MOLECULAR INSIGHTS INTO PROFILIN REGULATION AND ITS ROLE IN BREAST CANCER

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Dysregulation of actin-regulatory proteins is a hallmark feature of tumor malignancy. Along this line, it has been shown that actin-binding protein profilin-1 (Pfn1) is downregulated in human breast cancer. Contrary to its pro-migratory role in most physiological contexts, loss of Pfn1 promotes migration and invasion of breast cancer cells, and conversely, restoring Pfn1 expression in breast cancer cells by genetic overexpression suppresses these pro-metastatic traits. Based on these previous findings, we set out to a) determine whether Pfn1 regulates breast cancer cell motility through vascular endothelial cells (an obligatory step for intravasation and systemic dissemination of cancer cells) [Aim 1], b) identify small molecules that can elevate Pfn1 expression and suppress motility of breast cancer cells in a Pfn1-dependent manner [Aim 2], and c) gain fundamental insight into how Pfn1 expression is regulated in cells [Aim 3]. We established that loss of Pfn1 promotes transendothelial cell migration of breast cancer cells through enhancing VEGF secretion and barrier disruption of endothelial cells. Next, through a small-scale screen of small molecules, we identified several molecules that are capable of elevating Pfn1 expression including a broad-range kinase inhibitor Tyrphostin A9. The anti-migratory action of Tyrphostin A9 was further shown to be Pfn1-dependent. Finally, we

identified megakaryoblastic leukemia (MKL), a transcriptional co-activator of serum response factor (SRF), to be a major co-regulator of the expression of Pfn1, and its closely related isoform Pfn2 (also implicated in breast cancer), and established a novel MKL/STAT (signal transducer and activator of transcription) signaling axis in the context of Pfn regulation.

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ABBREVIATIONS AND ACRONYMS

ABPs: Actin-binding proteins

ADP: Adenosine-5’-diphosphate

ATP: Adenosine-5’-triphosphate

EC: Endothelial cell

ECM: Extracellular matrix

ER: Estrogen Receptor

ET-1: Endothelin-1

HEK-293: Human embryonic kidney 293

HER-2: Human epidermal growth factor receptor 2

HUVEC Human umbilical vein endothelial cells

IFN: Interferon

IL-8: Interleukin-8

MKL1: Megakaryoblastic leukemia **1**

MRTF-A: Myocardin related transcription factor A

NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B cells

Pfn: Profilin

Pfn1: Profilin-1

Pfn2: Profilin-2

PI3K: Phosphatidylinositol 3-kinase

PLP: Poly-L-proline

PPI: Phosphoinositide

PR: Progesterone receptor

Pyk2: Proline-rich tyrosine kinase 2

RhoA: Ras homology family member A

RTKs: Receptor tyrosine kinases

SAP: SAF-A/B, Acinus and PIAS

SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis

siRNA: Small interfering RNA

SOCS3: Suppressor of cytokine signaling 3

SRF: Serum response factor

STAT: Signal transducer and activator of transcription

TEM: Transendothelial migration

TGF-β: Transforming growth factor-beta

uPa: urokinase plasminogen activator

VASP: Vasodilator-stimulated phosphoprotein

VEGF: Vascular endothelial growth factor

PREFACE

Completion of the following thesis research would not be possible without the collective support of my academic mentors, family, and friends.

I would like to sincerely thank my advisor, Dr. Partha Roy, for providing me with this opportunity. I have learned so much over the past 7 years, beyond the scope of this thesis document. Thank you for presenting me with challenges and providing support and patience along the way. I would also like to thank my collaborative mentors from other departments at the University: Dr. Andreas Vogt, Dr. Andy Stern, Laura Vollmer, and Dr. Marina Kameneva. I am grateful to have been part of your research endeavors. Thank you to my committee members: Dr. Paul Monga, Dr. Steffi Oesterriech, Dr. Claudette St. Croix, and Dr. Bryan Brown. I appreciate your wisdom and input regarding my thesis; I hope you will be pleased with the final product.

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1.0 INTRODUCTION

1.1 BREAST CANCER AND CANCER METASTASIS

Breast cancer is currently the second leading cause of cancer-related death in women and an estimated 40,890 deaths will occur in 2016 as a result of breast cancer in the United States [1]. Breast cancer originates in epithelial cells comprising the duct or lobe components of the breast [2]. Prognostic factors predict the course of treatment and clinical outcome for cancer patients. In breast cancer, some of these factors include estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (HER-2), and Ki-67. ER positive (ER+) status is present in about 60% of breast cancers [3]. Risk of breast cancer can be determined by estrogen exposure [4]. Estrogen stimulates cell division and induces ER and PR on the surface of target cells. Progesterone regulates cell cycle and inhibits PRs. The status of both ER and PR influences the effectiveness of therapies targeting these hormones. HER-2 is a proto-oncogene that binds to cell growth factors and promotes cell proliferation. When in excess, HER-2 becomes an oncogene and causes uncontrolled growth and cell division; therefore, HER-2 is considered a poor prognostic factor. Ki-67 is another poor prognostic marker when in excess, which indicates elevated mitosis and abnormal tumor cell proliferation and tumor growth [5].

Approximately 90% of cancer-related deaths occur as a result of tumor cell metastasis from the primary tumor to a distant site [6]. The general mechanisms of tumor cell metastasis are

conserved in many different cancer types. Metastatic spread is a complex process that consists of the following steps: tumor cell invasion away from the primary site, intravasation into vasculature, survival within circulation, extravasation out of vasculature, and invasion to and survival at a secondary site (illustrated in **Fig 1**) [7].

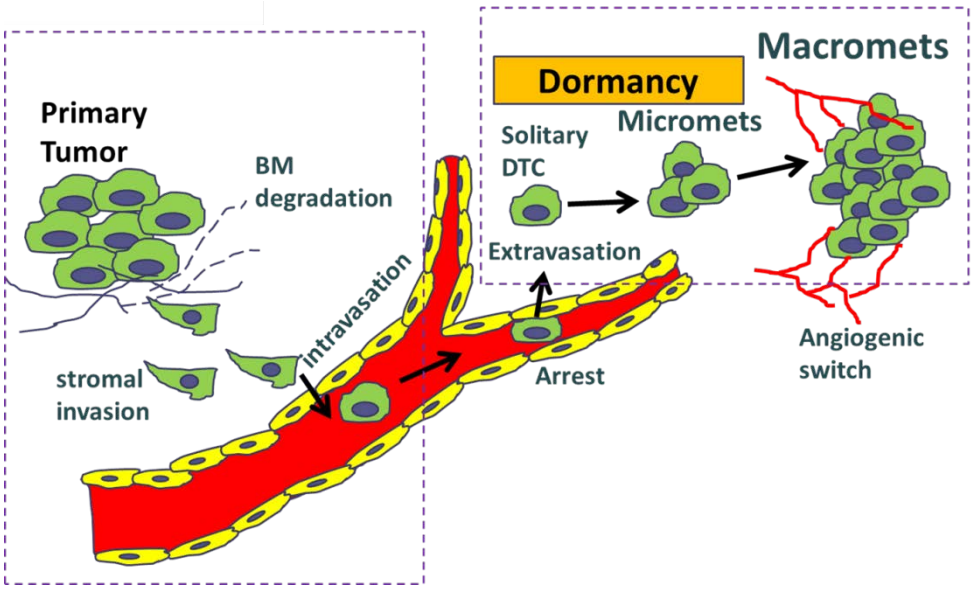

Figure 1. Schematic of the steps of metastasis.

There are numerous interactions between cells of the primary tumor and stromal cells in the very early stages of metastasis. Tumor and stromal cells secrete various factors that activate surrounding endothelial cells (EC) causing an increase in EC migration and protease expression [8]. Hypoxia inducible factor 1 (HIF-1) is activated under hypoxic conditions to induce the transcription of genes important for stimulating angiogenesis. Angiogenesis is the growth of new blood vessels from pre-existing blood vessels, which can be measured as a predictor of patient survival [9]. Angiogenesis promotes survival within the tumor and provides close access for tumor cells to escape into circulation.

Tumor cell dissemination involves tumor cell migration and invasion away from the primary site, and migration through ECs lining the blood vessels (intravasation). Invasion can occur as single cell invasion, where tumor cells are dispersed, or collective cell invasion, where the cancerous tissue pushes into the surrounding as a whole. Single invading cells often switch from an epithelial to mesenchymal phenotype, a process known as epithelial to mesenchymal transition (EMT), and facilitates invasion. Loss of E-cadherin and subsequent loss of intercellular contacts, and development of a spindle-like morphology, resembling fibroblasts, are hallmarks of EMT. Intravasation is a fundamental step leading to systemic spread of tumor cells, and has been considered the rate-limiting event of metastasis. This step is regulated by various signaling events between tumor and stromal cells and EC lining the blood vessels. VEGF (vascular endothelial growth factor) is secreted by tumor cells and binds to receptors on nearby blood vessel ECs to increase EC permeability. MMPs (matrix metalloprotease) aid in degrading both vascular junctions and the surrounding extracellular matrix, thereby promoting both invasion and intravasation. It is thought that migration through the EC barrier can occur by active or passive means. Active entry into blood vessels involves tumor cells following a gradient of nutrients or chemokines. Passive entry involves shedding of tumor cells into the leaky vasculature established in the tumor. [10-12].

The events that occur during tumor cell circulation remain poorly understood; however, it is accepted that very few tumor cells survive within circulation due to mechanical stress and host immune response. Extravasation is another step that is not well established. Tumor cells must attach to and migrate through endothelial cells to exit circulation. Platelets have been suggested to facilitate tumor cell adhesion to EC surface receptors [13]. Cancer cells that successfully extravasate into distant organs then face the challenge to thrive in a foreign environment.

Various microenvironmental factors, including adhesion molecules, growth factors, cytokines, inflammatory chemokines, and matrix-degrading proteases, influence cell survival and subsequently, formation of metastases within the secondary site. Furthermore the physical environment (matrix composition, stiffness, etc.) of the metastatic site can vary significantly from that of the primary tumor site and can have a large influence on cellular signaling and behavior [14-15].

1.2 CELL MOTILITY AND THE ACTIN CYTOSKELETON

1.2.1 Actin Cytoskeleton Dynamics

Cell motility plays an important role in various steps of the metastatic cascade. Cell migration away from the primary tumor site and invasion into surrounding tissues are critical events in metastatic dissemination [16].

Cell migration is driven by remodeling of the actin cytoskeleton (illustrated in **Fig 2**) [17, 18]. The cytoskeleton is a dynamic, self-assembled structure consisting of microfilaments, intermediate filaments, and microtubules. Actin is the main component of microfilaments, and is responsible for processes including cell migration, cell polarity, exo- and endocytosis, and morphogenesis [19]. The major steps of cell migration consist of cell protrusion, adhesion, cell body translocation, and de-adhesion. During protrusion, the force pushing the cell forward is generated by actin polymerization at the leading edge of the cell. These pushing forces can generate four types of protrusions: lamellipodia, filopodia, invadopodia, and blebbing [16]. Polymerization of G-(globular) to F-(filamentous) actin during protrusion formation is comprised

of three steps. Nucleation is the first step in which G-actin will assemble into dimers and trimers, and is the rate limiting phase. The next process is elongation, where G-actin monomers will attach to either end of the growing actin filament. Finally, steady state will be reached as G-actin will be both assembled and disassembled from the actin filament.

Various proteins influence actin dynamics, and are generally known as actin binding proteins (ABPs). The ABP complex Arp2/3/WASP (Wiskott Aldrich syndrome protein) and formins facilitate the nucleation phase of F-actin polymerization, gelsolin and cofilin assist in severing and depolymerization of actin filaments, profilins bind and activate actin monomers to speed up elongation, and Ena/VASP (vasodilator stimulated phosphoprotein) proteins aid in elongation at the leading edge [20]. A subset of these ABPs have been indicated in carcinogenesis, including profilins, the focus of the present research.

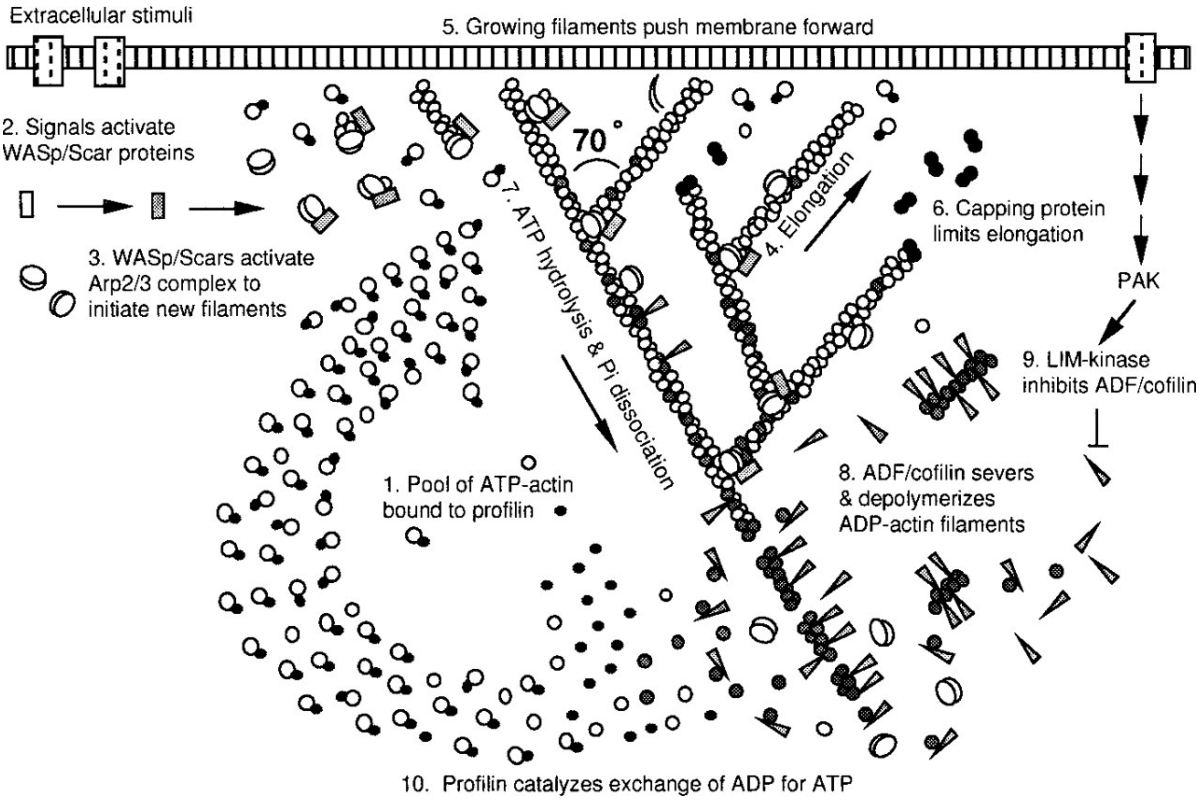

Figure 2. Steps of actin polymerization, illustrating the involvement of various actin binding proteins [18].

1.2.2 Regulation of the Actin Cytoskeleton.

Dynamic remodeling of actin cytoskeleton is an essential feature for many physiological processes. This is thought to partly depend on the gene expression program orchestrated by serum-response factor (SRF), a ubiquitously expressed and highly conserved transcription factor. SRF binds to the CArG [$CC(AT)_6GG$)] consensus sequence found in the control regions of a wide array of genes including SRF itself and many involved in regulating actin cytoskeletal, adhesion and contractility functions (*e.g.* actin, myosin, vinculin, filamin, integrin, calponin, Arp3, cofilin) [21]. Depending on the target gene, SRF-dependent gene expression is potently stimulated by two broad classes of transcriptional cofactors: Myocardin and TCF (ternary

complex factors). Myocardin-family transcriptional coactivators include myocardin (expressed exclusively in cardiac and smooth muscle cells) and broadly expressed two myocardin-related transcription factors, namely MRTF-A (also known as MAL or MKL1 [*megakryoblastic leukemia-1*] – originally identified in relation to its chromosomal translocation causing acute megakaryoblastic leukemia in children [22]) and MRTF-B (or MKL2). Loss-of-function studies have shown that depending on the context, the two isoforms of MKL either exhibit functional redundancy or have unique functions [23]. For example, in B16F0 cells (a poorly metastatic breast cancer cell line) constitutively active MKL1 elevated lung colonization from orthotopic xenograft assays [24]. In different cell types, an opposite effect of MKL1 has been observed. In epithelial cells and fibroblasts, MKL1 overexpression was capable of reducing proliferation in vitro, and minimizing tumor burden and liver metastasis in mice [25-26].

Contrasting the constitutively-active characteristic of myocardin, MKL function is highly signal responsive. MKL-SRF signaling axis is tightly regulated by the state of actin polymerization in cells. RhoA-MKL1 dependent changes in actin polymerization is a well-established pathway to induce SRF-mediated transcription (**Fig 3**) [27-28]. This pathway depends on the ability of MKL1 to bind G-actin in both the cytoplasm and in the nucleus. MKL1 bound G-actin keeps MKL1 localized in the cytoplasm and facilitates the export of nuclear MKL1 back to the cytoplasm. Rho-A is known to direct MKL1 localization by converting G- to F-actin during actin polymerization. This process occurs in the cytoplasm and nucleus causing a reduction in the G-actin pool in both cellular compartments. As a result, MKL1 is liberated from G-actin and unbound MKL1 localizes to the nucleus where it functions as a transcriptional co-activator of SRF [29]. In addition to the RhoA-MKL1 pathway, MKL1 activity is shown to be regulated by MAPK signaling. This mechanism involves Erk1/2 activation, which promotes

export of MKL from the nucleus, resulting in decreased MKL-SRF transcriptional activity [28]. When MKL1 is localized to the nucleus, MKL-SRF complex induces transcription of a variety of genes important for cell proliferation, migration, survival, apoptosis, and differentiation. Many of these genes are directly involved in cytoskeletal dynamics; therefore, MKL/SRF is considered a key pathway that connects dynamic reorganization of actin cytoskeleton to gene expression control. Since transcriptional targets of SRF include structural and regulatory components of actin cytoskeletal system, and MKL and SRF can influence each other's expression, a complex feedback loop exists between MKL-SRF signaling and actin polymerization in cells [30-33].

