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Functional analysis of the protein tyrosine phosphatase family in mammary epithelial cell models of breast cancer

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Abstract of the Dissertation

Functional analysis of the protein tyrosine phosphatase family in mammary epithelial cell models of breast cancer

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The protein tyrosine phosphatase (PTP) superfamily of enzymes is encoded by ~100 genes. In coordinating with protein tyrosine kinases (PTK), they regulate signaling pathways that control a broad spectrum of fundamental physiological processes and their disruption underlie human diseases. To date, however, the functional analysis of PTPs is not as advanced as for PTKs. Therefore, the ultimate goal of this dissertation is to develop the tools for global analysis of the function of PTPs through RNAi-mediated loss-of-function screens.

I constructed a PTP short-hairpin RNA (shRNA) library, which contains ~5 shRNAs specifically designed to target each of the PTPs. In collaboration with Min Yu, I studied the function of PTPRO in mammary epithelial cell morphogenesis by using these shRNAs. We found that suppression of PTPRO promoted cell proliferation and by recognizing the oncoprotein tyrosine kinase ErbB2 as a direct substrate, PTPRO augmented the ability of ErbB2 to induce multi-acinar structure in 3D culture. Preliminary analysis of other PTPs revealed different phenotypes consistent with distinct mechanisms of the enzymes.

I also employed an RNAi mediated loss-of-function screen to study systematically the role of the PTP family in mammary epithelial cell motility, in the absence or presence of ErbB2. I identified PTPs that either promoted or inhibited mammary epithelial cell motility. I further characterized 3 PTPs, PRPN23, PTPRG and PTPRR, which function to inhibit cell motility. I found that suppression of PTPN23, but not PTPRG or PTPRR, induced cell invasion. Suppression of PTPN23 increased E-cadherin internalization, impaired early endosome forward trafficking of E-cadherin and induced the expression of mesenchymal proteins. Moreover, I identified E-cadherin, β-catenin and Src as

direct substrates of PTPN23, and demonstrated that Src mediates the effects of suppressing PTPN23 on control cell motility and invasion.

In this dissertation, I have revealed novel functions for members of the PTP family. In addition, I illustrate how RNAi-mediated loss-of-function screen reveals new roles for PTPs in mammary epithelial cell motility and invasion. These findings highlight the specificity of PTPs in breast cancer, suggesting PTPs or the signaling controlled by them could be biomarkers or therapeutic targets for the treatment of breast cancer.

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Chapter 1: General Introduction:

Preface

In this dissertation I focus my study on characterizing the protein tyrosine phosphatase superfamily of enzymes. There are two major goals of this study. Firstly, I aim to study systematically the function of the PTP family using RNAi-mediated loss-of-function screens. Secondly, I characterize in more detail the function of PTPs selected in the screen. In order to accomplish these goals, I constructed a short hairpin RNA library that contains ~5 short hairpin RNAs (shRNAs) that target each member of the PTP family (Chapter 2). I characterized the functions of PTPRO in the morphogenesis of mammary epithelial cell through RNAi-mediated loss-of-function studies (Chapter 3). Furthermore, I deployed an RNAi-mediated loss-of-function screen to study systematically the function of the PTP family in mammary epithelial cell motility and invasion (Chapter 4). In the screen, I revealed novel functional insights into specific members of the PTP family that have remained uncharacterized to date including PTPN23, PTPRG and PTPRR. In summary, these studies demonstrate an approach to study systematically the function of the PTP family. Further applications of this approach will extend our understanding of this family of enzymes and benefit the development of therapeutic strategies that target PTPs for the treatment of breast cancer and other diseases.

1.1 Protein Phosphorylation

1.1.1 Protein phosphorylation is reversible:

Approximately one third of proteins in the cell are phosphorylated. Protein phosphorylation is the most important reversible post-transcriptional modification in the cell. It controls protein functions, such as activity, interaction, localization and stability. Phosphate was first discovered to be covalently linked to proteins in 1906 [1]. However, its role in manipulating cell signaling through reversible phosphorylation remained unknown until Edwin Krebs and Edmond Fischer's work in the mid-1950's to characterize phosphorylase, a glycogen metabolism enzyme [2, 3]. Briefly, there are two forms of phosphorylase, phosphorylase a and a less active phosphorylase b, that interconvert between each other in the cell [4, 5]. The interconversion of phosphorylase a and b is controlled by two enzymes. The phosphorylase kinase, which transfers the γ-phosphate from ATP to the phosphorylase with concomitant activation [3, 6] and the phosphorylase phosphatase, which removes the phosphate and inactivates the enzyme [7, 8]. These findings demonstrated the principle mechanism of reversible protein phosphorylation. Soon afterwards, the activity of the phosphorylase kinase was found to be controlled by another kinase, phosphorylase kinase kinase, otherwise known as the cyclic AMP-dependent kinase [9-11]. This finding introduced for the first time the idea that protein activity is modulated through a cascade of kinases regulated by reversible protein phosphorylation.

1.1.2 Protein tyrosine phosphorylation:

Protein phosphorylation usually occurs on amino acids that contain a hydroxyl group, such as serine, threonine and tyrosine residues. Serine is the most common amino acid that is phosphorylated, followed by threonine and tyrosine. Compared to serine or threonine phosphorylation, tyrosine phosphorylation is relatively rare in a protein. However, phosphorylation of tyrosine plays an important role in the control of protein functions. Protein tyrosine phosphorylation is controlled by two families of enzymes, protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs). pp60v-Src, a Rous sarcoma virus (RSV) transforming protein, was the first identified PTK [12]. It exclusively phosphorylates the tyrosine residues of proteins. Later, the EGF receptor (EGFR) was also identified as a tyrosine kinase, a receptor type was known (RTK). Following binding of EGF, its became phosphorylated predominantly on specific tyrosine residues through autophosphorylation [13]. To date, 90 unique tyrosine kinase genes have been identified in the human genome, including 58 receptor-type tyrosine kinases and 32 non-receptor tyrosine kinases [14]. PTP activity was also observed initially in parallel to the identification of the PTKs [13, 15, 16]. The first PTP to be purified to homogeneity was PTP1B, by Tonks [17, 18]. Later, the primary sequence of PTP1B was determined, and found to be homologous to the intracellular segment of the transmembrane receptor-like protein CD45, indicating that CD45 itself was a receptor-like PTPs [19-22]. These findings suggested that like the PTKs, PTPs exist as either non-receptor enzymes or transmembrane receptor-like enzymes

that have the potential to regulate signal transduction directly through dephosphorylation of tyrosine residues of proteins. Recently, it has been proposed that PTKs control the amplitude of a signaling response, whereas the PTPs are thought to play a greater role in controlling the rate and duration of the response [23, 24].

1.1.3 PTKs as therapeutic targets of human cancers:

Disruption of the coordinated and competing activities of PTKs and PTPs can alter the normal pattern of protein phosphorylation, resulting in the propagation of abnormal responses to extracellular stimuli and leading to the development of human diseases, including cancer. Thus, the ability to modulate specifically signaling pathways regulated by protein tyrosine phosphorylation holds enormous therapeutic potential. This rationale has recently led to the development of small molecular PTK inhibitors for the treatment of cancers, such as Gleevec, which targets Bcr-Abl, for the treatment of chronic myelogenous leukemia, and Iressa (Gefitinib) and Tarceva (Erlotinib), which both target EGFR, for the treatment of non-small cell lung cancer. In addition, antibody-based PTK inhibitors were also developed, such as Herceptin (Trastuzumab), which targets ErbB2, for the treatment of malignant breast cancer. However, the therapeutic success rate of these drugs is limited and patients develop resistance against the drug. A better understanding of protein tyrosine phosphorylation would benefit the development of new therapeutic strategies and the identification of novel therapeutic targets. However, most of the studies on protein tyrosine

phosphorylation have been focused on PTKs. Therefore, a systematic study of the function of the PTP superfamily of enzymes would not only complete our knowledge about protein tyrosine phosphorylation but also help to develop new therapeutic strategies and identify novel therapeutic targets for the treatment of cancers.

1.2 Protein Tyrosine Phosphatases

There are ~100 genes that encode PTPs that have been identified in the human genome. These include 21 receptor-like PTPs and 82 non-receptor PTPs [25]. PTPs are defined by the presence of an active site signature motif, $HC(X)_5R$ (X is any amino acid), in which the invariant Cys and Arg residues are essential for catalysis [26]. All members of the PTP family follow a two-step reaction to catalyze protein dephosphorylation (Figure 1-1). Firstly, the low-pKa cysteine residue (pKa = 4.7-5.4) [27] in the signature motif initiates a nucleophilic attack on the phosphate group of the substrate. The conserved Arg stabilizes the Cys-thiolate, assists substrate binding and maintains the transition-state of the phosphate. In addition, an invariant Asp (Asp 181 in PTP1B) serves as a general acid to protonate the tyrosyl leaving group of the substrate and leads to the formation of a cysteinyl-phosphate intermediate. In the second step, a water molecule is oriented by hydrogen bonding to a conserved glutamine residue (Gln 262 in PTP1B). The negatively charged Asp181 now serves as a general base to promote the nucleophilic attack of the water molecule. This leads to the

hydrolysis of the phosphocysteine intermediate and the release of the phosphate [26].

Based on their substrate specificity, PTPs are broadly divided into two groups, the classical PTPs (Figure 1-2) and the dual specificity phosphatases (DSPs) (Figure 1-3) [28]. The substrate specificity of the classical PTPs and the DSPs is determined by the architecture of the active site cleft. The catalytic cysteine lies at the bottom of the catalytic cleft. In the classical PTPs, there is an invariant Tyr (Tyr46 in PTP1B) that defines the depth of the catalytic cleft, thereby ensuring specificity for phosphotyrosine, which has a longer side chain. In contrast, the catalytic cleft of the DSPs is shallower because the residues that define the depth of the catalytic cleft are shorter and the important residues that interact with the aromatic ring of the substrate pTyr are missing [29-32]. This allows both phosphotyrosine and phosphoserine/threonine to access to the catalytic Cys, which lies at the bottom of the cleft. In PTEN, the active site cleft is wider than that of the DSPs. This accommodates the binding of the large inositol sugar ring of its substrates, phosphoinositides [32]. Interestingly, a PTEN mutation (G129E) that leads to the restriction of the dimension of the catalytic cleft causes the loss of lipid phosphatase activity but retains the protein phosphatase activity of PTEN [33], suggesting that the shape of the catalytic cleft plays a role in determining substrate specificity.

1.2.1 The Classical PTPs:

The classical PTPs consist of 37 genes in the human genome, including 23 transmembrane receptor-like PTPs and 14 non-transmembrane PTPs. They act exclusively on phosphotyrosine residues [12]. In addition to the catalytic domain, the non-transmembrane PTPs contain regulatory sequences that modulate the activity of the enzyme directly or indirectly through controlling the subcellular localization of the enzymes. For example, the SH2 domain of SHP2 is a phosphotyrosine binding domain that interacts with the phosphotyrosine residue of proteins, thereby targeting SHP2 to its substrate [34]. Upon binding to its substrates the catalytic cleft of SHP2 is exposed and activated. Therefore, the SH2 domain of SHP2 ensures the enzyme to be activated upon substrate binding in the correct place in the cell. The C-terminal tail of PTP1B contains a tail anchor signal, which localizes the enzyme to the cytoplasmic face of the endoplasmic reticulum (ER) [35]. In addition, HePTP, and two other receptor-like PTPs, PTPRR and STEP, contain a kinase-interaction motif (KIM), which targets the enzymes to their substrates, the mitogen-activated protein kinases (MAPKs) [36]. Another example is the C-terminal Proline-rich region of PTP-PEST that specifically interacts with the SH3 domain of its substrate, $p130^{CAS}$. Taken together, these findings illustrate that the presence of regulatory sequences provides an additional control mechanism to ensure that the PTPs target to the appropriate substrate at the correct time and place in the cell.

The receptor-like PTPs (RPTPs) are transmembrane proteins that possess extracellular segments of varying domains and extent of glycosylation that may regulate signaling pathways in response to ligand binding. Many RPTPs

contain two PTP domains, the membrane-proximal PTP domain (D1) and the membrane-distal PTP domain (D2). It has been suggested that the majority of the phosphatase activity resides in the D1 domain, whereas the D2 domain exhibits little or no activity [28]. Similar to the RTKs, the activity of the RPTPs has also been suggested to be controlled by ligand-regulated dimerization. However, in contrast to PTKs, the formation of RPTP dimers may inhibit the activity of the enzyme. This is converse to the mechanism for RTKs, in which ligand-induced dimerization causes trans-autophosphorylation and activation. The proposed model suggests that a juxtamembrane wedge motif N-terminal to the D1 domain, which is conserved in RPTPs, plays a central role in inhibiting RPTP activity in dimers by occluding the active site of the opposing D1 domain in the dimer and preventing access of the substrates to the active site.

1.2.2 The Dual Specificity Phosphatases:

DSPs consist of five groups of enzymes (Figure 1-3). They are defined as phosphatases that can dephosphorylate phosphotyrosine as well as phosphoserine/phosphothreonine residues. The group I of the DSPs consists of 28 proteins. The VH1-like DSPs are homologous to the viral protein VH1, which is the prototypic DSP. It was discovered in 1991 as a 20kDa protein encoded as the vaccinia virus *H1* open reading frame [37]. The human homologues of VH1, VH1-Related protein tyrosine phosphatase (VHR), was shown to negatively regulate the activity of the ERK and JNK signaling pathways by directly dephosphorylating and inactivating ERK and JNK [38-40]. Like VHR, MAP kinase

phosphatases (MKPs) also specifically dephosphorylate and inactivate MAPKs. They dephosphorylate both phosphothreonine and phosphotyrosine residue in the TXY motif of the MAPKs [41]. In addition to the catalytic domain, MKPs contain a cdc25-homology domain (CH2 domain) at the N-terminus [42]. The CH2 domain of MKP3 binds specifically to its physiological substrate, ERK [43]. The interaction of MKP3 to ERK induces a conformational change in MKP3, thereby enhancing its catalytic activity [44-46]. This illustrates that CH2 domain of the MKPs not only determines their substrate binding specificity, but also modulates the activity of the MKPs. In contrast to VHR and MKPs, which inhibit MAPK signaling pathways, JNK-Stimulatory Phosphatase-1 (JSP1) specifically increases the phosphorylation and activity of JNK, but not p38 or ERK [47]. However, the exact molecular mechanism by which JSP1 regulates JNK activity is unknown. It has been shown that MKK4 is required for JSP1-mediated JNK activation, suggesting that JSP1 acts upstream of the MAPKK level [47].

The group II of the DSPs consists of 10 enzymes. PTEN antagonizes the PI3K/Akt signaling pathway and regulates cell survival [33, 48, 49]. PTEN-like DSPs, TPTE and TPIP, are tissue-specific PTEN homologues. It has been shown that TPIP also possesses phosphatase activity toward phosphoinositides *in vitro* [50]. The Phosphatases in Regenerating Liver (PRLs) display low phosphatase activity *in vitro* [51]. PRLs are farnesylated at the C-terminal tail [52, 53], which may localize the enzymes to the membranes such as in the ER [54]. Growing evidence has revealed that PRLs may have oncogenic functions in human cancer, including breast cancer [55]. The group III of the DSPs contains the most

primordial DSPs that are most closely related to the tyrosine-specific enzymes. KAP is a single domain DSP with specificity for the dephosphorylation of Thr160 on the activation loop of Cdk2 and thereby inactivates Cdk2 [56-58]. Therefore, KAP is a negative regulator of the cell cycle progression.

In addition, there are other enzymes such as the MTMs, Cdc25s, LMW-PTP and Sac-domain containing phosphatases that are operationally grouped with the DSPs (Figure 1-3). The MTMs specifically target the 3- position of the sugar ring in phosphoinositides to regulate inositol phospholipid dependent signaling. Mutations of the *MTM1* gene, which is located on chromosome Xq28, have been connected to the etiology of X-linked recessive myotubular myopathy, a severe congenital muscular disorder characterized by hypotonia and generalized muscle weakness in newborn males [59, 60]. In addition, Mutations of *MTMR2* or *MTMR13* gene have been shown to cause the type 4B1 Charcot–Marie-Tooth (CMT) disease, a demyelinating neurological disorder arising from abnormal Schwann cell development [61, 62]. These findings highlight the importance of MTMs in the control of PtdIns-based cell signaling. Cdc25s are key regulators of cell cycle check point regulation. In higher eukaryotes, Cdc25s dephosphorylate cyclin-dependent kinases (Cdks) on residues equivalent to Thr14 and Tyr15 of cdc2 and promote cell cycle progression [63]. Human LMW-PTP has been shown to interact with several receptor tyrosine kinases and cell adhesion molecules and may regulate cell proliferation, adhesion and motility [64, 65]. Like the MTMs and PTEN, Sac-domain phosphatases are lipid phosphatases that control the phosphorylation of phosphoinositides [66].

1.2.3 Pseudophosphatases:

It is important to note that some members of the PTP family are catalytically inactive because they lack residues important for activity. These PTPs are called pseudophosphatases [28]. It has been demonstrated that pseudophosphatases, such as STYX and MK-STYX, can be converted to active enzymes by mutating the residues that are missing from the signature motifs to that of the consensus of the active enzymes [67, 68]. This highlights the conservation of the PTP domain structure in these pseudophosphatases. Many of the pseudophosphatases belong to the MTM family [69]. It has been shown that the catalytically inactive MTMR5 or MMTR13 heterodimerize with the active MTMR2 to regulate MTMR2 phosphatase activity and subcellular localization [70]. In 4B CMT patients, the phenotype caused by loss of MTMR13 is similar to that caused by loss of MTMR2, illustrating the functional importance of both the phosphatase and the pseudophosphatase MTMs.

In *C. elegans*, MBK2, a dual-specificity tyrosine-regulated kinase (DYRK) in the oocyte, is activated during oocyte-to-zygote transition. Two pseudophosphatases, egg-4 and egg-5, can function as substrate traps and inactivate MBKs by binding to the YTY motif in the activation loop of the kinase [71-73]. This finding reveals that pseudophosphatases can function as endogenous substrate traps, however, the mechanism by which they control the function of their “substrates” is unknown. One possibility is that binding of a

pseudophosphatase to its “substrates” either alters substrate activity or blocks the interaction of the substrate with downstream signaling proteins.

The D2 domain of many of the RPTPs is catalytically inactive and appears to be a pseudophosphatase domain (Figure 1-2). For example, in D2 of LAR only two point mutations are required to convert this catalytically inactive domain to a PTP with robust activity [74], suggesting a conservation of the PTP domain structure in D2 domain. In PTPRA (PTPα), it has been shown that the catalytic Cys residue in the D2 domain is more sensitive to reactive oxygen species than the D1 domain, suggesting that the D2 domain may function as a redox sensor. Oxidation of the catalytic Cys in the D2 domains also induced the formation of dimers through the generation of an S-S bond between the catalytic Cys of two D2 domains, thereby stabilizing PTPα in an inactive state [28].

1.3 PTPs and cancer

Due to the identification of the oncogenic capacity of PTKs, there has long been an interest in the potential role of PTPs as tumor suppressors. More recently, a role for specific PTPs to promote cell transformation has been illustrated. However, many of these studies have been focused on the identification of genomic or epigenomic abnormalities on chromosome loci containing PTPs. Only for a few PTPs, the underlying molecular mechanisms that connect their activity to the development of cancer have been characterized.

