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C-terminus of Hsc70-interacting protein regulates profilin1 and breast cancer cell migration

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ABSTRACT

Profilin1 (Pfn1) is a key mediator of actin polymerization and regulates cell migration. Low expression of Pfn1 is implicated in tumorigenesis of various cancers, including breast cancer. The regulatory mechanism behind Pfn1 levels has not yet been elucidated. In the present study, we find that Pfn1 is poly-ubiquitinated in human cell lines, and a portion of poly-ubiquitinated Pfn1 is regulated in a proteasome-dependent manner. C-terminus of Hsc70-interacting protein (CHIP), a co-chaperone E3 ligase, interacts with and ubiquitinates Pfn1, targeting it for proteasome-dependent degradation. Depletion of CHIP stabilizes Pfn1, suggesting that CHIP functions as a major E3 ligase for Pfn1. Stable expression of wild-type CHIP in the breast cancer cell line MDA-MB231 yielded downregulation of Pfn1 and enhanced cell migration. Pfn1 overexpression in MDA-MB231 cells expressing wild-type CHIP suppressed the enhanced cell migration. Taken together, our results demonstrate that CHIP regulates Pfn1 levels as an E3 ligase, and possibly plays a role in cell migration and metastasis of breast cancer.

1. Introduction

Profilins are key mediators of actin polymerization, and essential for cell morphology and migration [1–3]. Among the four profilin genes found in mammals, profilin1 (Pfn1) is ubiquitously expressed while others show tissue specific expression [1,3]. Silencing of Pfn1 inhibits proliferation and migration of endothelial cells [4], and a study that utilized Pfn1 knockout mice showed that Pfn1 is essential for cell survival and cell division in early development [5]. Pfn1 has also been implicated in tumorigenesis as a tumor suppressor. Pfn1 has been shown to be downregulated in human pancreas, breast, liver, and bladder cancers [6–9], and depletion of Pfn1 led to faster migration of the breast cancer cell line MDA-MB231 [10]. More importantly, downregulated Pfn1 levels correlate with the metastasis of breast cancer [11]. The molecular mechanism underlying diminished levels of Pfn1 in various cancer tissues is not known. Though, recent studies have shown that phosphorylation of Pfn1 by ROCK1 leads to its degradation in a proteasome-dependent manner [12] as well as expression of expanded polyglutamine-targeted Pfn1 for proteasomal degradation and altered actin dynamics [13]. These studies suggest that proteasome activity is involved in the regulation of Pfn1 levels, but the molecular mechanism behind this regulation remains to be elucidated.

C-terminus of Hsc-70 interacting protein (CHIP) is a co-chaperone E3 ligase that was originally shown to ubiquitinate misfolded proteins presented by chaperones such as heat shock protein 70 (Hsp70), thereby implicating CHIP in the pathogenesis of protein aggregation diseases [14]. Recent studies have shown that CHIP also regulates specific target proteins. For example, CHIP regulates oncogenic proteins such as steroid receptor co-activator 3 [15] in breast cancer, p65 in gastric and colorectal cancer [16,17], and androgen receptor in prostate cancer [18]. CHIP levels are typically downregulated in late stages of various cancers, including breast cancer [15–17,19]. These findings indicate that CHIP plays a regulatory role in tumorigenesis through regulating oncogenic proteins.

In this work, we investigated potential Pfn1–CHIP interactions. We examined the role of CHIP as an E3 ligase that regulates Pfn1 levels via the ubiquitin proteasome system. Finally, we investigated a role for CHIP in breast cancer cell migration through regulation of Pfn1.

Abbreviations: Pfn1, profilin1; CHIP, C-terminus of Hsc70-interacting protein; Hsp, heat shock protein; TPR, tetratricopeptide; BiFC, bimolecular fluorescence complementation; GFP, green fluorescent protein; CHX, cycloheximide; DAPI, diamidino-2-phenylindole; EDTA, ethylenediaminetetraacetic acid.

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2. Materials and methods

2.1. Cell culture and RNA interference

HeLa cells were maintained in MEM (Welgene) supplemented with 10% FBS at 37 °C in a humidified 5% CO_2 atmosphere. HEK293, MDA-MB231, and SH-SY5Y cells were maintained in DMEM (Welgene) supplemented with 10% FBS. HEK293 or HeLa cells were transfected with 100 pmol of CHIP-targeting siRNA (si-CHIP, Dharmacon) or scrambled RNA (Ambion, Cat. #4635) using Lipofectamine 2000 (Invitrogen). After 48 h, cells were harvested and lysed for western blotting (WB). The sequence used for siCHIP is as follows: CGCUGGUGGCCGUGUAUUA.