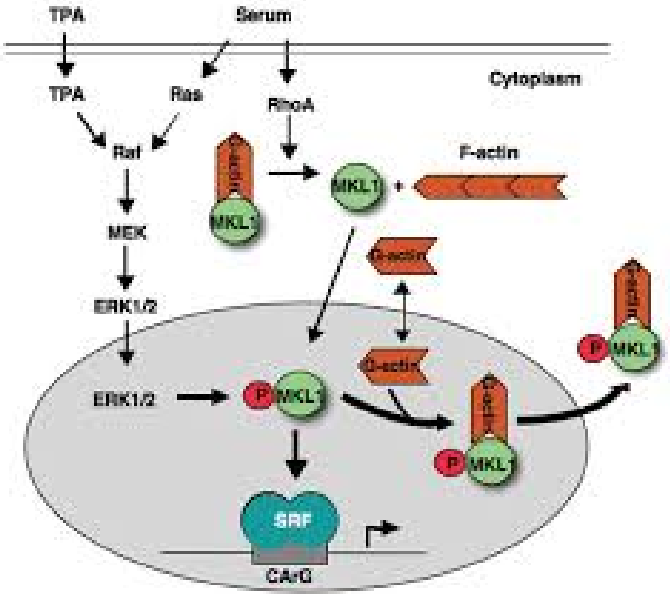

Figure 3. Schematic of RhoA-MKL1-SRF signaling axis [28].

1.3 HUMAN PROFILIN ISOFORMS

1.3.1 Biochemistry and Function

Profilins (Pfns) belong to a class of small 14-17kD actin-binding proteins that are found in virtually every eukaryotic organism as well as some viruses [34]. There are four known Pfn isoforms (Pfn1, Pfn2, Pfn3, Pfn4) that are encoded by distinct genes located at different genomic loci. Pfn1 (the most widely studied form) is ubiquitously and abundantly expressed in all cell types with the exception of skeletal muscle cells. Pfn2 is expressed primarily in the brain, in lower amounts in skeletal muscle and kidney, and in much lower levels in other cell types. Pfn3 and Pfn4 are even more tissue restricted, located exclusively in the testes, and share only 30% amino acid sequence similarity with each other and with Pfn1 and Pfn2 [35]. Pfns have been functionally implicated in many actin-dependent cellular functions ranging from cell migration to cytokinesis, vesicle trafficking and transcription/splicing control of genes (**Fig 4**) [36].

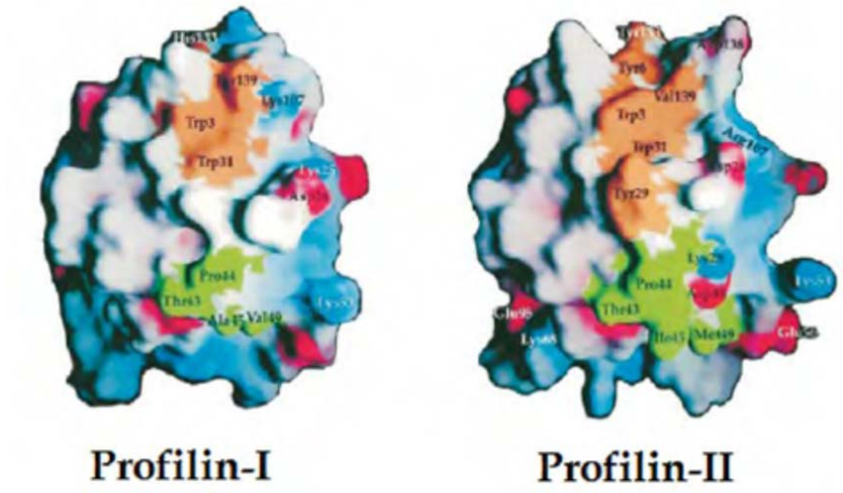

Figure 4. Structure of human profilin-1 and –2. Colored regions highlight amino acid residues that are different in profilin-I and -II. Non-conserved residues are shown in blue; conserved residues are shown in brown [36].

Pfns are largely known as actin binding proteins with two major functions in regulating actin dynamics. The first function of Pfn is to bind monomeric G-actin and induce nucleotide exchange (ADP to ATP), thereby activating actin and enhancing polymerization of F-actin at the growing end of the filament. However, when capping proteins (block F-actin polymerization at the growing end) are present, Pfn can no longer bind G-actin to the filament. In this case, Pfn acts to sequester G-actin, leading to depolymerization and reduction in actin nucleation. Aside from its interaction with actin, Pfns have other binding partners including phosphoinositides (PPI) and ligands with proline-rich sequences. Communication between the cytoskeleton and cell membrane is facilitated through Pfns interaction with PPIs. Pfn's interaction with poly-L-proline (PLP) domains is thought to mediate activities such as cytoskeletal dynamics and membrane trafficking. Vasodilator stimulator protein (VASP) binds with Pfn through its PLP domain and serves as an important interaction for facilitating Pfn binding with various other ligand partners. These three major ligand types are conserved among the Pfn isoforms; however, their affinity for these ligands varies. This variance occurs because of dissimilarities in isoform amino acid sequences, while their three-dimensional structure is conserved. For example, Pfn1 has been shown to bind actin with a fivefold higher affinity than Pfn2, while Pfn2 has a higher affinity for dynamin-1, revealed in studies comparing the biochemical characteristics of Pfn isoforms [37-38].

The two predominant Pfn isoforms, Pfn1 and Pfn2, have been functionally compared during development. It has been indicated that there is no compensatory function for Pfn1 and Pfn2 isoforms during development. This was revealed in mice where Pfn1 knockout mutation proved to be lethal. Furthermore, heterozygous mice with a 50% reduction in Pfn1 level were able to survive but to a reduced extent compared to wild type mice, collectively illuminating an

important role for Pfn1 in mouse development [39]. Another study found that Pfn1, but not Pfn2, is required for C. eleagans and Dictyostelium amoebae development, and during embryogenesis, expression patterns revealed differences in cellular localization of the two isoforms [40]. Aside from development, Pfns have been evaluated in vascular-related pathology and various forms of cancer, as discussed in the following chapter.

1.3.2 Role of Profilins in Cancer and Disease

Regulators of cytoskeletal dynamics have been indicated in tumor progression and metastasis in a variety of cancers. Specific to the current study, dysregulated expression of the two main Pfn isoforms (Pfn1 and Pfn2) have been correlated with several forms of cancer and other diseases.

Pfn1 plays an essential role in migration and proliferation of most normal cell types [41-43]. Seemingly, contrary to Pfn1's conventional pro-migratory function, downregulation of Pfn1 expression has been indicated in several adenocarcinoma cell types including breast, hepatic, pancreatic, and bladder [44-47]. Previous research efforts have shown that Pfn1 expression is downregulated in human breast cancer. Clinical correlation studies from our laboratory have found that the most dramatic Pfn1 downregulation is presented in human breast tumors with significant lymph node infiltration and/or distant metastases. Consistent with these clinical correlation findings, our in vivo studies have demonstrated that blood-burden of tumor cells in mice bearing mammary tumors induced by MDA-MB-231 xenografts is substantially enhanced when Pfn1 expression is suppressed, even though depleting Pfn1 expression does not affect the growth of primary tumor. We have also found that loss of Pfn1 expression promotes migration and invasion and conversely, increasing cellular Pfn1 level suppresses motility and invasiveness

of breast cancer cells in vitro [48-51]. Collectively, these finding suggest that loss of Pfn1 enhances the disseminative potential of breast cancer cells.

Aside from cancer, Pfns have been studied in the context of other diseases. Mutations of the Pfn1 gene have been linked to familial amyotrophic lateral sclerosis [52]. Shao et al. identified a potential role for Pfn isoforms in the aggregation of huntingtin (Htt) protein in Huntington's disease. They found that overexpression of Pfn1 or Pfn2 inhibited Htt aggregation in a dose-dependent manner [53]. Both Pfn1 and Pfn2 have been evaluated in the neurological disorder, fragile X syndrome. The two isoforms were revealed to have different functions in the stages of spine development Pfn1 was identified as the isoform critical for early development and shown to have a role in fragile X syndrome, whereas Pfn2 was important for adult spine plasticity and had no role in the disease [54].

1.3.3 Regulation of Profilin Expression

Given the role of Profilins in various diseases, it is essential to understand the underlying mechanisms responsible for regulating Pfn expression. This knowledge could establish a conceptual strategy to treat some of these diseases through targeting the master regulators of Pfn expression. However, little is known about the molecular pathways that control Pfn expression.

MicroRNA mediated regulation of Pfn1 expression has been explored by at least one group with specific focus on miR-182 in breast cancer cells. This study employed a luciferase reporter gene assay to identify Pfn1 as a target gene of miR-182. They also found that MDA-MB-231 cells transfected with a miR-182 inhibitor had significantly increased Pfn1 protein levels, revealing that miR-182 is a negative regulator of Pfn1 expression [55]. Another group evaluated the role of Pfn1 in bone morphogenetic protein (BMP)-mediated osteoblast

differentiation and bone regeneration. They found that BMP is a negative transcriptional regulator of Pfn1 expression and revealed a potential Pfn1-BMP negative feedback loop that controls osteoblast differentiation [56]. Other transcriptional regulatory mechanisms involve the signal transducer and activator of transcription (STAT) family members. STAT3 was identified as a positive transcriptional regulator of Pfn1 in rat aortic endothelial cells [57]. STAT1 has been suggested to directly target the Drosophila homolog of Pfn, chic, in germline stem cells [58].

1.4 HYPOTHESIS AND SPECIFIC AIMS

Regulators of cytoskeletal dynamics have been indicated in tumor progression and metastasis in a variety of cancers. Profilins are essential control elements of actin polymerization and are found to have altered expression levels in cancer and other diseases. Along this line, recent studies have established causal relationships between downregulated expression of important actin-binding proteins, profilin-1 and -2 (Pfn1, Pfn2), and increasing invasive ability of breast cancer cells. Previous studies from our laboratory revealed that Pfn1 depletion enhances tumor cell blood burden, without affecting grown of the primary tumor, suggesting that loss of Pfn1 enhances the disseminative potential of breast cancer cells. Therefore, I hypothesize that loss of Pfn1 promotes tumor cell migration through vascular endothelial cells (intravasation), which is a necessary step for systemic dissemination of cancer cells. In addition to evaluating the effects of Pfn1 depletion, previous findings indicate that restoring Pfn1 expression in breast cancer cells by genetic overexpression suppresses pro-metastatic traits. As a result, I hypothesize that restoring Pfn1 expression could serve as an effective strategy to limit breast cancer cell motility and invasion. Elevation of Pfn1 and Pfn2 have both, independently, been shown to suppress features

of aggressive breast cancer. Despite the importance of Pfns in actin cytoskeletal remodeling and disease relevance, the regulatory mechanisms involved in Pfn expression is poorly understood. Identification of a common regulatory pathway of the two isoforms could provide a novel conceptual strategy to suppress metastatic potential of breast cancer cells in a Pfn-dependent manner. The following specific aims were formulated to address these research gaps and hypotheses.

Specific Aim 1: To determine whether Pfn1 regulates breast cancer cell motility through vascular endothelial cells (an obligatory step for intravasation and systemic dissemination of cancer cells).

Specific Aim 2: To identify small molecules that can elevate Pfn1 expression and suppress motility of breast cancer cells in a Pfn1-dependent manner.

Specific Aim 3: To gain fundamental insight into how Pfn expression is regulated in cells.

The presence of tumor metastasis leads to poor prognosis for breast cancer patients. The proposed research will clarify the effect of Pfn1 downregulation on tumor cell intravasation, a critical step during dissemination, and identify small-molecule inducers of Pfn1 expression with anti-migratory activity. These studies could further implicate Pfn1 as a new prognostic marker in breast cancer and expose a promising pharmacological point of attack in the context of metastatic breast cancer. A major gap in Pfn-related research is a limited understanding of the molecular mechanisms responsible for regulating Pfn expression in both normal and cancerous cells. Identification of novel regulatory pathways involved in Pfn expression will not only advance knowledge in the field, but could provide therapeutic approaches for treating certain cancers and diseases.

2.0 MATERIALS AND METHODS

2.1 CELL CULTURE AND TREATMENTS

MDA-MB-231 and HEK 293 cells were cultured in Eagle's minimal essential medium and Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and antibiotics. HmVEC cells (ATCC, CRL-3243) were cultured in MCDB131 (Life Technologies) growth medium [10% (v/v) FBS, 100U/mL Penicillin, 100µg/mL Streptomycin, 10ng/mL EGF, 1µg/mL Hydrocortisone, 10mM L-Glutamine]. MDA-MB-231 cells were treated with 10 µM working concentration of Tyrphostin A9, Purvalanol A (both purchased from Sigma-Aldrich and dissolved in DMSO to make a 10 mM stock solution), or DMSO control for 48 hours before being analyzed for single cell motility or Western blot analyses. HEK-293 cells were treated with 10µM MG-132 or 10mM ammonium chloride for 12 hours before protein extraction.

2.2 SIRNA AND PLASMID TRANSFECTIONS

Cells were transfected using the following siRNAs at indicated working concentrations: 50nM-100nM smart-pool control siRNA (ThermoFisher, Hudson, NH, USA), 50nM Pfn1 siRNA (Fisher Scientific, M-012003-01-0005), 50nM Pfn2 siRNA (Santa Cruz, sc-78482), 100nM VEGF siRNA (Santa Cruz Biotechnology, Dallas, TX, USA), 100nM MKL1 siRNA (Santa

Cruz, Dallas, TX, USA, sc-43944), 100nM MKL2 siRNA (Santa Cruz, sc-61074), 100nM SRF siRNA (Santa Cruz, sc-36563), 100nM STAT1 siRNA (Santa Cruz, sc-44123), 100nM STAT3 siRNA (Santa Cruz, sc-29493). siRNAs were transfected using either Transfection Reagent 1 (Dharmacon) or Transfection Reagent 2 (Dharmacon) for 293/HmVEC or MDA-231, respectively, following manufacturer's instructions. All subsequent experiments were performed 48 h after transfection. All MKL1 and SRF constructs were generous gifts from Ron Prywes. Flag-MKL1 3 point mutant was generated on Flag-MKL1 plasmid using the following primers: K237A (sense: 5'- GAAGAAGCTCGCGTACCACCAGT-3'), K237A Y238A (sense: 5'-GAAGAAGCTCGCGGCCCACCAGT-3'), and K237A Y238A H239A (sense: 5'-GCTCGCGGCCGCCCAGTACATCC-3'). STAT1α-FLAG construct was kindly provided by Jennifer Grandis. Stat3 Flag pRc/CMV was a gift from Jim Darnell (Addgene Plasmid #8707). Plasmid DNA transfections for HEK-293 and MDA-231 cells were done using XtremeGENE HP transfection reagent (Roche, Basel, Switzerland) and Lipofectamine 3000 (Life Technologies, Carlsbad, PA, USA) according to the manufacturer's instructions.

2.3 PROTEIN EXTRACTION AND IMMUNOBLOTTING

Total cell lysate was extracted with a modified RIPA buffer [25 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1% (v/v) NP-40, 5% (v/v) glycerol, 1 mM EDTA, 50 mM NaF, 1 mM sodium pervanadate, protease inhibitors, and 6x sample buffer diluted to 1x]. Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes. The following antibodies were used for immunoblotting: monoclonal Pfn1 (Abcam, Cambridge, England, 1:3000), monoclonal GAPDH (Biorad, Hercules, CA, USA, 1:3000), monoclonal α-tubulin (1:2000; Sigma, T5168),

monoclonal p27kip1 (BD Biosciences, Frankin Lakes, NJ, USA, 1:2000), polyclonal MKL1 (Santa Cruz, 1:500), polyclonal MKL2 (Santa Cruz, 1:500), polyclonal SRF (Santa Cruz, 1:500), polyclonal Pfn2 (Santa Cruz, 1:500), monoclonal STAT1 (Cell Signaling, Danvers, MA, USA, 1:1000), monoclonal STAT3 (Cell Signaling, 1:1000), monoclonal phospho-STAT1 (Cell Signaling, 1:1000), and monoclonal Flag (Sigma Aldrich, 1:3000). Protein bands were visualized with horseradish peroxidase-conjugated secondary antibodies (Jackson Immunoresearch) and an enhanced chemiluminescence reagent (BioRad).

2.4 TRANSENDOTHELIAL MIGRATION ASSAY

Early passage (within three passages) HUVEC were grown to confluence on the upper side of collagen-coated 3 μm-pore transwell inserts (BD Biosciences). Fifty thousand MDA-231 cells were seeded on top of HUVEC monolayer and allowed to migrate toward a serum gradient for 24 h. At the end of the experiment, cells on the upper side of transwells were removed, and transmigrated MDA-231 cells located on the bottom side of the transwells were fixed and imaged at multiple fields (at least 5 fields/transwell from 2–3 replicate transwells/group). Immunostaining of β-catenin in HUVEC/MDA-231 co-culture was performed according to the published protocol36. Images of stained cells were acquired with a × 20 objective on an Olympus IX-71 epifluorescence microscope (Olympus America, Center Valley, PA, USA).

2.5 ANTIBODY ARRAY

Conditioned media of serum-starved MDA-231 cells was collected and concentrated in centrifugation filter units (molecular weight cutoff: 3 kDa; Millipore) at 2500 g for 40 min before analyzing on a commercially available human angiogenesis antibody array (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's protocol. Chemiluminescence signals on the array spots were quantified using ImageJ software (NIH Image, Bethesda, MD, USA).

2.6 TIME-LAPSE CELL MOTILITY

MDA-MB-231 cells were treated 10 µM working concentration of Tyrphostin A9 or Purvalanol A (both purchased from Sigma-Aldrich and dissolved in DMSO). Twenty-four h following initial treatment, cells were re-plated in collagen-coated 48-well plates for single-cell motility assays, and were again treated with 10 µM compound. For single cell motility, cells were imaged the following day for 3 h at 60 s time intervals between successive image frames. During imaging, the culture plate was placed in an incubation chamber (LiveCell™ System, Pathology Devices Inc.) to maintain appropriate environmental conditions (37°C/pH 7.4). Cell trajectory was generated by frame-by-frame analysis of the centroid positions (x, y) of cell nuclei (assumed to be the representations of cell bodies). 20-40 individual cells were scored in each experiment. All images were acquired and quantified using Metamorph and NIH ImageJ software, respectively.

