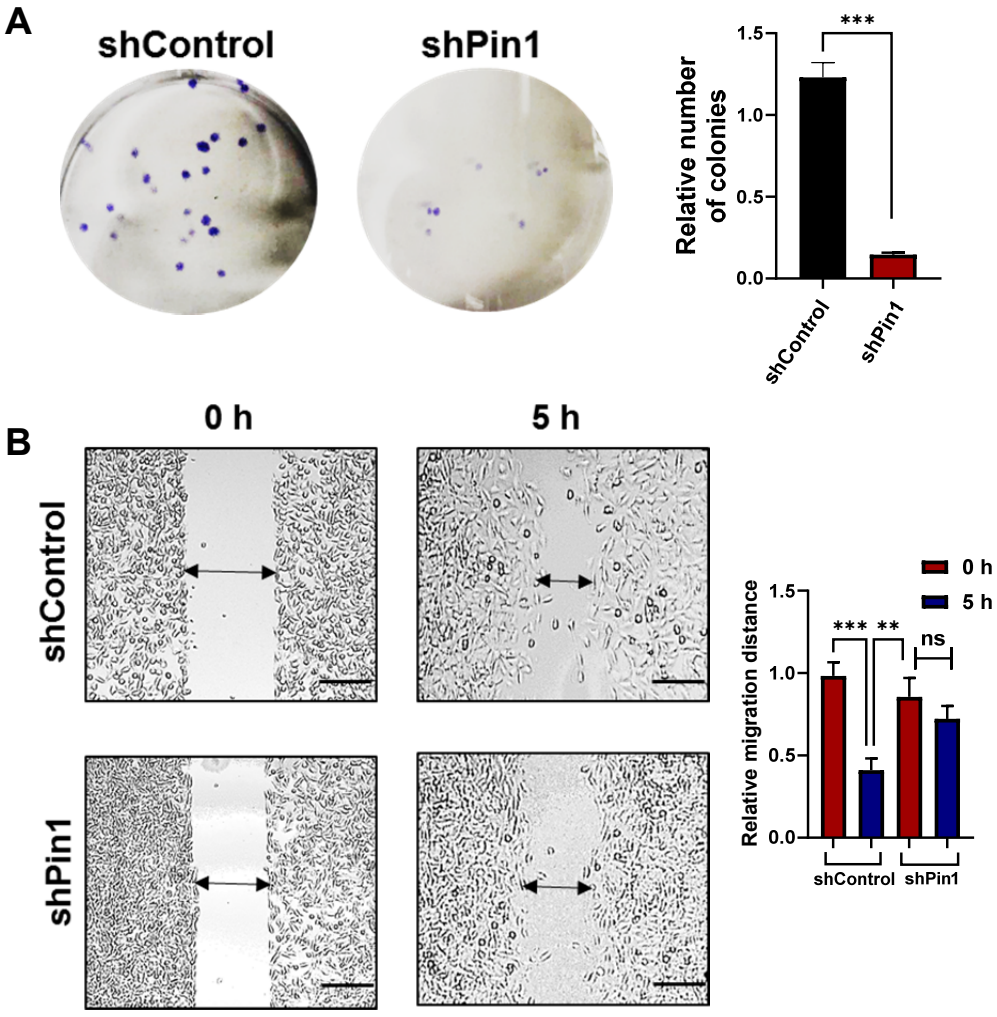


Figure 3-2. Nuclear accumulation of Pin1 and Nrf2

(A) Co-localization of Pin1 and Nrf2 in human breast tumor tissues and H-Ras transformed human breast epithelial cells in culture. (A) Co-localization of Pin1 and Nrf2 in human breast cancer tissues determined by immunofluorescence analysis. Breast cancer specimens were exposed to anti-Nrf2 and anti-Pin1 antibodies. Scale bar: 200 μm. (B and C) Comparative expression of Pin1 and Nrf2 proteins in the whole lysate, cytoplasmic and nuclear fractions of MCF10A and MCF10A-*Ras* cells. ***$p < 001$. (D) Comparison of mRNA expression of Pin1 and Nrf2 in non-oncogenic MCF10A and MCF10A-*Ras* cells. ***$p < .001$. (E) Immunofluorescence staining of Pin1and Nrf2 in MCF10A and MCF10A-*Ras* cells. Scale bar: 200 μm.

3.3. Silencing of Pin1 attenuates the clonogenic and migratory capability of MCF10A-*Ras* cells

The regulation of MCF10A-*Ras* cell growth and proliferation by Pin1 was evidenced by marked reduction in colony formation (**Fig. 3-3A**) and migration (**Fig. 3-3B**) by stable knockdown of its expression using shRNA. Further, silencing of *Pin1* decreased the expression of Nrf2 protein, but not its mRNA transcript (**Fig. 3-3C**). In contrast, knockdown of *Nrf2* failed to suppress Pin1 expression (**Fig. 3-3D**).