2.2. Antibodies and co-immunoprecipitation

Anti-Myc (Millipore), anti-profilin1 (Youngin Frontier), anti-HA (Covance), anti-GST (Santa Cruz Biotechnology), and anti-actin (Bethyl) were used for WB or co-immunoprecipitation (co-IP) analyses. For co-IP, lysates were incubated with the indicated antibody overnight at 4 °C. Immunocomplexes recovered with protein-A Sepharose (Sigma) were washed three times with washing buffer (20 mM Tris pH 8.0, 150 mM NaCl, 1 mM EDTA, 0.1% Triton X-100) and analyzed by WB.

2.3. Protein half-life

HeLa cells were co-transfected with Pfn1-mychis and empty vector, HA-CHIP WT, or HA-CHIP K30A expression plasmids. Twenty-four hours after transfection, cells were treated with 200 μg/ml cyclohexamide (CHX) and harvested at indicated time points. Cells were lysed with RIPA buffer (150 mM NaCl, 50 mM Tris pH 7.4, 0.1% SDS, 1% Triton X-100, 0.5% sodium-deoxycholate) and analyzed by WB.

2.4. GST pull-down assay

GST-CHIP, Pfn1-6His, and Hsp70-6His expression plasmids were generated by cloning into pGEX4T-1 and pET23a vectors. Recombinant proteins were produced in bacteria and purified. GST or GST-CHIP on glutathione-agarose beads (GE Healthcare) was incubated with Pfn1-6His or Hsp70-6His in lysis buffer (0.1 M Tris pH 8.0, 100 mM NaCl, 2 mM EDTA, and 5% glycerol) for 2 h at 4 °C in a rotator. The glutathione beads were washed three times, and precipitated proteins were analyzed by WB.

2.5. Bimolecular fluorescence complementation (BiFC) assay

Pfn1 and ubiquitin (Ub) genes were cloned into the VN 173 and VC 155 vectors (a kind gift from Dr. Lee K.H., Chosun Univ.), respectively. HeLa or SH-SY5Y cells were co-transfected with VN173-Pfn1 and VC155-Ub. Imaging and analysis protocols were previously described [30].

2.6. Wound healing assay

MDA-MB231 cells stably expressing empty vector, HA-CHIP WT, or HA-CHIP ΔU Box were seeded onto a 60 mm culture plate to generate a monolayer. The monolayer of cells was gently scratched with sterile tips and then incubated in DMEM with 2% FBS. Phase-contrast images of three random locations were acquired at indicated time points using a digital camera and microscope (Olympus). Wound closure areas were measured and a percentage change was calculated with the formula: Area (%) = 100 × (wound closure area at 24 h ÷ wound closure area at 0 h).

3. Results and discussion

3.1. A portion of poly-ubiquitinated Pfn1 in human cells is regulated in a proteasome-dependent manner

As intracellular Pfn1 ubiquitination and its mechanism have not yet been described, we first examined if Pfn1 is ubiquitinated in human cells. We performed BiFC assays to examine *in vivo* Pfn1 ubiquitination [20,21]. Bright GFP signals, indicating Pfn1 ubiquitination, were observed mainly in the cytoplasm, and in nuclei with lower intensities, in HeLa and human neuroblastoma (SH-SY5Y) cell lines (Fig. 1A). Next, we co-expressed Pfn1-mychis and HA-Ub in HeLa cells and performed a ubiquitination assay. WB analysis revealed numerous bands above the IgG heavy chain that indicate poly-ubiquitination of Pfn1 (Fig. 1B). Poly ubiquitination of Pfn1 increased upon MG132 treatment (Fig. 1B). These results indicate that Pfn1 is poly-ubiquitinated in human cell lines, but only a portion of poly-ubiquitinated Pfn1 is targeted to the proteasome. This is interesting because it was expected that Pfn1 ubiquitination would proceed only in the presence of MG132, and previous reports have shown MG132 to affect Pfn1 stability [12,13]. Our results suggest that the pool of ubiquitinated Pfn1 might involve different sub-types of ubiquitination, namely one for proteasomal degradation and another for use as regulatory signals. Consistent with previous reports [22], we observed that the majority of GFP-Pfn1 was cytoplasmic prior to MG132 treatment (Fig. 1A), but became nuclear and cytoplasmic in the presence of MG132 (Fig. 1C). These results collectively suggest that the pool of ubiquitinated Pfn1 might consist of different types of ubiquitination, and particular types of ubiquitinated Pfn1 might provide signals for protein localization and subsequent proteasome-mediated degradation.