I will introduce some of these PTPs as examples to highlight the importance of PTPs in cancer.

1.3.1 PTEN, a well known tumor suppressor:

PTEN (phosphatase and tensin homologue deleted on chromosome 10) was first identified in 1997. It localizes on human chromosome 10q23.3, which is a region known to be frequently deleted in high-grade prostate and brain tumors [75, 76]. PTEN is a VH1-like DSP that possesses catalytic activity toward protein substrates as well as lipid substrates, in particular phosphoinositides (Figure 1-3) [77]. By dephosphorylating phosphatidyl inositol- (3, 4, 5) - trisphosphate, PTEN antagonizes the PI3K/AKT signaling pathway [33, 48, 49]. The *Pten* gene is frequently mutated or decreased in expression in human sporadic cancers such as lung, prostate, endometrium, breast and glioblastoma (reviewed in [78]). Germline mutation of PTEN leads to hereditary disorders such as Cowden disease, Bannayan-Zonana syndrome, Lhermitte-Ducros disease and Proteus syndrome, all of which are cancer predisposition syndromes [79-81]. PTEN contains a C2 domain C-terminal to the catalytic domain, which has been shown to interact with and stabilize the tumor suppressor protein p53, suggesting a tumor-suppressing function independent of its phosphatase activity [82].

1.3.2 SHP2, an oncogenic PTP:

SHP2 is a non-transmembrane PTP that contains two SH2 domains N-terminal to the catalytic domain (Figure 1-3) (reviewed in [34]). There are two tyrosine

residues in the C-terminal tail (C-tail) of SHP2, which can be phosphorylated by PTKs, and are critical for the regulation of SHP2 activity. It has been proposed that the N-terminal SH2 domain (N-SH2) associates with the catalytic cleft of the PTP domain and thereby blocks the entry of the substrate to the catalytic cleft. This maintains SHP2 in an inactive state. SHP2 is activated by two proposed mechanisms. In the first mechanism, the SH2 domains of SHP2 interact with the phosphotyrosine in particular sequence motifs in another protein, which releases and exposes the catalytic cleft of SHP2 to its substrate. In the second mechanism, the SH2 domains can bind to the phosphorylated tyrosine residues on the C-tail and release the catalytic cleft of SHP2. Germline mutations of *SHP2* have been identified in ~50% of Noonan Syndrome (NS) patients, who have a high incidence of leukemia [83, 84]. In addition, point mutations on *SHP2* gene were detected in 20 to 25% of sporadic juvenile myelomonocytic leukemia (JMML). Mutations identified in JMML and NS cluster at the interface between the N-SH2 and PTP domain in the structure of SHP2. These mutations abolish the association of N-SH2 with the PTP domain, thus generating a constitutively active SHP2 that promotes oncogenic signaling through activating the Ras/MAPK and Src signaling pathways. SHP2 can activate Ras either by blocking the binding of the negative regulator RasGAP to the RTKs or by inactivating Sprouty, which inhibits Ras activity. In addition, SHP2 could activate Src family of kinases (SFKs) to enhance oncogenic signal. It has been shown that SHP2 could dephosphorylate the transmembrane protein PAG/CBP and blocks the

recruitment and activation of Csk, which phosphorylates the inhibitory tyrosines (Tyr527 on mouse Src) and inactivate SFKs [34].

1.3.3 CDC25s and cancer:

Cdc25 is an important regulator of cell cycle progression. It is conserved in eukaryotes from yeast to human. In budding yeast, Cdc25 promotes mitosis by removing the inhibitory phosphate on Thr14 and Tyr15 of the cyclin-dependent kinase (Cdc2) [85, 86]. There are three *cdc25* genes, *cdc25A, B and C,* in the human genome. The encoded proteins of these three genes, Cdc25A, B and C, are DSPs. They have been shown to target the inhibitory phosphorylation sites of cyclin-dependent kinases (Cdks) that are equivalent to Thr14 and Tyr15 of Cdc2, and control cell cycle progression. CDC25s are overexpressed in a wide variety of human cancers, including breast, liver, esophageal, endometrial and colorectal cancers and their overexpression is frequently correlated with more aggressive disease and poor prognosis [87]. It has been shown that in collaboration with oncogenic Ras, ectopic expression of CDC25A and CDC25B can transform normal fibroblast [88], highlighting the oncogenic potential of these enzymes. As a consequence, Cdc25-specific inhibitors are being explored as potential therapeutic reagents for the treatment of cancer [89, 90].

1.3.4 PTPs and breast cancer:

PTKs have been shown to play an important role in the etiology of breast cancer, suggesting that PTPs may play an equally important role in the

development of breast cancer. However, to date, the role of most PTPs in the development of breast cancer is still unknown. Analysis in cellular or animal model has shown that some PTPs, such as PTPRE, DEP1, and PTP-BAS might play a role in the development of breast cancer [91]. Abnormal expression of several PTPs has been observed in breast tumors e.g. PTPRA is overexpressed in ~30% of breast tumors [92]. Expression of PRL3 [93] and LAR [94] was found to be up-regulated in metastatic breast cancer. In contrast, the expression of PTPRG is lower in breast tumors [95]. These observations raise the possibility that expression of certain PTPs could be used as prognostic markers. Genomic deletions or mutations in PTPs, such as *PTEN* [96], *PTPN12* (PTP-PEST) [97], and *PTPRF* (LAR) [98] have also been identified in breast tumors. Breast tumors with *PTEN* loss display decreased sensitivity to Herceptin, suggesting that PTEN status may serve as indicator for the susceptibility of breast tumor to the drug [99, 100]. Moreover, expression of some PTPs may also be regulated in response to stimuli or oncogene activation such as estrogen [101] or ErbB2 [102, 103], respectively. Therefore, the change in certain PTPs may be useful as a prognostic/diagnostic marker in breast cancer.

1.4 PTPs characterized in this dissertation

1.4.1 PTPRO:

PTPRO is a transmembrane receptor like PTP (Figure 1-2) that was first discovered in rabbit renal glomerular epithelial cells (podocytes) [104]. Because

of the usage of different promoters and alternative splicing, there are six mRNA variants that have been identified. The expression of PTPRO variants is highly tissue specific, suggesting that the expression of this enzyme is tightly regulated *in vivo*. PTPRO knockout mice are normal, except for a reduced glomerular filtration rate [105]. No chromosomal abnormalities at the PTPRO locus have been found in human tumors. In hepatoma and lung cancer cell lines, the expression of PTPRO is found to be suppressed by CpG island methylation on the PTPRO locus. Reintroduction of PTPRO to these cell lines rescues these cells from transformation [106, 107]. In the A549 cell line and carcinomic human alveolar basal epithelial cells, expression of PTPRO inhibits its anchorage-independent growth, delays its cell cycle progression, and increases its susceptibility to apoptosis [107]. Overexpression of PTPROt, an alternatively spliced form of PTPRO that is expressed in hematopoietic cells, induces G0/G1 arrest in B cells and suppresses the transformation of myelogenous leukemia cell line K562 [108]. Taken together, these findings suggest a role of PTPRO as a tumor suppressor. However, the role of PTPRO in the development of breast cancer has not been explored.

1.4.2 DEP1:

DEP1 is a receptor-like transmembrane PTP that is structurally similar to PTPRO (Figure 1-2). It was first cloned in the Tonks lab as a PTP that is up-regulated in cells grown in confluent culture [109], suggesting that DEP1 regulates density dependent cell growth arrest. Genomic studies have revealed

that DEP1 is the only gene that presents in the mouse colon cancer susceptibility locus *Scc1* (Susceptibility to colon cancer 1) and is frequently deleted in human cancers, such as colon, lung and breast cancer [110]. The receptor PTK, Met, which is aberrantly up regulated in several human tumors, is a substrate of DEP1 [111]. In addition, genomic mutations in DEP1 have been detected in tumor types associated with aberrant Met signaling. This implies a functional connection between DEP1 and Met in the progression of cancers. Moreover, re-expression of DEP1 in DEP defective cancer cell lines, including breast cancer cell lines rescues cell transformation phenotypes [112-114]. Taken together, these findings suggest that DEP1 plays a role as a tumor suppressor in the development of cancers, including breast cancer. However, the underlying molecular mechanism is still unknown.

1.4.3 PTPN23:

PTPN23 (HD-PTP) is a non-transmembrane classical PTP (Figure 1-2) [28, 115, 116]. The *Ptpn23* gene localizes to human chromosome region 3p21.3 [115], a hot spot of loss-of-heterozygosity (LOH) in many types of solid tumors [117]. This implies that PTPN23 is a potential tumor suppressor. PTPN23 is a multi-domain protein that consists of a BRO1 domain, a proline rich region, a His-domain (HD domain), a catalytic domain and a PEST motif. The BRO1 domain exhibits ~45% identity to yeast BRO1 [115, 116], which is required for the internalization of cell surface proteins [118, 119]. In mammalian cells there are two BRO1 domain-containing proteins, PTPN23 and its paralog Alix [120]. Alix

interacts with proteins in the endosomal sorting complex required for transport (ESCRT) machinery and is involved in the sorting of cell membrane receptors to multivesicular bodies (MVBs) [121-123]. Interestingly, PTPN23 also interacts with ESCRT proteins, such as CHMP4 and TSG101 [120, 124]. Suppression of PTPN23 blocks the biogenesis of MVBs [124], suggesting that PTPN23 plays a role in the regulation of endosomal sorting machinery through the interaction of its BRO1 domain with proteins of the ESCRT complex.

The His-domain is localized to amino acid residues 770-1130, which contains a total of 14 His and 2 Cys residues that are linked by proline-rich segments [115]. This special arrangement of amino acids indicates the potential of this domain to form a zinc-finger like structure [115]. However, the function of the His-domain is still unknown. The PEST motif is known as a destabilizing sequence. The presence of the PEST motif implies that PTPN23 may be a short-lived protein *in vivo* [115].

It is a subject of debate in the field as to whether PTPN23 possesses catalytic activity because the active site signature sequences of the PTP domain in PTPN23 deviate from the consensus of other active members of the PTP family (Figure1-4). These deviations include the Ala to Ser conversion (the second amino acid after the catalytic Cys) and the Asp to Glu conversion (the conserved Asp of PTPs that equivalent to Asp181in PTP1B). Biochemical studies that examined the catalytic activity of the recombinant PTPN23 PTP domain towards artificial substrates suggested that PTPN23 is inert [125]. A point mutation that converts the Ser back to Ala can enhance the catalytic activity of

PTPN23 [125], suggesting that this residue play a role in determining PTPN23 activity [125, 126]. However, the effect of the Asp to Glu conversion on PPN23 activity has not been characterized. It has been shown that the phosphatase activity of the Rat ortholog of PTPN23 is important to suppress RAS-mediated transformation of fibroblasts [116]. In an endothelial cell line, PTPN23 has been shown to interact with FAK and suppression of PTPN23 induces tyrosine phosphorylation of FAK and endothelial cell motility [127]. Taken together, these findings suggest that it is unclear whether PTPN23 is an active enzyme and whether catalytic activity is required for its biological function. This study I addressed in the experiments reported in this thesis.

1.4.4 PTPRG:

Like PTPN23, the *PTPRG* gene is also mapped at human chromosome 3p21, a hot spot for deletion in breast cancer [128]. Therefore, PTPRG was proposed to have tumor suppressor functions. The protein architecture of PTPRG is characterized by an extracellular segment that contains an N-terminal carbonic anhydrase-like domain and an FNIII domain(Figure 1-2) [128, 129]. No ligand has been identified for PTPRG. However, pleiotrophin was identified as the ligand of PTPRZ1, which is structurally similar to PTPRG [130, 131]. It has been shown that PTPRZ1 is activated upon the binding of its ligand, pleiotrophin. Since PTPRG shares a similar extracellular domain with PTPRZ1, the activity of PTPRG may also be regulated by ligand binding. PTPRG knockout animals display no obvious phenotype [132], suggesting functional redundancy of this

enzyme. In human cancers, the expression of PTPRG is reduced in lung, ovarian and breast tumors, consistent with the potential of PTPRG to function as a tumor suppressor [128, 133-135]. Epigenomic studies have identified changes in methylation of the *PTPRG* gene in cutaneous T cell lymphoma [136] and gastric cancer [137]. In the context of breast cancer, the *PTPRG* gene has been shown to be an estrogen-regulated tumor suppressor [95, 101, 138]. Overexpression of PTPRG inhibits cell proliferation and anchorage-independent growth and impairs the mitogenic effects of estrogen stimulation in MCF7 cells [139]. Recently, it was shown that ectopically expresses PTPRG in MCF7 cells suppresses tumor formation by the MCF7 cells in nude mice [140]. These findings suggest that PTPRG plays a role in the development of breast cancer. However, the precise mechanism to explain the functions of PTPRG in breast cancer is unexplored.

1.4.5 PTPRR:

The expression of PTPRR was first detected in mouse brain [141] and a neuronal cell line [142, 143]. Due to the use of distinct promoters, alternative sites of translation initiation and alternative splicing, there are four isoforms of PTPRR [144]. The largest isoform, PTP-BR7, is a transmembrane RPTP, whose activity is suppressed upon oligomerization in the membrane [145]. This is consistent with the proposed model for RPTPs to form oligomers in which activity is suppressed [28]. All isoforms of PTPRR contain a KIM (Kinase Interaction Motif) sequence (Figure 1-2), which is important for its interaction with MAPKs [146]. In *PTPRR*-deficient mice, phosphorylation of p42/p44 MAPKs is enhanced

in the brain [147]. Moreover, a spliced isoform of PTPRR, PTP-SL, has been shown to regulate the phosphorylation and nuclear translocation of ERK5 [148]. These results suggest that PTPRR is a negative regulator of the MAPK signaling pathways. In the context of cancer, there is a chimeric *TEL/PTPRR* gene was identified in Acute Myelogenous Leukemia. However, PTPRR activity is not associated with the protein product of this chimeric gene [149]. To date, there are no indications of the involvement of PTPRR in breast cancer.

1.4.6 PTP1B:

PTP1B, the prototypic PTP. It is a non-transmembrane PTP (Figure 1-2). The C-terminal 35 residues are predominantly hydrophobic and function to target the enzyme to the cytoplasmic face of the ER membrane [35]. Many proteins have been reported as direct substrates for PTP1B, e.g. insulin receptor [150], EGFR [151, 152], PDGFR [151, 153], Janus kinase family of kinase JAK2 and TYK2 [154] and Src [155], suggesting that PTP1B is involved in controlling a wide variety of biological functions in the cell. Interestingly, the PTP1B knockout mice display a restricted phenotype, in which there is enhanced sensitivity to insulin and resistance to high fat diet-induced obesity and diabetes [156, 157], suggesting that PTP1B is a potential target for the treatment of these diseases [26, 158]. Recently, studies from the groups of Tremblay and Neel pointed out the important role of PTP1B in ErbB2 signaling [159, 160]. In their studies, they crossed the transgenic mice that expressed an activated form of ErbB2 with the PTP1B null mice. Interestingly, breast tumor development was delayed in the

absence of PTP1B. Moreover, development of lung metastasis was also attenuated, indicating that PTP1B plays a positive role in promoting ErbB2-induced tumor growth and metastasis. However, the mechanism by which loss of PTP1B attenuates breast tumor formation and metastasis is unclear, although it has been suggested that PTP1B normally promotes Ras/MAPK signaling by dephosphorylating p62Dok, thereby destabilized RasGAP.

1.5 Study the function of PTP in an *in vitro* model of breast cancer

Epithelium is a sheet of well-arranged cells that form the surface of the body and provides a physical barrier that separates the outside environment and the interior cavities. In addition, it constitutes the glandular tissue of organs, such as breast, lung and prostate gland. Among all cell types, epithelial cell is the type that is most susceptible to tumorigenesis. Under a microscope, epithelial cells feature the presence of polarized morphology, specialized cell-cell contact and the attachment to an underlying basement membrane in a 3 dimensional (3D) environment. This 3D architecture tightly controls the physiology of the epithelial cells, such as proliferation, differentiation, survival, migration and secretion. The disruption of this well-organized architecture is considered as the first step of the pathogenesis of epithelial tumors. In order to study the biology of epithelial cell under its *in vivo* architecture, methods that involve growing epithelial cell in reconstituted extracellular matrix have been developed. In particular, the MCF10A 3D culture system has been widely used to study the morphogenesis

and tumorigenesis of mammary epithelial cell. Thus, I used MCF10A cells and its application in the 3D culture system as biological systems to analyze the function of the PTP family.

1.5.1 The MCF10A 3D culture system:

MCF10A is an immortal mammary epithelial cell line derived from MCF10 cell line, which was isolated from human breast fibrocystic mammary tissue [161]. It inherits the normal breast epithelium characteristics of the MCF10 cells, which include: (i) lack of tumorigenicity in nude mice (ii) hormone- and growth factor-dependent growth (iii) lack of anchorage-independent growth and (iv) form the domes when confluent [161]. Most importantly, MCF10A cells forms spheroid structures, called acini, when grown on extracellular matrix (ECM). These acini are characterized by the presence of a single layer of polarized, growth-arrested outer cells and a hollow lumen inside, which recapitulates the architecture of the mammary epithelial cells *in vivo* [161, 162]. The morphogenetic process of MCF10A acini usually takes about 14 days (Figure 1-5). At the early stage, the outer layer of cells, which have direct contact with the ECM, undergo apical-basal polarization, which is maintained during the entire morphogenesis process. In addition, the proliferation signal in these cells, which is responsible for the growth of the acini, is down-regulated when the acini become mature. The inner cells, which have no contact with the ECM, undergo apoptotic cell death. This creates and maintains the hollow lumen inside the acini. The coordination of the

proliferation signal in the outer cells and the apoptosis signal in the inner cells maintains the morphology of the acini [163, 164].

1.5.2 MCF10A 3D culture system for the study of cell signaling and breast tumorigenesis:

It has been shown that abnormal expression or activation of oncogenic proteins can disrupt the normal development of MCF10A 3D acini. Ectopic expression of cyclin D1 or HPV16 E7 (E7 oncoprotein from human papillomavirus 16) promotes MCF10A cell proliferation and leads to the formation of bigger acini. In addition, the apoptosis signals in these acini are elevated. Therefore, the acini still maintain the hollow lumen inside [164]. Suppression of the anti-apoptotic Bcl protein, BCL2, in these acini leads to the repopulation of the luminal cells [164]. In contrast, activation of AKT, a serine/threonine kinase that is expressed exclusively in the outer cells of the mature acini, not only enhances the proliferation signal, but also suppresses apoptosis of the inner cells. Therefore, it leads to the formation of bigger acini without the hollow lumen inside [165].

The function of oncogenes, such as ErbB1, ErbB2, CSF-1R and Met, to induce mammary epithelial cell tumorigenesis has also been studied in the MCF10A 3D culture system. As described, activation of a chimeric ErbB2 through homodimerization in MCF10A 3D acini results in the formation of multi-acinar structures that resemble the phenotype of ductal carcinoma in situ (DCIC) lesions [166-168]. In contrast, activation of ErbB1 through homodimerization

does not induce the multi-acinar structures [166]. However, upon heterodimerization of ErbB1 and ErbB2, promotes the formation of multi-acinar structures and induces cell invasion [169]. Importantly, this phenomenon is only observed in the MCF10A cells grown in the 3D culture system but not in the 2D culture system, illustrating the advantage of using the MCF10A 3D culture system to study cell signaling in the early development of breast cancer. In contrast, overexpression of Met or CSF-1R in MCF10A acini induces a more robust phenotype including the disruption of the localization of E-cadherin to the cell-cell contact region and the enhancement of cell invasion [170].