A
shControl
shPin1
Relative number of colonies
***
1.5
1.0
0.5
0.0
shControl
shPin1
B
0 h
5 h
shControl
shPin1
Relative migration distance
0 h
5 h
*** **
ns
1.5
1.0
0.5
0.0
shControl
shPin1

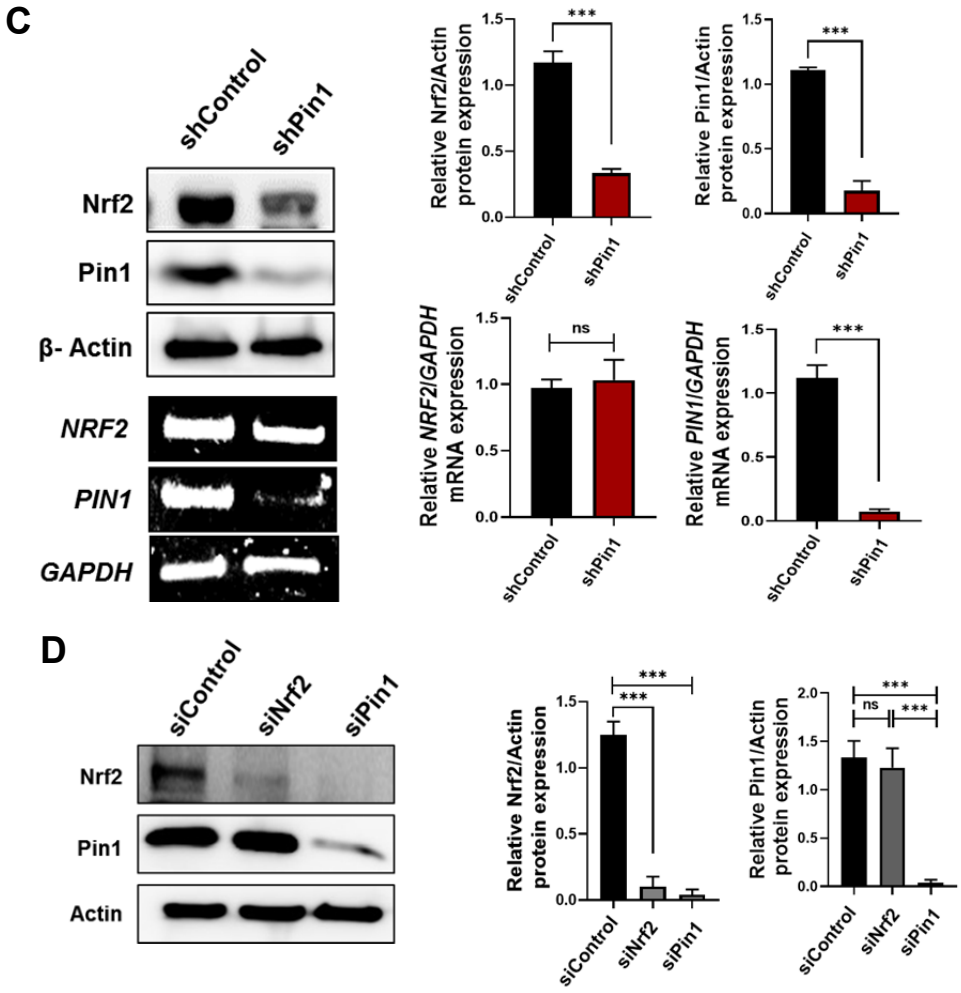


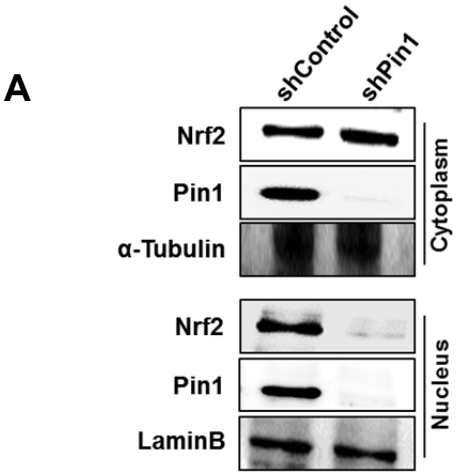
Figure 3-3. Effects of Pin1 on the clonogenicity and migration of MCF10A-*Ras* cells

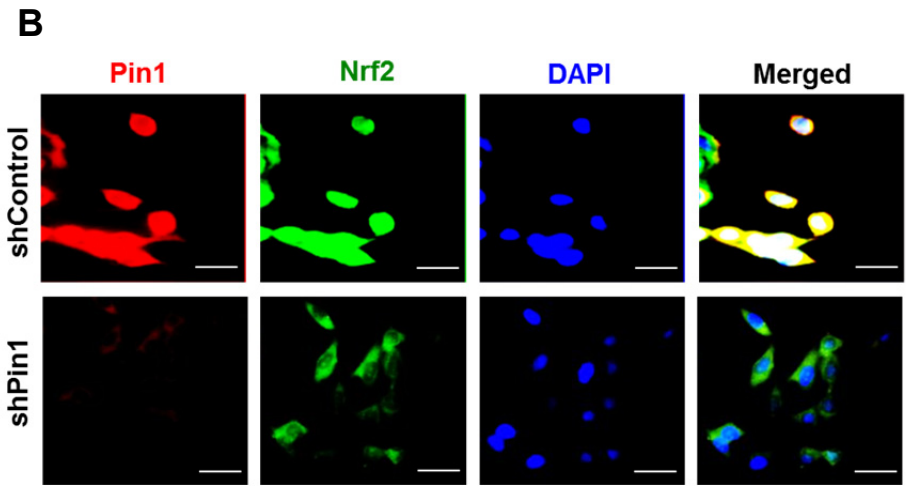
(A) Control or shPin1 MCF10A-*Ras* stable cells were seeded in 6-well plates and treated as described in Materials and Methods. Attached cells were photographed after crystal violet staining, and the proportion of attached cells was quantified by counting the number of colonies. A representative set of images from three

independent experiments is shown. Data are shown as the mean ± SD, and the statistical significance was determined by Student's t-test. ***$p < .001$. (B) Stable MCF10A-*Ras* cells were generated with control or Pin1 Lentivirus shRNA. Then, cell migration was visualized under a microscope. **$p < .01$ and ***$p < .001$. ns, not significant. Scale bar: 200 μm. (C) Effects of Pin1 knockdown on Nrf2 protein and mRNA expression in MCF10A-*Ras* cells. ***$p < .001$. ns, not significant. (D) Differential effects of silencing Pin1 and Nrf2 on expression of Nrf2 and Pin1, respectively. ***$p < .001$. ns, not significant.