Various types of ubiquitination, involving different isopeptide linkages formed using seven lysine (K) residues of Ub, have been suggested [23,24]. Among them, the most widely accepted canonical ubiquitination involves the formation of isopeptide linkages via K48 of Ub, providing a signal for proteasomal degradation. Therefore, we tested whether ubiquitination of Pfn1 involves K48-mediated isopeptide linkages by using a Ub K48R mutant in which the K48 residue is mutated to arginine (R). A ubiquitination assay using HEK293T cells showed that Ub K48R affected poly-ubiquitination of Pfn1 in a similar manner as Ub WT (Fig. 1D). This indicates that Pfn1 ubiquitination is not dependent on K48, and non-K48-mediated isopeptide linkages might be formed for use as regulatory signals as well as proteasome targeting. Correspondingly, recent studies have reported that non-K48-mediated isopeptide linkages are utilized for targeting proteins to the proteasome as well as production of regulatory signals [24]. Taken together, these results demonstrate that in human cells, Pfn1 is poly-ubiquitinated via non-K48-mediated isopeptide linkages and a portion of ubiquitinated Pfn1 serves as a signal for proteasome-dependent degradation.

3.2. Pfn1 interacts with CHIP via the TPR domain

Next, we searched for a candidate E3 ligase of Pfn1. Bacterial Hsp70 is believed to participate in quality control of cytoskeletal proteins in conjunction with its cognate chaperones [25]. Indeed, Pfn1 has been shown to interact with Hsp70 in human T-cell leukemia [26]. Additionally, CHIP is an E3 ligase that associates with molecular chaperones (Hsp70 or Hsp90), and is known to ubiquitinate the client proteins of molecular chaperones. Considering these