2.7 3D COLLAGEN INVASION

MDA-MB-231 cells were treated with each agent at 10uM working concentration. 24 h following initial treatment, cells were re-plated, and again treated with 10 uM compound concentration, for collagen invasion assay. Collagen-I (Type I Rat Tail; BD Biosciences, San Jose, CA), 10x M199 medium and cells were well mixed and poured into duplicate wells of a 24-well plate. Final collagen and cell concentrations were 2.5 mg/ml and 2×106 /ml, respectively. The collagen solution was allowed to polymerize for 30 minutes at 37°C and then overlaid with complete growth medium containing 50 ng/ml EGF and 50 ng/ml PMA. Real-time imaging of cells was performed, using an incubation chamber (LiveCell™ System, Pathology Devices Inc.), at 10 minute intervals for a total duration of 30 hours. The average invasion speed was scored in the same manner as in the single cell motility assay.

2.8 SUBCELLULAR FRACTIONATION

Cells were extracted from 10 cm dishes at 80% confluence. Extraction was performed for 15 min in Buffer A (10mM HEPES, 10mM KCl, 0.1mM EDTA, 0.1% NP-40, 1mM DTE, protease and phosphatase inhibitors, pH 7.9). Samples were then centrifuged at 5,000 rpm for 10 min and the supernatant was collected as the cytoplasmic fraction. The remaining cell pellet was washed and centrifuged (5,000 rpm, 5 min) 3x with Buffer A. The pellet was then re-suspended in Buffer B (20mM HEPES, 0.4mM NaCl, 1mM EDTA, 10% glycerol, 1mM DTE, protease and phosphatase inhibitors, pH 7.9) and vigorously vortexed for 1 h at 4 °C. Samples were centrifuged at 13,000 rpm for 10 min and the supernatant was collected as the nuclear fraction.

Purity was confirmed by immunoblots positive for Histone H-1 in the nuclear fraction and negative GAPDH in the cytoplasmic fraction.

2.9 MRNA EXTRACTION AND QPCR

Total RNA was extracted from cell cultures using the RNeasy Mini Kit (QIAGEN) according to the manufacturer's instructions. cDNA was synthesized from 1μg RNA using the QuantiTect Reverse Transcription Kit (QIAGEN) following the manufacturer's instructions. Each PCR reaction was prepared with 50μg cDNA, 12.5μL SYBR Select Master Mix (Thermofisher Scientific), 1μM (final concentration) forward and reverse primers, and water for a total volume of 25μL. Thermal cycling and data analysis was performed using the StepOne Plus Real-Time PCR System and StepOne Software (Applied Biosystems) to detect quantitative mRNA expression of Pfn1, Pfn2, and GAPDH (endogenous control). The primer sequences for GAPDH were 5′-CGGAGTCAACGGATTTGGTCGTAT-3′ (sense), 5′-AGGCTTCTCCATGGTGGTGAAGAC-3′ (antisense). The primer sequences for Pfn1 were 5′-CGAGAGCAGCCCCAGTAGCAGC-3′ (sense), and 5′-ACCAGGACACCCACCTCAGCTG-3′ (antisense). The primer sequences for Pfn2 were 5′-TGTCGGCAGAGCTGGTAGAGTCTT-3′ (sense), and 5′-GCAGCTAGAACCCAGAGTCTCTCAA-3′ (antisense). The PCR cycling conditions for GAPDH, Pfn1, and Pfn2 were 95˚C (30s), 55˚C (30s), and 72˚C (1min) for a total of 35 cycles.

2.10 IMMUNOSTAINING

Cells were washed with PBS, fixed with 3.7% formaldehyde for 15 min, and permeabilized with 0.5% Triton-X 100 for 5 min. Cells were then blocked with 10% goat serum in PBS at room temperature for 30 min. Cells were incubated with monoclonal Flag antibody (Sigma Aldrich; 1:100) diluted in 5% goat serum for 1 hr at room temperature. After washing cells two times with 0.02% tween-20 and twice with PBS, cells were incubated with FITC-conjugated secondary antibody (Jackson Immunoresearch, West Grove, PA) for 1 hr. Cells were again washed with 0.02% tween-20 and PBS, two times each, before mounting with Duolink Mounting Medium with DAPI (Sigma Aldrich). Slides were imaged using a 20× objective on an Olympus IX71 inverted microscope. Quantification of Flag-MKL1 localization was performed manually by overlaying green fluorescence (Flag-MKL1) and DAPI fields. Cells were scored based on the presence of Flag-MKL1 either completely within the nucleus, completely outside of the nucleus, or present in both compartments. Percentage of cells within each compartment was calculated for data representation.

2.11 STATISTICS

Statistical analyses of cell motility, invasion, and transendothelial migration were performed using ANOVA, followed by Tukey-Kramer post-hoc test for multiple comparisons and P-values less than 0.001 were indicated as significant. For motility/invasion Box and whisker plots were used to represent experimental data (box: 25th and 75th percentile; whisker: 10th and 90th percentile; line: median). Immunoblots were quantified using ImageJ to calculate and normalize

band area and intensity. qRT-PCR data was generated using StepOne Software (Applied Biosystems) and was expressed as mean +/- standard deviation. Differences between groups were determined using Student's t-test and P-values less than 0.01 were considered statistically significant. All statistical tests were performed with Stata/SE software (StataCorp. 2011. Stata Statistical Software: Release 12. College Station, TX: StataCorp LP.).

3.0 LOSS OF PROFILIN-1 EXPRESSION PROMOTES TRANSENDOTHELIAL MIGRATION OF BREAST CANCER CELLS IN VITRO

3.1 INTRODUCTION

The contents of this section are published. Reference: **Ding, Z**; Joy, M., Bhargava R, Gunsaulus M, Lakshman N, Miron-Mendoza M, Petroll M, Condeelis J, Wells A & Roy, P. Profilin-1 downregulation has contrasting effects on early vs late steps of breast cancer metastasis. Oncogene. 2013; doi: 10.1038/onc.2013.166.

Tumor metastasis is the most significant characteristic of aggressive cancer progression and is responsible for most cancer-related deaths. Consequently, prevention of metastasis is a fundamental target of cancer research and therapeutics. Metastatic spread is a complex process, consisting of tumor cell invasion away from the primary site, intravasation into vasculature, survival within circulation, extravasation out of vasculature and finally, invasion to and survival at a secondary site. Disrupted actin cytoskeleton is a feature of oncogenic transformation and malignant phenotype. Along this line, our lab has found that expression of Pfn1 is, in fact, downregulated in human breast cancer. Clinical correlation studies indicate that the most dramatic Pfn1 downregulation is presented in human breast tumors with significant lymph node infiltration and/or distant metastases. Consistent with these clinical correlation findings, in vivo studies demonstrate that blood-burden of tumor cells in mice bearing mammary tumors induced by MDA-MB-231 xenografts is substantially enhanced when Pfn1 expression is suppressed,

even though depleting Pfn1 expression does not affect the growth of primary tumor. Our lab has also shown that loss of Pfn1 expression promotes migration and invasion and conversely, increasing cellular Pfn1 level suppresses motility and invasiveness of breast cancer cells in vitro [48-51]. Collectively, these finding suggest that loss of Pfn1 enhances the disseminative potential of breast cancer cells. Transendothelial migration is an obligatory step for tumor cells to gain entry into or exit from circulation during the disseminative process and is regulated by various signaling events between tumor cells and endothelial cells (EC) lining the blood vessels. Various tumor cell secretions, including cytokines, inflammatory chemokines, growth factors and matrix-degrading proteases, are indicated in disrupting endothelial ECM and cell-cell junctions providing tumor cells with access to vasculature. Specific to this study, vascular endothelial growth factor (VEGF), a pro-angiogenic signaling protein, is known to increase vascular permeability by disrupting endothelial cell-cell junctions. The current study will address the role of Pfn1 in transendothelial migration of breast cancer cells.

3.2 RESULTS

3.2.1 Loss of Profilin-1 Expression Reduces Transendothelial Migration of Breast Cancer Cells

In an effort to understand how loss of Pfn1 might enhance the intrinsic ability of breast cancer cells to disseminate into the circulation, we have conducted studies examining the effect of Pfn1 KD on tumor-vascular endothelial cell interactions. Since tumor cell migration through an endothelial cell monolayer is a key requirement for tumor cell intravasation leading to systemic

dissemination, we used a transendothelial cell migration assay to model the dissemination process in vitro. We found that TEM of MDA-231 cells through a monolayer of human umbilical vein endothelial cells (HUVEC) is increased by nearly 2-fold upon Pfn1 KD, which could be rescued by re-expression of GFP-Pfn1 (**Fig 5A-B**).

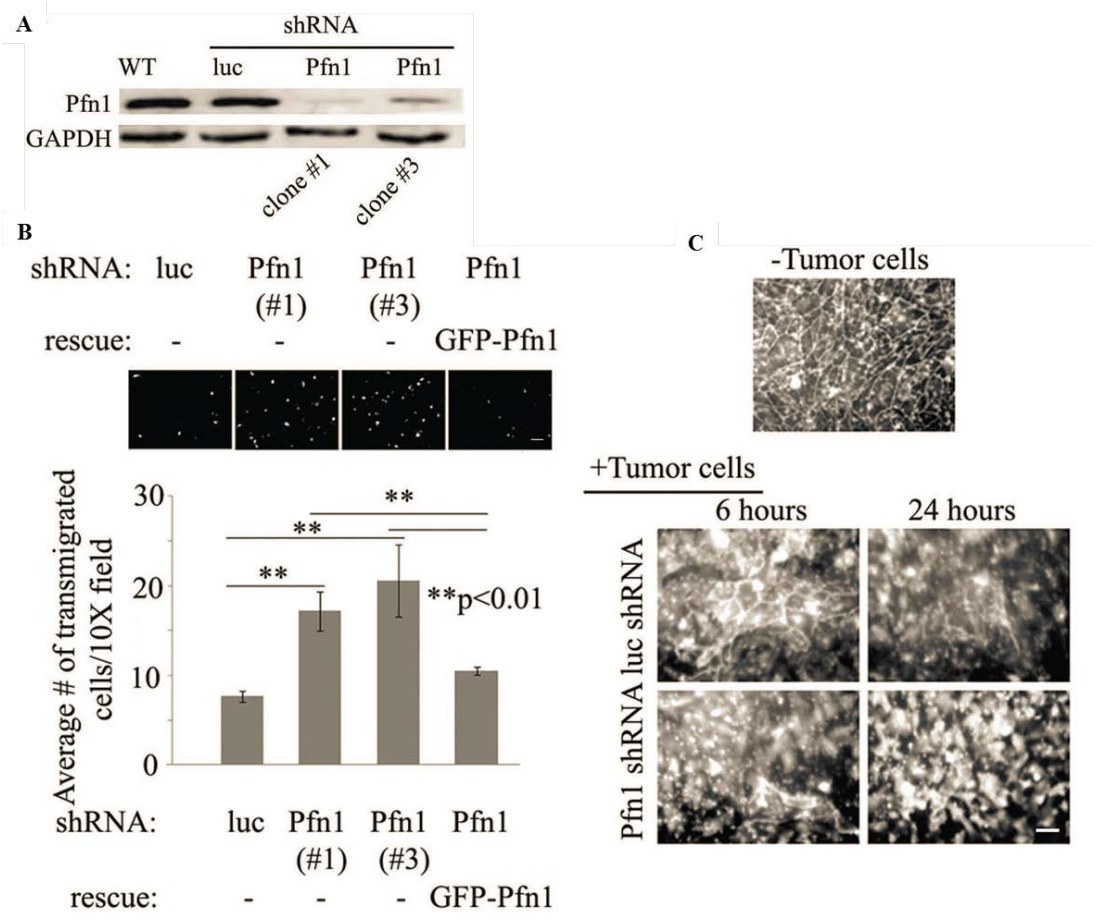

Figure 5. Pfn1 KD promotes transendothelial migration ability of MDA-231 cells. (A) Pfn1 immunoblot of total lysates derived from MDA-231 cells stably expressing either luc (control)- or Pfn1-shRNA (#1, 3—two independent Pfn1-shRNA-bearing clones). GAPDH blot serves as the loading control. (B) A bar graph showing that average number of transmigrated MDA-231 cells through HUVEC monolayer (per 10 × field of observation) for the indicated experimental perturbations (the inset shows the representative images; Bar=100 μm). (C) β-catenin immunostaining of HUVEC without or with co-culture of MDA-231 cells for the indicated time period (Bar=50 μm).

3.2.2 Loss of Profilin-1 Expression Promotes Transendothelial Migration through Disruption of Endothelial Cell-Cell Junctions

Since tumor cells must disrupt endothelial cell-cell junctions in order to transmigrate, the HUVEC monolayers were examined following cancer cell transmigration. In order to transmigrate through HUVEC monolayers, tumor cells must disrupt endothelial cell intercellular junctions. Immunostaining for β-catenin revealed that MDA-231 cells induce prominent junctional disruption of HUVEC as expected; however, the extent of HUVEC junctional disruption appears to be more pronounced when in co-culture with Pfn1 KD cells than with control cells (**Fig 5C**).

3.2.3 Loss of Profilin-1 is Associated with Increased Secretion of Several Pro-Metastatic Factors

Our observation of MDA-231 induced endothelial cell junctional disruption led to the postulate that loss of Pfn1 expression in breast cancer cells promotes secretion of certain factors that compromise endothelial barrier function. Antibody array-based analyses of conditioned media from MDA-231 culture revealed that Pfn1 KD is associated with increased secretion of several pro metastatic factors including uPA, IL-8, VEGF and endothelin-1 (**Fig 6A**).

3.2.4 Profilin-1 Depletion Increases Breast Cancer Cell Proficiency to Cross Endothelial Barriers in a VEGF-Dependent Manner

Given that VEGF is one of the most potent disruptors of endothelial cell-cell junctions, we next silenced VEGF expression by RNAi to observe any change in TECM. We found that VEGF knockdown completely abrogated TECM differential between control and Pfn1 KD cells (**Fig 6B-C**). These results suggest that breast cancer cells acquire an increased proficiency to cross through the endothelial barrier upon loss of Pfn1 expression in a VEGF-dependent manner.

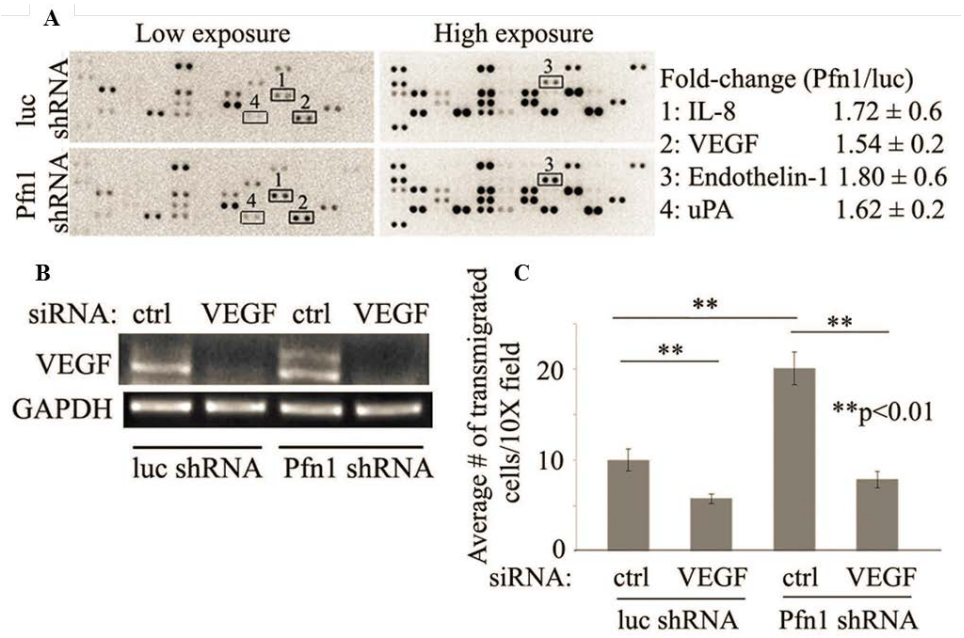

Figure 6. Profilin-1 Depletion Increases Breast Cancer Cell Proficiency to Cross Endothelial Barriers in a VEGF-Dependent Manner. (A) Antibody microarray analyses (two different exposures of blots are shown) of conditioned media from control vs Pfn1 KD MDA-231 cells show differential secretion of interleukin-8, VEGF, endothelin-1 and uPA between the two groups (the fold changes for these factors, averaged from five independent experiments, are tabulated on the right). (B) RT–PCR data show effective silencing of VEGF expression by RNAi (GAPDH RT–PCR serves as loading control). (C) Bar graph summarizing the effect of VEGF silencing on TECM of control and Pfn1 KD cells. All TECM experiments were repeated at least three times for statistical comparison.

3.3 DISCUSSION

The current research has established a potential mechanism by which loss of a traditionally conceived pro-migratory molecule, Pfn1, can augment motile behavior of breast cancer cells. We reveal that loss of Pfn1 can promote the ability of breast cancer cells to cross through vascular endothelial barrier, a critical aspect of tumor cell intravasation, by modulating VEGF expression. This finding provides new mechanistic insight into how Pfn1 negatively regulates an important step in breast cancer dissemination. Furthermore, we found that secretion of several additional pro-metastatic factors (uPA, interleukin-8, endothelin-1) are elevated upon Pfn1 knockdown. This finding highlights the importance of further efforts to investigate the effect of Pfn1 on tumor-stromal interactions.