3.4. Pin1 depletion inhibits the nuclear accumulation of Nrf2

At the subcellular levels, *Pin1* knockdown did not affect the expression of cytoplasmic Nrf2 protein (**Fig. 3-4A** upper). However, the nuclear accumulation of Nrf2 was abolished in the *Pin1* silenced cells (**Fig. 3-4A** lower). An immunofluorescence assay also verifies that Pin1 depletion inhibits the nuclear translocation of Nrf2 (**Fig. 3-4B**).





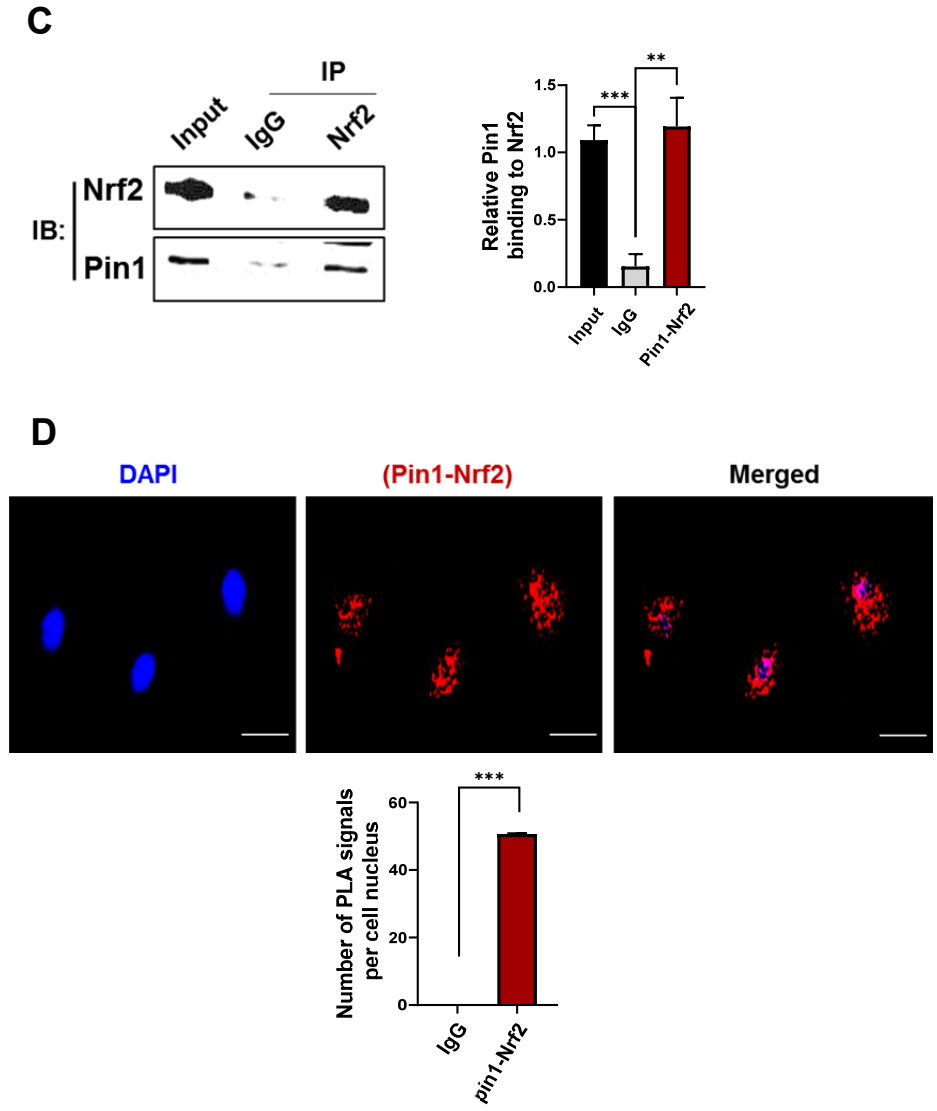
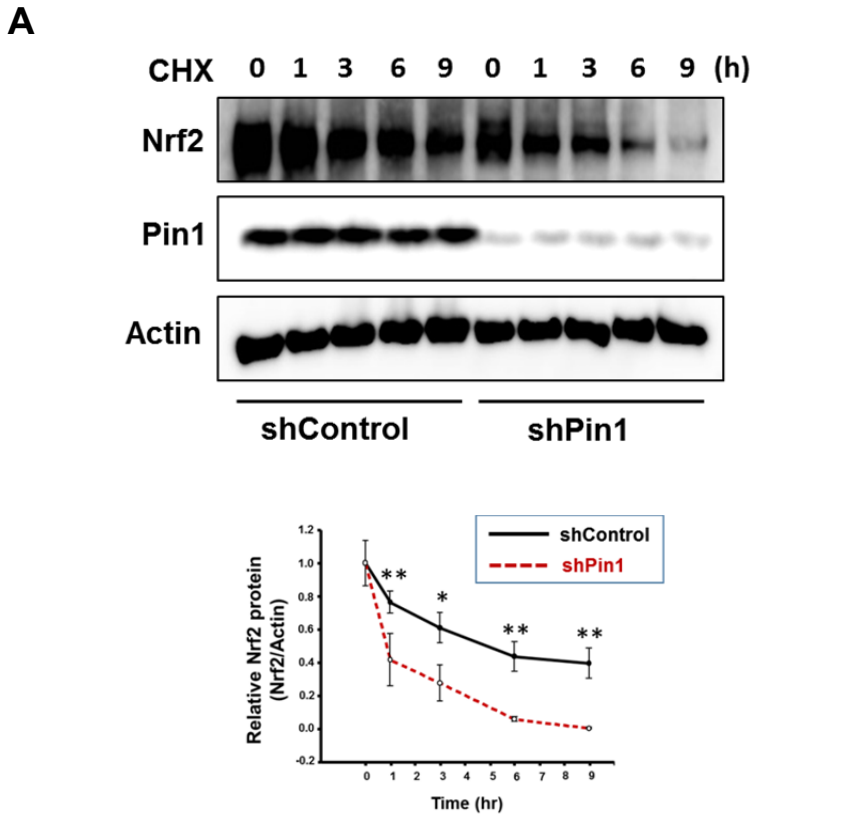