1.6 ErbB2 signaling and breast cancer

1.6.1 ErbB2 signaling:

ErbB2 (HER2/neu) belongs to the human epidermal growth factor receptor family of receptor tyrosine kinases, which comprises ErbB1 (EGFR/HER1), ErbB2, ErbB3 (HER3) and ErbB4 (HER4). The ErbB receptor family is activated by the binding of the EGF family of ligands such as EGF, neuregulins (NRGs), epiregulin, and betacellulin. Each ErbB receptor has its ligand preference. Upon ligand binding to their extracellular domains, ErbB receptors undergo homo- or heterodimerization with members of the ErbB receptor family and transphosphorylation of their intracellular domains. These phosphotyrosine residues provide docking sites for signaling molecules, such as SH2 domain containing proteins, to activate downstream signaling pathways, such as the

MAPK, PI3K/AKT and STAT signaling pathways (reviewed in [171, 172]). To date, no ligand has been found to bind ErbB2. Moreover, unlike other ErbB receptors, ErbB2 does not undergo transition between active and inactive conformations. It exists constitutively in an active conformation [173, 174]. Studies have shown that ErbB2 signaling might be controlled through its formation of heterodimers with other ligand-bound ErbB receptor members [175]. In addition, under conditions in which ErbB2 is overexpressed, ErbB2 activation has been reported to involve ligand-independent homodimerization [176-179]. However, the differential regulation of the downstream signaling controlled by ErbB2 homo- and heterodimers has not been clearly delineated.

1.6.2 ErbB2 and breast cancer:

The transforming activity of ErbB2 was first observed in experiments with its rat homologue, neu, which induces transformation of the mouse embryonic fibroblast cell line, NIH3T3 [180, 181]. Overexpression or amplification of ErbB2 is observed in ~25% of breast cancers, which correlates with more aggressive tumor development and poor prognosis [182, 183]. Moreover, ErbB2 amplification is observed in ~50% of ductal carcinoma *in situ* (DCIS), an early stage benign tumor [184, 185]. This suggests that ErbB2 is an oncoprotein kinase with an important role in the development of breast cancer. The exact molecular mechanisms by which ErbB2 overexpression promotes breast tumor formation are not clear. As I have mentioned, activation of ErbB2 is achieved through the formation of homo- or heterodimers. One hypothesis suggests that

ErbB2 is the preferred heterodimerization partner for all four ErbB receptors [186]. Overexpression of ErbB2 increases the formation of the ErbB2 heterodimers and enhances and prolongs downstream signaling [187-189]. In addition, overexpression of ErbB2 also results in the formation of homodimers or high-order homopolymers [190, 191]. Muthuswamy et al. have generated a cell line, MCF10A/ErbB2, that expresses a chimeric form of ErbB2, which can be activated trough homodimerization by a small molecule dimerizer, AP1510 (Figure 1-6 and [166]). Activation of ErbB2 homodimers in MCF10A/ErbB2 cells grown in a 3-dimensional culture system causes the formation of atypia-like multiple-acini structures, which resemble the morphology of DCIS [166]. This underlines the importance of ErbB2 homodimer in the development of early stage breast tumors. Interestingly, activation of ErbB2 homodimers does not induce cell invasion. In contrast, activation of ErbB2/ErbB1 heterodimers in MCF10A acini not only causes the formation of atypia-like multiple-acini structures, but also promotes an invasive phenotype [169]. Moreover, it has been shown that inhibition of ErbB2 with TKIs induces the expression of ErbB3, which activates PI3K/AKT signaling pathway and contributes to the development of tumors that are resistant to anti-ErbB2 therapy [192, 193]. Taken together, these findings suggest that there is functional diversity between the ErbB2 homo- and heterodimers on the development of breast cancer. In addition, it also indicates that upon dimerization with different partners, ErbB2 confers distinct functions, presumably by exerting distinct effects on the activation of signaling pathways. In

this dissertation, I aim to focus my study on the function PTPs on ErbB2 homodimers in the development of the early stage breast cancer.

1.6.3 ErbB2 as therapeutic target:

Cancer cell lines that overexpress ErbB2 become growth-arrested and die if the expression of ErbB2 is suppressed [194-198]. Transgenic mice that overexpress an oncogenic mutant of ErbB2, *neuT*, under the control of the MMTV promoter, develop breast tumors and their metastatic tumor. Withdrawal of *neuT* expression in these mice causes regression of the *neuT*-induced primary tumors and their metastatic tumors [199]. Thus, ErbB2 is a potential therapeutic target. To date, more than 100 monoclonal antibodies against ErbB2 have been generated. Among them, trastuzumab (Herceptin) is the most successful drug [200]. Clinically, its best effect is in the treatment of early stage *ErbB2*-amplified breast cancer patients. Addition of trastuzumab to the chemotherapy regimens of these patients significantly prolongs disease-free survival and reduces the chances of disease recurrence [201, 202]. For patients with metastasis, trastuzumab also shows therapeutic potential. However, it is not curative as the tumor relapses after a median duration of ~5 months despite continuous trastuzumab treatment [203]. Another anti-ErbB2 mAb, pertuzumab, is currently undergoing clinical testing. Pertuzumab binds to the extracellular domain of ErbB2 and prevents the dimerization of ErbB2 with other ErbB receptor members [204]. Preliminary evidence suggests that it can enhance the therapeutic effect of trastuzumab, but its effect as a single agent on *ErbB2*-amplified breast tumor is

still being tested (reviewed in [205]). In addition to the anti-ErbB2 mAbs, ErbB receptor tyrosine kinase inhibitors, such as Gefitinib, Erlotinib (Tarceva), Lapatinib (Tykerb) and Canertinib may also have anti-cancer activity in ErbB2 positive cancer (reviewed in [205, 206]). The most clinically advanced anti-ErbB2 small inhibitor is lapatinib. Lapatinib is a dual ErbB1 and ErbB2 tyrosine kinase inhibitor [207]. Preliminary clinical studies suggest that lapatinib increases progression-free survival in patients with advanced ErbB2-amplified breast cancer [205].

1.6.4 Regulation of ErbB2 signaling by PTPs:

To date, there is very limited understanding of the roles of PTPs in the regulation of ErbB2 signaling. It has been shown that certain PTPs, such as PTP-BAS and PTPN9 directly dephosphorylate the C-terminal phosphotyrosine of ErbB2, thus negatively modulating ErbB2 signaling in breast cancer cell lines [208, 209]. It has been shown in transgenic mouse models that knockout of PTP1B in mice that expressed activated ErbB2 delays and attenuates ErbB2 tumor development and metastasis [160, 210]. This implies that PTP1B plays a positive role in the development of ErbB2-induced breast tumor [211, 212]. In an *in vitro* model of breast cancer, activation of ErbB2 leads to the formation of atypia-like structures [166]. Interestingly, the expression of PTP1B is required for the development of these atypia-like structures [159]. This confirms that PTP1B is a positive regulator in ErbB2-induced breast cancer. In summary, these results suggest that PTPs are important regulators of ErbB2 signaling. Nevertheless, the

function of most PTPs in the regulation of the ErbB2 signaling and their role in the development of breast cancer is still unknown. Therefore, the ultimate goal of this dissertation is to study systematically the function of the PTP family of enzymes in mammary epithelial cell models of breast cancer in the absence or presence of ErbB2 signaling. Hopefully, this study will reveal novel therapeutic targets or strategies which can complement current anti-ErbB2 therapy for the treatment of breast cancer.

1.7 Loss of E-cadherin and cancers

1.7.1 Functions of E-cadherin:

E-cadherin, the major type of cadherin, is a Ca^{2+}-dependent transmembrane protein that constitutes the adherens junctions of epithelial cells [213]. In the presence of Ca^{2+}, the extracellular domain of the E-cadherins trans-dimerizes with the extracellular domain of other E-cadherins on the membrane of the adjacent cells to provide the mechanical force that tethers the cells together. The cytosolic end of E-cadherin interacts with the actin network through adhesion molecules, including α-, β-, γ- and δ-catenin, vinculin and α-actinin, to stabilize cell-cell adhesion [214]. The association of E-cadherin with β-catenin is also involved in the regulation of the canonical Wnt signaling pathway [215, 216]. In Wnt signaling, the cytosolic β-catenin is functioning as a co-regulator of the TCF (T-cell factor) family of transcription factors (reviewed in [217]). In the absence of Wnt, the cytosolic β-catenin forms a complex with axin and adenomatosis

polyposis coli (APC) protein (Figure 1-7A). At the same time, CK1 and GSK3β are also recruited to the complex where they phosphorylate β-catenin on Ser37/Thr41in the N-terminal of the protein. N-terminal phosphorylation of β-catenin promotes its polyubiquitination and degradation and thus silences the Wnt signaling. In the presence of Wnt, Wnt brings Fz and LRP6 together to form the Fz/Wnt/LRP6 complex, which recruits Dishevelled (Dvl), and resulting in LRP6 phosphorylation and activation and the recruitment of the Axin complex to the receptors. These events inhibit the Axin-mediated β-catenin phosphorylation, thereby increasing β-catenin stability and nuclear localization (Figure 1-7B). Down-regulation of E-cadherin releases β-catenin from the cell adhesion to the cytosoplasm and thus activates the canonical Wnt signaling pathway.

1.7.2 Loss of E-cadherin and cancers:

The E-cadherin gene, *CDH1*, is localized to human chromosome 16q22.1, which is a loss-of-heterozygosity (LOH) site in sporadic breast cancer [218]. Epigenetic studies indicate that methylation of the E-cadherin promoter region silences the expression of the E-cadherin [219]. Ectopic expression of E-cadherin in metastatic breast and prostate cancer cells inhibits cell invasion and metastasis [220]. These findings suggest that E-cadherin can function as a tumor suppressor. In addition to the genomic or epigenomic abnormalities at the E-cadherin locus, loss of E-cadherin is observed in cells undergoing epithelial-mesenchymal transition (EMT). Loss of E-cadherin expression is the hallmark of EMT, which plays an important role in embryonic development as well as

tumorigenesis. EMT proteins such as Snail, Slug and the ZEB family of proteins (ZEB1 and ZEB2) are transcription factors that bind to the E-boxes of the E-cadherin promoter and suppress the expression of E-cadherin [221-224]. During EMT, the expression of these transcription factors is up-regulated, which suppresses the expression of E-cadherin. Loss of E-cadherin function also promotes the EMT phenotypes, including cell scattering, increase in mesenchymal protein expression, and increase in β-catenin activity in mammary epithelial cell lines [225].

1.7.3 Internalization of E-cadherin disrupts adherens junctions:

Although E-cadherin is an adhesion molecule located on the cell surface, it is not constitutively anchored to the plasma membrane. It has been shown that fluorescence-labeled cell surface E-cadherins are internalized after a short period of time. Later on, the internalized labeled E-cadherins were recycled back to the cell surface [226], suggesting that E-cadherin is dynamically trafficking back and forth from the plasma membrane. There are several steps involved in the intracellular trafficking of E-cadherin: (1) Internalization of E-cadherin to the early endosome through the endocytosis machinery [227-230]. (2) Sorting of internalized E-cadherin to the late endosome and lysosomes or recycling endosomes. (3) Degradation of late endosomal and lysosomal E-cadherin. (4) Recycling of the internalized E-cadherin back to the cell surface by the Rab11-containing recycling vesicles [230]. It has been shown that internalization of E-cadherin can cause the depletion of cell surface E-cadherin and lead to the

disruption of cell adherens junctions as well as cell motility and invasion. However, the exact role of E-cadherin internalization in cancer development and metastasis is still unknown.

1.7.4 Regulation of E-cadherin internalization:

In mammalian cells, E-cadherin is internalized via either clathrin-dependent endocytosis or the caveolin-1 mediated endocytosis, depending on cell type or the property of the stimuli [227, 231-234]. For example, activation of TGF-β and MAPK signaling triggers the internalization of E-cadherin through clathrin-dependent endocytosis [235, 236]. In contrast, activation of EGF signaling induces E-cadherin internalization through caveolae-1-mediated endocytosis [237]. Growing evidence suggests that internalization of E-cadherin is specifically regulated. In endothelial cells, internalization of VE-cadherin is inhibited upon ectopic expression of p120 catenin [234], suggesting that p120 catenin suppresses E-cadherin endocytosis. In keratinocytes, activation of Rac1 promotes the endocytosis of E-cadherin [231]. In *Drosophila* models, it has been shown that Cdc42 can regulate E-cadherin endocytosis through the recruitment of dynamin, WASP and Arp2/3 complex to adherens junctions [238, 239]. Taken together, these results suggest the involvement of the small GTPases in the regulation of E-cadherin endocytosis. Moreover, activation of ARF6 promotes clathrin-dependent internalization of E-cadherin at the basolateral cell surface of MDCK cells [232]. Tyrosine phosphorylation and ubiquitination of E-cadherin also induces its endocytosis [240]. E-cadherin is phosphorylated by Src at

Tyr755/Tyr756. Phosphorylation on these two sites recruits the binding of an E3 ubiquitin-ligase, Hakai, which ubiquitinates E-cadherin and eventually leads to the internalization and lysosomal degradation of E-cadherin [240].

1.7.5 Regulation of E-cadherin recycling:

The recycling of the internalized E-cadherin to the cell surface is shown to be highly regulated. It has been shown that E-cadherin colocalizes with Rab11 positive recycling vesicles in live HeLa and MDCK cells [241],suggesting that the internalized E-cadherin is recycled by the Rab11-positive vesicles. Expression of a constitutively active form of Rab11 causes the accumulation of E-cadherin in the cytoplasm. In contrast, expression of dominant-negative Rab11, leads to mislocalization of the recycling E-cadherin to the apical region [241]. These data suggest that Rab 11 activity could control the spatial recycling of the internalized E-cadherin. Internalization of E-cadherin also activates a small GTPase protein, Rap1, through the activation of Src. Activation of Rap1 suppresses recycling of the Rab11 positive vesicles [242], suggesting a negative feedback mechanism to control the recycling of E-cadherin through Src activation. In *Drosophila*, membrane-localized Armadillo, the *Drosophila* homologue of β-catenin, is required for recycling of the E-cadherin-containing exocyst complex to the cell surface [243, 244]. This indicates that the localization of β-catenin can direct spatial recycling of E-cadherin. In *Drosophila*, mutations of *par6*, *aPKC* or *cdc42* cause defects in E-cadherin endocytosis [238, 245, 246], suggesting that the

Par6-aPKC cell polarity protein complex, is involved in the regulation of the spatial recycling of E-cadherin as well.

1.8 Implications of this dissertation to understanding the function of the PTP family

In this dissertation, I report the construction of a PTP short hairpin RNA (shRNA) library, which contains shRNAs that target each member of the PTP family. By using the shRNA, I revealed a novel function of PTPRO in mammary epithelial cell morphogenesis and the regulation of ErbB2 signaling. In addition, I conducted an RNAi-mediated screen to study systematically the functions of the PTP family in mammary epithelial cell motility and invasion. My study revealed functional insights into the members of the PTP family, including PTPN23, PTPRG, and PTPRR as well as some members of the DSPs in mammary epithelial cell motility. I characterized further three PTPs, PRPN23, PTPRG and PTPRR, which inhibited cell motility. I found that suppression of PTPN23, but not PTPRG or PTPRR, induced cell invasion. Suppression of PTPN23 increased E-cadherin internalization, impaired early endosome forward trafficking of E-cadherin, induced the expression of mesenchymal proteins and caused cell scattering. Moreover, I identified E-cadherin, β-catenin and Src as direct substrates of PTPN23. The activity of β-catenin and Src was elevated when PTPN23 was suppressed. Inhibition of Src with the small molecule inhibitor, SU6656, blocked the effects of PTPN23 depletion, suggesting that loss of

PTPN23 may modulate the activity of the Src and E-cadherin/ β-catenin interaction to promote tumor growth and metastasis in breast cancer.

All in all, this study illustrates a loss-of-function screen to reveal new roles for PTPs in mammary epithelial cell motility and invasion. I identified PTPRO as an important regulator in mammary epithelial cell morphogenesis and the regulation of ErbB2 signaling. In addition, I identified PTPN23 as a novel regulator of mammary epithelial cell motility, invasion and scattering. In the future, further application of this approach will extend our understanding of this family of enzymes and benefit the development of therapeutic strategies that target PTPs for the treatment of breast cancer.