A recent study found that Pfn1 overexpression enhances chemotherapy induced tumor cell death by complete inhibition of NFkB. They further revealed that NFkB-dependent genes, including VEGF, Angiotensin I, and IL-8, are suppressed in Pfn1 overexpressing cells [59]. Another group found that inhibition of NFkB leads to reduced VEGF transcript levels in MDA-231 cells [60]. Given this information, future evaluation of the potential link between Pfn1, NF-kB, and VEGF might reveal a new pathway by which loss of Pfn1 facilitates transendothelial migration. Furthermore, translational efforts targeting this pathway would provide a novel therapeutic avenue for prevention of specific steps of metastasis such as intravasation or extravasation.

Tumor cell dissemination involves tumor cell migration and invasion away from the primary site, and migration through ECs lining the blood vessels (intravasation). Invasion can occur as collective or single cell invasion, where single invading cells often switch from an epithelial to mesenchymal phenotype, a process known as epithelial to mesenchymal transition

(EMT). Loss of E-cadherin and subsequent loss of intercellular contacts, and development of a spindle-like morphology, resembling fibroblasts, are hallmarks of EMT. Intravasation is a fundamental step leading to systemic spread of tumor cells, and has been considered the rate-limiting event of metastasis [61]. This step is regulated by various signaling events between tumor cells, stromal cells, including fibroblasts and immune cells, and EC lining the blood vessels. VEGF is secreted by tumor or stromal cells and binds to receptors on nearby blood vessel ECs to increase EC permeability. MMPs aid in degrading both vascular junctions and the surrounding extracellular matrix, thereby promoting both invasion and intravasation. It is thought that migration through the EC barrier can occur by active or passive means. Active entry into blood vessels involves tumor cells following a gradient of nutrients or chemokines, whereas passive entry involves shedding of tumor cells into the leaky vasculature established in the tumor. Aside from the influence of biochemical signaling, the physical environment (matrix composition, stiffness, etc.) of the tumor microenvironment can also affect tumor cell behavior. The complexity of such metastatic events presents significant technical research limitations in modeling physiologically relevant representations and analysis of tumor metastasis. Transendothelial migration assays, as used in the current study, fail to recognize the complexity of cellular intravasation, extravasation, or interactions within the microenvironment during the translocation of tumor cells from one distinct tissue site to a second distinct site. Recent attempts to tackle the issue of understanding various events of cancer metastasis in vitro have been developed as alternatives. One of these assays utilize a microfluidic chip, which allows incorporation of a primary site for single cell tumors to invade through and intravasate into a fluidic pathway and subsequently attach to an endothelial monolayer [62]. Another method involves the use of chemokine-releasing tissue engineering scaffolds to model the inflammatory

aspect of cancer [63]. Similar methods could be employed to provide a more physiologically relevant model of the early steps of intravasation, before embarking on in vivo models of metastasis.

4.0 RESTORING PROFILIN-1 EXPRESSION VIA CHEMICAL THERAPEUTICS IS AN EFFECTIVE STRATEGY TO LIMIT BREAST CANCER CELL MOTILITY

4.1 INTRODUCTION

The contents of this section are published. Reference: **Joy, M.**, Vollmer, LL., Hulkower, K., Stern, AM., Peterson, CK., Boltz, RC., Roy, P., and Vogt, A. A high content, multiplexed screen in human breast cancer cells identifies profilin-1 inducers with anti-migratory activities. PLoS One 2014; 9(2). doi: 10.1371/journal.pone.0088350.

Tumor metastasis is a complex process, consisting of tumor cell invasion away from the primary site, intravasation into vasculature, survival within circulation, extravasation out of vasculature and finally, invasion to and survival at a secondary site. Cell motility plays an important role in some of these events. Therefore, small-molecule mediated inhibition of tumor cell motility could be a promising strategy to reduce migratory and invasive activity in the treatment of metastatic cancers [64]. We have found that Pfn1 depletion in breast cancer cells promotes migration, invasion and transendothelial migration *in vitro* and vascular dissemination of breast cancer cell *in vivo* [48-49]. Conversely, increasing cellular Pfn1 level suppresses motility and invasiveness of breast cancer cells [50-51]. Collectively, these data suggest that restoring Pfn1 expression could be an effective strategy to reduce breast cancer cell motility and present therapeutic potential in limiting metastatic dissemination in breast cancer. The current research will examine

whether small-molecule mediated induction of Pfn1 is capable of limiting breast cancer cell motility.

4.2 RESULTS

4.2.1 Small Molecule Tyrphostin-A9 and Purvalanol-A are Identified as Anti-Migratory and Profilin-1 Inducers

In collaboration with the University of Pittsburgh Drug Discovery Institute, we performed a high throughput screen (HTS) of 1280 pharmacologically active compounds with known biological activities (Library of Pharmacologically Active Compounds, LOPAC) to identify small-molecules that were both antimigratory and capable of increasing Pfn1 expression levels in breast cancer cells. We identified four minimally cytotoxic agents that were anti-migratory and had elevated Pfn1 levels based on fluorescence readouts including purvalanol A, tyrphostin A9, 5-azacytidine and indirubin-3-oxmie. These results were confirmed by Western blot analysis where tyrphostin A9 and purvalanol A increased Pfn1, whereas 5-azacytidine and indirubin-3-oxime did not (**Fig 7A**). The anti-migratory phenotypes of purvalanol A and tyrphostin A9 in MDA-231 cells was confirmed in a single-cell motility assay. We found that at the 10uM concentration, both agents significantly reduced cell motility compared with vehicle control (**Fig 7B-C**). Based on our previous findings of Pfn1′s inhibitory effect on collagen invasiveness of MDA-MB-231 cells, we also tested the invasiveness of MDA-231 cells upon compound treatment using a random 3D collagen invasion assay. We confirmed that both purvalanol A and tyrphostin A9 significantly reduced collagen invasiveness of MDA-MB-231 cells (**Fig 7D**).

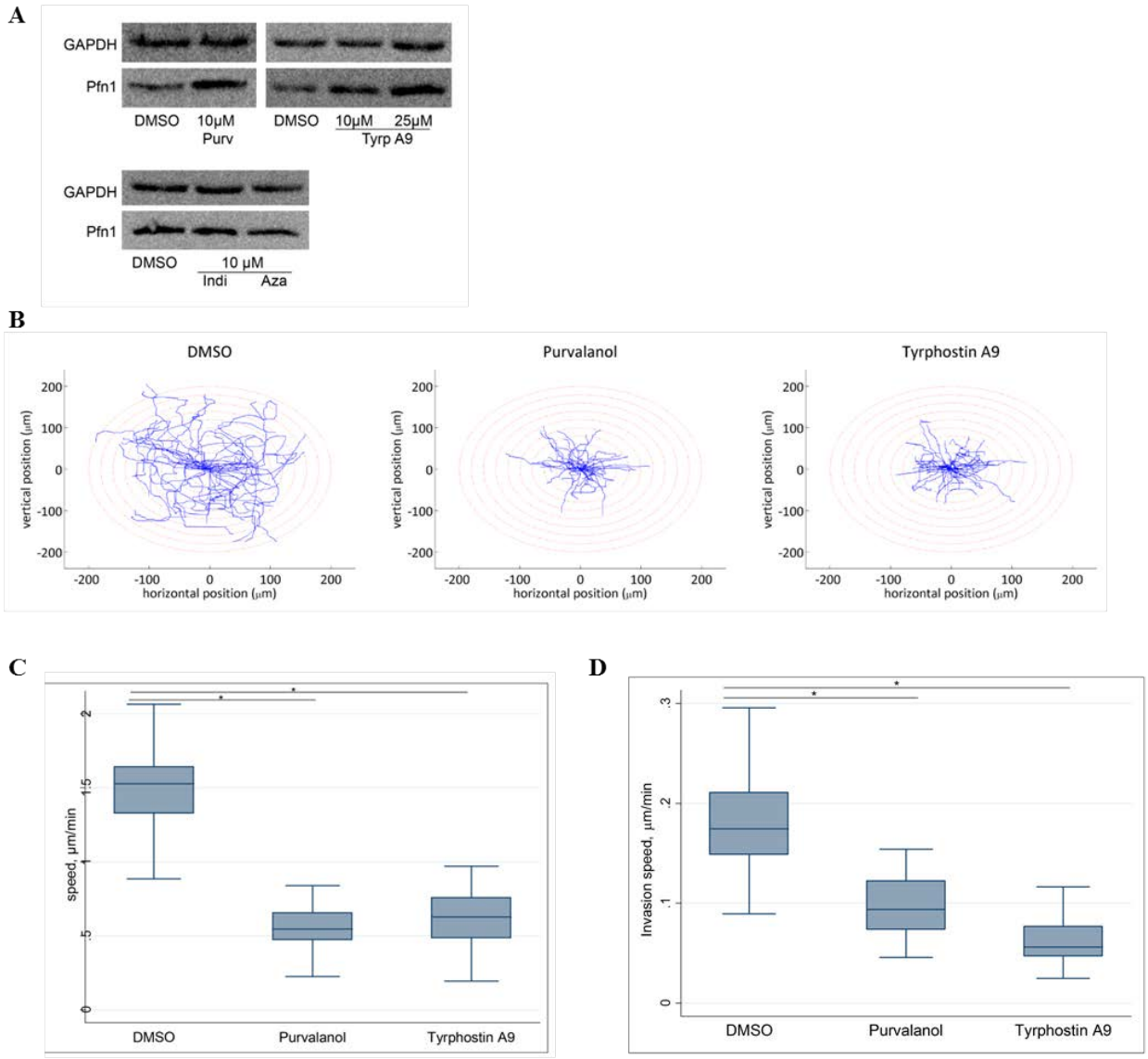

Figure 7. Confirmation of anti-migratory and anti-invasive activity by profilin-1 inducing agents. (A) Western blot analysis confirms elevated profilin-1 expression by purvalanol and tyrphostin A9. (B) Rose plots illustrate motility patterns of individual cells. Each line represents the trajectory of individual MDA-MB-231 cells of different experimental groups from time-lapse motility over a period of 48 h. Data are from a single experiment that has been repeated once with identical results. (C) Box and whisker plots representing the average speed of migration of MDA-MB-231 cells treated with DMSO (control) vs. 10 μM of either purvalanol or tyrphostin A9. Treatments were performed for 48 hours before assessing motility. Box, 25th and 75th percentiles, whiskers, 10th and 90th percentiles; line, median. Data are pooled cell data from two independent experiments (n=20 cells per group; *p<0.001). (D) The effect of purvalanol and tyrphostin A9 on invasion of MDA-MB-231 cells. Box and whisker

plots represent the average speed of invasion of cells treated with DMSO (control) vs. 10 μM of either purvalanol or tyrphostin A9. Box, 25th and 75th percentiles; whiskers, 10th and 90th percentiles; line, median. Data are the combined values from two independent experiments comprising of 37 (DMSO), 38 (purvalanol) and 40 (tyrphostin A9) individual cells (*$p<0.001$).

4.2.2 Profilin-1 is Mechanistically Linked to the Anti-Migratory Activity of Tyrphostin-A9

We then questioned if Pfn1 is functionally involved in the anti-migratory activities of purvalanol A and tyrphostin A9. If the anti-migratory effects of each compound occur through a Pfn1 dependent mechanism, one would expect that their activity should be abrogated, or at least substantially diminished, upon Pfn1 depletion. Therefore, single-cell motility assays were performed to compare the effects of tyrphostin A9 and purvalanol A on cell motility in Pfn1-proficient vs. -depleted conditions. In the presence of control siRNA, both purvalanol and tyrphostin A9 significantly reduced cell motility, as expected. When Pfn1 was silenced, DMSO-treated cells migrated faster, consistent with our previously published data. Importantly, knockdown of Pfn1 abolished the anti-migratory activity of tyrphostin A9 but not of purvalanol (**Fig 8A-B**). Western blots confirmed elevated levels of Pfn1 after compound treatments with control siRNA but not Pfn1 siRNA (**Fig 8C**). Taken together, the results suggest Pfn1 is mechanistically linked to cell migration inhibition by tyrphostin A9, providing biological validation to the analytical approach.

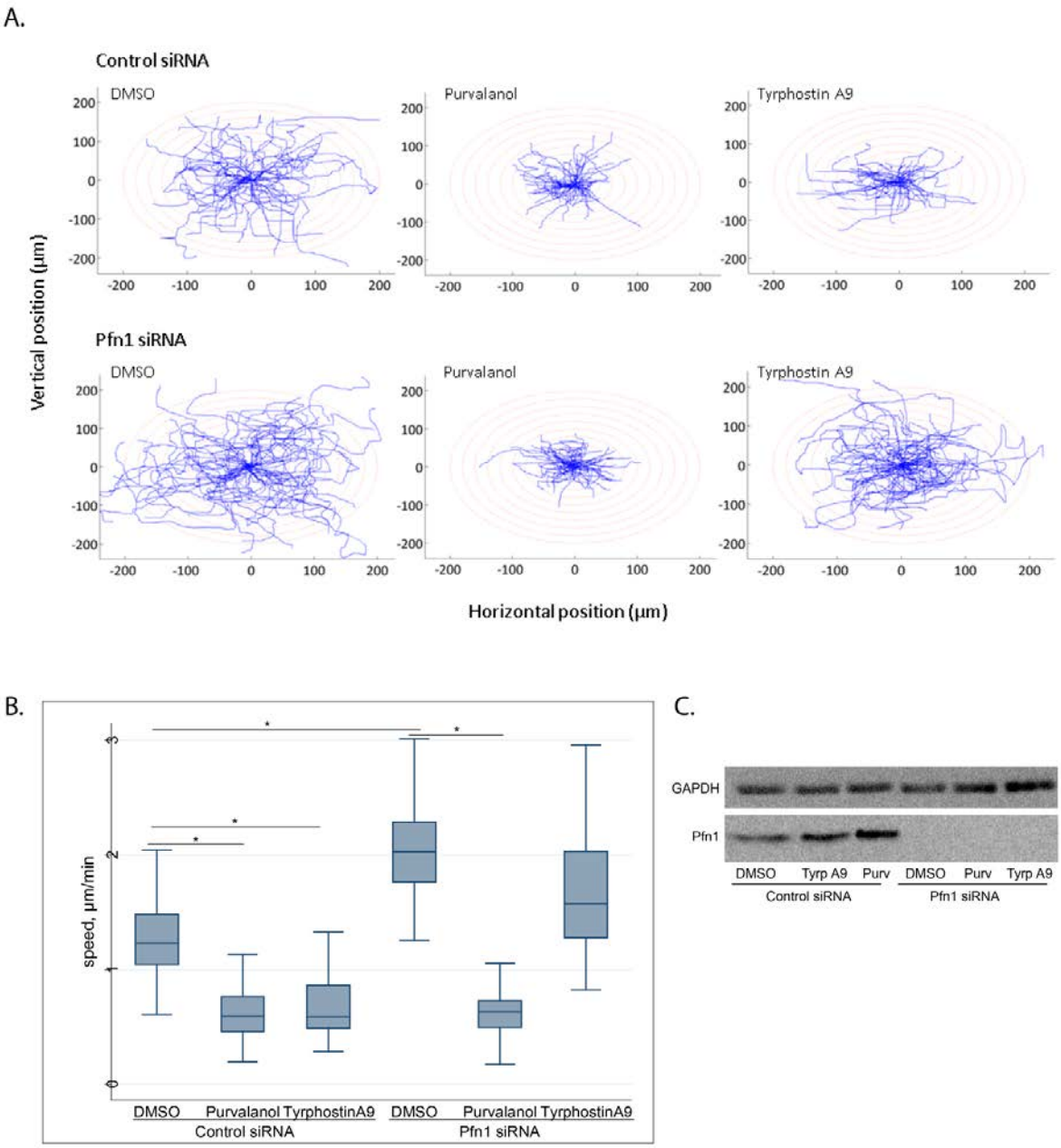

Figure 8. Profilin-1 knockdown abolishes anti-migratory activity of tyrphostin A9 but not purvalanol. (A) Trajectories of individual cells treated with vehicle (DMSO), 10µM purvalanol, or 10µM tyrphostin A9. (B) Box and whiskers plot documenting significant reduction in cell motility by both agents and reversal of anti-migratory activity by profilin-1 siRNA of tyrphostin A9 but not purvalanol. Data are pooled from two independent experiments comprised of 28 cells each. *, $p<0.0001$. (C) Western blot analysis of profilin-1 expression in the presence or absence of profilin-1 siRNA. Data are from a single experiment that has been repeated twice with identical results.

4.3 DISCUSSION

Previous studies reveal that loss of Pfn1 facilitates breast cancer cell motility, ECM degradation by increasing MMP secretion, invasion, transendothelial cell migration, and blood burden from orthotopic xenografts. Conversely, genetic overexpression of Pfn1 inhibits tumor formation in vivo, and reduces cell migration and invasion in breast cancer cells [48-51]. Pfn1 overexpression has also been shown to reduce endothelial cell migration and angiogenesis [65]. Collectively, these data suggest that elevation of Pfn1 could provide therapeutic value in limiting the early steps of tumor progression and metastasis. Currently, there are no Pfn1-targeted pharmacological strategies available to evaluate the effect of Pfn1 elevation on breast cancer phenotype.

The overall goal of this study was to identify compounds that inhibit cell motility through upregulation of Pfn1 using a multiplexed phenotypic screen. In collaboration with the University of Pittsburgh Drug Discovery Institute, 1280 compounds with known biological activities were screened using the Oris^{TM} Pro 384 cell migration platform to identify agents that increased Pfn1 expression greater than two-fold over vehicle controls and exerted anti-migratory effects in the absence of overt cytotoxicity. A subset of the anti-migratory hits induced the expression of Pfn1. Two compounds, purvalanol A and tyrphostin A9, were confirmed by Western blot and in single-cell motility assays. Both compounds had previously been found to inhibit cell motility. siRNA knockdown experiments revealed that one of them (tyrphostin A9) lost anti-migratory activity in Pfn-1 depleted cells, indicating a functional involvement of Pfn-1 in its anti-migratory activity. Overall, the study reveals that small molecule mediated induction of Pfn1 is capable of limiting breast cancer cell motility in vitro. Given this knowledge, elevation of Pfn1 expression could have therapeutic potential in the treatment of metastatic breast cancer.