C
IP
Input
IgG
Nrf2
IB:
Nrf2
Pin1
Relative Pin1 binding to Nrf2
1.5
1.0
0.5
0.0
***
**
Input
IgG
Pin1-Nrf2
D
DAPI
(Pin1-Nrf2)
Merged
Number of PLA signals per cell nucleus
60
40
20
0
***
IgG
pin1-Nrf2
E
siControl
siRas
GTP-H-Ras
Nrf2
Pin1
Actin
F
siControl
siRas
IB:
Nrf2
Pin1
IP: Nrf2

Figure 3-4. Pin1 regulates Nrf2 through physical interaction

(A) Effects of stable knockdown of Pin1 on Nrf2 protein expression in the cytoplasmic and nuclear fractions of MCF10A-*Ras* cells. (B) Immunofluorescence staining of Nrf2 in control and Pin1 silenced MCF10A-*Ras* cells. Scale bar: 200 μm. (C) Interaction between Pin1 and Nrf2 in MCF10A-*Ras* cells. The Pin1-Nrf2 complex was detected by immunoprecipitation with anti-Nrf2 antibody followed by immunoblot analysis with an antibody against Pin1 $^{**}p < .01$ and $^{***}p < .001$. (D) Detection of Pin1-Nrf2 interaction in situ. The interaction of Pin1 with Nrf2 was visualized by Duolink analysis (PLA) that allows in situ detection protein interactions with high specificity and sensitivity. Pin1 and Nrf2 were co-labeled with corresponding antibodies. Each red spot represents a single interaction, and nucleus was stained with DAPI. Scale bar: 200 μm. $^{***}p < .001$. (E and F) Effects of H-Ras silencing on the protein expression of Nrf2 and Pin1 as well as H-Ras (E) and interaction between Pin1 and Nrf2 (F) in the MDA-MB-231 cells. Scale bar: 200 μm.

3.5. Pin1 stabilizes Nrf2 in human breast cancer cells

After confirmation of the direct binding of Pin1 to Nrf2, we investigated whether this could affect the stability of Nrf2 in MCF10A-*Ras* cells. MCF10A-*Ras* cells express a higher level of Nrf2 than the parental MCF10A cells (**Fig. 3-2B and Fig. 3-2C**), but equivalent levels of its mRNA transcript (**Fig. 3-2D**). Moreover, silencing of *Pin1* had no effect on the expression of *Nrf2* mRNA, but markedly inhibited its protein expression (**Fig. 3-3C**). These findings suggest that Pin1-induced accumulation of Nrf2 is mediated through stabilization of the Nrf2 protein rather than stimulation of gene transcription. In order to test this possibility, shControl and sh*Pin1* stable cells were treated with CHX to block *de novo* synthesis of proteins. As shown in **Fig. 3-5A**, *Pin1*-silenced MCF10A-*Ras* cells exhibited a significantly elevated degradation rate of pre-existing Nrf2 compared with the shControl group. Treatment with a proteasome inhibitor, MG-132 abolished the degradation of Nrf2 induced by ATRA, a pharmacologic inhibitor of Pin1 (**Fig. 3-5B**). The knockdown of *Pin1* substantially increased the level of ubiquitinated Nrf2 (**Fig. 3-5C**), corroborating the stabilization of Nrf2 by Pin1.


A
CHX 0 1 3 6 9 0 1 3 6 9 (h)
Nrf2
Pin1
Actin
shControl
shPin1
shControl
shPin1
Relative Nrf2 protein (Nrf2/Actin)
Time (hr)
B
ATRA - + - +
MG132 - - + +
Nrf2
Pin1
Actin
Relative Pin1/Actin protein expression
Relative Nrf2/Actin protein expression