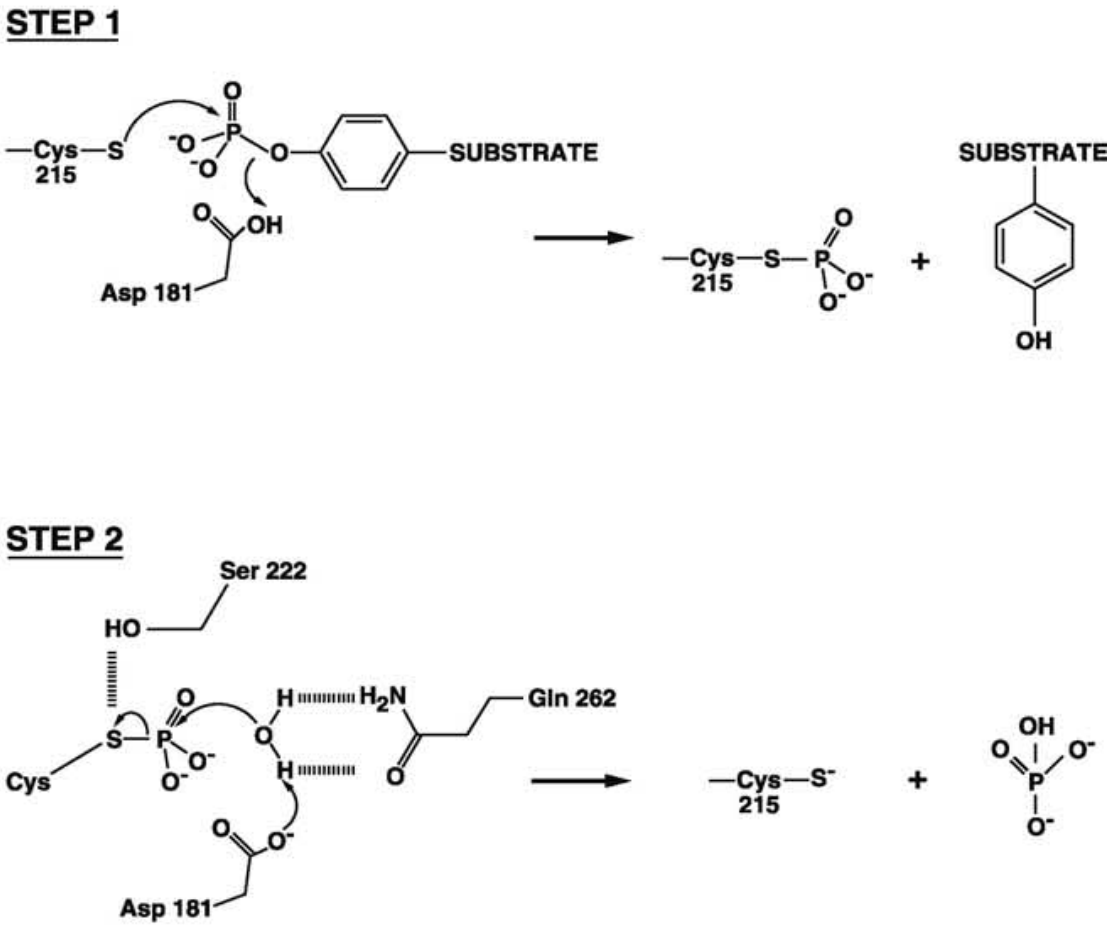

Figure 1-1: General catalytic mechanism of PTPs. (Tonks NK, FEBS Lett. 2003. 546(1):140-8)

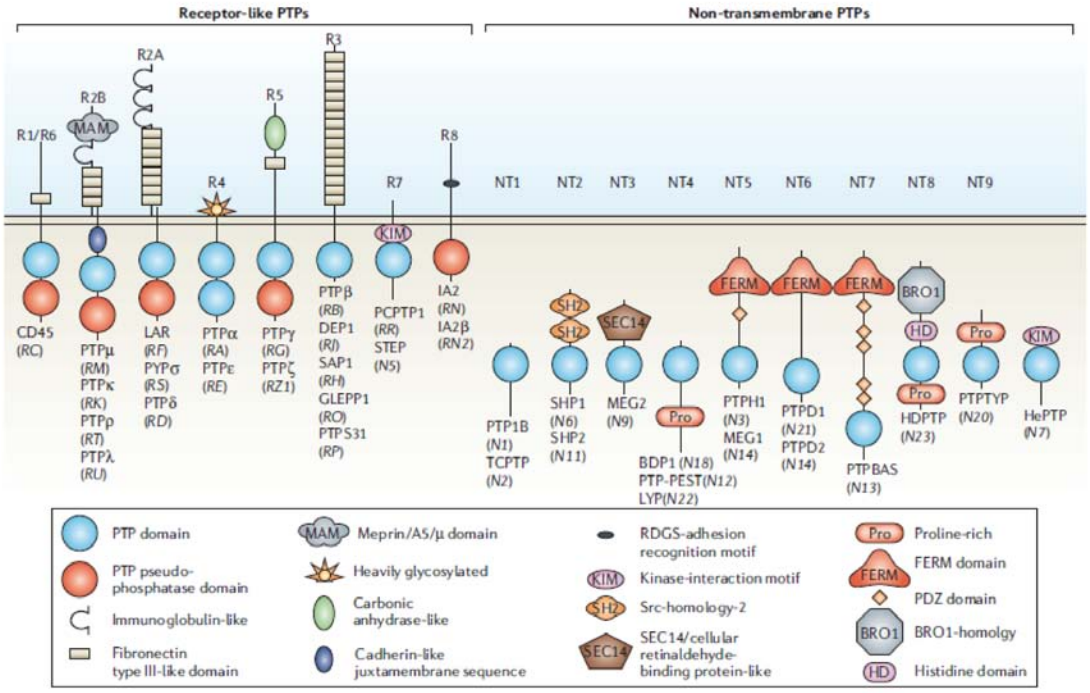

Figure 1-2: The classical protein tyrosine phosphatases (Tonks NK, *Nat Rev Mol Cell Biol*. 2006. 7(11):833-46).

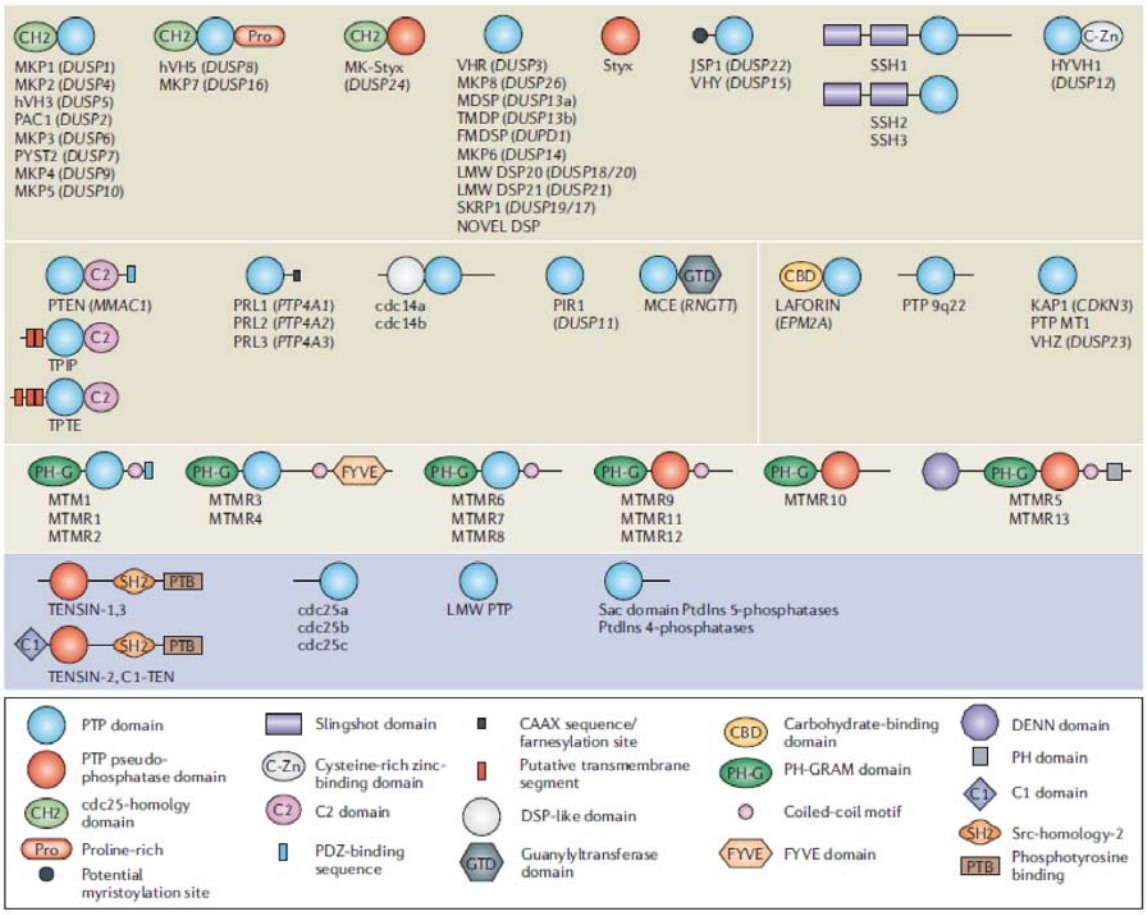

Figure 1-3: The Dual specificity phosphatases (Tonks NK, *Nat Rev Mol Cell Biol*. 2006. 7(11):833-46).

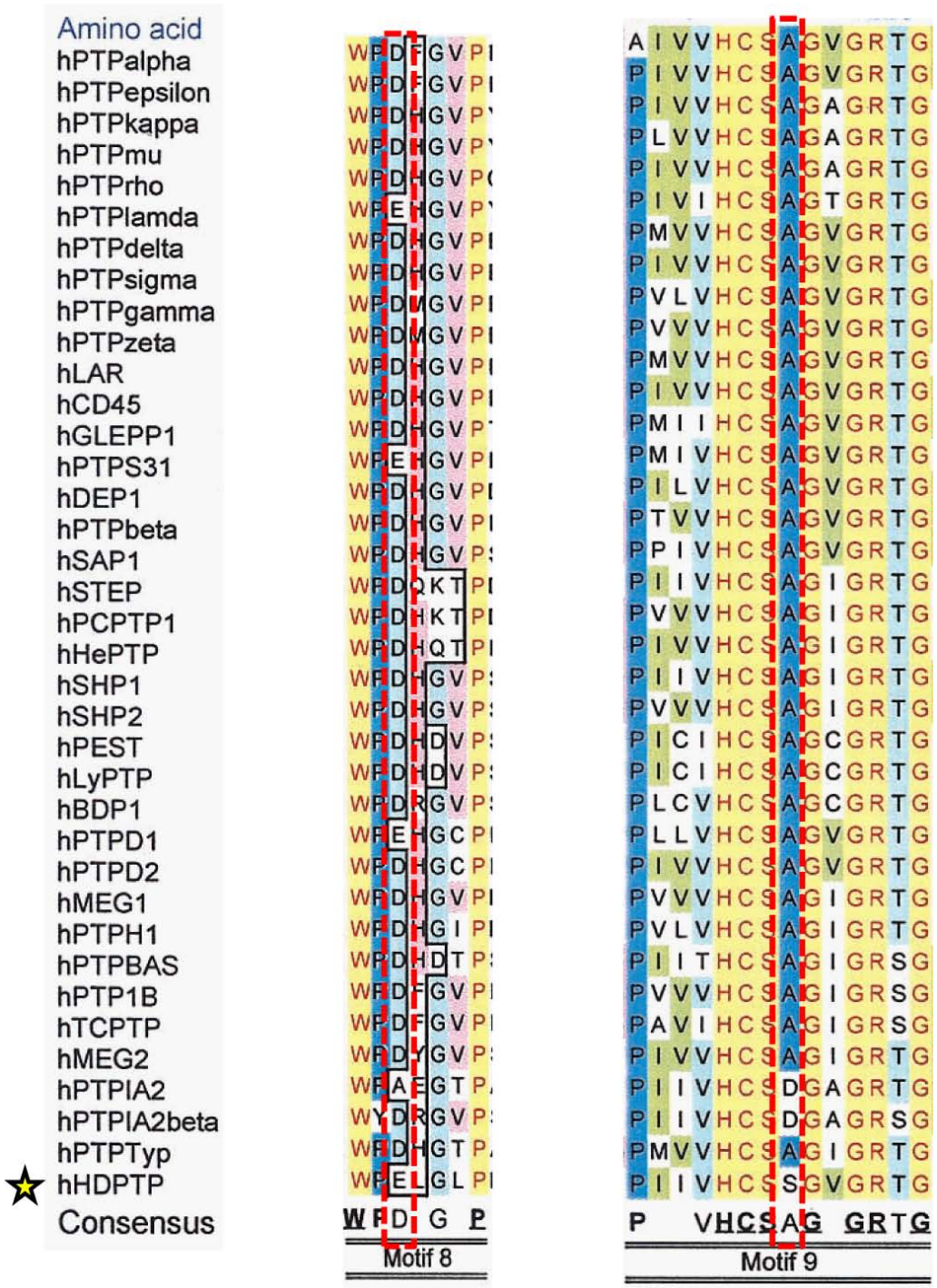

Figure 1-4: The active site signature sequence of the PTP domain in PTPN23 deviates from the consensus of other active members of the PTP family. Star indicates PTPN23 (HDPTP). Amino acids deviated in PTPN23 are highlighted by red-dotted boxes. (From Andersen et. al., *Mol Cell Biol.* 2001. 21(21):7117-36)

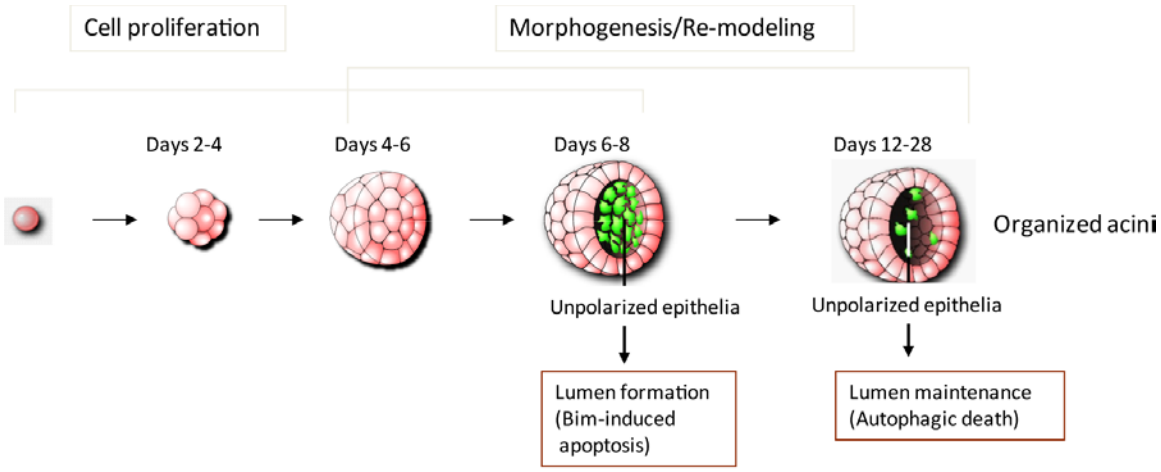

Figure 1-5: Morphogenesis of the MCF10A acini in 3D culture. (From Muthuswamy SK)

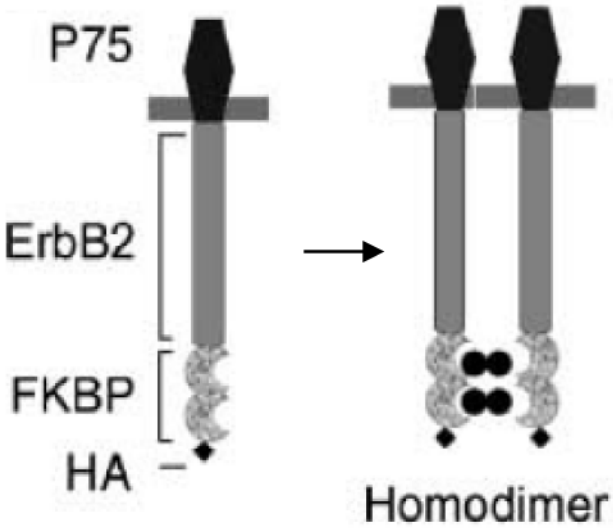

Figure 1-6: Structure map of the chimeric ErbB2. (Muthuswamy SK. Nat Cell Biol. 2001. 3(9):785-92)

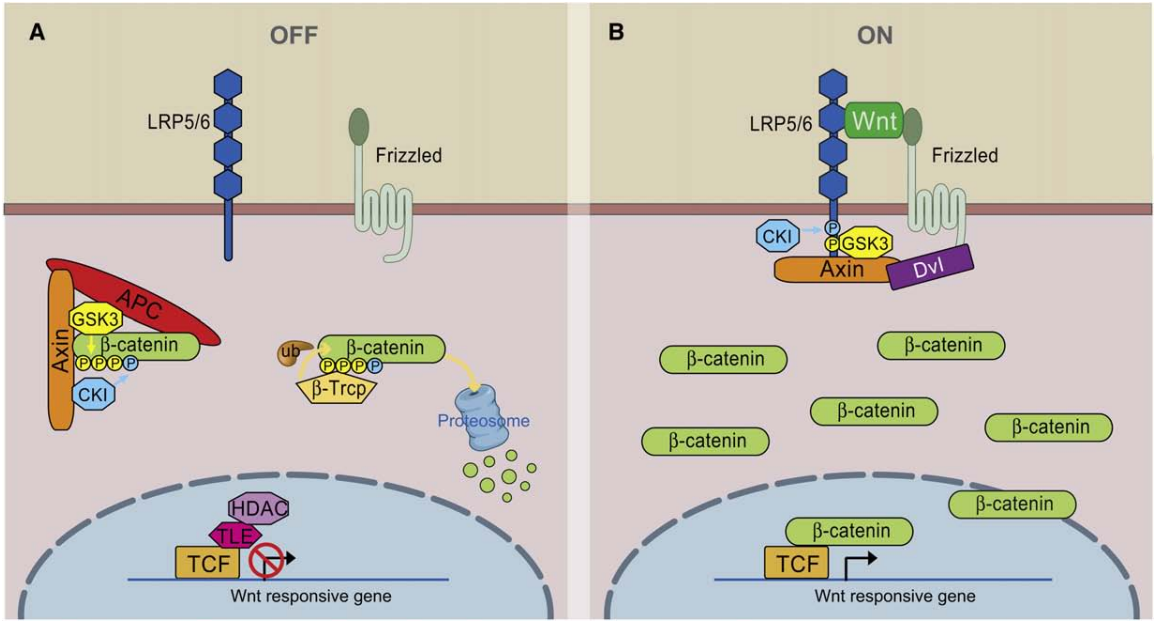

Figure 1-7: Overview of Wnt/β-Catenin Signaling. (From MacDonald et. al., Dev. Cell. 2009. 17(1):9-26)

Chapter 2: Construction of PTP shRNA Library.

In Chapter I, I have introduced the importance of PTPs as regulators in the progression of human cancers including breast cancer. However, for most of the PTPs, their function in the progression of breast cancer has remained uncharacterized. Therefore, the ultimate goal of my study is to develop a method to globally examine the function of PTP family in mammary epithelial models of breast cancer. Based on recent development in RNA interference (RNAi) technology, to date, it is possible to study gene or protein function through RNAi mediated loss-of-function studies. In order to study systematically the function of PTP family in the progression of breast cancer, I have constructed a short hairpin RNA (shRNA) library, which contains ~5 shRNAs that specifically designed to target each of the PTPs in the PTP family. There are ~500 shRNA in this shRNA library. In this chapter, I will demonstrate the construction of the PTP shRNA library.

2.1 RNA Interference

2.1.1 microRNAs:

RNAi was first identified as a defense mechanism triggered by double-stranded RNAs (dsRNAs) to protect cells from parasitic nucleic acids in lower organisms. Later, a conserved RNAi mechanism was also been found in higher eukaryotic cells. In general, RNAi involves the cleavage of the dsRNA into ~22nt

dsRNA, which target its homologous RNAs for destruction. MicroRNAs (miRNAs) are endogenous small non-protein-coding regulatory RNAs. Computational analysis reveals that there are up to ~1% of predicted miRNA genes in worm, fly, and human genome, suggesting that they are abundant classes of regulatory genes [241]. It has been shown that miRNAs could control a variety of functions. In flies, it has been discovered that miRNA could control cell proliferation, cell death, and fat metabolism [242, 243]. In plants, it could control the development of leaf and flower [244-247]. In mammalian cells, miRNAs has also been shown to involve in the regulation of a wide variety of processes. Interestingly, miRNAs are also expressed in tumors. Moreover, the expression of some miRNAs is highly specific to different tumor type or state, suggesting the potential to use miRNAs expression as diagnosis marker in cancers [248].

2.1.2 Biogenesis of miRNA:

The expression of miRNAs is tightly controlled in different tissue or during the developmental stages, suggesting miRNA expression is controlled through a highly specific program. However, the molecular mechanism that depicts miRNA biogenesis has remained uncharacterized. Interestingly, some miRNA genes have been found in clusters in the genome, suggesting that these miRNAs may be transcribed as a single transcriptional unit [249]. In general, miRNAs are generated as primary miRNAs (pri-miRNAs) in the nucleus (reviewed in [250, 251]). Many pri-miRNAs are predicted to fold into elaborated secondary structure which includes multiple stem-loop structures. Pri-miRNAs are digested by the

RNase III enzyme Drosha to produce a ~70nt stem-loop structured precursor miRNA (pre-miRNA). The pre-miRNAs contain a two-nucleotide 3' overhang, which is a general consequence of RNase III digestion. The pre-miRNA is transported to the cytoplasm by exportin-5, a Ran-GTP-dependent nuclear export factor that recognizes the two-nucleotide 3' overhang end structure of pre-miRNA. In the cytoplasm, pre-miRNA is processed further by Dicer to remove the loop structure and generate a mature ~22nt miRNA or siRNA.