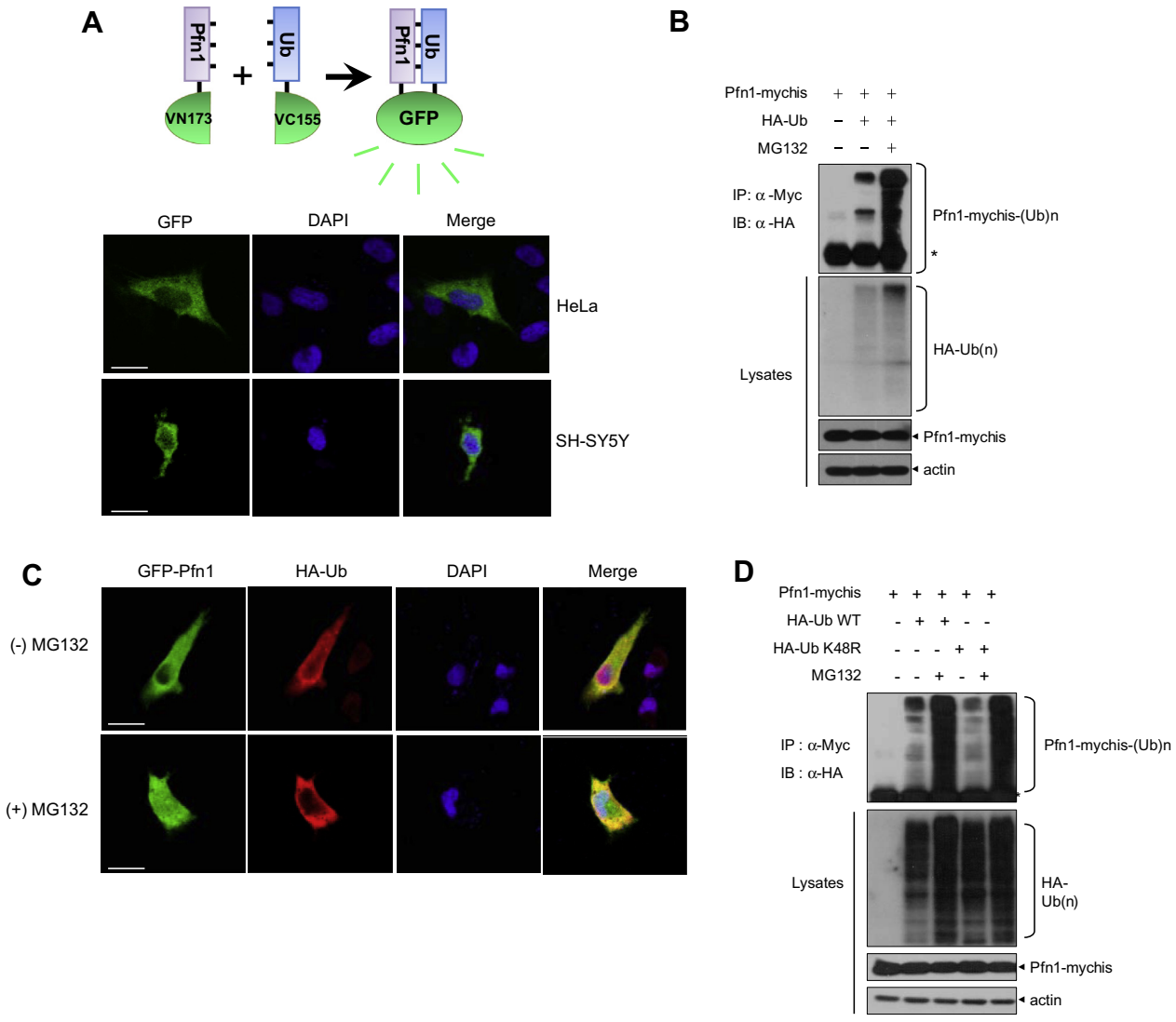

Fig. 1. Pfn1 is poly-ubiquitinated in human cell lines. (A) Bright GFP signals were detected when VN173-Pfn1 and VC155-Ub were co-expressed in HeLa (top) and SH-SY5Y (bottom) cells. Nuclei were stained with DAPI (blue). Scale bar, 10 μm. (B) Pfn1 ubiquitination was revealed by co-IP with anti-Myc and WB with anti-HA antibodies. *IgG heavy chain. (C) Differential localization of GFP-Pfn1 depending on the presence of MG132. HeLa cells were co-transfected with GFP-Pfn1 and HA-Ub. 18 h after transfection, either DMSO or MG132 (20 μM) was added to cell media for 6 h, and followed by examination with confocal microscopy. Nuclei were stained with DAPI (blue). Scale bar, 10 μm. (D) Ubiquitination of Pfn1 in the presence of Ub WT or Ub K48R mutant. Ubiquitination assay was performed by co-IP with anti-Myc and WB with anti-HA antibodies. *IgG heavy chain. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

findings, we hypothesized that CHIP might function as an E3 ligase for Pfn1.

First, we investigated if Pfn1 interacts with CHIP. Co-IP results showed strong binding of CHIP with Pfn1 (Fig. 2A). Intriguingly, we found that CHIP K30A mutant, which does not bind to Hsp70 [27], bound to Pfn1 with slightly less affinity than CHIP WT. To test our hypothesis that Hsp70-associated CHIP interacts with and ubiquitinates Pfn1 in an Hsp70-dependent manner, we further investigated the detailed interaction between Pfn1 and CHIP using CHIP deletion mutants (Fig. 2B). For this, we used a CHIP U-Box deletion mutant (HA-CHIP ΔU Box) that cannot bind to E3 ligases, and a TPR deletion mutant (HA-CHIP ΔTPR) that lacks the domain responsible for Hsp70 interaction. Co-IP experiments using these mutants showed that Pfn1 binds to the U-Box deletion mutant, but not the TPR deletion mutant, indicating that Pfn1 interacts with CHIP through the TPR domain (Fig. 2C). Considering that CHIP K30A interacted with Pfn1 (Fig. 2A), it is highly possible that the TPR domain-mediated interaction between CHIP and Pfn1 might be independent of Hsp70.

Indeed, certain target proteins such as BER [28], Runx1 [29], and CtBP2 [30] are regulated by CHIP in an Hsp70-independent manner, and both Smad1/5 [31] and IRF-1 [32] have been shown to interact with CHIP directly. Therefore, we investigated if CHIP directly interacts with Pfn1 via its TPR domain in an Hsp70-indepent manner using GST pull-down assays. These experiments revealed that while Hsp70-6His bound to GST-CHIP WT on glutathione beads, Pfn1-6His did not (Fig. 2D), indicating that Pfn1 proteins do not directly interact with CHIP. Complementarily, accumulating evidence suggests that the interaction of CHIP and target proteins is mediated by unknown factors, and not heat shock proteins [28–30]. Taken together, our results demonstrate that Pfn1 interacts with CHIP via its TPR domain and suggest that this interaction may be assisted by an unknown mediator, rather than Hsp70.