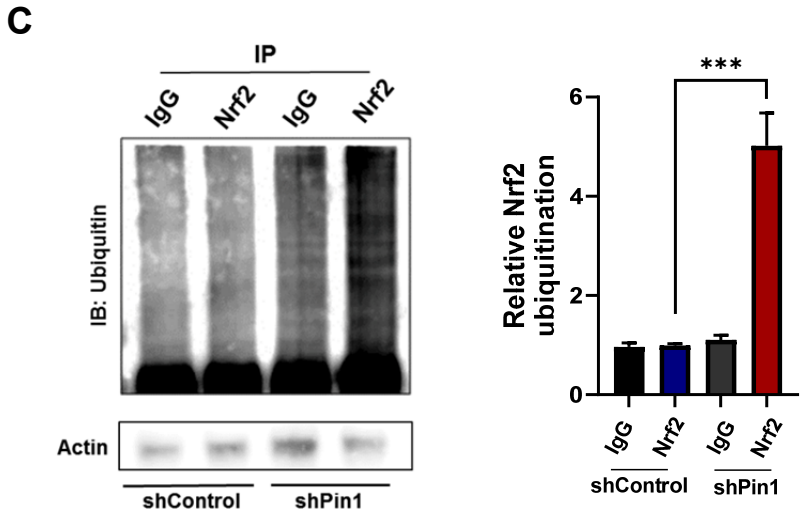


Figure 3-5. Regulation of Nrf2 stability by Pin1

(A) The reduced stability of Nrf2 by Pin1 silencing. shControl and shPin1 MCF10A-*Ras* stable cells were exposed to CHX (10 μM) for the indicated time periods. Cell lysates were subjected to Western blot analysis with anti-Pin1 and anti-Nrf2 antibodies. *p < .05 and **p < .01. (B) Effects of pharmacologic inhibition of Pin1 on Nrf2 protein accumulation in MCF10A-*Ras* cells with or without exposure to the proteasome inhibitor MG-132. After treatment with ATRA (50 μM) for 48 h, cells were exposed to MG-132 (20 μM) for additional 4 h, and subjected to Western blot analysis. *p < .05, **p < .01 and ***p < .001. (C) Effects of Pin1 silencing on ubiquitination of Nrf2 in control and Pin1 knockdown MCF10A-*Ras* cells. Nrf2 ubiquitination was determined by immunoprecipitation of Nrf2 with anti-ubiquitin antibody. ***p < .001.

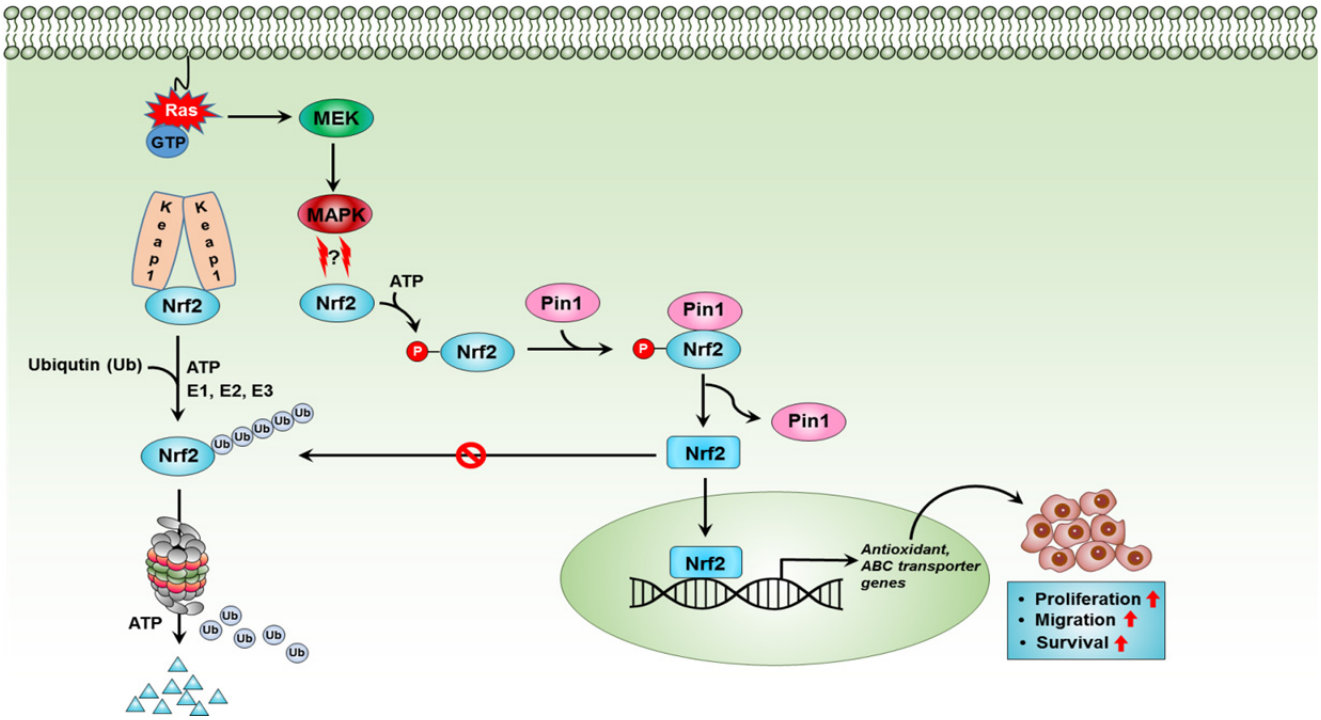


Figure 3-6. Proposed scheme for Nrf2 stabilization by complex formation with Pin1 in breast cancer cells with activated H-Ras

In a resting state, Nrf2 is sequestered in the cytoplasm as an inactive complex with Keap1 (Kelch-like-ECH-associated-protein 1), an adaptor for a cullin-3 (Cul3)-based ubiquitin ligase, responsible for proteasomal degradation of Nrf2. H-Ras in a GTP bound form activates MAPKs via the MEK pathway. Some MAPKs (e.g., ERK) have been known to phosphorylate Nrf2 at specific serine/threonine residues, especially those present in the WW domain binding motif. This facilitates Pin1 binding to Nrf2, which may alter structural conformation of this transcription factor. As a result, Nrf2 escapes from sequestration by Keap1 and hence Cul3-mediated

ubiquitination and degradation. The stabilized Nrf2 translocates to nucleus where it regulates the transcription of antioxidant and other stress responsive genes encoding proteins (e.g., glutamate cysteine ligase, heme oxygenase, ABC transporters, etc.) essential for cancer cell proliferation, migration, and survival.