After Dicer cleavage, the ~22nt miRNA or siRNA is immediately incorporated into the RNA-induced silencing complex (RISC) effectors complex. RISC is a ribonucleoprotein complex that includes the PAZ-Piwi-domain Argonaute family of proteins, miRNA or siRNA, and the mRNA that homologous to the miRNA or siRNA. To be incorporated into the RISC, the ~22nt miRNA or siRNA binds directly to the PAZ domain of an Argonaute protein. Depending on its specific components, RISC may target homologous mRNA for degradation or delay homologous mRNA translation.

2.2 Application of RNAi in loss-of-function studies

The analysis of loss-of-function phenotypes are an important approach to link biological functions to genes. Recent developments in RNAi technology have enabled its use for large scale loss-of-function genetic screens in mammalian cells (reviewed in [252]). In general, this involves the systematical manipulation of the endogenous miRNA biogenesis pathway to target genes of interested.

There are three methods to trigger the RNAi machinery artificially to suppress the expression of a specific gene (reviewed in [253]). Briefly, the first method involves the use of double strand RNAs (siRNAs) that are complementary to the mRNA of the target gene to trigger the RNAi machinery. The second method is to trigger the endogenous microRNA (miRNA) machinery by transfecting the cells with a construct that expresses a short hairpin RNA (shRNA), which is structurally similar to the stem-loop structured endogenous pre-miRNA. The third method is to transfect the cells with artificial pri-miRNAs that contain sequence homologous to the target mRNAs under the control of Pol II or Pol III transcription machinery. To accommodate the third method, human miR30 was selected as a scaffold for the expression of artificial shRNA because of the following advantages [254, 255]. First of all, it can be expressed under the control of Pol II promoter in the transfected mammalian cells. Secondly, the biogenesis of miR30 is not affected by sequence changes within its precursor stem as long as the size and the predominantly double-stranded nature are preserved. Lastly, the stem precursor sequence can be replaced by any artificial double-stranded sequence of similar length to generate the artificial sequence as a ~22-nt artificial siRNA (summarized in [256]). By using such a design, Zeng *et al.* [254] replaced the stem precursor sequence with the sequence of polypyrimidine tract binding protein (PTB) (nucleotides 1179–1201) in miR30 and expressed this construct using the CMV promoter in 293 cells. They generated shRNAs that targeted PTB and suppressed the expression of PTB to 70%-80% [254].

2.3 Short hairpin RNA expression constructs:

Recently by using the miR30 design, Silva et al. [257] and Dickins et al. [258] in the Cold Spring Harbor Lab have developed artificial shRNA expression systems. Silva *et al.* designed a library which contains all predicted genes in human and mouse genomes using a construct called pSM2 [257]. pSM2 is a U6 promoter driven vector that expresses designed shRNAs in the miR30 precursor, miR30 cassette. The pSM2 construct shows a dramatic elevation of gene suppression when transiently transfected in the HEK 293 cells [257]. The design of Dickins et al. [258], the pMSCV-LTRmiR30-PIG (pMLP or LMP) (Figure 2-1A and C), is also follow the miR30 cassette shRNA expression strategy. Briefly, pMLP is based on the backbone of the pMSCV-Puro (Clontech). The shRNAs are incorporated into the sequence of the miR30 cassette and are expressed under the control of viral LTR (Pol II promoter). Downstream of the shRNA expression cassette there is a puromycin resistant marker and an EGFP marker for the generation and indication of the stable cell line (Figure 2-1B). By using pMLP to express shRNAs target p53, Dickins et al. have shown successful suppression of p53 expression in mammalian cells [258].

2.4 Results and Discussions:

2.4.1 The design of the PTP shRNA library:

In order to analyze the biological functions of the PTP superfamily phosphatases systematically, I constructed a PTP shRNA expression library by using the design of the Dickins et al. I cloned the PTP shRNA containing miR30 cassette into the retroviral shRNA expression vector pMLP. The PTP shRNA library contained ~500 distinct shRNAs, which were designed according to the mRNA sequence of all the PTP superfamily of enzymes. Five shRNAs were designed to target each member of the PTP family, which includes the classical PTPs, VH1-like DSPs, cdc25s, LMW-PTP, myotubularin-like DSPs and the Sac-domain phosphatases (Figure 2-2). The sequence of the PTP shRNAs were designed by using the RNAi Codex program (http://cancan.cshl.edu/cgi-bin/Codex/Codex.cgi) that was developed by Gregory Hannon, Ravi Sachidanandam, Richard McCombie, and Stephen Elledge's lab. The designed PTP shRNAs were either obtained from OpenBiosystem (same sequence but in different expression constructs) or were synthesized as 97-nucleotide DNA oligos (Figure 2-3B).

The 97-nucleotide DNA templates (from 5' to 3') sequentially consisted of a common 5'miR30 flanking sequence, a sense strand targeting sequence (Figure 2-3A and B), a common miR30 loop sequence, an antisense strand targeting sequence (Figure 2-3A and B), and a common 3' miR30 flanking sequence. The oligo encodes a RNA transcript that can form a stem–loop

structure based on the complementary sequence of the sense and the antisense siRNA sequence. The common 5' and 3' miR30 flanking sequence are a part of the naturally existing micro RNA miR30, which could be recognized and digested by Drosha (Figure 2-3C). In addition, the common miR30 flanking sequence contains the XhoI and EcoRI sites for cloning to the expression vector pMLP (Figure 2-3B).

RNA Codex provides multiple possibilities for shRNA sequences. In choosing five oligos toward a single PTP gene, the following criteria were used to select the sequences from the RNAi Codex results.

1. Choose a region that is conserved among different transcription or splicing variants.
2. Choose the sequences that are also conserved in the mouse homologues.
3. Let the five oligos be equally distributed along the gene.
4. Choose the short hairpins that contain G/C in 5' and A/T in 3' on the sense strand [259].

2.4.2 The construction of the PTP shRNA library:

To clone the PTP shRNAs from the 97-nucleotide DNA oligos, the DNA oligos were synthesized and then pooled into 4 pools. The oligos in the same pool were amplified at the same time because they all carry a common 5' and 3' miR30 flanking sequence, which can be used as primer annealing site. To clone the PTP shRNAs obtained directly from OpenBiosystem, I grew bacteria that

carry the construct in pools. The plasmid DNA was extracted from each of the bacterial pools and then was used as templates in the PCR amplification.

There were four steps to PCR amplify the PTP shRNA sequences and clone them to the expression vector, pMLP (Figure 2-4): (i) Amplification of the PTP shRNAs from oligo pools or from plasmid DNA pools by PCR. (ii) Cloning the purified PCR fragments into the pMLP vector. (iii) Sequencing the clones. (iv) Identification of the correct clones. After one cycle of the cloning procedure, the correct clones were selected and the PTP shRNAs that have not been cloned were pooled into other 4 pools and then were PCR amplified and cloned again. The procedures were repeated until all the PTP shRNA were successfully cloned into pMLP.

i) Amplification of the PTP shRNAs from oligo pools or from plasmid DNA pools by PCR.

The designed shRNA oligos were synthesized by Sigma-Genosys. The synthesized oligos were pooled into four groups with each group containing an equal number of oligos. These pools of oligos were amplified in a PCR reaction. On the other hand, plasmid DNA pools extracted from bacterial clones obtained from OpenBiosystem were also used as template for PCR. A parallel PCR reaction that did not contain the oligo template was used as a negative control to present the specificity of the PCR.

ii) Cloning the purified PCR fragments into the pMLP vector.

The PCR products were purified and digested with XhoI and EcoRI. In a parallel reaction the pMLP vector was also digested by XhoI and EcoRI. The restriction enzyme digested PCR fragments and pMLP were purified by QIAEX II Gel Extraction Kit, and ligated. The ligation products were transformed to E. coli strain DH5α and grown on ampicillin plates. Before sequencing the clones, their quality was measured by XhoI and EcoRI digestion of the mini-prep DNA extracted from the clones. If more than 50% of the clones examined contained the insert the clones were ready for sequencing.

iii) Sequencing the clones.

The DNA sequencing of the clones was performed in Share Resource DNA Sequencing Facility at CSHL. For the first three runs of cloning, the regular sequencing method was used, through which I identified 337 independent clones. However, for the fourth run, the sequencing success rate fell off to ~10%, with indications from the data that a strong propensity to form secondary structure in the remaining clones caused interference to the sequencing enzyme. After adding this issue in the DNA sequencing facility, the remaining clones were identified successfully.

iv) Identification of the correct clones.

The sequence within the shRNA-mir cassette region between the XhoI and EcoRI site of the selected clones was compared to the sequence

of their oligo templates. The sequence information of the correct clones were selected and collected in an Excel file. The clones were stored at -80℃.

After six runs of cloning procedures, about 3,500 clones were selected and sequenced. Among these, 820 clones were confirmed to contain correct insert and all of the PTP shRNAs were isolated.

I have constructed a PTP shRNA library, which contains ~5 shRNAs that specifically designed to target each member of the PTP superfamily of enzymes (~500shRNAs in total). Hopefully, further application of this shRNA library will help us to better understand the function of the PTP superfamily of enzymes.

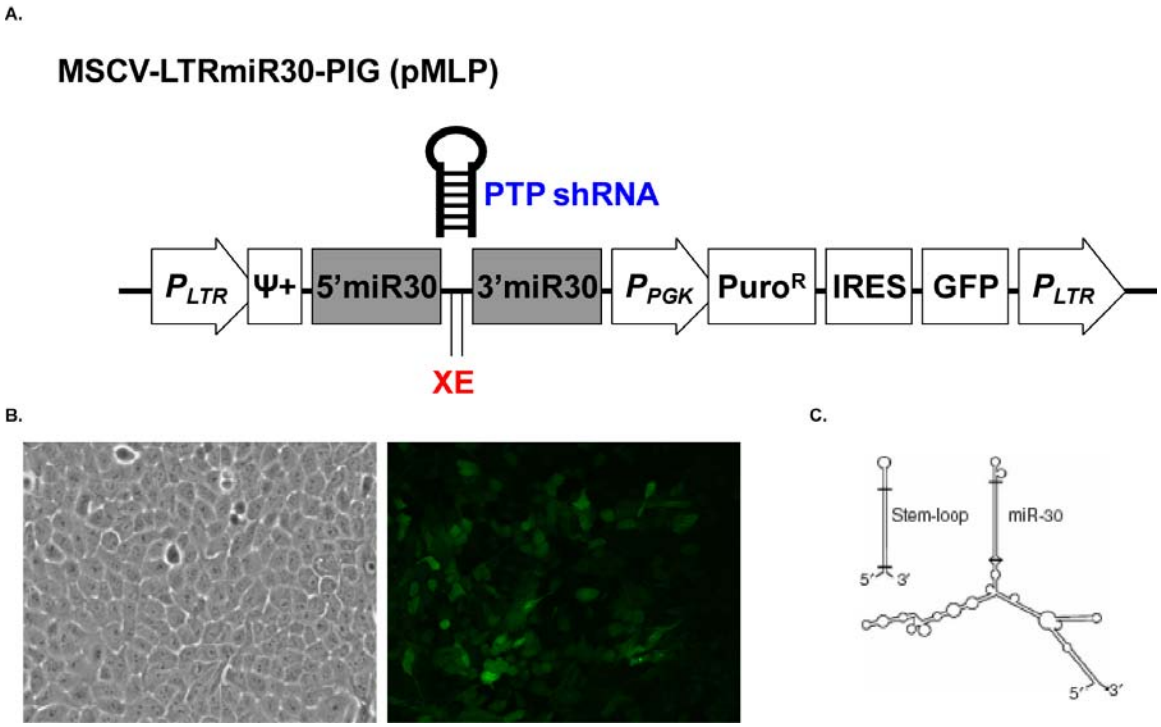

Figure 2-1: The shRNA expression construct, pMLP. (A) Structure map of pMLP. (B) MCF10A cells transfected with empty pMLP. The expression of GFP marked the shRNA-expressing cells. (C) Predicted secondary structure of miR30 shRNA.

PTP	Number
Classical PTPs:	37
Dual Specificity Phosphatases:	43
Myotubularin Phosphatases:	14
Sac-Domain Inositol Phosphatases:	5
CDC25s:	3
Low Molecular Weight PTP:	1
Total:	103

Figure 2-2: PTPs included in the PTP shRNA Library.

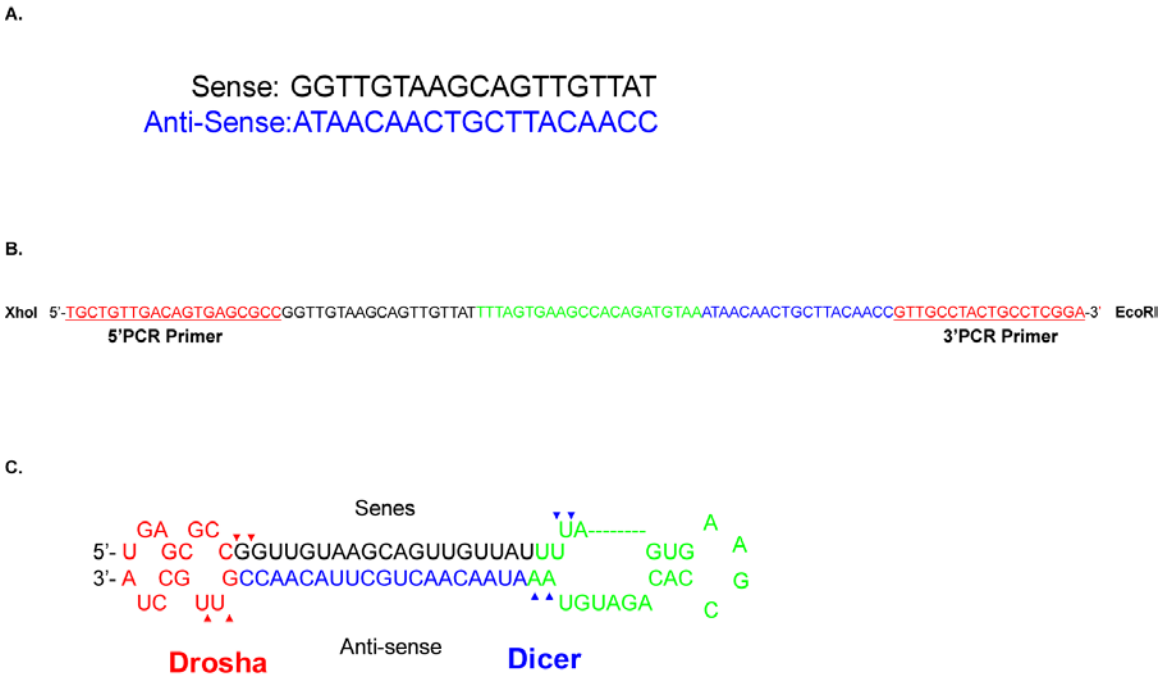

Figure 2-3: Structure of the PTP shRNA. (A) The sense and anti-sense shRNA sequences that target PTP1B. (B) Sequence arrangement of the 97-nt oligo template. (C) Stem-loop structure of the shRNA expressed in the cells.

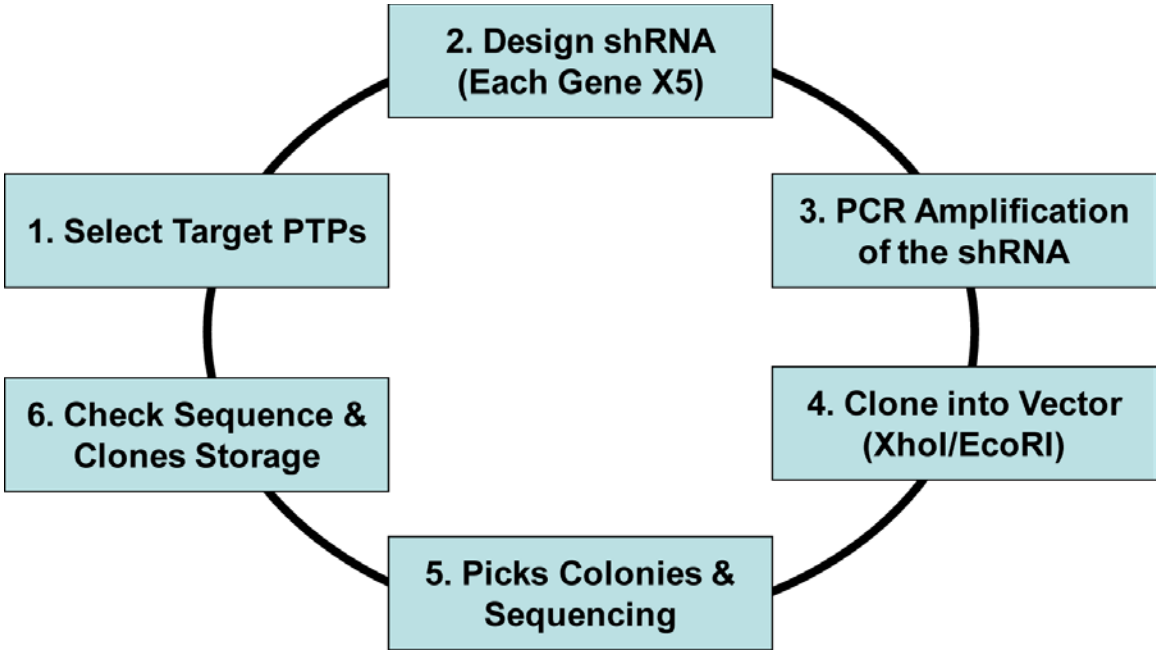

Figure 2-4: Flowchart of PTP shRNA Library construction.

Chapter 3: Functional studies of PTPRO in MCF10A three-dimensional culture system.

MCF10A cells form acini-like spheroids, when subjected to 3D culture on artificial extracellular matrix. These structures recapitulate the architecture of mammary epithelial *in vivo*. Therefore, it has been widely used as an *in vitro* model to study cell signaling in mammary epithelial cell. Recently, it has been found that the expression of PTP1B is up-regulated in the MCF10A 3D acini. In addition, the expression of PTP1B is required for ErbB2 to induce the formation of the multi-acinar structures [159]. These findings reveal that PTPs may be important regulators in MCF10A 3D acini morphogenesis. In collaboration with Min Yu in the Muthuswamy Lab, we characterized the function of PTPRO, in the morphogenesis of the MCF10A 3D acini. PTPRO is a transmembrane receptor like PTP. As I have introduced in chapter 1, genetic or epigenetic data have suggested that PTPRO may be a potential tumor suppressor. However, its role in the morphogenesis and tumorigenesis of mammary epithelial cell has not been characterized.

To characterize PTPRO in detail, I collaborated with Min Yu from the Muthuswamy Lab to examine the effect of shRNAs that target PTPRO on the morphogenesis of the MCF10A acini in 3D culture. We found that suppression of PTPRO induced the formation of larger acini. This is because the proliferation signal in the PTPRO depleted cells was prolonged. Moreover, suppression of PTPRO augmented the effect of ErbB2 to induce the formation of multi-acinar

structures. We also demonstrate that ErbB2 is a direct physiological substrate of PTPRO. Suppression of PTPRO increased the tyrosine phosphorylation of ErbB2, suggesting that PTPRO regulates ErbB2 activity directly through modulating the phosphorylation status of ErbB2. In a preliminary experiment, I observed that suppression of DEP1, which is structurally similar to PTPRO, induced the formation of multi-acinar structures independent of ErbB2 activation, implicating that DEP1 may be a tumor suppressor in breast cancer. In summary, we characterized the function of two structurally similar PTPs in MCF10A acini morphogenesis. Suppression of these two PTPs caused different effect on MCF10A acini morphogenesis. This is consistent with specificity in the function of PTPs as regulators of signaling.