Tyrphostin A9 has been identified as an inhibitor of Pyk2 (proline-rich tyrosine kinase 2) phosphorylation [66]. Pyk2 is indicated in breast cancer proliferation and invasion [67]. Similar to our study, one group found that inhibition of Pyk2 by Tyrphostin A9 reduced migration and invasion of MCF7 breast cancer cells [68]. Another group identified a role for Pyk2 in the regulation of Pfn expression in glomerular cells [69]. These studies illuminate the possibility that Pyk2 could be involved in Tyrphostin A9-induced elevation of Pfn1 and reduced cell motility. A potential pathway linking Pyk2 with Pfn1 should be explored in future studies by means of a specific inhibitor or siRNA mediated knockdown of Pyk2. However, other avenues should be explored as well, since Tyrphostin A9 inhibits many other tyrosine kinases besides Pyk2.

Our data build a strong foundation to explore a more targeted approach to upregulate Pfn1 expression, since Tyrphostin A9 is a very broad RTK inhibitor. This approach could be accomplished by performing a kinase-inhibitor/RNAi library screen. Targeted screening would allow further examination of whether induction of Pfn1 through chemical means can inhibit other metastatic events, including transendothelial migration in vitro, using similar methods as in the current study, as well as intravasation in vivo. These studies would provide valuable information regarding the specificity of chemical agents and would present a novel approach to modulate cellular Pfn1 in breast cancer cells.

5.0 MYOCARDIN-RELATED TRANSCRIPTION FACTOR MKL CO-REGULATES THE EXPRESSION OF PROFILIN ISOFORMS IN AN SRF-INDEPENDENT MANNER

5.1 INTRODUCTION

The contents of this section have been submitted. Reference: **Joy M**., Gau D., Castellucci N., Roy P. Myocardin-related transcription factor MKL co-regulates the expression of profilin isoforms in an SRF-independent manner.

Recent studies have established that downregulation of Pfn1 or Pfn2 expression is associated with increased motility and invasiveness of breast cancer cells and conversely, genetically elevating expression of these Pfn isoforms suppresses breast cancer cell aggressiveness in vitro [49, 70]. Therefore, identification of a common signaling pathway linking the regulation of these two Pfn isoforms may lead to a novel conceptual strategy to suppress metastatic potential of breast cancer cells in a Pfn-dependent manner. Despite its crucial importance in actin cytoskeletal remodeling and disease relevance, how expression of Pfn is regulated in cells is poorly understood. It was previously shown that Pfn1 can influence nuclear localization of MKL and transcriptional activity of SRF [30, 32].

MKL/SRF-mediated gene transcription is a highly conserved mechanism that connects dynamic reorganization of actin cytoskeleton to gene expression control. MKL/SRF transcriptional unit regulates a wide range of genes including several important structural and regulatory components of actin cytoskeleton. MKL has been identified to have a role in a variety

of cancers, given its vast effects on many cellular activities. In mouse mammary epithelial cells, MKL knockdown was shown to reduce transcript levels of Eplin-α, an indicated cytoskeleton-associated tumor suppressor in breast cancer [71]. In ERα positive breast cancer cell lines (MCF7 and T47D), the actin/MKL1 pathway is shown to be suppressed, whereas MKL1 is active in ERα negative cells (MDA-231 and HMT-3522 T4-2). This study further revealed that forced MKL1 expression in MCF7 cells caused downregulation of both ERα expression and E2-induced transcription, leading to elevated signaling of certain growth factors involved in tumor progression [72]. MKL1 overexpression in 4T1 (mouse breast cancer cell line) cells has been shown to accelerate both mammary tumor growth and formation of spontaneous lung metastases from orthotopic xenografts in mice [73]. MKL1 also has a role in other cancers, aside from breast cancer. MKL1 was found to be upregulated in metastatic thyroid tumor tissue compared to non-metastatic tumors [74]. Constitutive activation of MKL1 in src-transformed or oncogenic-ras intestinal epithelial cells was shown to suppress tumor formation and liver metastases from orthotopic xenografts in mice [26].

We herein demonstrate that despite their different genomic locus, the two major isoforms of profilin (Pfn1 and Pfn2) are co-regulated by a common signaling axis involving MKL and, surprisingly, this occurs post-transcriptionally and does not appear to be dependent on the traditional SRF-related activity of MKL. Given that a) MKL and SRF can regulate each other, and b) Pfn can modulate MKL localization and SRF activation, this study highlights a novel feedback system between MKL/SRF signaling and Pfn, adding another complexity in the current model of actin/MKL/SRF signaling.

5.2 RESULTS

5.2.1 Loss of function of MKL but not SRF leads to concomitant downregulation of Pfn isoforms

To determine whether MKL/SRF signaling influences Pfn expression, we first performed gene silencing of MKL1 in two different cell lines including HEK-293 (a human embryonic kidney epithelial cell line) and MDA-MB-231 (MDA-231 - a human breast cancer cell line), and analyzed the changes in Pfn expression. In both HEK-293 and MDA-231 cells, knockdown (KD) of MKL1 significantly downregulated the expression of Pfn1 and Pfn2 as well as SRF (this is consistent with MKL's ability to promote SRF activity and in turn stimulate SRF expression) (**Fig 9A**). Pfn1 expression was reduced by 60% and 50% in HEK-293 and MDA-231 cells, respectively, in response to MKL1 KD; the corresponding reduction in Pfn2 was 70% and 50% in HEK-293 and MDA-231 cells, respectively. To further determine whether alteration in the expression of one Pfn isoform can affect the expression of the other isoform, we performed isoform-specific KD and overexpression (O/X) of Pfn in both HEK-293 and MDA-231 cells. Selective KD of Pfn1 had no appreciable effect on the expression of Pfn2 and vice-versa (**Fig 9B**). These data argue against the regulation of one Pfn isoform downstream of the other, and further suggest that MKL promotes the expression of Pfn1 and Pfn2 in a coordinated fashion through a common upstream mechanism.

Given the parallel between expression of SRF and Pfn upon perturbation of MKL, we next evaluated the effect of silencing SRF on Pfn expression in HEK-293 and MDA-231 cell lines. Pfn. Surprisingly, when SRF was directly knocked down by siRNA, we did not see any

appreciable change in either Pfn1 or Pfn2 expression even though the KD efficiency of SRF was 70% (**Fig 9C**). Collectively, our gene silencing experimental data demonstrate that loss-of-function of MKL but not SRF can trigger downregulation of Pfn expression.

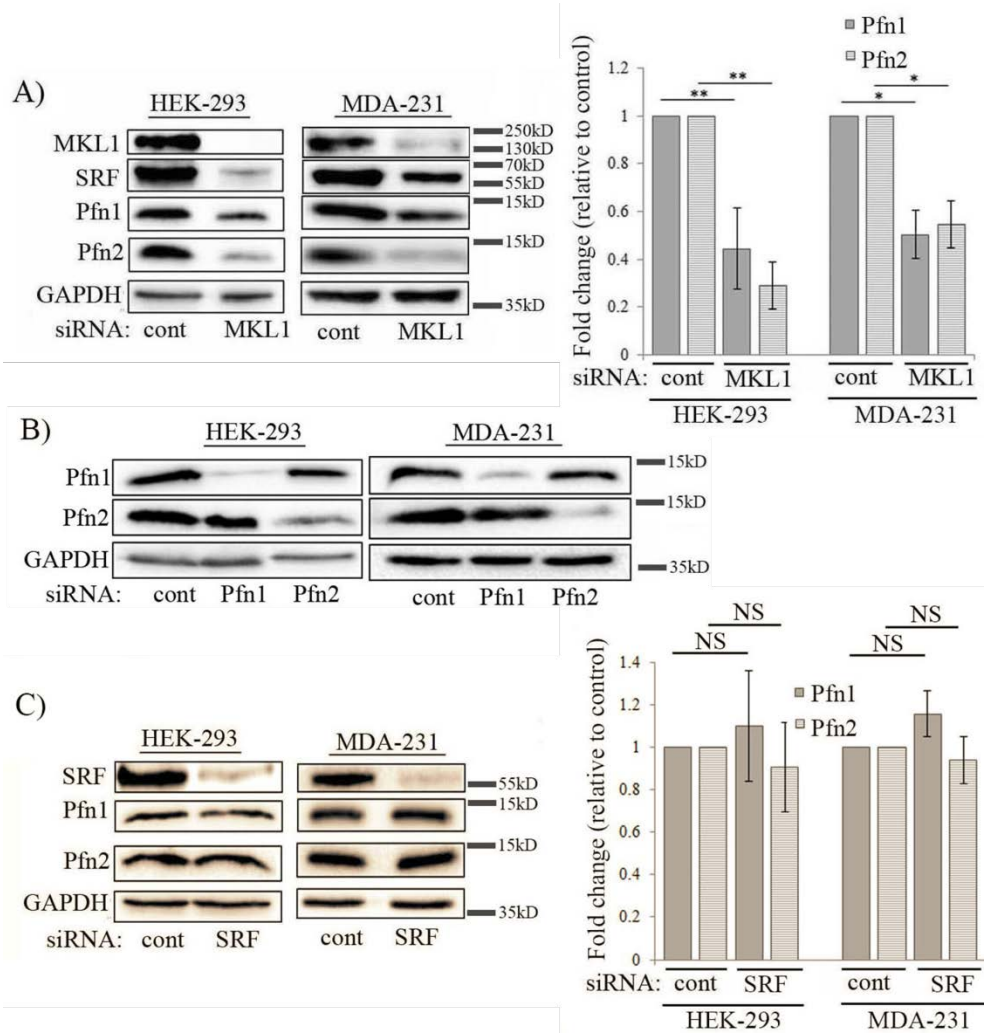

Figure 9. MKL, but not SRF, co-regulates Pfn isoforms. (A) Immunoblot analyses of MKL1, SRF, Pfn1 and Pfn2 expression in HEK-293 and MDA-231 cells 72 hrs after transfection with twith either MKL1- or control siRNA. (B) Immunoblot analyses of Pfn1 and Pfn2 expression in HEK-293 and MDA-231 cells following transfection with either Pfn1- or Pfn2- or control-siRNA. (C) Immunoblot analyses of SRF, Pfn1 and Pfn2 expression in HEK-293 and MDA-231 cells following SRF KD. The bar graphs alongside show the average fold-changes in Pfn1 and Pfn2 expression with respect to the corresponding control conditions (data summarized from at least 3 experiments; *p<0.05, **p<0.01). GAPDH blots serve as the loading control.

5.2.2 MKL regulates Pfn expression through its SAP domain activity in an SRF-independent manner

Although the foregoing SRF KD experimental data provided an initial indication that downregulation of SRF activity may not be the contributing factor for Pfn downregulation upon MKL1 KD, since SRF KD not was not complete in our experiments, we were not able to conclusively rule out SRF's involvement in regulating Pfn expression from those experiments.

We next performed SRF overexpression (OX) experiments which led to increase in Pfn levels in both HEK-293 and MDA-231 cells; however, SRF O/X was also associated with elevated MKL1 level in these cell lines (this was not unexpected since MKL promoter has SRF-binding sites) (**Fig 10A**). We hypothesized that SRF OX induced elevation of Pfn expression is an indirect effect of MKL upregulation rather than SRF being directly responsible for mediating MKL-dependent induction of Pfn. To test this hypothesis, we initially performed pilot experiments to examine the changes in Pfn expression in response to graded OX of MKL1 in HEK-293 cells. In these experiments, we transfected HEK-293 cells with increasing doses of a plasmid (ranging from 0.25-5 μg for a 35 mm dish culture) encoding full-length (FL) MKL1 (FL-MKL1) as a flag-tagged fusion protein. These pilot experiments revealed an interesting biphasic trend in Pfn expression in response to forced elevation of MKL1 (**Fig 10B-C**). Up to a certain level of MKL1 OX (corresponding to a range of 0.25-1 μg plasmid amount with 1 μg plasmid transfection estimated to upregulate MKL1 level by approximately ~30-fold), Pfn isoforms were found to be co-elevated compared to the vector control group, and these data are consistent with the effect of MKL1 KD on Pfn expression. However, at the higher plasmid doses (2.5 and 5 μg), Pfn isoforms showed a reverse trend reaching to levels comparable to those in the vector control group at the highest plasmid dose. We also noticed that MKL1 level did not correlate with the plasmid dosage

especially in the latter half of our dose range (1-5 μg). We speculated that this complex response of Pfn to MKL1 elevation and the apparent saturating trend in MKL1 level at higher plasmid doses possibly reflects an internal adaptive feedback mechanism between MKL1 and Pfn triggered by some threshold levels of MKL1 and Pfn in cells (this is discussed later). Based on these pilot data, we performed all of our subsequent MKL1 OX studies in a transfection setting (1 μg plasmid transfection for a 35 mm culture dish) that led to Pfn induction mimicking the effect of SRF OX and allowing us to examine SRF's direct involvement in MKL1's regulation of Pfn through the following sets of experiments.

First, following our transfection protocol, we confirmed that MKL O/X led to concomitant elevation of SRF and Pfn expression in both HEK-293 and MDA-231 cells (**Fig 10D**). On an average, Pfn1 expression increased by 2.3- and 1.7-folds in HEK-293 and MDA-231 cells, respectively; the corresponding increase in Pfn2 expression in these two cell lines were ~2.5- and 2.4-folds respectively. Since these results were based on transient transfection experiments where transfection efficiency is typically around 70-80%, these fold-change values were likely underestimated to some extent.

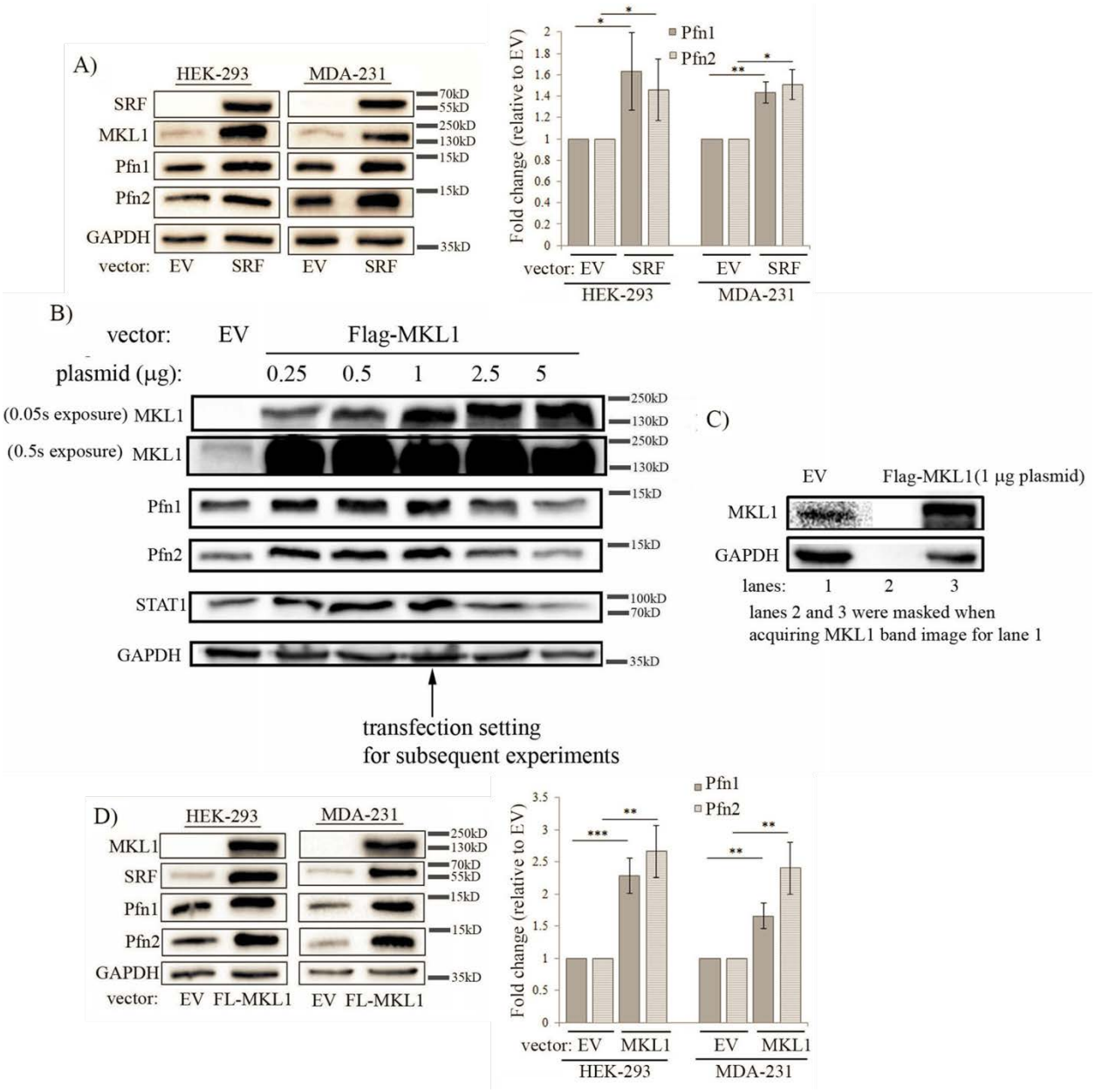

Figure 10. Effect of MKL1 and SRF OX on the expression Pfn isoforms in HEK-293 cells. (A) Immunoblot analyses of SRF, Pfn1 and Pfn2 expression in HEK-293 and MDA-231 cells following empty vector (EV) or SRF O/X. (B) Immunoblot analyses of MKL1, Pfn1, Pfn2, STAT1 and GAPDH (loading control) expression in HEK-293 cells 48 hrs after transfection with either empty vector (EV) or increasing amounts of flag-tagged FL-MKL1 OX vector (transfection was carried out in 35 mm culture dish). Two different exposures (0.05s, 0.5s) of MKL1 blot are shown. The 0.05s exposure blot allowed us to visualize exogenous flag-MKL1 protein level as a function of the plasmid concentration without signal saturation but the exposure was too low to detect the

endogenous MKL1 level. The higher exposure blot revealed the endogenous MKL1 band but the exogenous MKL1 lanes were saturated (representative of 3 experiments). (C) To assess the level of MKL1 OX, extracts of EV and FL-MKL1 (1.0 μg plasmid) expressers were re-run on a SDS-PAGE and immunoblotted with MKL1 and GAPDH antibodies, and endogenous MKL1 band was imaged with masking of OX lane. Relative GAPDH levels were used for normalization purpose. (D) Immunoblot analyses of MKL1, SRF, Pfn1 and Pfn2 expression in HEK-293 and MDA-231 cells 48 hrs after transfection with either empty vector (EV) or flag-tagged FL-MKL1. The bar graphs alongside show the average fold-changes in Pfn1 and Pfn2 expression with respect to the corresponding control conditions (data summarized from at least 3 experiments; *$p<0.05$, **$p<0.01$, ***$p<0.001$). GAPDH blots serve as the loading control.