4. DISCUSSION

Breast cancer is a heterogeneous malignancy consisting of different subtypes that are characterized by distinct histopathological features, specific genetic and epigenetic alterations, and diverse aggressive behaviors (Vargo-Gogola et al., 2007). Mutations of the *Ras* oncogene are among the most frequent genetic alterations in human tumors. The single point mutation at amino acid residue 12 (Gly to Asp) of H-*Ras* is more frequently found in mammary carcinoma (Franks et al., 1997). In contrast, K-*Ras* and N-*Ras* mutations are more predominant in other types of cancers, such as bladder, ovarian, thyroid, lung, colon and rectum, and pancreatic carcinoma; neuroblastoma (Bentires-Alj et al., 2006; Cichowski et al., 2001; Rochlitz et al., 1989; Tartaglia et al., 2005; Watzinger et al., 1999).

It has been reported that non-cancerous human mammary epithelial MCF10A cells transfected with H-*Ras* acquires invasive and migratory phenotypes (Moon et al., 2000). Manifestation of an invasive phenotype by H-*Ras* was also observed in the MDA-MB-453 human breast cancer cell line (Yong et al., 2011). The divergence among the Ras isoforms is attributable to the final 23 to 24 C-terminal amino acids, the so called 'hypervariable region (HVR)' that retains the signals responsible for correct plasma membrane localization of Ras (Jaumot et al., 2002). The C-terminal HVR of H-*Ras*, especially the flexible linker domain with two consecutive proline residues (Pro173 and Pro174), has been shown to play a critical

role in the activation of H-*Ras* and its invasive potential in human breast epithelial cells (Yong et al., 2011).

Pin1 is involved in the majority of main cellular processes of breast cancer development and progression. Pin1 is often overexpressed in breast cancers and associated with worse clinical outcome (Reineke et al., 2008). The WW domain of Pin1 binds to a pSer-Pro or pThr-Pro motif, in a sequence-dependent manner (Lu et al., 1999; Lu et al., 2002). It is noticeable that the C-terminal HVR of H-Ras harbours Pro173 preceded by Ser, which may comprise a WW binding motif, providing a potential binding site for Pin1. Overexpression of Pin1 correlates with upregulation/activation of distinct oncoproteins, such as Cyclin D1, β-catenin, AKT, NF-κB/p65 and PKM2. Pin1 not only binds to phosphorylated c-Jun, but also dramatically increases its ability to transactivate the Cyclin D1 promoter in cooperation with either activated JNK or oncogenic H-Ras (Gianni et al., 2009; Liao et al., 2009; Lu et al., 2014; Moretto-Zita et al., 2010; Wei et al., 2015).

H-*Ras* and some other oncogenic signaling molecules, such as HER2, PI3K and p38, have been shown to induce *Pin1* mRNA expression (Kamimura et al., 2011; Ryo et al., 2002; Ryo et a.l, 2009). Notably, Pin1 overexpression in non-transformed human breast epithelial cells led to neoplastic transformation and also greatly enhanced the acquisition of the transformed phenotype induced by oncogenic H-*Ras* (Ryo et al., 2002). Moreover, Pin1 overexpression disrupts cell cycle coordination leading to centrosome amplification, chromosome instability and breast cancer development (Wei et al., 2015). In contrast, *Pin1* knockout mice

prevented mammary tumorigenesis, even that induced by activated oncogenes including *Ras* (Wulf et al., 2004). In breast cancer, overexpression of both Pin1 and Cyclin E contributes to centrosome amplification, and oncogenic H-Ras activity (Rustighi et al., 2017). Our current study also reveal that PIN1 plays a role in clonogenicity and migration of MCF10A-*Ras* cells as its knowckdown attenuated both events.

Nrf2 plays a central role in cellular stress response. In unstressed conditions, Nrf2 is ubiquitinated by the Keap1-Cul3 complex and subsequently degraded by the proteasomes. Once the cells are exposed to electrophiles or ROS, some sensor cysteine residues of Keap1 is modified, which instigates its inactivation and consequently renders Nrf2 stabilized. Nrf2 then translocates to the nucleus and activates the transcription of cytoprotective genes by binding to ARE/EpRE. However, aberrant overactivation of Nrf2 in tumor tissues is significantly associated with a poor clinical outcome in various cancers (Itoh et al., 1999; Lu et al., 2017; Motohashi et al., 2004; Taguchi et al., 2017; Wang et al., 2008; Yu et al., 2000).

Because Nrf2 potentiates the cellular antioxidant capacities, constitutive activation of Nrf2 in cancer cells promotes their survival against oxidative stress and confers resistance to chemo- and radiotherapy which are mainly dependent on the ROS generation (Itoh et al., 1999; Lu et al., 2017; Taguchi et al., 2017). It is speculated that oncogenic Ras may regulate both pro-oxidant and antioxidant programs depending on the redox status of the tumor cells, in order to promote their growth and progression (Lim et al., 2019). In maintaining the redox balance through

the complementary role of both pro- and antioxidant pathways, Ras may cooperate with Nrf2. Further studies will be necessary to explore the coordinated function of H-Ras and Nrf2-Pin1 complex in the breast cancer progression.

In this study, we have shown that the expression of Pin1 is positively correlated with the accumulation of Nrf2 in human breast tumor tissues as well as in H-*Ras* transformed human breast epithelial cells. It has recently been reported that Pin1 interacts with Nrf2, and the resulting complex co-localizes in the nucleus in pancreatic cancer cells (Liang et al., 2019). Consistent with this observation, we also found that Pin1-Nrf2 complex predominantly accumulates the nucleus in MCF10A-*Ras* cells. The genetic inhibition of *Pin1* markedly reduced the levels of Nrf2 protein. However, silencing of *Pin1* had no significant effect on the mRNA expression of Nrf2, indicating that Pin1 may regulate Nrf2 in a post-translational way. Our data indicate that Pin1 stabilizes Nrf2 by inhibiting its ubiquitination and degradation. Although silencing of Pin1 expression by siRNA decreased the Nrf2 accumulation, knockdown of *Nrf2* had no significant effect on the expression of Pin1, indicating that the Pin1-Nrf2 interaction is unidirectional. Further, H-*Ras* deficiency abolished the interaction between Pin1 and Nrf2 in breast cancer cells. It is likely that H-Ras activation facilitates the association between Pin1 and Nrf2, and thereby stimulates growth and progression of human mammary epithelial cells. We speculate that H-Ras activates some MAP kinases (e.g., ERK), which in turn phosphorylates Nrf2 at specific serine/threonine residues, especially those present in the WW domain binding motif. This promotes Pin1 binding to Nrf2, but elucidation of a more detailed mechanism will be necessary.