3.1 Materials and methods:

Cell Culture

MCF10A and MCF10A/ErbB2 cell lines were obtained from the Muthuswamy lab in CSHL [166]. They were maintained as previously described [162]. To activate ErbB2 in MCF10A/ErbB2 cell line, MCF10A/ErbB2 cells were serum starved for16 hours in assay medium and were then stimulated with 1μM of AP1510 for the indicated length of time.

Three-Dimensional Morphogenesis Assays

For the 3D morphogenesis assay, eight-well chamber slide (BD Biosciences) pre-coated with 70 μl of Matrigel (BD Biosciences) was used. Briefly, at day 1st, 4,000 cells were grown in a well of the eight-well chamber slide in the presence of 5ng/ml of EGF [266]. To activate ErbB2, 1μM of AP1510 was added to the culture medium at Day 4th. Cell morphology was photographed on Day 6th, 10th and 16th.

Acinar size quantification:

Phase images of the MCF10A or MCF10A/ErbB2 3D acini were taken on the indicated date with a 10X objective. The structure area was measured using Axiovision 4.4 software (Zeiss). At least 200 acini were quantified. The experiment was repeated for three times. Their average was plotted.

Immunofluorescence

For the 3D invasion structures, day 10 acini without ErbB2 activation were fixed and stained as previously described [162]. Briefly, cells were grown in 3D and then were fixed and stained on the indicated date. Cells were fixed in 5% formalin in 1XPBS for 30 minutes. After fixation, cells were permeabilized with 0.5% Triton X-100 for 10 minutes and then were blocked in blocking solution (130 mM NaCl, 7 mM Na_2HPO_4, 3.5 mM NaH_2PO_4, 7.7 mM NaN_3, 0.1% bovine serum albumin, 0.2% Triton X-100, 0.05% Tween-20 and 10% goat serum) for 1 hour at room temperature. Following blocking, cells were stained by first antibodies diluted in blocking solution at 4ºC overnight and then stained with secondary antibodies at room temperature for 1 hour. Cell nucleus was highlighted by DAPI. The cells were mounted by mounting solution, Prolong Antifad (Molecular Probe, Invitrogen).

Microscopy

Images were taken by Zeiss Axiovert 200M using AxioVison 4.4 software.

Protein Preparation and Western Blotting

To harvest total cellular lysate from 3D acini, MCF10A cells were grown in 3D for the indicated date. The 3D acini were washed 3 times with 1XPBS and then resuspensed in 400µl of cell recovery solution (BD Biosciences) on ice for 30 minutes. After resuspension, cells were spin at 6000 rpm for 3 minutes to remove cell recovery buffer. After washing twice with 1X PBS, cells were lysed in 1%NP40 lysis buffer (150 mM NaCl, 20 mM Hepes pH 7.5, 1% NP40, 50 mM

NaF, 1 mM Na_3VO_4, 10% glycerol, protease inhibitor cocktail from Roche) for 30 minutes at 4 ºC. Cell debris was removed by centrifuging at 14000 rpm for 10 minutes. For the Western blot analysis, equal amount of protein was resolved using SDS-PAGE. The protein was transferred onto nitrocellular membrane. The membrane was incubated at 4 °C overnight with primary antibody, followed by incubation with the horseradish peroxidase-conjugated secondary antibody. Chemiluminescent detection reagents (GE Healthcare Bio-Sciences Co.) were used to detect immunoreactive protein.

Construction, Expression and Purification of recombinant His-tagged PTP domain of PTPRO

Wild type full length PTPRO expression construct was purchased from ORIGENE. Point mutation D1102A was introduced to the PTP domain of PTPRO using QuickChange Site-Directed Mutagenesis kit (Stratagene). Following mutagenesis, the PTP domain of the wild type and D1102A full length PTPRO was sub-cloned to bacterial expression vector, pET28a, to generate wild type and D1102A PTPRO PTP domain protein expression constructs. These constructs were then introduced into the bacterial strain BL21-RIL for recombinant protein production. Briefly, 500 µl of the bacterial overnight culture was refreshed in 500 ml of LB medium until OD 600 reached 0.6. Protein expression was induced by addition of 0.5 mM of IPTG at room temperature overnight. Bacteria harvested from the overnight culture were lysed in 50 ml of 1X PBS by sonication. After sonication, cell debris was removed by centrifugation.

His-tagged recombinant proteins were purified using Ni-NTA column (QIAGEN) according to the manufacturer's instructions. After purification, protein concentration was measure using Bradford Protein Assay (Bio-Rad).

Co-immunoprecipitation

To co-immunoprecipitate ErbB2 using recombinant PTPRO, indicated amounts of the purified recombinant PTPRO (wild type or D1102A) were prebound to the Ni-NTA beads. After washing, the Ni-NTA beads were incubated with 100 µg of pervanadate treated MCF10A/ErbB2 lysate at 4 ºC overnight. After co-immunoprecipitation, the Ni-NTA beads were washed by lysis buffer and then were eluted by sample buffer.

Antibodies and Reagents:

Antibodies used in this study were against: Ki-67 (Zymed), Activated Caspase 3 (Cell Signaling), Phosphotyrosine PT66 (Sigma), Phosphotyrosine 4G10 (upstate, Millipore), Phosphotyrosine G104 (our lab stock), PTPRO (Ari Elson, Weizmann), HA tag (COVANCE) and His tag (QIAGEN). In addition Alexa-Fluor-conjugated secondary antibodies (Molecular Probes, Invitrogen) were used for the immunofluorescence staining.

Statistics

All statistics were performed using a standard Student's *t*-test.

3.2 Results:

3.2.1 The expression of PTPRO is up-regulated in matured MCF10A acini.

In the Muthuswamy Lab, Min Yu had found that the expression of PTPRO mRNA was up-regulated in the mature MCF10A acini by using microarray analysis. This suggests that PTPRO may regulate the morphogenesis of MCF10A acini in 3D culture. In collaboration with Jim Hicks, we analyzed the expression of PTPRO mRNA in 32 human breast tumors. We found that ~1/3 of the breast tumors expressed at least 2.0-fold less of PTPRO mRNA than the normal tissue (Figure 3-1). This is consistent with the proposed function of PTPRO as a tumor suppressor. Moreover, it suggests that PTPRO may play a role in the development of breast cancer. In collaboration with Min Yu, we further characterized the function of PTPRO in the morphogenesis of MCF10A acini in 3D culture. We first confirmed the microarray data by analyzing the expression of PTPRO protein in the premature (day 8) and mature (day 16) acini. We found that the expression of PTPRO was up-regulated in the Day 16 acini (Figure 3-2), consistent with the microarray data. In addition, expression of shRNA that target PTPRO blocked the detection of the enzyme, confirming the identity of the PTPRO observed on the blot. Taken together, these data verify that the expression of PTPRO is up-regulated in matured MCF10A acini.

3.2.2 Suppression of PTPRO increases the size of MCF10A acini.

We characterized further the function of PTPRO in the morphogenesis of MCF10A acini using shRNAs that target PTPRO (shROs). We identified two shRNAs, shRO-1 and shRO-4, that increased the size of the MCF10A acini, whereas one shRNA, shRO-5, did not (Figure 3-3A). We validated the suppression efficiency of these shRNAs. As expected, the ability of these shRNAs to suppress PTPRO was consistent with their effect to increase the size of the acini (Figure 3-3B). This confirms that the phenotype we observed is specific to the suppression of PTPRO. We further quantified the size distribution of the acini that expressed the control shRNA (shRNA targets firefly luciferase), shRO-4 (Figure 3-3 and 3-4A) or shRO-5. As expected, the size distribution of the shRO-4 acini (Figure 3-4B; green line) shifted to right side by comparing to the size distribution of the control (Figure 3-4B; blue line) or the shRO-5 expressing acini (Figure 3-4B; red line). This indicates that the suppression of PTPRO increased the size of MCF10A acini.

3.2.3 Suppression of PTPRO delays cell proliferation arrest, but does not affect cell apoptosis.

Cell proliferation and apoptosis are two major mechanisms that contribute to the regulation of the size of the MCF10A acini. We examined whether cell proliferation or cell apoptosis was affected in the shRO-4 acini. We found that the proliferation signal, as shown by Ki-67 staining, was down regulated at day 10^{th} and was totally suppressed at day14^{th} in the control acini (Figure 3-5A; upper). In contrast, the proliferation signal remained strong on day10^{th} in shRO-4 acini and

remained detectable on day14th (Figure 3-5A; lower). This suggests that suppression of PTPRO delays the proliferation arrest in the morphogenesis of MCF10A acini. In contrast, the apoptosis signal, as shown by activated caspase 3 staining, was not affected upon depletion of PTPRO (Figure 3-5B). All together, these results suggest that suppression of PTPRO delays cell proliferation arrest, but does not affect cell apoptosis.

3.2.4 Suppression of PTPRO augments the effect of ErbB2 activation.

The combination of loss of tumor suppressor and hyper-activation of oncogenes has been considered as the major cause of cancers. ErbB2, an oncogenic PTK, is overexpressed or amplified in ~25% of breast cancers. Its overexpression or amplification is usually correlated with more aggressive tumor development and poor prognosis [182, 183]. Previously the Muthuswamy Lab showed that the activation of ErbB2 in MCF10A/ErbB2 cells grown in 3D culture induces the development of multi-acini structures, which resemble the early stage breast tumor.[166]. Therefore, we characterized further that whether suppression of PTPRO can enhance the effect of ErbB2 activation in MCF10A/ErbB2 acini. We generated MCF10A/ErbB2 cells that either expressed the control shRNA or shRO-4. As shown in figure 3-6, activation of ErbB2 induced the development of multi-acinar structures (Figure 3-6; lower left). Suppression of PTPRO also increased the size of the MCF10A/ErbB2 acini (Figure 3-6; upper right). Combination of PTPRO suppression and ErbB2 activation increased the size of the ErbB2-induced multi-acinar structures (Figure

3-6; lower right), indicating that suppression of PTPRO augments the effect of ErbB2 activation.

3.2.5 Suppression PTPRO enhances ErbB2-induced cell proliferation.

We have demonstrated that suppression of PTPRO enhanced cell proliferation in MCF10A acini. Next, we examined the effect of PTPRO depletion on ErbB2-induced cell proliferation. As expected, suppression of PTPRO or activation of ErbB2 separately, enhanced cell proliferation signal (Figure 3-7A; upper right or lower left, respectively). The combination of these two manipulations further promoted cell proliferation signal (Figure 3-7A and quantified in 3-7B). Taken together, these findings suggest that suppression of PTPRO augments the effect of ErbB2 activation.

3.2.6 Suppression PTPRO enhances ErbB2 phosphorylation.

ErbB2 is an oncogenic RTK. Activation of ErbB2 induces protein tyrosine phosphorylation in the cell. We have shown that suppression of PTPRO augmented the effect of ErbB2. Therefore, we examined whether suppression of PTPRO amplified ErbB2 induced protein tyrosine phosphorylation. As shown in Figure 3-8, suppression of PTPRO induced protein tyrosine phosphorylation in the MCF10A/ErbB2 acini (Figure 3-8; upper right). This is consistent with the function of PTPRO as a phosphatase. As expected, activation of ErbB2 induced protein tyrosine phosphorylation (Figure 3-8A; lower left). Suppression of PTPRO in ErbB2 activated acini further promoted ErbB2 induced protein tyrosine

phosphorylation (Figure 3-18; lower right), suggesting that suppression of PTPRO enhances ErbB2 signaling. On the images, we noted that the phosphotyrosine signal was detected in the cell-cell contact, which colocalized with ErbB2. This highlights the possibility that ErbB2 may be a downstream effector of PTPRO. As expected, we found that the basal phosphorylation of ErbB2 is higher when PTPRO was suppressed (Figure 3-8B; 0 min; arrow indicates chimera ErbB2). In addition, suppression of PTPRO elevated the plateau phosphorylation of ErbB2 (Figure 3-8B; 10, 30 and 60 min.), indicating that suppression of PTPRO promotes ErbB2 phosphorylation. Interestingly, the most robust increase in ErbB2 phosphorylation induced by shRO-4 was observed on a degradation form of the chimeric ErbB2 (Figure 3-8B; stars), suggesting that the degradation of ErbB2 upon activation of the PTK may be also affected when PTPRO is suppressed.

3.2.7 ErbB2 is a direct substrate of PTPRO.

We have revealed that suppression of PTPRO induced ErbB2 phosphorylation. Because ErbB2 and PTPRO localized to the membrane, implicating that ErbB2 could be a direct substrate of PTPRO. Therefore, we examined the direct enzyme-substrate interaction between these two enzymes. It has been shown in our lab that the point mutation of the conserved Asp181 residue of PTP1B, which plays a crucial role in the catalytic activity of PTP1B [152], impairs the catalytic activity of PTP1B but maintains the affinity of PTP1B to its substrates. Thus, we are able to co-immunoprecipitate the substrates of

PTP1B by using this PTP1B D181A mutant, or substrate trapping mutant [26]. We overexpressed and purified the recombinant His-tagged PTP domain of PTPRO either in the format of wild type or substrate trapping mutant (D1102A mutant). As shown in Figure 3-11, the recombinant PTP domains of PTPRO were used to pull down their substrates form pervanadate pre-treated MCF10A/ErbB2 lysate. We found that ErbB2 was only co-immunoprecipitated by the D1102A mutant, but not by the wild type PTPRO PTP domain (Figure 3-9), demonstrating a direct enzyme-substrate interaction between PTPRO and ErbB2. Therefore, it suggests that ErbB2 is a direct substrate of PTPRO. PTPRO augments the effect of ErbB2 through modulating directly the tyrosine phosphorylation status of ErbB2.

3.2.8 Suppression of DEP1 induces the formation of multi-acinar structures independent of ErbB2 activation.

DEP1 is a receptor-like transmembrane PTP that is structurally similar to PTPRO. Like PTPRO, DEP1 has also been proposed to be a tumor suppressor. Therefore, we analyzed whether suppression of DEP1 could induce abnormal development of the MCF10A 3D acini. We expressed the shRNAs that target DEP1 in MCF10A cells. Strikingly, our preliminary data revealed that the suppression of DEP1 induced the formation of the multi-acinar structures independent of ErbB2 activation (Figure 3-10), suggesting that the loss of DEP1 could induce tumorigenesis in mammary epithelial cell independent of the activation of ErbB2.

3.3 Discussion:

We have found that the expression of PTPRO is up regulated in matured MCF10A 3D acini, suggesting that PTPRO may be a regulator of the morphogenesis of the MCF10A acini. Suppression of PTPRO promoted proliferation and delayed the proliferation signal arrest and led to the formation of bigger acini. The size of cells in these acini was not changed (Figure 3-5 and 3-6), suggesting that suppression of PTPRO only increase the number of cells per acini, but not affected the size of cells. Interestingly, suppression of PTPRO was not sufficient to induce the formation of the multi-acinar structures. In contrast, suppression of DEP1, which is structurally similar to PTPRO, induced the formation of the multi-acinar structures independent of ErbB2 activation. I suppressed two structurally similar PTPs and observed two different phenotypes, suggesting that these two PTPs are regulating distinct downstream signaling pathways. This is consistent with PTPs as specific enzymes. It is important to note that these phenotypes was only observed in MCF10A cells that were grown in 3D culture system, but not those grown in the traditional 2D culture, suggesting the advantage of studying the function of PTPs using the MCF10A 3D culture system.

Overexpression or amplification of ErbB2 is observed in ~25% of breast cancers, which is usually correlated with more aggressive tumor development and poor prognosis [182, 183]. Moreover, ErbB2 amplification is seen in ~50% of ductal carcinoma *in situ* (DCIS), an early stage benign tumor [184, 185]. However, the exact molecular mechanism by which ErbB2 overexpression promotes breast

tumor formation is unclear. It has been shown that PTPs, such as PTP-BAS and PTPN9 directly dephosphorylate the C-terminal phosphotyrosine of ErbB2, and thus, negatively modulates ErbB2 signaling in breast cancer cell lines [208, 209]. Here, we have identified PTPRO as a novel regulator of ErbB2 signaling. Suppression of PTPRO augmented ErbB2 induced cell proliferation and the formation of multi-acinar structures. This implies that loss of PTPRO may accelerate the development of ErbB2 positive breast tumor. Therefore, the expression of PTPRO could be a biomarker for the prognosis of ErbB2 positive breast tumors.

We have demonstrated that PTPRO is an important regulator in the morphogenesis of MCF10A acini. Our study has also revealed that PTPRO is a direct regulator of ErbB2. In addition, our preliminary data also suggests that DEP1 may play an important role in the regulation of MCF10A 3D morphogenesis. Taken together, this data shows that PTPs are important regulators in the 3D morphogenesis of MCF10A acini. Therefore, it would be interested to characterize systematically the function of the PTP superfamily of enzymes in the morphogenesis of MCF10A acini using RNAi-mediated loss-of-function screens in the absence or presence of ErbB2 activation.