3.3. CHIP regulates Pfn1 stability

Next, we investigated if Pfn1 is ubiquitinated by CHIP. HEK293T cells were co-transfected with Pfn1-mychis, HA-Ub, and HA-CHIP

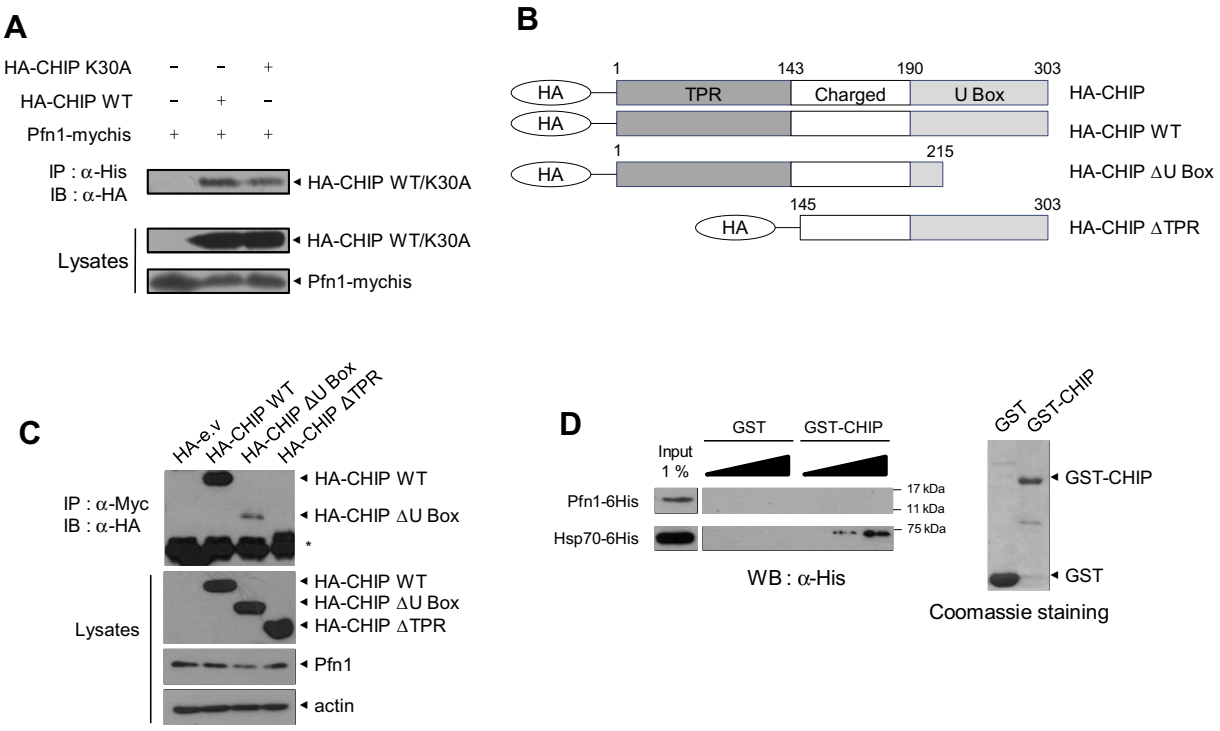

Fig. 2. Pfn1 interacts with CHIP via the TPR domain. (A) Pfn1 interacts with CHIP in an Hsp70-independent manner. HEK293T cells were co-transfected with Pfn1-mychis and either HA-CHIP WT or HA-CHIP K30A. 24 h after transfection, co-IPs were performed with anti-His and WB with anti-HA antibodies. (B) Schematic diagram of CHIP WT and CHIP deletion mutants. (C) HEK293T cells were co-transfected with Pfn1-mychis and the HA-CHIP mutants. Co-IPs were performed with anti-Myc and WB with anti-HA antibodies. *IgG heavy chain. (D) Purified Pfn1-6His or Hsp70-6His proteins were incubated with GST-CHIP- or GST-immobilized agarose beads. After incubation, beads were analyzed by WB using anti-His antibody.