Previous studies have shown that the basic-rich B1 domain of MKL1 (also contains one of the two nuclear localization signal (NLS) sequences) mediates its interaction with SRF (a schematic representation of MKL1 with its different domains is shown in **Fig 11A**). Mutagenesis studies have shown K237, Y238, H239 and Y241 residues located in the basic-rich B1 domain of MKL1 are critical for its SRF interaction, and alanine substitution on these residues abrogates MKL1:SRF interaction and MKL1-mediated SRF activation [75]. First, to determine whether nuclear localization of MKL is important for induction of Pfn, we analyzed the effects of overexpression of either FL-MKL1 or two different deletion mutants of MKL1 (ΔB1-MKL1, ΔN100-MKL1) on Pfn expression in HEK-293 cells. Deletion of the B1 region (ΔB1-MKL1) substantially interferes with the nuclear localization and transcriptional activity of MKL1and impairs MKL1:SRF interaction. Due to the absence of actin-binding RPEL motif, ΔN100-MKL1 cannot be sequestered in the cytoplasm by the action of actin monomers. ΔN100-MKL1 and tends to predominantly localize in the nucleus activating gene transcription in a constitutive manner (*i.e.* even in the absence of any serum stimulation) [28, 75]. By subcellular fractionation and immunofluorescence methods, we confirmed that in the growing culture of HEK-293 cells in

the presence of serum, both FL-MKL1 and ΔN100-MKL1 predominantly localized in the nucleus but nuclear localization of ΔB1-MKL1 was visibly impaired (**Fig 11B-C**). We found that deletion of the entire B1 domain blocks MKL1's ability to induce Pfn isoforms when overexpressed in HEK-293 cells. By contrast, deletion of the N-terminal actin-binding RPEL domain (ΔN100-MKL1) led to increased SRF expression (an indirect readout of SRF-activation by MKL) and coordinated upregulation of Pfn isoforms similar to the action of FL-MKL1 (**Fig 11D**). These data are consistent with the requirement of nuclear localization of MKL1 for its ability to promote Pfn expression in cells.

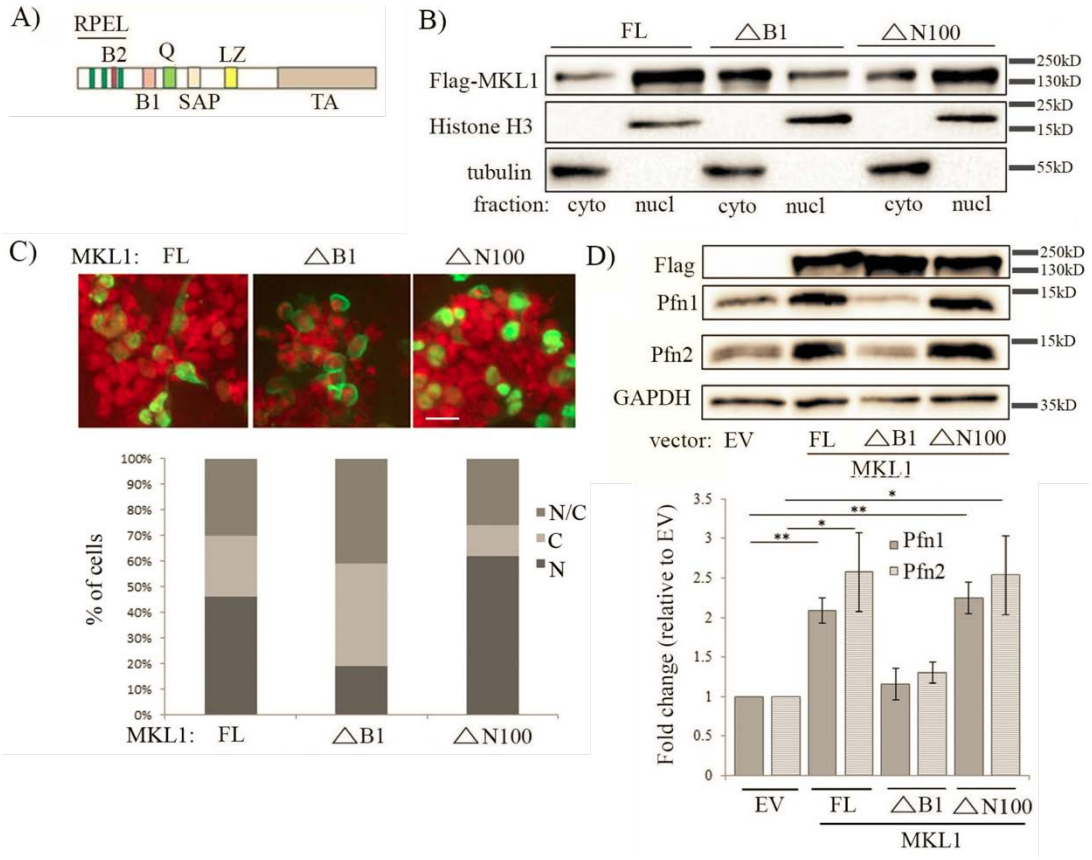

Figure 11. Nuclear localization of MKL1 is important for its ability to induce Pfn expression. (A) Schematic of MKL structure. RPEL: actin-binding region that has basic rich B2 region containing a NLS; B1: basic region that has a second NLS and SRF binding site; Q: glutamine-rich domain; SAP: DNA-binding domain; LZ:

leucine zipper (dimerization) domain; TA – transcriptional activation domain). (B) Anti-Flag immunoblot analyses of nuclear vs cytoplasmic fractions prepared from HEK-293 cells 48 hrs after transfection with the indicated Flag-tagged MKL constructs. Histone-H3 and tubulin blots serve as loading controls for nuclear and cytoplasmic fractions, respectively. (C) HEK-293 cells expressing various Flag-tagged MKL1 constructs (FL, ΔN100 (lacks the first 100 amino acids), ΔB1 (internal deletion of amino acids 222-237)) were immunostained with anti-flag (green) antibody and DAPI (red) and scored for % cells for sub-cellular localization of flag-MKL1 as summarized in the graph below (N- exclusively nuclear, C- exclusively cytoplasmic, N/C – localized in both cytoplasmic and nuclear compartments; 'n' indicates the number of cells analyzed in each group). (D) Immunoblot analyses of Pfn1 and Pfn2 expression in HEK-293 cells 48 hrs after transfection with the indicated MKL constructs (the Flag blot shows comparable expression levels of the various MKL1 constructs).

We next analyzed the effect of overexpression of either FL-MKL1 or a triple-point-mutant version of MKL1 (K237A/Y238A/H239A – denoted as MKL1$^{3p(mut-B1)}$) on Pfn expression in HEK-293 cells. Contrasting the effect of FL-MKL1, O/X of MKL1$^{3p(mut-B1)}$ mutant did not result in elevation of SRF expression thus confirming its lack of ability to activate SRF; however, Pfn isoform levels were comparably elevated by both FL-MKL1 and MKL1$^{3p(mut-B1)}$ overexpression (**Fig 12A**). In a complimentary experiment, we found that silencing SRF expression failed to block Pfn elevation in response to MKL1 O/X (**Fig 12B**). Collectively, these results demonstrate that MKL1 regulates Pfn expression in an SRF-independent manner.

A previous study demonstrating differential gene expression and phenotypic distinctions between MKL- and SRF-knockout megakaryocytes suggested that MKL can have SRF-independent transcriptional activity in cells [76]. It has been shown that MKL can transcriptionally regulate gene expression in breast cancer cells in an SRF-independent manner, directly utilizing its SAP-domain (a homology domain that is found in SAF-A/B, Acinus and PIAS proteins, and thought to facilitate DNA-binding of MKL) activity [77- 78]. Since our

findings suggested that MKL promotes Pfn expression without SRF's involvement, we further asked whether SAP-domain function of MKL1 might be important in the context of Pfn regulation. To address this question, we investigated the effect of deletion of either the SAP or the transcriptional activation (TA) domain on the ability of MKL1 to promote Pfn expression. We found that deletion of either the SAP (ΔSAP-MKL1) or the transcriptional activation (ΔTA-MKL1 – this construct is completely impaired in transcriptional activity) domain prevents MKL1-induced Pfn elevation in HEK-293 cells (**Fig 12C**). These data are consistent with a scenario that MKL regulates Pfn expression through its SAP-domain directed transcriptional activity.

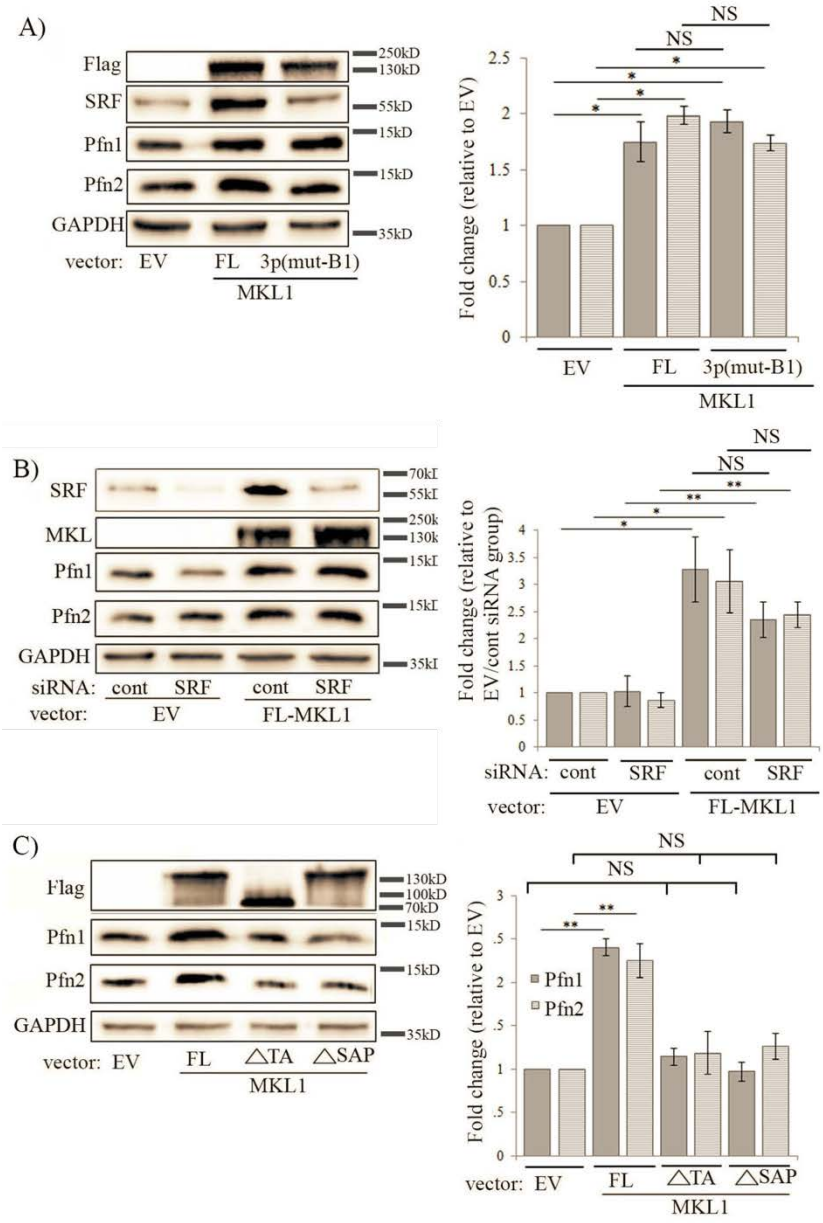

Figure 12. MKL regulates Pfn in an SRF-independent manner utilizing its SAP domain function. (A) Immunoblot analyses of Pfn1 and Pfn2 expression in HEK-293 cells transfected with either FL-MKL1 or MKL1$^{3p(mut-B1)}$ or EV (as control). (B) Immunoblot analyses of Pfn1 and Pfn2 expression in HEK-293 cells following co-transfection of the indicated siRNAs (Control or SRF siRNA) and plasmids (EV or FL-MKL1). (C) Immunoblot analyses of Pfn1 and Pfn2 expression in HEK-293 cells transfected with either FL- or the indicated deletion (ΔTA or ΔSAP) mutant forms of MKL1. The bar graphs alongside show the average fold-changes in Pfn1 and Pfn2 expression with respect to the corresponding control conditions (data summarized from at least 3 experiments; *p<0.05, **p<0.01, NS-no significance). GAPDH blots serve as the loading control.

5.2.3 MKL regulates Pfn expression through controlling the level of STAT

Our next goal was to search for alternative pathways that might link SAP-domain function of MKL to Pfn regulation. A previous study revealed that conditional deletion of MKL1 gene in mouse mammary gland is associated with alterations in the transcript levels of certain STAT (signal transducer and activator of transcription)-pathway associated genes [27]. There is also evidence that STAT3 can be recruited to the promoter region and transcriptionally regulate the expression of Pfn1 in rat aortic endothelial cells, specifically, in response to diabetic-condition mimicking stimuli. Furthermore, in drosophila, STAT (note that unlike in mammals, drosophila has only one STAT isoform, and is structurally similar to STAT3/STAT5) has been shown to bind to the promoter of *chickadee*, the Pfn1 homolog. Based on these findings, we explored whether there could be a potential connection between MKL, STAT and Pfn. To test this, we first examined the effects of MKL1 KD and OX on the expression levels of two major STAT isoforms, namely STAT1 and STAT3. In both HEK-293 and MDA-231, MKL1 KD resulted in downregulation of STAT1 expression. STAT3 expression also followed a similar trend in response to MKL KD although STAT3 downregulation in MDA-231 cells was not as pronounced as observed in HEK-293 (**Fig 13A**). Consistent with KD experiment results, both STAT1 and STAT3 isoforms were prominently elevated when MKL1 was overexpressed in either HEK-293 or MDA-293 cells (**Fig 13B**). Contrasting the effects of MKL KD, expression of STAT isoforms was not affected in MDA-231 or HEK-293 cells when SRF was silenced (**Fig 13C**). Accordingly, interfering with its SRF interaction did not affect MKL's ability to upregulate STAT expression, as judged by the comparable levels of STAT1 expression in HEK-293 cells upon OX of either FL-MKL1 or $MKL1^{3p(mut\text{-}B1)}$ (**Fig 13D**). However, deletion of either the SAP or the TA domain completely abrogated MKL1's ability to upregulate STAT isoforms

(**Fig 13E**). Collectively, these data demonstrate that MKL is an important positive regulator of STAT expression and that MKL promotes STAT expression through an SRF-independent, SAP-domain function-directed manner.

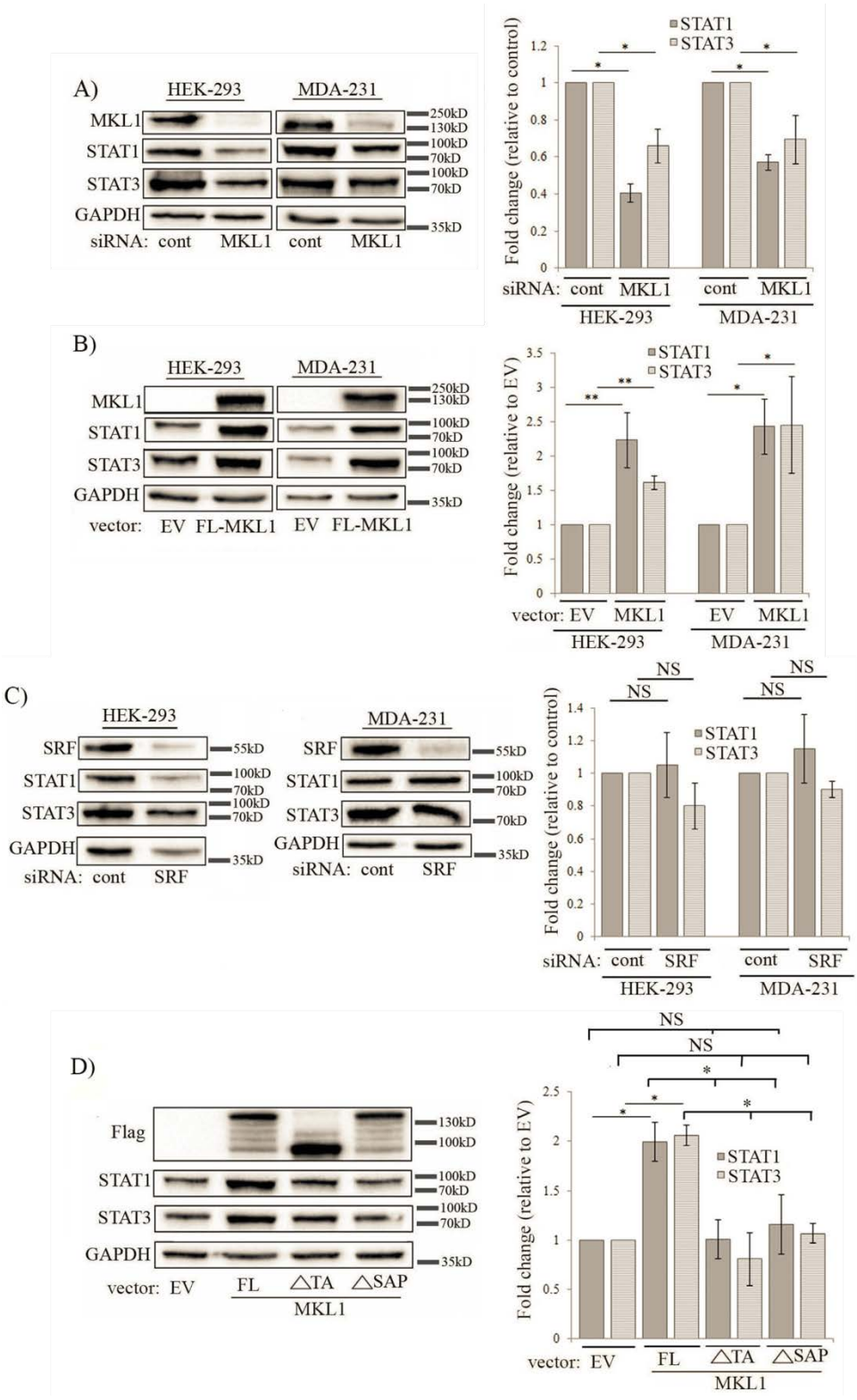

Figure 13. MKL promotes the expression of STAT isoforms in an SRF-independent manner utilizing its SAP domain function. (A) Immunoblot analyses of STAT1 and STAT3 expression following KD of MKL1 in HEK-

293 and MDA-231 cells. (B) Immunoblot analyses of STAT1 and STAT3 expression following O/X of MKL1 in HEK-293 and MDA-231 cells. (C) Immunoblot analyses of SRF, STAT1 and STAT3 expression in HEK-293 and MDA-231 cells following transfection with either control or SRF-siRNA. (D) Immunoblot analyses of STAT1 and STAT3 expression in HEK-293 cells following transfection of either FL- or the indicated deletion (ΔTA or ΔSAP) mutant forms of MKL1. The Flag blot shows comparable expression levels of the various MKL1 constructs. The bar graphs alongside show the average fold-changes in Pfn1 and Pfn2 expression with respect to the corresponding control conditions (data summarized from at least 3 experiments; *p<0.05, **p<0.01, NS-no significance). GAPDH blots serve as the loading control.