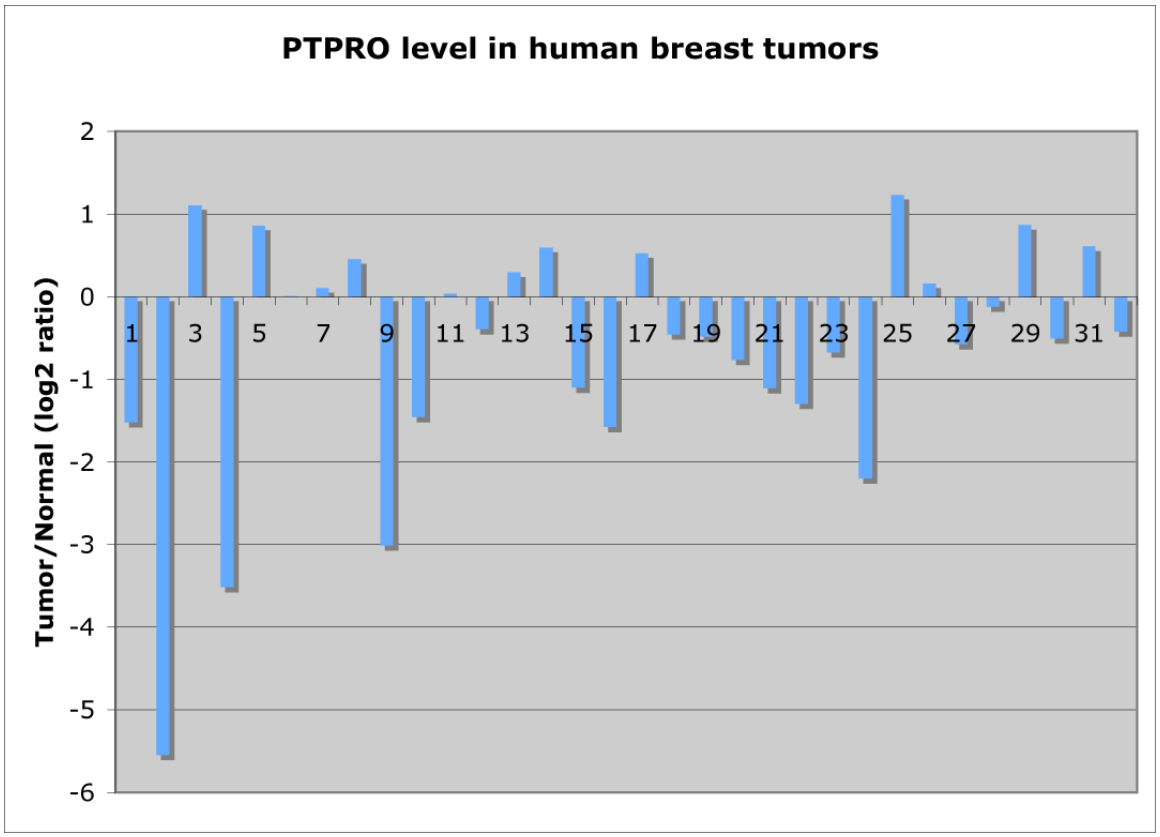

Figure 3-1: Expression of PTPRO mRNA in breast tumors vs. normal tissues. (In collaboration with Jim Hicks)

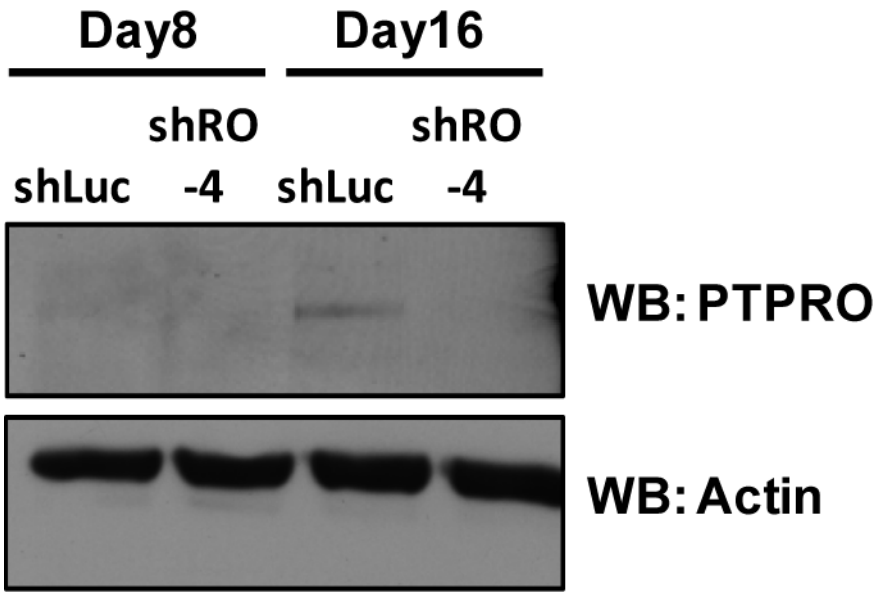

Figure 3-2: The expression of PTPRO is up-regulated in matured MCF10A acini. The expression of PTPRO was detected in the total cell lysate harvested from MCF10A acini manipulated as indicated. The expression of β-Actin was used as the loading control. (In collaboration with Prof. Ari Elson, Weizmann Institute of Science, Israel)

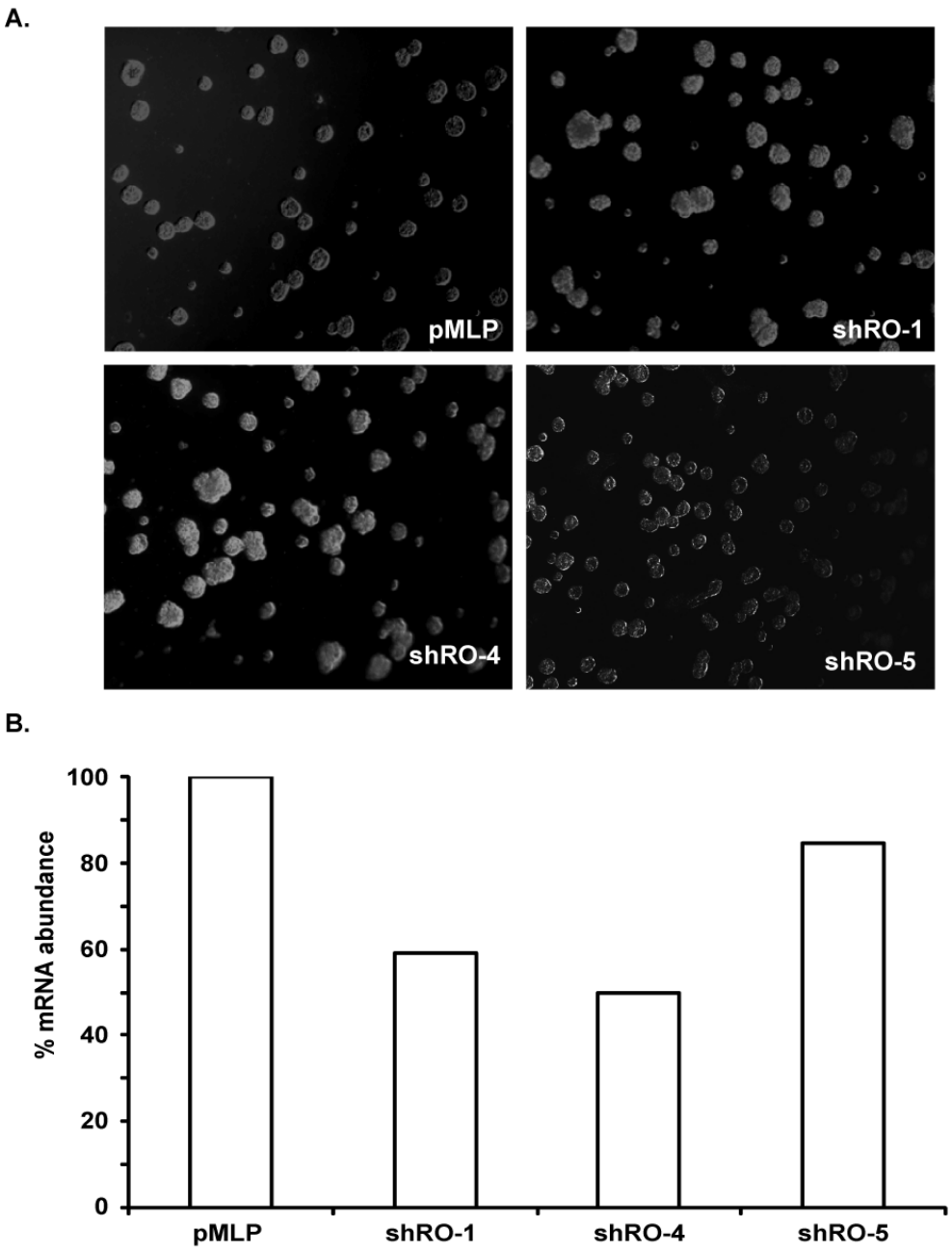

Figure 3-3: Suppression of PTPRO increases the size of MCF10A acini. (A) Phase images of the MCF10A acini that expressed the indicated shRNA. (B) Suppression efficiency of the PTPRO shRNAs. The PTPRO mRNA expression level of the indicated MCF10A cell was measured by qRT-PCR.

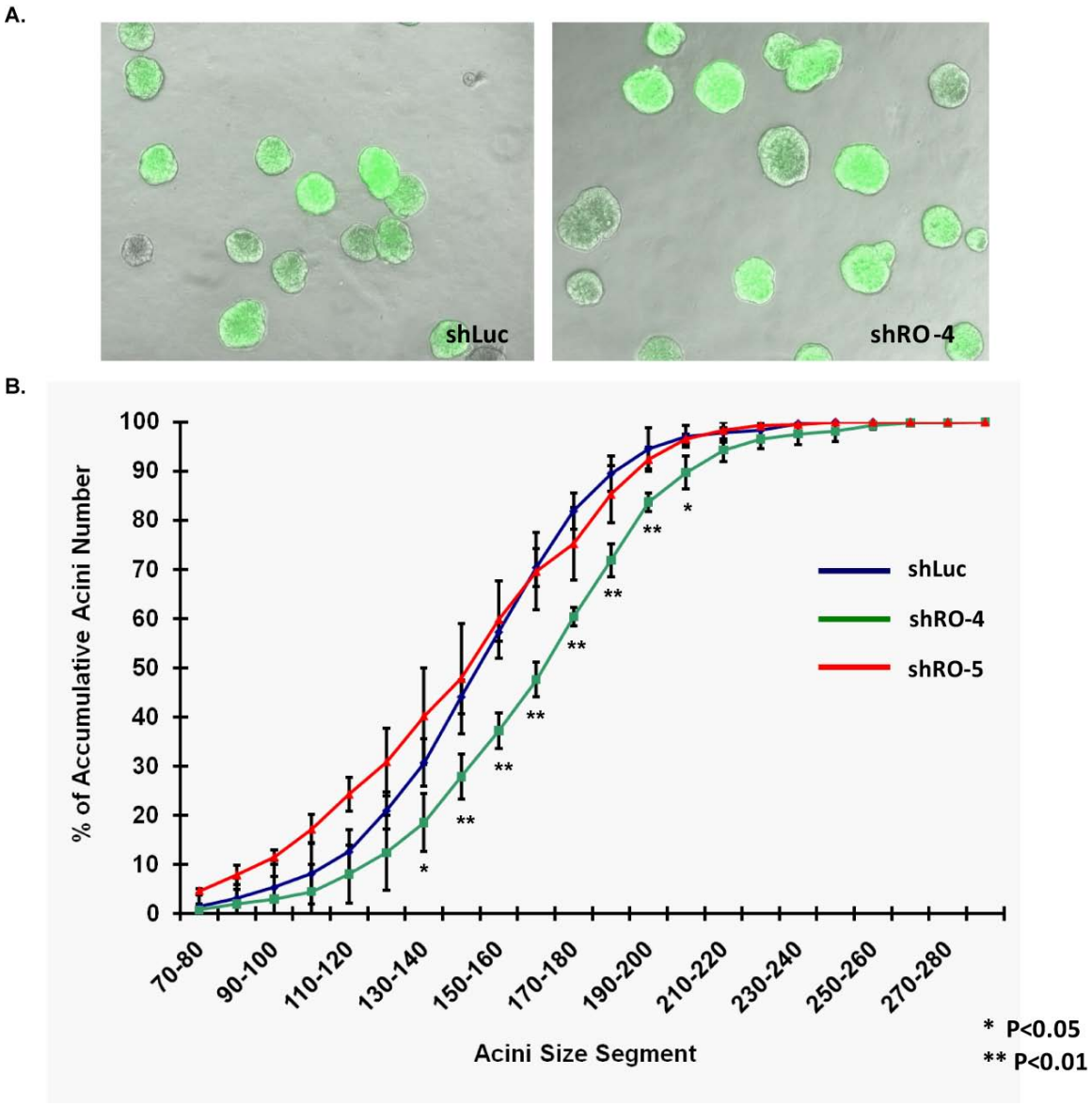

Figure 3-4: Suppression of PTPRO increases the size of MCF10A acini. (A) Phase images of the MCF10A acini that expressed the indicated shRNA. (B) Quantification of the size distribution of the MCF10A acini that expressed the indicated shRNA. Error bars represent S.E.M. (n=3; "*" P<0.05; "**" P<0.01).

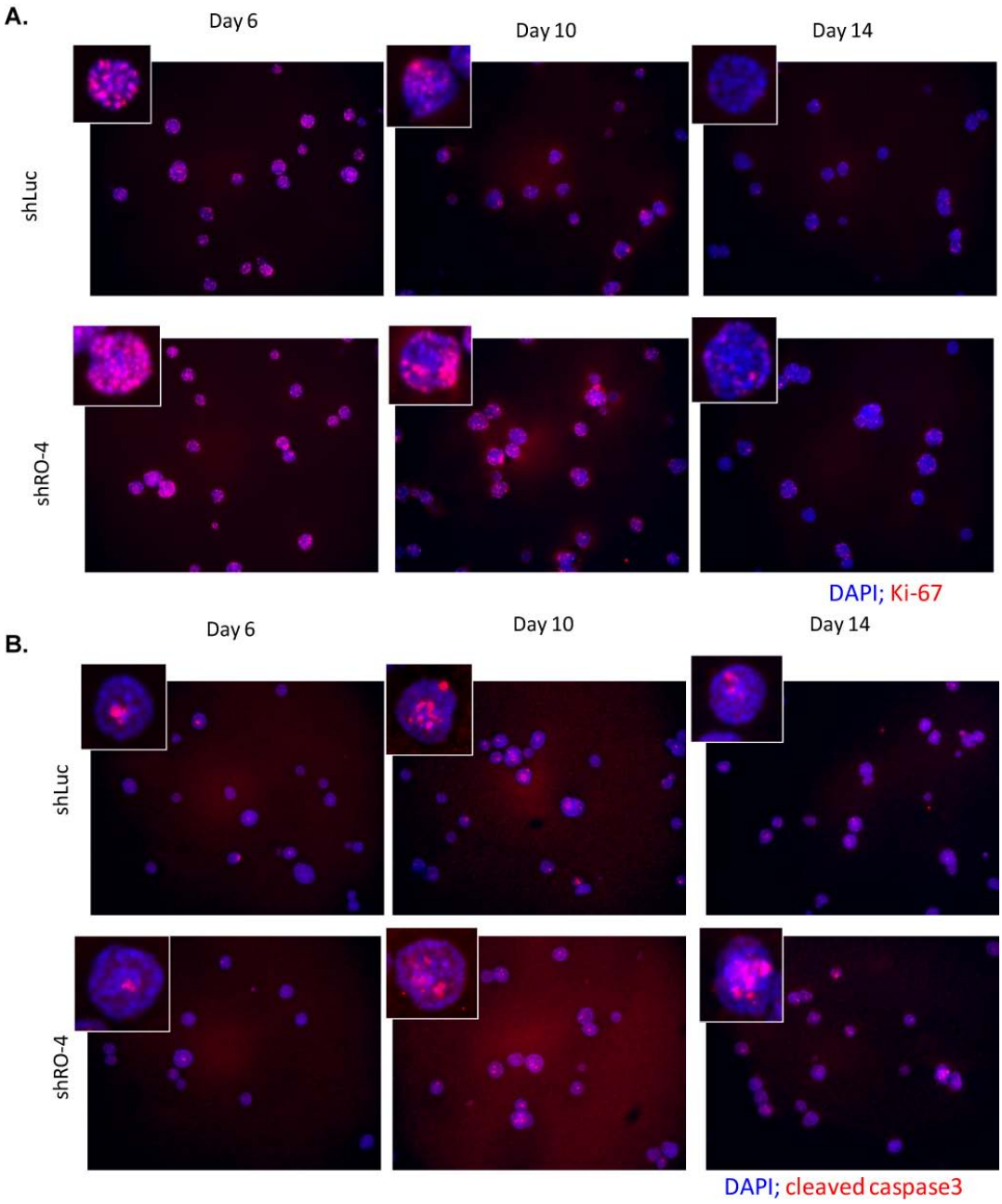

Figure 3-5: Suppression of PTPRO delays cell proliferation arrest, but does not affect cell apoptosis. MCF10A acini were manipulated as indicated. (A) Cell proliferation in control or shRO-4 MCF10A acini was detected by Ki67 antibody (Red). (B) Cell apoptosis in control or shRO-4 MCF10A acini was detected by activated caspase 3 antibody. Cell nucleus was highlighted by DAPI staining (Blue).

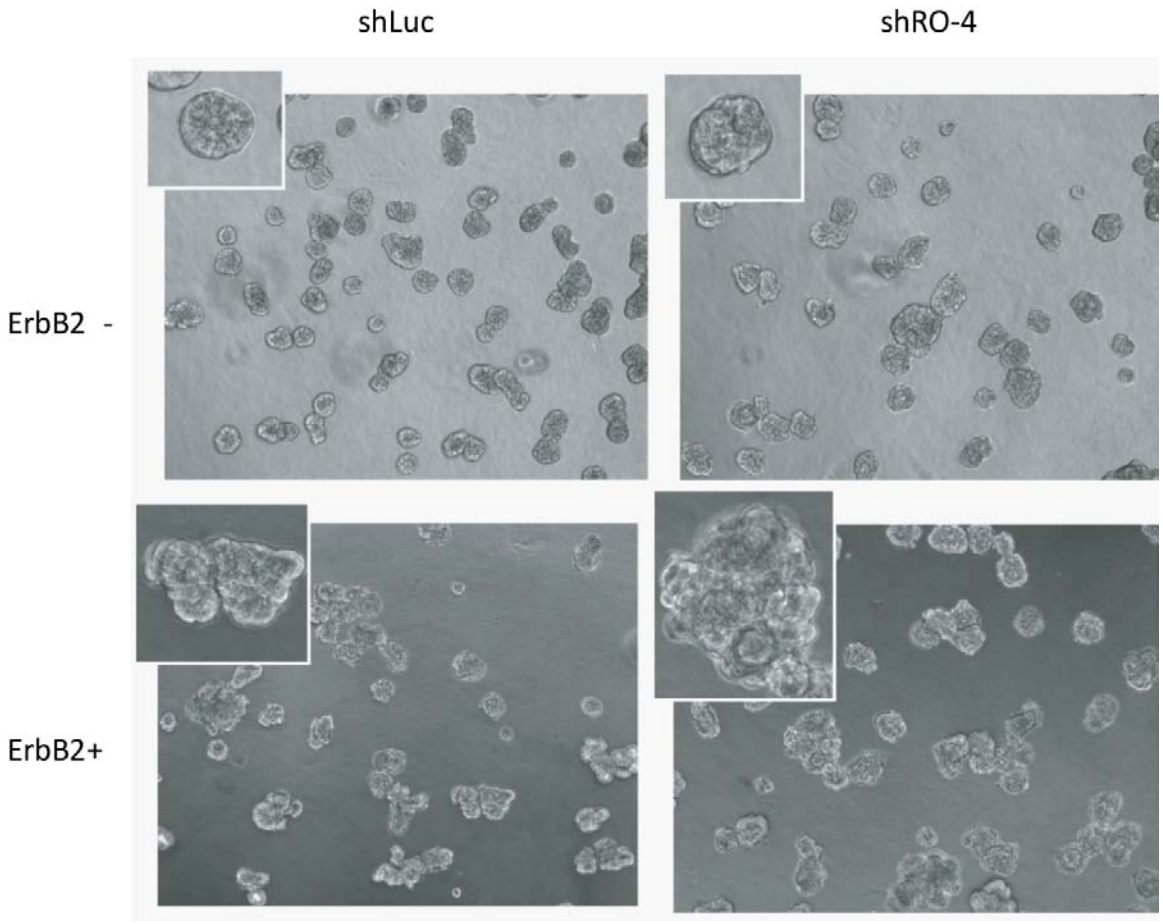

Figure 3-6: Suppression of PTPRO augments ErbB2-induced multi-acinar structure. Phase images of the MCF10A acini manipulated as indicated. Images were taken on Day12.