WT or HA-CHIP K30A for 18 h, and then treated with MG132 for another 6 h. Ubiquitination assays revealed that ubiquitinated Pfn1 accumulated upon MG132 treatment, indicating that CHIP-ubiquitinated Pfn1 was targeted to the proteasome for degradation (Fig. 3A). The CHIP K30A mutant generated similar levels of Pfn1 ubiquitination, revealing that Hsp70 is not necessary for CHIP-mediated Pfn1 ubiquitination or the interaction between CHIP and Pfn1 (Fig. 2A).

Additionally, we investigated the stability of endogenous Pfn1 after CHIP WT or CHIP K30A mutant was expressed in HEK293T cells. As can be observed in Fig. 3B, levels of endogenous Pfn1 were downregulated after CHIP WT expression, but before CHX treatment. Additionally, the half-life of Pfn1 after CHIP K30A mutant expression was reduced about 50% when compared to control vector expression (Fig. 3B). These results indicate that Pfn1 levels are regulated by CHIP in an Hsp70-independent manner. Finally, when

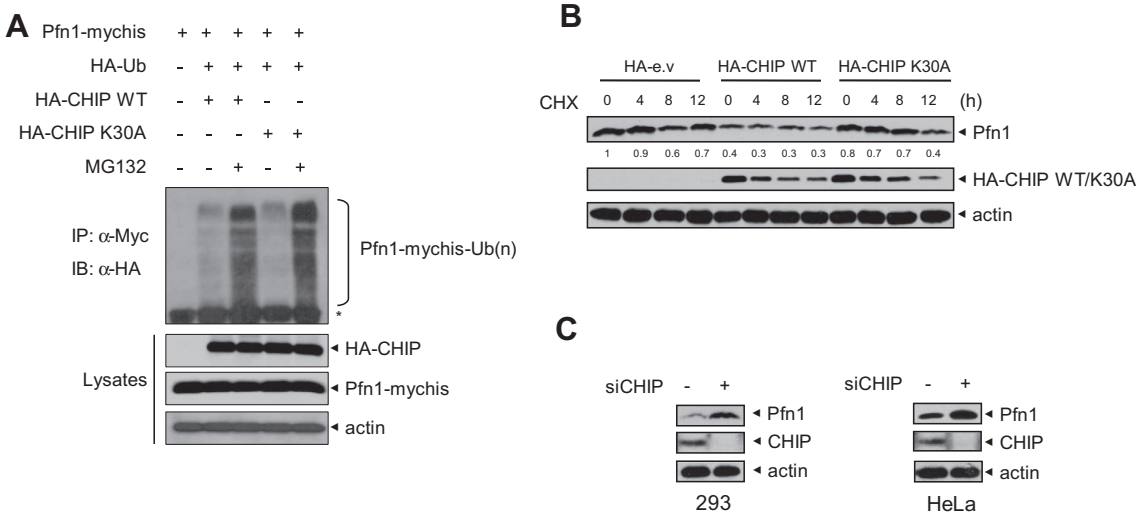

Fig. 3. Pfn1 stability is regulated by CHIP. (A) CHIP ubiquitinates Pfn1 in an Hsp70-independent manner. Ubiquitination assay was performed by co-IP with anti-Myc and WB with anti-HA antibodies. *IgG heavy chain. (B) HeLa cells were transfected with empty vector, HA-CHIP WT, or HA-CHIP K30A. 24 h after transfection, cells were treated with 200 μg/ml CHX and then harvested at the indicated time points. Expression of Pfn1 relative to actin is shown. (C) The levels of Pfn1 in CHIP-depleted cells. HEK293 or HeLa cells were transfected with scRNA or siCHIP for 48 h. Cell lysates were analyzed by WB with antibodies against Pfn1, CHIP, and actin.