We next investigated the effects of isoform-specific KD and O/X of STAT on Pfn expression. In HEK-293 cells, KD of only STAT1 (but not STAT3) resulted in prominent downregulation of Pfn isoforms. In MDA-231cells, KD of either STAT1 or STAT3 caused prominent downregulation of Pfn isoforms mimicking the effect of loss-of-function of MKL (**Fig 14A**). In this cell type, KD of one STAT isoform also led to concomitant downregulation of the other isoform (not unexpected since STAT isoforms can be cross-regulated). Consistent with KD results, forced O/X of either STAT1 or STAT3 led to co-elevation of Pfn isoforms (note that STAT1 O/X promoted STAT3 expression and vice-versa) in MDA-231 cells. In HEK-293 cells, Pfn expression was elevated upon O/X of only STAT1 but not STAT3 (**Fig 14B**). In fact, even when STAT3 expression was forcibly knocked down, STAT1 O/X was still able to result in Pfn elevation in HEK-293 cells (**Fig 14C**). Collectively, these results demonstrate an importance of STAT in the regulation of Pfn expression. Although HEK-293 cell data suggest that STAT1 alone is sufficient to promote Pfn expression, we do not rule out a possibility of STAT3 as a collaborator in a cell-type specific context).

Given the ability of MKL to elevate expression levels STAT1 and Pfn isoforms simultaneously, we further asked whether STAT1 plays any role for MKL-dependent regulation of Pfn. To test this, we overexpressed FL-MKL1 in control vs. STAT1-siRNA transfected cells and analyzed the changes in Pfn expression. We found that silencing STAT1 expression prevented MKL1-induced elevation of both Pfn1 and Pfn2 in HEK-293 cells (**Fig 14D**). These results suggest that MKL-mediated regulation of Pfn occurs through a STAT1-dependent mechanism.

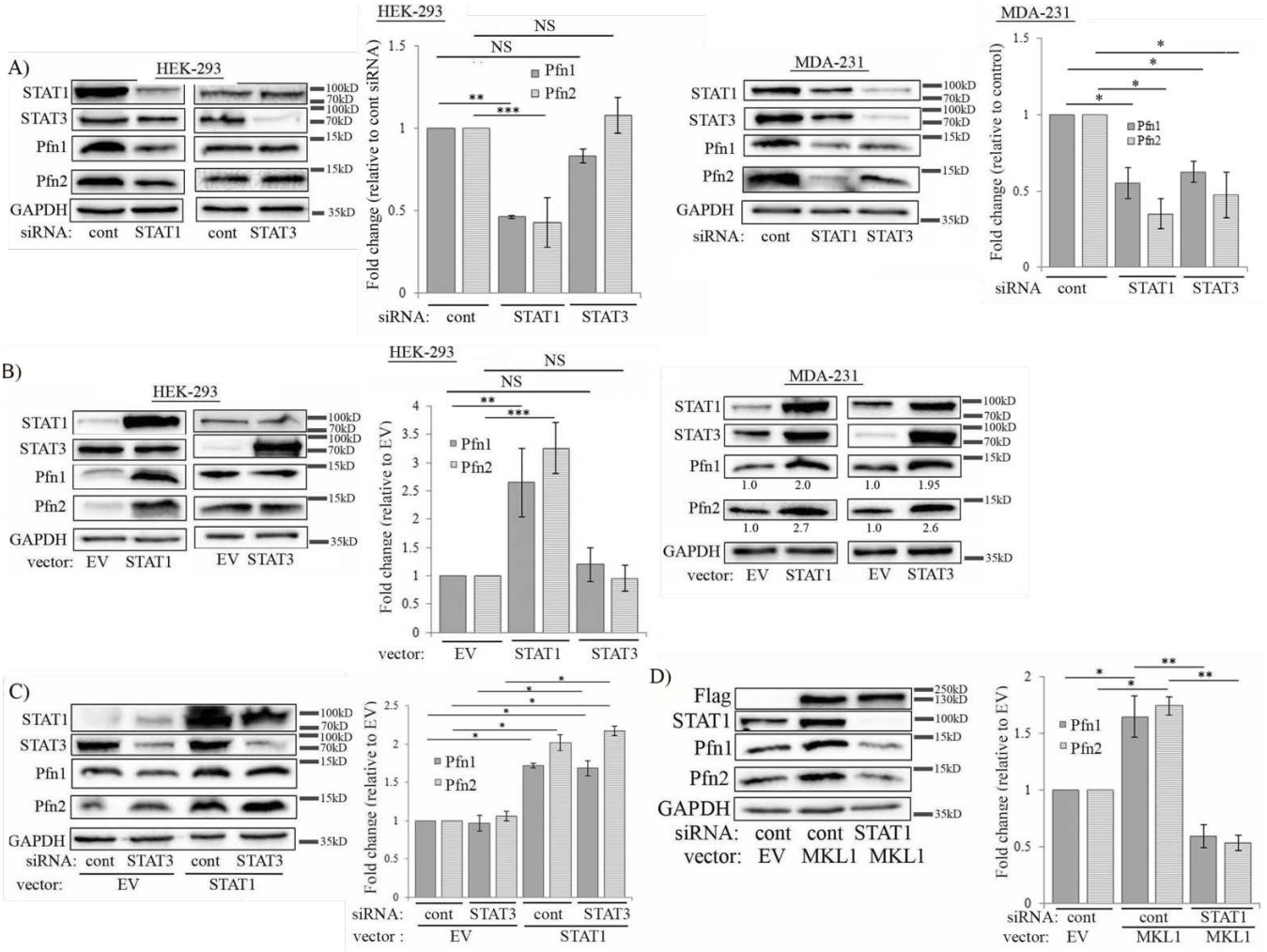

Figure 14. MKL1 promotes Pfn expression through STAT. (A) Immunoblot analyses of STAT1, STAT3, Pfn1, and Pfn2 expression in HEK-293 and MDA-231 cells following transfection with either control or STAT1 or STAT3 siRNA. (B) Immunoblot analyses of STAT1, STAT3, Pfn1, and Pfn2 expression in HEK-293 and MDA-231 cells 48 hrs after transfection with either EV or Flag-tagged-STAT1 or -STAT3 overexpression vectors. (C)

Immunoblot analyses of STAT1, STAT3, Pfn1 and Pfn2 in HEK-293 cells following co-transfection of the indicated siRNAs (control or STAT3) and overexpression vectors (EV or STAT1). (D) Immunoblot analyses of STAT1, Pfn1 and Pfn2 in HEK-293 cells following co-transfection of the indicated siRNAs (control or STAT1) and overexpression vectors (Flag-FL-MKL or EV as control). The bar graphs alongside show the average fold-changes in Pfn expression with respect to the corresponding control transfection condition (data summarized from 3 experiments; * $p<0.05$, **: $p<0.01$; ***: $P<0.001$; NS – not significant). GAPDH blots serve as the loading control.

5.2.4 MKL1 regulates Pfn expression in a post-transcriptional manner

In order to gain insight into the mode of regulation of Pfn by MKL, we analyzed the mRNA levels of Pfn in control vs MKL1 siRNA transfected HEK-293 cells by quantitative RTPCR. Although silencing of MKL1 elicited robust changes in the expression of Pfn isoforms at the protein level, surprisingly, we found no significant difference in the mRNA level of Pfn1 between control and MKL1 KD HEK-293 cells (**Fig 15A**). Similarly, the mRNA levels of Pfn isoforms were also not significantly affected when STAT1 expression was knocked down (**Fig 15B**). These data suggest that Pfn isoforms are primarily post-transcriptionally downregulated upon loss of either MKL1 or STAT1. We also explored whether altered protein stability might be responsible for MKL1-dependent changes in Pfn expression. To evaluate protein stability, we treated control- and MKL1-siRNA transfected HEK-293 cells with either proteasome inhibitor MG-132 or DMSO (vehicle control) and analyzed the changes in Pfn expression. Interestingly, downregulation of Pfn isoforms upon MKL1 depletion could not be reversed by MG-132 treatment (the efficacy of MG-132 was confirmed by upregulation of $p27^{kip1}$, a cell-cycle protein that is known to be downregulated by ubiquitin-mediated proteolysis) (**Fig 15C**). Lysosomal mediated protein degradation was also evaluated by ammonium chloride treatment in HEK-293. We found that inhibition of the lysosome was unable to reverse the effect of MKL1 KD on Pfn

levels (**Fig 15D**). Based on these results, it is unlikely that accelerated protein turnover, via lysosomal- or proteasomal- mediated degradation pathway, accounts for the reduction in Pfn expression upon loss of MKL. We also noticed that MKL1 KD affects the expression of only endogenous Pfns, but not epitope-tagged Pfns (e.g. myc-Pfn1, GFP-Pfn2) that were ectopically expressed by transfected cDNAs (**Fig 15E**). Insensitivity of exogenous Pfn to MKL depletion seems to be consistent with the overall idea of protein stability control not accounting for MKL-dependent changes in Pfn expression.

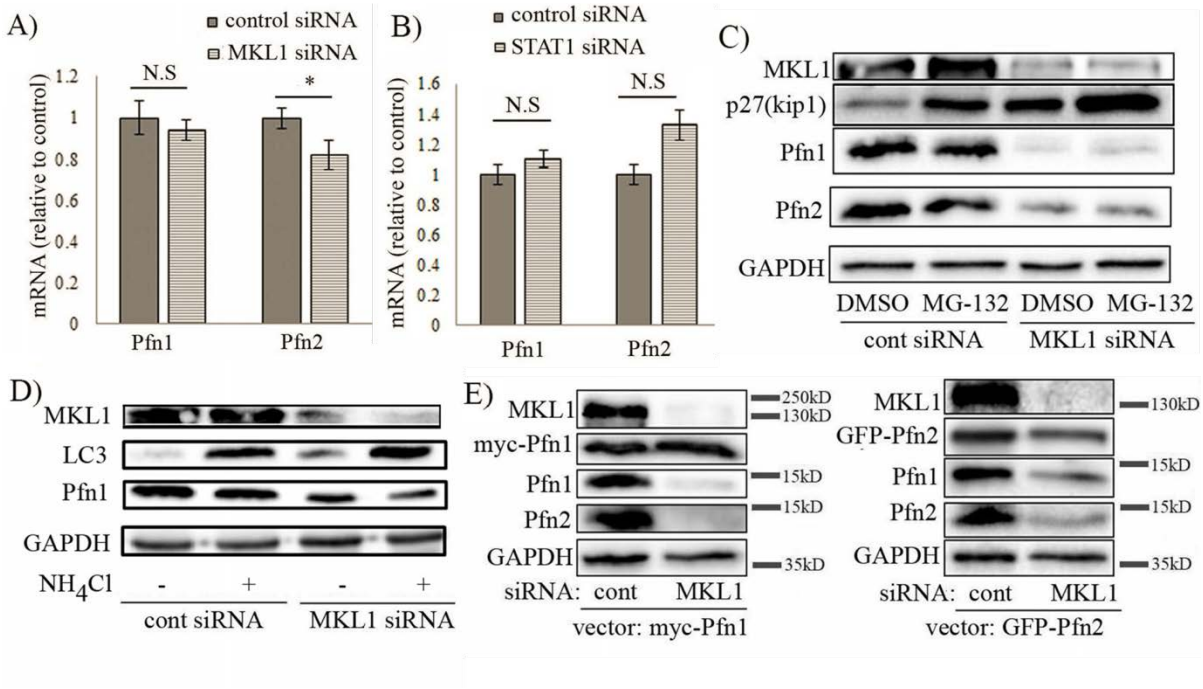

Figure 15. MKL co-regulates Pfn isoforms in a post-transcriptional manner that does not likely involve accelerated protein turnover. (A) Relative mRNA levels of Pfn1 and Pfn2 between control vs MKL1-siRNA transfected HEK-293 cell as determined by qRT-PCR analyses. (B) Relative mRNA levels of Pfn1 and Pfn2 between control vs STAT1-siRNA transfected HEK-293 cell as determined by qRT-PCR analyses. (C) Immunoblot analyses of MKL1, Pfn1, Pfn2 and p27(kip1) expression in HEK-293 cells 72 hrs after transfection with the indicated siRNAs and being treated with either 5uM MG-132 or DMSO (vehicle control) for 12 hrs. p27kip1, a cell-cycle protein that is rapidly turned over by proteasomal degradation, shows elevation upon MG-132 treatment

serving as a positive control in these experiments (data representative of 3 experiments). (D) Immunoblot analyses of MKL1, Pfn1, LC3 and GAPDH (loading control) expressions in HEK-293 cells 72 hrs after transfection with the indicated siRNAs and being treated with either 10 mM NH_4Cl (blocks lysosomal degradation pathway) or vehicle control for 12 hrs. LC3, an autophagy protein that is subjected to lysosomal degradation, shows elevation upon NH_4Cl treatment serving as a positive control in these experiments (data representative of 2 experiments). (E) Immunoblot analyses of extracts prepared from HEK-293 cells transfected with either myc-Pfn1 (left panel) or GFP-Pfn2 (right panel) along with the indicated siRNAs to reveal differential effects of MKL1 KD on endogenous vs exogenous Pfn. GAPDH blot serves as the loading control.

5.2.5 MKL/SRF can be also regulated by Pfn

Consistent with Pfn1's important role in facilitating actin polymerization in cells, Triesman group had previously shown that overexpressing Pfn1 in fibroblasts promote nuclear localization of MKL and transcriptional activity of SRF, at least measured in a transfected -reporter assay, and these effects are dependent on Pfn1's ability to interact with actin [30, 32]. To further extend these observations, we next studied the effect of perturbations of Pfn on MKL and SRF (as SRF is a transcriptional target of SRF itself) levels in MDA-231 cells. We found co-depletion of Pfn isoforms led to a significant reduction in both MKL1 and SRF levels in MDA-231 cells (**Fig 16A**) Consistent with these observations, OX of Pfn1 alone resulted in elevation of both MKL1 and SRF levels in MDA-231 cells (**Fig 16B**). Taken together with our foregoing results of MKL1's ability to control Pfn, these findings suggest that MKL and Pfn can regulate each other's expression.

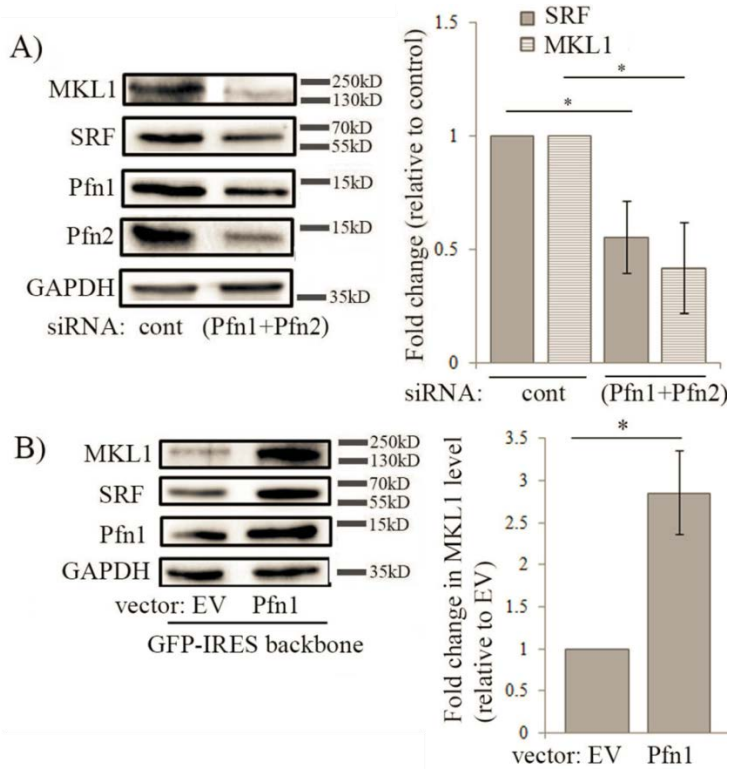

Figure 16. Expressions of MKL and SRF are sensitive to perturbations of Pfn. (A) Immunoblot analyses of MDA-231 extracts showing the effect of co-depletion of Pfn isoforms (via transfection of pooled siRNAs targeting Pfn1 and Pfn2) on MKL1 and SRF levels. (B) Immunoblot analyses of MDA-231 extracts showing the effect of OX of Pfn1 (cloned into GFP-IRES backbone vector) on MKL1 and SRF levels (cells transfected with the GFP-IRES backbone vector served as a control group). The bar graphs alongside show the average fold-changes in Pfn expression with respect to the corresponding control transfection condition (data summarized from 3 experiments; * $p<0.05$,). GAPDH blots serve as the loading control.