In conclusion, Nrf2 can be a novel partner protein of Pin1 and the Pin1-Nrf2 interaction facilitated in the presence of H-*Ras* may contribute to human breast cancer development and progression (**Fig. 3-6**). Therefore, the Pin1-Nrf2 axis can be considered a novel therapeutic target, especially for H-*Ras* mutated breast cancer.

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국문 초록

인체 유방암에서 Peptidyl-prolyl cis-trans isomerase NIMA-interacting 1 (Pin1)과의 직접 결합을 통한 Nrf2 의 안정화 연구

단백질의 인산화는 세포내 신호전달 단백질들의 활성에 영향을 미치며, 세포내 기능 조절을 위해 매우 중요한 역할을 담당한다. 인산화 부위 중 세린/트레오닌 다음에 특이적으로 프롤린 (pSer/Thr-Pro)이 위치할 경우 CDKs, MAPKs, and GSK-3β등과 같은 proline directed kinase 들 (에 의해 인산화된다. 프롤린은 *cis* 나 *trans* 의 conformation 을 모두 채택할 수 있어 단백질의 접힘이나 기능 활성을 조절한다. Pin1 (peptidyl-prolyl isomerase family of proteins)은 프롤린 잔기의 *cis/trans* 의 이성화반응을 촉매하는 효소로서 인산화된 세린/트레오닌 다음에 프롤린이 오는 motif 에 유일하게 결합하여 *cis/trans* 이성질화를 촉진한다. Pin1 의 구조는 N-말단에 있는 WW 도메인과 C-말단에 있는 PPIase 도메인으로

이루어져 있으며, WW 도메인은 기질 단백질과 결합하고 PPIase 도메인은 인산화된 세린/트레오닌-프롤린 (pSer/Thr-Pro) 모티프의 프롤린 이성질화를 통한 구조 변화에 관여한다. Pin1 에 의한 기질 단백질의 구조 변화는 단백질의 활성, 단백질 상호결합 및 세포내 분포와 안정성에 영향을 미친다. Pin1 은 세포 주기 발달에서 중요한 역할을 하는 단백질들을 조절하는 것으로 알려져 세포내의 Pin1 단백질 발현 수준의 변화는 세포의 과도한 증식과 관련된 질병인 암의 발생 및 진행에 영향을 줄 수 있다

Nrf2 는 산화적 스트레스에 의해 유발되는 염증, 암 발생, 심혈관계 질환, 및 당뇨등의 병리학적 과정에 관여하여 세포를 보호해 준다. 정상적인 상태에서 Nrf2 는 저해단백질인 kelch-like ECH-associated protein 1 (Keap1)에 결합된 비활성 상태로 세포질내에 존재한다. 하지만 세포가 활성산소종 또는 친전자성 물질에 의해 자극되면 Nrf2 의 인산화 또는 Keap1 이 thiol modification 을 통해 Keap1 으로부터 Nrf2 가 분리된다.

이후 Nrf2 는 핵내로 이동하고, 핵내에 존재하는 small Maf 단백질과 복합체를 이룬후 항산화 효소의 프로모터 지역에 결합함으로써 phase II 항산화/해독화 효소의 발현을 조절한다. 그러나 스트레스에 반응하여 일시적으로 활성화되는 정상세포의 경우와 달리 악성 종양 세포에서 Nrf2 의 과도한 지속적인 발현은 암세포의 성장을 촉진시키고 항암치료의 효과를 감소시킨다. Nrf2 단백질에도 다수의 Pin1 결합부위 (pSer/Thr-Pro motifs)가 존재하므로, Pin1 과 Nrf2 과의 상호작용과 이를 통한 유방암 증식 및 진행에 미치는 영향에 관하여 연구하였다. 특히, Nrf2 단백질의 세린 215, 408 and 577 의 인산화 잔기가 Pin1 과의 결합 부위임을 확인하였고, Pin1 과 Nrf2 의 결합은 Nrf2 의 프로테아좀에 의한 분해를 억제함으로써 Nrf2 의 안정화에 관여함을 알 수 있었다.

한편, Nrf2 의 주요 억제 단백질인 Keap1 은 유방암 세포내에서 세린 104 번 잔기와 트레오닌 277 번 잔기에서의 인산화가 이루어짐을 ESI-LC-MS 분석을 통하여 확인하였고, 세린 104 번 잔기와 트레오닌 277 번

잔기의 인산화가 차단된 돌연변이 세포는 Pin1 과의 직접적 결합이 Keap1 wild type 세포에 비해 현저히 감소함을 확인 할 수 있었다. 또한 Pin1 siRNA 를 처리한 MDA-MB-231 세포에서는 control siRNA 그룹에 비해 Nrf2 와 Keap1 의 결합이 증가하였으며, Keap1 knockout 마우스 배아에서 분리한 섬유아세포(embryonic fibroblasts)에서는 Nrf2 와 Pin1 의 결합이 증가되었다. 이를 통하여 Pin1 과 Nrf2 는 Keap1 과의 결합에 있어서 서로 경쟁적 역할을 함을 확인하였다. 그러므로 Pin1 은 Nrf2 와 Keap1 사이의 결합을 방해하며 Nrf2 단백질의 활성화와 안정화에 관여할 것으로 사료된다.