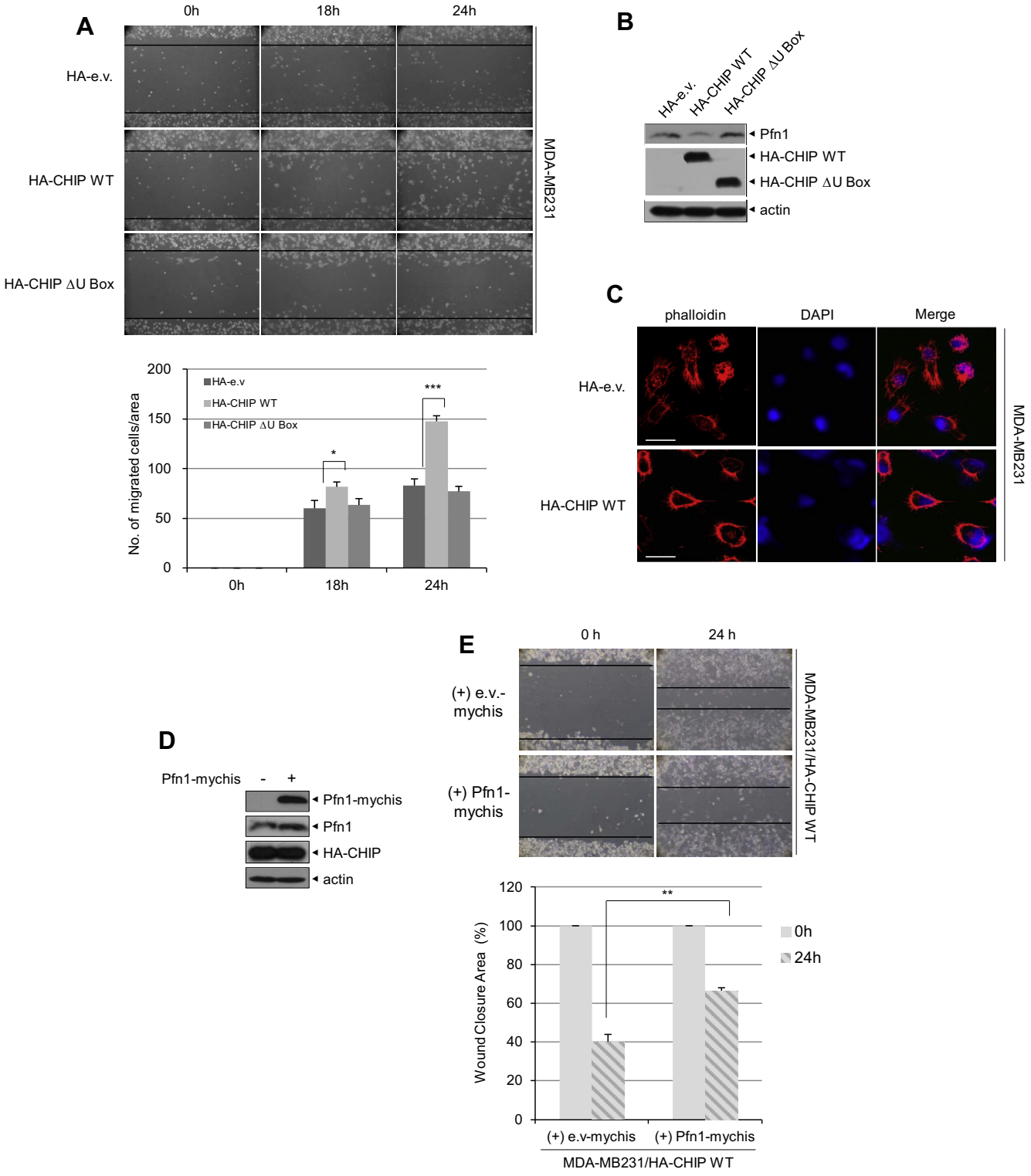

Fig. 4. CHIP-regulated Pfn1 levels affect migration of breast cancer cells. (A) MDA-MB231-HA-CHIP WT cells show accelerated cell migration (top). The migrating cells were counted and mean ± SEM for 0, 18, and 24 h time points are given ($n = 3$ each). $^{*}p < 0.05$; $^{***}p < 0.001$; unpaired t-test (bottom). (B) Lysates were analyzed by WB with antibodies against Pfn1, HA, and actin. (C) MDA-MB231-HA-CHIP WT or control cells were plated on coverslips, and F-actin was stained with phalloidin for 1 h. Cells were examined with confocal microscopy. Nuclei were stained with DAPI (blue). Scale bar, 10 μm. (D) Lysates were analyzed by WB with antibodies against Pfn1, Myc, HA, and actin. (E) Pfn1 overexpression suppressed migration of MDA-MB231-HA-CHIP WT cells (top). Wound closure areas were measured at 0 and 24 h, and percentage change calculated with the formula: Area (%) = 100 × (wound closure area at 24 h ÷ wound closure area at 0 h). Mean ± SEM are given for 0 and 24 h time points ($n = 3$ each). $^{**}p < 0.01$; unpaired t-test (bottom). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

we inhibited CHIP expression in HEK293 or HeLa cells using RNAi, we found that the levels of endogenous Pfn1 were upregulated. Taken together, these results indicate that CHIP functions as an E3 ligase for Pfn1 in human cell lines, and stability of Pfn1 is regulated by CHIP via ubiquitination and proteasome-dependent degradation.