5.2.6 Loss of MKL1 promotes breast cancer cell motility through downregulation of Pfn expression

Finally, to explore a possible functional connection between MKL1 and Pfn, we next asked whether Pfn plays any role in MKL-mediated regulation of actin-dependent cellular events such as cell migration. Specifically, we investigated the effect of MKL1 KD on random motility of

MDA-231 cells without or with OX of Pfn1. We found that MKL1 KD reduced the basal level of Pfn1 as expected and increased the average speed of MDA-231 cells by ~1.9 fold and this is consistent with our previously published finding of enhanced motility of MDA-231 cells upon Pfn1 KD [49]. Hypermigratory phenotype of MDA-231 cells upon MKL1 depletion was completely reversed when Pfn1 expression was forcibly elevated thus suggesting that downregulation of Pfn1 expression contributed to enhanced motility of MKL1-silenced MDA-231 cells (**Fig 17**). These data provided a proof-of-principle of MKL's ability to impact cellular phenotype through modulating Pfn expression.

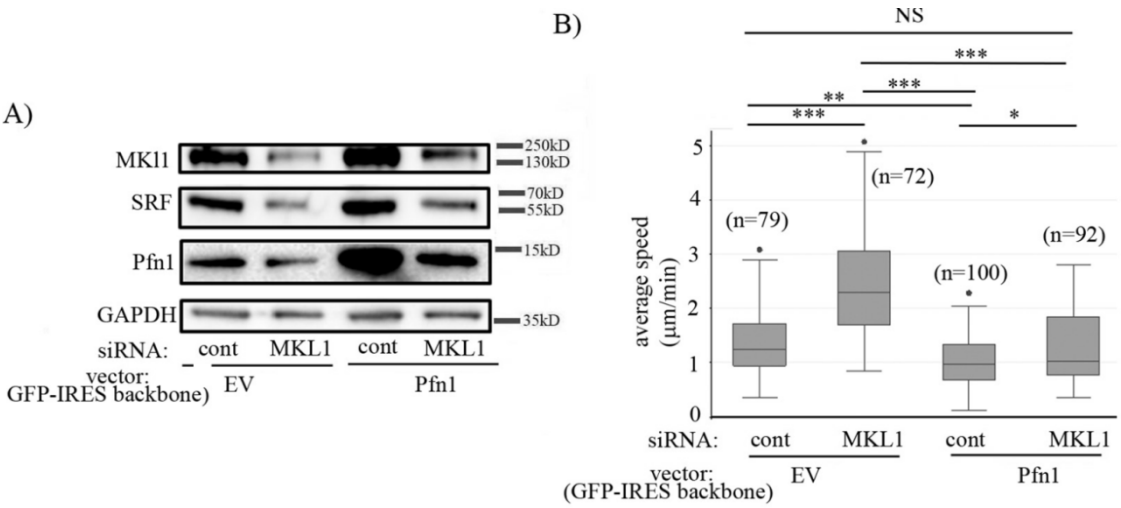

Figure 17. Loss of function of MKL1 promotes MDA-231 cell motility through downregulating Pfn1 expression. (A) Immunoblot analyses of MKL1, SRF, Pfn1 and GADPH (loading contr\ol) expression in MDA-231 cells co-transfected with the indicated siRNAs (control vs MKL1) and OX vectors (GFP-IRES backbone (EV) vs GFP-IRES-Pfn1). (B) A box-whisker plot summarizing the average speed of migration of these four groups of cells (transfected cells were identified by GFP fluorescence) in random-motility assays. In the plot, the middle line, the upper and lower hinges of the box represent the median, 75th and 25th percentile of data and the whiskers represent the maximum and minimum values (data summarized from 3 experiments; n: number of cells analyzed in each group pooled from 3 experiments; * $p<0.05$, **: $p<0.01$; ***: $P<0.001$; NS – not significant).

5.3 DISCUSSION

Although Pfns are important control elements of actin polymerization, very little is known about how their expression is controlled in cells. In this study, we report several novel findings. First, we show that despite their different genomic locations, the two main cellular isoforms of Pfn can be co-regulated and this regulation occurs through a common signaling pathway involving the action of the transcriptional coactivator MKL and importantly, this mechanism is generalizable across different cell types. Second, contrasting the conventional paradigm of transcriptional control of cytoskeletal genes by the MKL/SRF axis, we herein demonstrate that MKL regulates Pfn isoforms post-transcriptionally and in an SRF-independent manner. Third, we identify STAT1 as an important upstream regulator of Pfn expression and further show that MKL regulates Pfn expression through controlling the level of STAT1 via its SAP-domain (a putative DNA-binding domain of MKL that has been implicated in SRF-independent transcriptional control of MKL [77-78]) function. SAP-domain-directed transcriptional regulation by MKL is recently gaining attention; however, the key downstream players in this process remain to be identified. Since STAT isoforms are regulated by the SAP-domain-activity of MKL, it further raises the possibility of a STAT-centric gene expression control downstream of SRF-independent, SAP-domain activity of MKL that can extend beyond and above the context of regulation of Pfn.

In rat aortic endothelial cells, there is evidence of STAT3's ability to directly bind to the promoter of Pfn1 and stimulate its transcription [57]. However, STAT3 recruitment to the Pfn1 promoter occurs only in response to certain stimulus (such as oxysterol treatment as shown in that study). Similarly, *drosophila* STAT (structurally similar to mammalian STAT3 or STAT5) binds to a region near the promoter of the chickadee (*Drosophila* homolog of Pfn1) gene;

however, mutating the STAT binding site does not affect the *chickadee* promoter activity [58]. While these findings suggest that STAT3 has the potential to transcriptionally promote Pfn expression under certain conditions, it is not the main regulator of Pfn under basal conditions. Potential roles of other STAT family members in the context of Pfn regulation have not been explored previously. This study shows that STAT1 is a key upstream regulator of Pfn expression even under basal conditions, and that STAT1 can promote Pfn expression without requiring STAT3. Although loss of STAT1 does not affect the mRNA level of Pfn isoforms, transcriptional activity of STAT1 may still be important for regulating an upstream controller of Pfn expression.

The exact details of how MKL controls STAT expression and in turn post-transcriptionally regulate Pfn isoforms are still unclear. However, several possible mechanisms can be explored in the future. In a previous study, differential transcriptome analyses of MKL1 knockout and wild-type mammary tissue demonstrated robust transcriptional upregulation of SOCS3 (suppressor of cytokine signaling 3) gene upon loss of MKL1 [27]. Since SOCS proteins negatively regulate JAK-STAT signaling, it is possible that MKL regulates STAT expression through modulating SOCS3 transcription (either directly or indirectly). There is also evidence in the literature that MKL1 and STAT3 can physically interact and transactivate gene expression in breast cancer cells [79]. Whether other STAT members can directly interact with MKL1, or at least co-complex with MKL1:STAT3, are not known. Since STAT family members can homo- as well as hetero-dimerize to regulate the expression of their own and other STAT members, it is possible that MKL:STAT interaction may play a role in regulating STAT expression. As for Pfn, our MG-132 data (supported by our observation that ectopically expressed epitope-tagged Pfn is insensitive to MKL KD) tend to suggest that accelerated proteasome-mediated protein

degradation does not underlie Pfn downregulation upon loss of MKL. Similarly, lysosomal mediated protein-degradation does not appear to mediate MKL1-induced Pfn regulation shown by ammonium chloride data. However, it is possible that structural changes in Pfn as a result of epitope tagging can affect its protein stability, so we cannot absolutely rule out the possibility of protein stability control of Pfn downstream of MKL function at this point. Since *in silico* analyses predict the potential of many common microRNAs targeting the 3' untranslated regions of Pfn1 and Pfn2, an alternative possibility is that signaling downstream of MKL/STAT may be somehow linked to translational control of Pfns through the action of a common microRNA targeting the two isoforms of Pfn. These issues will need to be addressed in future studies.

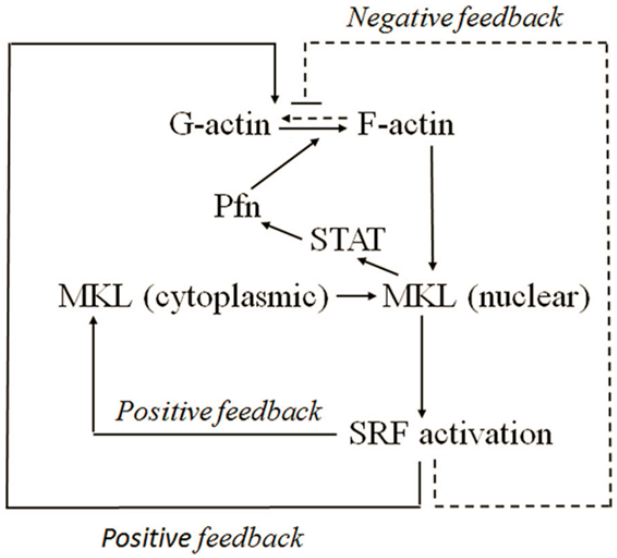

Figure 18. A proposed schematic model of actin/MKL/Pfn/SRF signaling circuit. This model integrates the current findings of Pfn being regulated downstream of MKL in an SRF-independent manner through STAT and Pfn's ability to also modulate MKL and in turn SRF activity/expression, thus enabling a positive feedback loop. SRF activation can either elicit a feedforward (through promoting actin polymerization, MKL expression) action amplifying the response or a negative feedback action (through elevating G-actin level) thus dampening the response beyond a certain limit.

It has been reported that O/X of Pfn1 can promote nuclear localization of MKL1 and increase SRF activation, and this effect of Pfn1 on MKL/SRF signaling requires its actin-binding function [30, 32]. MKL and Pfn1 can also compete for G-actin binding [33]. Therefore, Pfn1 likely promotes MKL/SRF activation via inhibiting MKL:actin complex formation through direct competition with MKL and/or promoting actin polymerization. Since MKL and SRF can regulate each other's expression, the present finding of MKL1's positive regulation of Pfn1 through STAT1, taken together with Pfn1's effect on MKL/SRF signaling, suggests that there is a feedback loop between MKL/SRF, Pfn1, and dynamic control of actin polymerization involving the action of STAT (**Fig 18**). According to this feedback model, enhanced nuclear localization of MKL1 induced by Pfn1 overexpression would be expected to cause an increase in Pfn2 expression; clearly, this was not observed in our studies. A potential explanation could be that since all of our O/X experiments were done in serum-containing culture where MKL was predominantly nuclear (as judged by the subcellular fractionation analyses of flag-MKL1), additional contribution from Pfn1 overexpression in terms of regulating MKL localization may be inconsequential. Given that SRF can bind to a conserved intronic region of both mouse [80] and human Pfn1 (Joseph Miano, University of Rochester – personal communication), there could also be a negative feedback signal from hyperactivated SRF somehow dampening the autoregulatory loop. The proposed model also illuminates a potential role for G-actin in the regulation of Pfn expression, since actin depolymerization can trigger MKL1 inhibition. Future studies investigating the effects of actin-modifying drugs or other actin-polymerization regulatory proteins on Pfn expression will shed further insight into this signaling pathway.

6.0 CONCLUSIONS

Dysregulation of actin-regulatory proteins is a hallmark feature of tumor malignancy. Along this line, recent studies have established causal relationships between downregulated expression of important actin-binding proteins, profilin-1 and -2 (Pfn1, Pfn2), and increasing invasive ability of breast cancer cells. Previous studies from our laboratory revealed that Pfn1 depletion enhances tumor cell blood burden, without affecting grown of the primary tumor, suggesting that loss of Pfn1 enhances the disseminative potential of breast cancer cells. This hypothesis was addressed by evaluating the effect of Pfn1 KD on tumor-vascular endothelial cell interactions. Results from the study establish how loss of a traditionally conceived pro-migratory molecule, Pfn1, can promote intravasation of breast cancer cells through modulating VEGF expression, providing new mechanistic insight into how Pfn1 negatively regulates an important step in breast cancer cell dissemination.

Another goal of the study was to determine whether restoring Pfn1 expression could serve as an effective strategy to limit breast cancer cell motility. We performed a small-molecule screen for anti-migratory compounds capable of elevating Pfn1 expression. Tyrphostin A9 was identified from the screen and orthogonal assays confirmed the functional involvement of Pfn1 in the anti-migratory activity of Tyrphostin A9. Overall, the study reveals that small molecule mediated induction of Pfn1 is capable of limiting breast cancer cell motility in vitro.

Recent studies have established that genetically elevating Pfn1 and Pfn2 expression suppresses aggressiveness of breast cancer cells. Therefore, identification of a common signaling pathway linking the regulation of these two Pfn isoforms may lead to a novel conceptual strategy to suppress metastatic potential of breast cancer cells in a Pfn-dependent manner. However, the molecular pathways responsible for Pfn downregulation in cancer are not known. In fact, very little is understood regarding Pfn regulation in normal cells. The current study identified megakaryoblastic leukemia 1 (MKL1), a transcriptional co-activator of serum response factor (SRF), to be a major regulator of Pfn isoform expression. We also established an important role for STAT1 in the pathway of MKL1-mediated regulation of Pfn isoforms. The present finding of MKL1's positive regulation of Pfn1 through STAT1, taken together with Pfn1's effect on MKL/SRF signaling, illuminates a potential feedback loop between MKL/SRF, Pfn1, and dynamic control of actin polymerization involving the action of STAT. Our findings not only demonstrate that Pfn isoforms can be co-regulated by an unconventional MKL1 signaling pathway involving the action of STAT1, but this study could open up promising pharmacological points of attack in the context of metastatic breast cancer.

7.0 FUTURE DIRECTIONS

7.1 EVALUATE THE EFFECT OF PFN1 DOWNREGULATION ON INTRAVASATION OF BREAST CANCER CELLS IN VIVO

We have found that human breast tumors involving significant lymph node infiltration and/or distant metastasis present the most dramatic downregulation in Pfn1 expression. Aligned with these clinical findings, our *in vivo* experiments have further revealed that Pfn1 depletion increases blood burden of tumor cells from xenograft-induced mammary tumors but has no effect on the growth of the primary tumor, suggesting that loss of Pfn1 promotes hematogenous dissemination of breast cancer cells. Transendothelial migration (TEM) is an obligatory component of tumor cell intravasation leading to vascular dissemination of cancer cells, and our *in vitro* studies have shown that Pfn1 KD enhances TEM of breast cancer cells. Therefore, future studies will aim to identify the effect of Pfn1 downregulation on the actual intravasation of breast cancer cells *in vivo*. Intravital microscopy will be performed to visualize tumor cell intravasation in mammary tumors formed by MDA-231 xenografts and MMTv-pyMT in mice. Intravital imaging will utilize a point-scanning multiphoton microscope capable of high-magnification imaging of tumor cell invasion/intravasation events as well as various components of the tumor microenvironment at a single-cell resolution [81].

7.2 IDENTIFY SPECIFIC KINASE INHIBITOR AGENTS THAT ARE CAPABLE OF REDUCING TRANSENDOTHELIAL MIGRATION BY ELEVATING PFN1 EXPRESSION

Through a LOPAC screen, we have already identified that Pfn1 expression can be robustly increased in breast cancer cells by Tyrphostin A9. Since Tyrphostin A9 is a broad RTK inhibitor, as a next step we will perform a kinase inhibitor library (targets >100 kinases; commercially available) screen to derive a more targeted approach to enhance Pfn1 expression (these experiments will be performed in collaboration with U. Pitt Drug Discovery Institute as a part of our ongoing collaboration). The overall procedure will be similar to that used in our preliminary studies. Essentially, MDA-MB-231 cells will be plated in the wells of 384-well plate and subjected to treatments of either 10 uM of different kinase inhibitors or DMSO (control) for 48 hours. Cells will be then fixed and stained for Pfn1 and fluorescence images will be analyzed to derive the initial hits of Pfn1 inducers (z score >=2). Pfn1 induction by these selected initial hits will be confirmed by orthogonal assays (western blot) and further tested for dose-dependence. A parallel screen will be conducted for transendothelial migration (TEM – will be measured as per the procedure adopted in our study) with this library of compounds. Those compounds which simultaneously increase Pfn1 expression and inhibit TEM will be selected and further tested for mechanistic links using Pfn1 shRNA cells as controls similar to what we had done for assessing anti-migratory effect of Tyrphostin A9. Essentially, if a compound inhibits TEM through Pfn1 upregulation, its inhibitory action should be greatly diminished in Pfn1 shRNA cells. Overall, these studies would provide valuable information regarding the specificity of chemical agents and would present a novel approach to modulate cellular Pfn1 in breast cancer cells.

7.3 IDENTIFY THE REGULATORY LINK BETWEEN STAT1 AND PFN ISOFORMS

The current research aimed to identify regulatory pathways involved in Pfn isoform expression, which is a major gap in Pfn-related research. We found that STAT1 is a positive regulator of Pfn protein level, but the exact link between STAT1 and Pfn1 was not addressed. STAT1 has a well-established role in inflammation and immune response, and in cancer. STAT1 is generally classified as a tumor suppressor, likely through both its immune regulatory and tumor cell-specific functions [82]. Inflammation is necessary for normal processes such as wound healing or response to infection. However, aberrant immune response or chronic inflammation may facilitate tumor growth and dissemination. STAT1 is a known mediator of inflammatory signals from various cytokines, including interleukins and interferons (IFNs). IFNs are major activators of STAT1, and are often used as anti-tumor agents by targeting the immunological components of the disease [83]. In order to understand the link between STAT1 and Pfn, it is important to identify whether STAT1 level or activity is responsible for Pfn induction. IFNγ treatment would be a logical starting point to evaluate the effect of active STAT1 on Pfn expression. From there, one could further evaluate specific genes transcribed by the IFN-STAT1 pathway in relation to Pfn expression. In general, inflammatory processes involved in breast cancer should be considered in future studies on Pfn regulation since STATs are largely involved in these pathways.

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