키워드: Pin1, Nrf2, Keap1, 단백질-단백질 상호작용, 유방암

Soma Saeidi

Address. #142-412, 1 Gwanak-ro, Gwanak-gu, Seoul, South Korea 08826

Tel. +82-10-6712-4612

E-Mail. saeidi@snu.ac.kr

Born. 28th July 1986, Sanandaj city, Kurdistan Province, Iran

Based in Seoul, South Korea

Education

03/2015 –08/2020 **Ph.D. course (Thesis Adviser: Prof, Young-Joon Surh)**; Department of Molecular Medicine and Biopharmaceutical Sciences, Seoul National University, Seoul, South Korea

- Thesis title: **Peptidyl-Prolyl *cis-trans* isomerase NIMA-interacting 1 (Pin1) directly binds and stabilizes Nrf2: Implications for breast cancer growth and progression**

03/2013 – 02/2015 **Master of science;** Department of Molecular Medicine and Biopharmaceutical Sciences, Seoul National University, Seoul, South Kore

- Thesis title: **Protective Effects of Pyropia yezoensis Glycoprotein against Ethanol-induced Chronic Gastric Injury in the Rat**

09/2005– 02/2009 **Bachelor's of engineering, Food Science and Nutrition,** College of Food Sciences and Nutrition, Islamic Azad University, Kurdistan, Sanandaj, Iran

Research Interest

- Cancer prevention research, Cancer treatment research, Cancer genomics research, Drug development research.
- Protein-Protein interactions, Molecular mechanism of tumor microenvironment, Targeting immune suppression pathways in cancer, Functional genomic screening, Drug targeted therapies.

Publication

- Soma Saeidi, Su-Jung Kim, Hyeong-Jun Han, SeongHoon Kim, Jie Zheng, Han-Byoel Lee, *et al*. H-Rasinduces Nrf2-Pin1 interaction: Implications for breast cancer progression. Toxicol Appl Pharmacol **2020**;115121.
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- Bang HY, Park SA, Saeidi S, Na HK, Surh YJ. Docosahexaenoic Acid Induces Expression of Heme Oxygenase-1 and NAD(P)H:quinone Oxidoreductase through Activation of Nrf2 in Human Mammary Epithelial Cells. Molecules

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Selected Conference Paper and Presentation

- Bang HY, Saeidi S, et al. Docosahexaenoic acid induces expression of heme oxygenase-1 and NAD(P)H:quinone oxidoreductase through activation of Nrf2 in human mammary epithelial cells. *Molecules*, 22, pii: E969.
- Soma Saeidi, et al. (2014), Protective Effects of Pyropia yezoensis Glycoprotein against Ethanol-induced Chronic Gastric Injury in the Rat. *Kor. J. Fish. Aquat. Sci*, 47, 765-769.
- Poster presentation in IUBMB 2018(24th IUBMB Congress and 15th FAOBMB Congress), "The Peptidyl Prolyl Isomerase Pin1 stabilizes Nrf2 in H-*ras* Transformed Mammary Epithelial Cells" (Jun. 4th- 8th, 2018, COEX, Seoul, South Korea)- "Integrating Science for Bio-Health Innovation"
- Oral presentation in International Conference of the 8th Asiatox2018 (Asian Society of Toxicology), "Pin1 stabilizes Nrf2 in *ras*-transformed human mammary epithelial cells: Implications for its role in breast cancer progression" (June. 17th- 20th, 2018, Pattaya, Thailand)
- Oral presentation in The 9th Biennial Meeting of Society for Free Radical Research-Asia (SFRR-Asia) "Pin1 exerts an oncogenic function by directly

binding and stabilizes Nrf2 in *ras*-transformed human mammary epithelial cells" (April. 4th- 7th, 2019, Kyoto, Japan)

- Oral and poster presentation in The Environmental Response V international conference"Pin1 directly binds and stabilizes Nrf2 in Ras-transformed human mammary epithelial cells: Implications for its role in breast cancer growth and progression" (September. 12th- 14th, 2019, Sendai, japan)

Honors and Awards

- Travel award from the 9th Biennial Meeting of Society for Free Radical Research-Asia (SFRR-Asia), April 4th-7th, Kyoto, Japan
- Best Poster Presentation award on the occasion of the International Conference, 21th Annual Meeting of the Korean Society of Cancer Prevention (KSCP), Dec 8th- 9th, 2016, Seoul, South Korea
- Young Investigator Award at the International Conference of the Korean Society of Cancer Prevention (KSCP), July 6th- 8th, 2017, Osaek, South Korea
- Young Scientist Award at the 12th International Conference and 5th Asian Congress on Environmental Mutagens (in conjunction with the 33rd Annual Meeting of KSOT/KEMS), Nov, 12th- 16th, 2017, Incheon, South Korea
- Young Investigator Award at the Summer Symposium of the Korean Society of Cancer Prevention (KSCP), July 5th- 7th, 2018, Osaek, South Korea

- Outstanding poster presentation award from the International Journal of Molecular Sciences on the occasion of The 10th International Conference on Heme Oxygenase, October, 31th – November, 3rd, 2018, Seoul, South Korea
- Travel award from the 9th Biennial Meeting of Society for Free Radical Research-Asia (SFRR-Asia), April 4th-7th, Kyoto, Japan
- Young Investigator Award at the Summer Symposium of the Korean Society of Cancer Prevention (KSCP), July 5th- 7th, 2019, Osaek, South Korea
- Poster selected award at The Environmental Response V conference, September 12^{th} – 14^{th},2019, Sendai, Japan