3.4. Pfn1 levels, as regulated by CHIP, affect migration of breast cancer cells

Loss of Pfn1 expression enhances breast cancer cell motility [33] and is associated with increased metastatic potential of breast cancer [11]. Therefore, we hypothesized that CHIP expression might lead to enhanced migration of breast cancer cells by regulating Pfn1. For our experiments, we chose to use the breast cancer cell line MDA-MB231 because it is known to express low levels of CHIP [15]. We generated stable cell lines of MDA-MB231 expressing control vector (MDA-MB231-HA-e.v.), CHIP WT (MDA-MB231-HA-CHIP WT), or CHIP U-Box deletion mutant (MDA-MB231-HA-CHIP ΔU Box) and then examined cell migration using wound healing assays. Our results show that MDA-MB231-HA-CHIP WT cells migrated about twofold faster after 24 h compared to control or MDA-MB231-HA-CHIP ΔU Box cells (Fig. 4A). Additionally, Pfn1 levels were downregulated in MDA-MB231-HA-CHIP WT cells compared to control or MDA-MB231-HA-CHIP ΔU Box cells (Fig. 4B), indicating that CHIP WT regulates Pfn1 in MDA-MB231 cells. These results suggest a correlation between cell migration and CHIP-regulated levels of Pfn1 in breast cancer cell lines.

In a previous study, silencing of Pfn1 resulted in reduced F-actin structure and enhanced cell migration of MDA-MB231 cells [33]. We wondered if CHIP expression might also lead to alteration in F-actin structure. To investigate this notion, we compared the F-actin structure of control MDA-MB231 and MDA-MB231-HA-CHIP WT cells using phalloidin staining. MDA-MB231-HA-CHIP WT cells showed much reduced and simpler F-actin structure when compared to MDA-MB231 control cells (Fig. 4C). Considering that Pfn1 is a key factor controlling F-actin polymerization, these results suggest that reduced CHIP-regulated Pfn1 levels might cause the pro-migratory organization of F-actin, thus enhancing migration of MDA-MB231-HA-CHIP WT cells.

Next, we investigated if CHIP-regulated Pfn1 levels affect the migration of MDA-MB231-HA-CHIP WT cells by overexpressing Pfn1-mychis (Fig. 4D) and analyzing cell migration using wound healing assays. Pfn1 overexpression suppressed the migration of MDA-MB231-HA-CHIP WT cells, and measurement of the wound closure area revealed a 25% reduction in this migration (Fig. 4E). These results suggest that Pfn1 levels affect the migration of breast cancer cells, and that CHIP might play a role in the migration of breast cancer cells through regulation of Pfn1.

In the present study, we demonstrate that CHIP functions as an E3 ubiquitin ligase, targeting Pfn1 to the proteasome as well as regulating breast cancer cell migration. Interestingly, we found that Pfn1 can be differentially ubiquitinated for proteasomal degradation and/or for use as a regulatory signal. Therefore, we assume that multiple E3 ligases may regulate Pfn1 in different contexts, and CHIP is likely to be a major E3 ligase that regulates levels of Pfn1. Considering that Pfn1 is a key regulator of actin polymerization, CHIP might play a regulatory role in various cellular events requiring F-actin structures, such as cytokinesis, endocytosis, and membrane trafficking, as well as migration.

CHIP has been shown to play contrasting roles as either a tumor suppressor or a tumor promoting protein in different cancers [15–17,19,34,35]. As a tumor suppressor, CHIP levels have an inverse correlation with malignancy of human breast cancers [15]. However, results from the present study imply that CHIP might play a tumor promoting role in the process of breast cancer metastasis because CHIP expression enhanced breast cancer cell migration through downregulation of Pfn1. Interestingly, previous work has shown that Pfn1 has a dichotomous function in breast cancer metastasis [11]. Specifically, Pfn1 downregulation leads to enhanced cell migration in the early phase of breast cancer. Conversely, Pfn1 downregulation inhibits metastatic colonization in late phase breast cancer. Considering either low or no expression of CHIP was observed in late stages of breast cancer [15], the present study may better describe processes in the early phase of breast cancer, when high or normal levels of CHIP result in downregulated Pfn1 and subsequently enhance cell migration. Collectively, these findings suggest that CHIP might be an important modulator for the progression of breast cancer, and regulation of CHIP levels might be useful as a therapeutic strategy.

Acknowledgments

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