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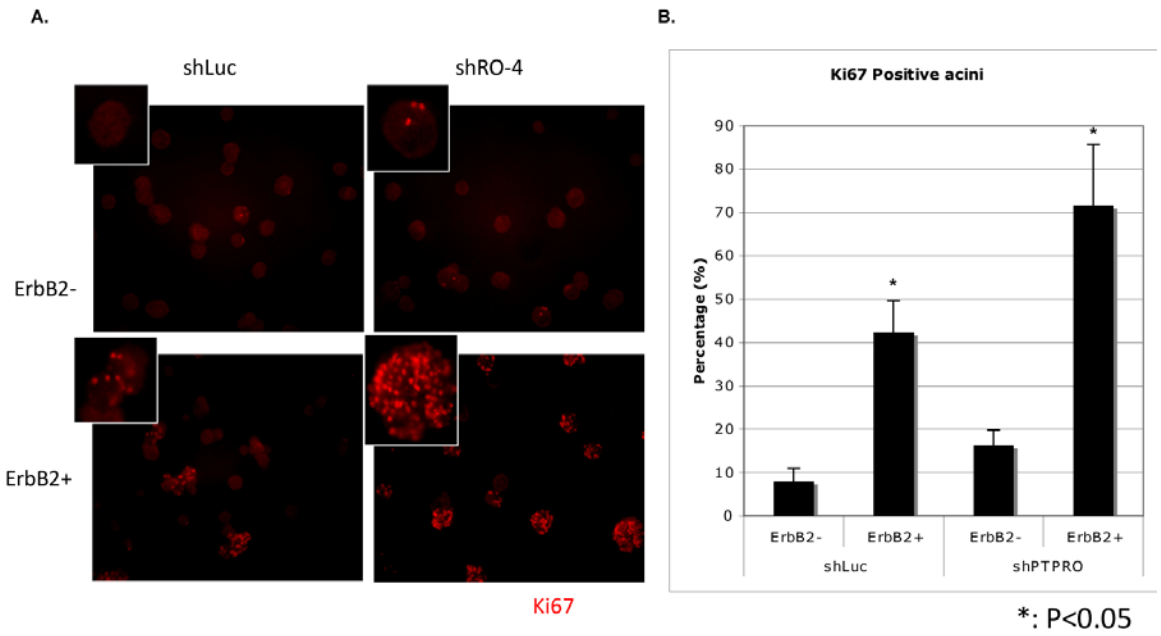

Figure 3-7: Suppression of PTPRO augments ErbB2-induced cell proliferation. (A) MCF10A/ErbB2 acini were manipulated as indicated. Cell proliferation was detected by Ki67 antibody (Red). (B) Quantification of Ki-67 positive MCF10A acini. MCF10A/ErbB2 acini contain more than three Ki67 positive cells were scored as positive. The percentage of Ki67 positive MCF10A/ErbB2 acini was plotted. Error bars represent S.E.M. (n=3; "*" P<0.05).

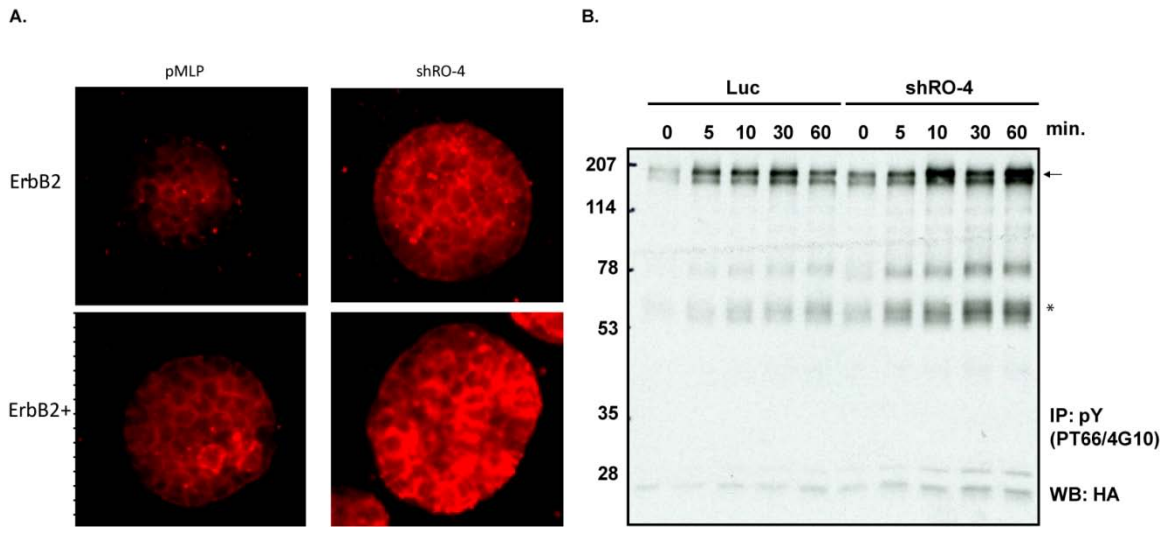

Figure 3-8: Suppression of PTPRO increases ErbB2 phosphorylation. (A) MCF10A acini were manipulated as indicated. Protein tyrosine phosphorylation was detected by anti-phosphotyrosine antibody. (B) Phosphorylation of ErbB2 in the control or shRO-4 MCF10A/ErbB2 cells upon activation of ErbB2 by AP1510. Total phosphotyrosine protein was immunoprecipitated by anti-phosphotyrosine antibody. Chimeric ErbB2 was detected by anti-HA antibody. Arrow indicates chimeric ErbB2. Star indicates degradation form of the chimeric ErbB2.

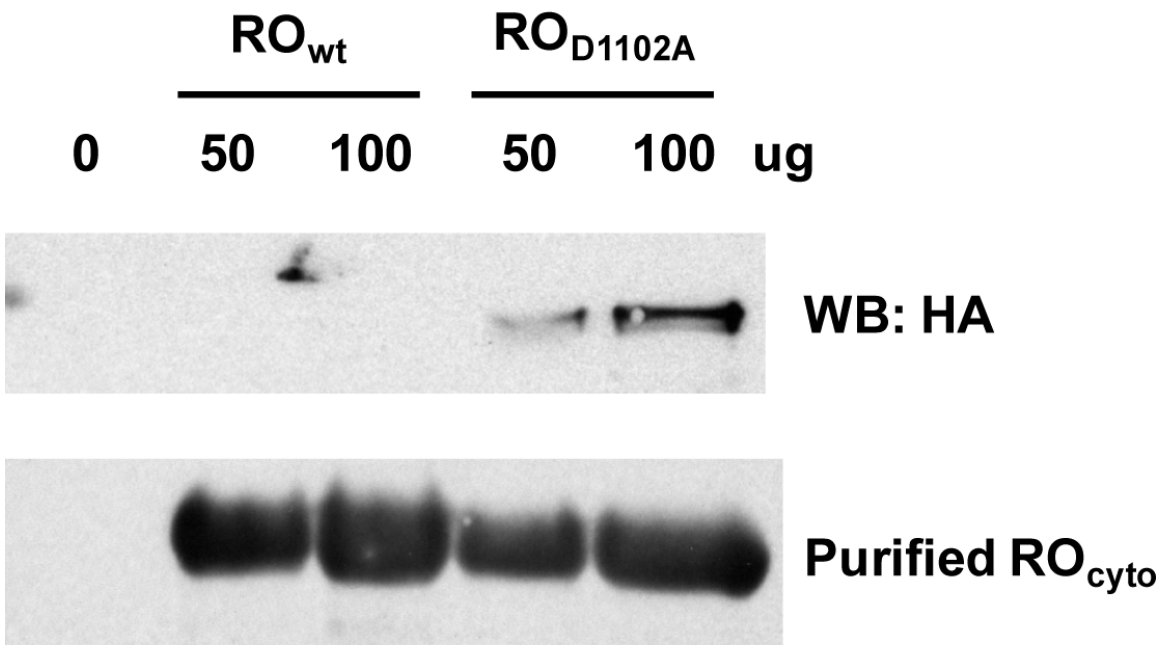

Figure 3-9: ErbB2 is a direct substrate of PTPRO. Recombinant PTPRO PTP domain wt or D1102A mutant was used to precipitate its substrates from pervanadate-treated MCF10A/ErbB2 cell lysate. The coprecipitated chimeric ErbB2 was detected by anti-HA antibody. The recombinant PTPRO PTP domain was detected by anti-His-tag antibody.

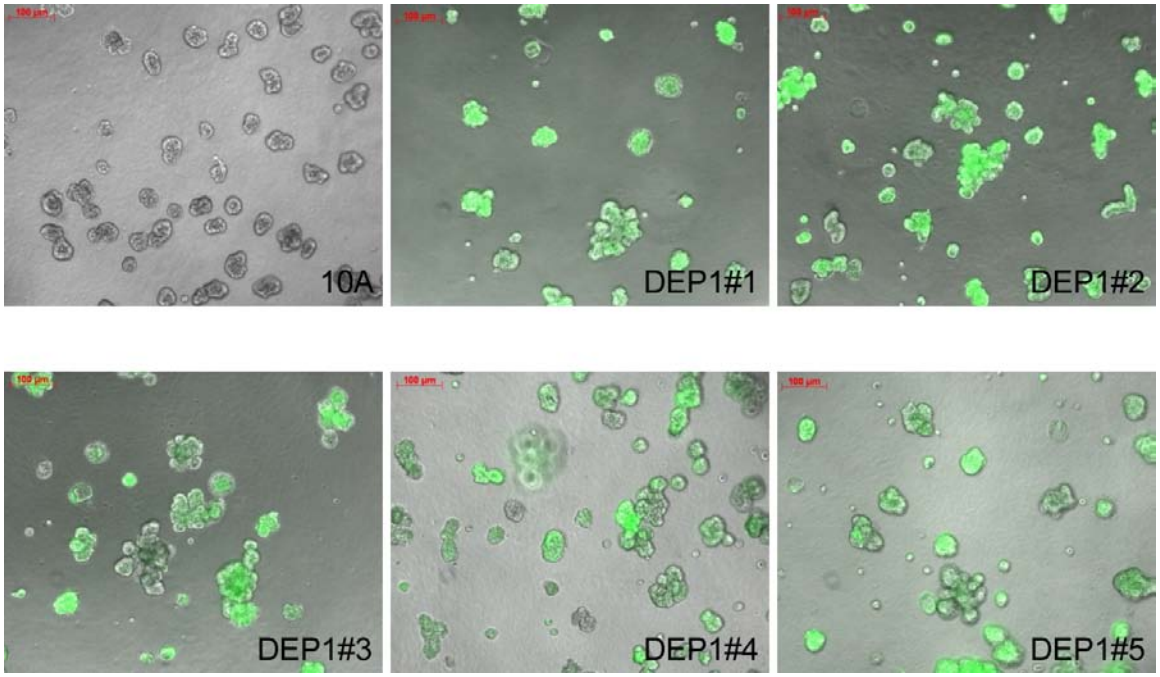

Figure 3-10: Suppression of DEP1 induces the formation of multi-acinar structures independent of ErbB2 activation. MCF10A acini were manipulated as indicated. Phase images were taken on Day12. GFP expression indicates the expression of the shRNA (Green).

Chapter 4: Identification of PTPN23 as novel regulator of cell invasion in mammary epithelial cells from a loss-of-function screen of the "PTP-ome".

Breast cancer is the most common type of cancer among women in the United States. Each year, more than 192,000 women are diagnosed with breast cancer. (From the National Cancer Institute, at http://www.cancer.gov). In general, tumor metastasis happens in the late stage of cancer and is the major cause of the lethality cases of cancers. Therefore, treatments that inhibit tumor metastasis hold enormous therapeutic potential to prevent the lethality of cancers. Cell invasion is an essential step of metastasis. It describes a process that cells gain the ability to migrate and invade through the extracellular matrix that surrounding them. To date, the roles of PTPs in cell invasion have remained uncharacterized. As the major goal of this chapter, I aimed to study the function of PTP superfamily of enzymes in mammary epithelial cell motility and invasion.

Overexpression or amplification of ErbB2 is observed in ~25% of breast cancers, which is usually correlated with more aggressive tumor development and poor prognosis [182, 183]. However, ErbB2 amplification is seen in ~50% of ductal carcinoma *in situ* (DCIS), an early stage benign tumor [184, 185], suggesting that not all the ErbB2 positive tumors could become invasive. It has been shown in MCF10A 3D culture system that activation of ErbB2 induced the formation of DCIS-like structures, but there is no cell invasion [166]. Interestingly, simultaneous activation of TGFβ and ErbB2 promotes the development of cell invasion [267], suggesting the presence of a mechanism that prevents ErbB2

positive tumors from becoming invasive. Therefore, I also explored the role of PTP family in ErbB2-induced mammary epithelial motility and invasion. I expected that based on my study I could identify PTPs by itself or in conjunction with ErbB2 to regulate mammary epithelial cell motility and invasion.

To achieve these goals, I employed an RNAi mediated loss-of-function screen to study the function of PTPs in the regulation of mammary epithelial cell motility in the absence or presence of ErbB2 activation. I identified three PTPs, PTPN23, PTPRG and PTPRR, which inhibited mammary epithelial cell motility. Interestingly, of these only PTPN23 was found to modulate mammary epithelial cell invasion. In addition, suppression of PTPN23 induced caveolin-1 mediated internalization and blocked early endosome vesicles trafficking, which led to the accumulation of E-cadherin in early endosomes. Loss of E-cadherin from the cell surface may induce the impairment of cell-cell adherens junctions, cell scattering, and expression of mesenchymal proteins, which promoted cell motility and invasion. I also demonstrated that PTPN23 is a direct negative regulator of E-cadherin, β-catenin and Src. PTPN23 may regulate E-cadherin/β-catenin/Src signaling complex to modulate cell motility, invasion and scattering.

4.1 Materials and methods:

Construction of the PTP shRNA library:

The construction of the PTP shRNA library is described in Chapter 2. Briefly, I designed ~5 shRNA that target each of the PTPs. Then the designed shRNAs were cloned to shRNA expression construct, pMLP [264].

Cell Culture

MCF10A/ErbB2 cell line was obtained from the Muthuswamy lab in CSHL [166]. They were maintained as previously described [162]. 293-B2 cell line was also obtained from the Muthuswamy lab in CSHL. They were maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Invitrogen), containing 10% FBS (Gibco, Invitrogen) and 1% penicillin/streptomycin. To activate ErbB2 in 293-B2 cell line, 293-B2 cells were serum starved for16 hours and were then stimulated with 1μM of AP1510 for the indicated length of time.

Transwell-Chamber Migration and Invasion Assays

To examine cell motility, Cell Culture Insert for 6-well plates with 8.0 μm pore size (BD Falcon) was used. Briefly, $1X10^6$ of the MCF10A/ErbB2 cells were grown in the Cell Culture Insert in the absence or presence of 1μM of AP1510. After 48 hours, cells that migrated to the other side of the membrane were fixed with 5% formalin diluted in 1XPBS for 30 minutes and were then stained with 1:50 dilution of KARYOMAX Giemsa Stain (Gibco, Invitrogen) according to the manufacturer's

instructions. For the transwell-chamber invasion assays, BD BioCoat™ BD Matrigel™ Invasion Chamber, 8.0 μm pore size (BD BioCoat™) was used. Briefly, $1X10^6$ of the MCF10A/ErbB2 cells were grown in the Invasion Chamber in the presence of 5ng/ml of EGF. After 48 hours, cells invaded to the other side of the membrane were fixed and stained as described before. To quantify cell motility and invasion, the average of the number of the migrated or invaded cells counted in at least 5 random fields were measured. The assay was repeated for three times. The average of three experiments was plotted.

Three-Dimensional Cell Invasion Assays

For the 3D invasion assay, eight-well chamber slide (BD Biosciences) pre-coated with 70 μl of 1:1 mixture of Matrigel and Collagen I (BD Biosciences) was used. Briefly, at day 1, 4,000 cells were grown in a well of the eight-well chamber slide in the presence of 5 ng/ml of EGF [266]. To activate ErbB2, 1 μM of AP1510 was added to the culture medium at Day 4. Cell morphology was photographed on Day 6, 10 and 16.

Immunofluorescence

The acini grown in 3D culture were fixed and stained as described previously [162]. Cells grown in 2D culture were fixed in 3.6% formaldehyde diluted in 1X PBS for 15 minutes. After washing with 1X PBS, the cells were incubated in 100% ice-cold methanol at -20 ºC for 10 minutes. Following fixation, cells were incubated in blocking solution (5% goat serum and 0.3% Triton-X 100 in 1X PBS)

at room temperature for 1 hour, and were then incubated with indicated first antibodies diluted in blocking solution at 4 ºC overnight. Following washing with 1X PBS, the cells were stained with secondary antibodies diluted in blocking solution at room temperature for 1 hour. The cell nucleus was highlighted by DAPI. The cells were mounted by mounting solution, Prolong Antifad (Molecular Probe, Invitrogen).

Microscopy

The phase images were taken by Zeiss Axiovert 200M using AxioVison 4.4 software. Immunofluorescence staining images were taken by Perkin Elmer Spinning Disc Confocol Microscope using velocity 5 software.

Protein Preparation and Western Blotting

MCF10A/ErbB2 or 293-B2 cells were lysed in either 1%NP40 lysis buffer (150 mM NaCl, 20 mM Hepes pH 7.5, 1% NP40, 50 mM NaF, 1 mM Na_3VO_4, 10% glycerol, protease inhibitor cocktail from Roche) or modified RIPA buffer (150 mM NaCl, 50 mM Tris-Cl pH 7.4, 1% NP40, 1% sodium deoxycholate, 0.1% SDS, 50 mM NaF, 1 mM Na_3VO_4, 10% glycerol, protease inhibitor cocktail from Roche) at 4 ºC for 30 minutes. Cell debris was removed by centrifugation. Protein concentration was measured by Bradford Protein Assay (Bio-Rad). For the Western blot analysis, equal amount of protein was resolved using SDS-PAGE. The protein was transferred onto the nitrocellular membrane. The membrane was incubated at 4 °C overnight with primary antibody, followed by incubation with the

horseradish peroxidase-conjugated secondary antibody. Chemiluminescent detection reagents (GE Healthcare Bio-Sciences Co.) were used to detect immunoreactive protein.

Zymogram

Cells were grown in 3D invasion assay using 4-well chamber-slides (BD-Biosciences). To activate ErbB2, 1 μM of AP1510 was added to the 3D culture at day 4. To generate conditional medium for the detection of MMP activity, one day before the indicated time points, the culture medium was changed to 150 μl of additives free DMEM/F12 medium. After 16 hours, the condition medium was recovered. Cell debris was removed by centrifugation. The conditional medium was denatured in sample buffer (don't boil) and was then resolved in 8% SDS-PAGE that contained gelatin substrate (1 mg/ml) in the separation gel. After electrophoresis, gel was incubated in re-nature buffer (2.5% Triton-X 100, 50 mM Tris, 200 mM NaCl, 5uM $ZnCl_2$, 5 mM $CaCl_2$ and 0.02% NaN_3, pH7.5) for 60 minutes, and buffer was changed four times to renature the MMPs. After renaturing, gel was incubated in the developing buffer (50 mM Tris, 200 mM NaCl, 0.005 mM $ZnCl_2$, 5 mM $CaCl_2$ and 0.02% NaN_3, pH7.5) at 37ºC overnight to allow MMPs to digest the gelatin. The activity of MMPs was visualized by staining the gel with Coomassie brilliant blue R-250.

Co-immunoprecipitation

To co-immunoprecipitate Src by the purified recombinant PTPN23 PTP domain, 1 μg of the purified recombinant PTPN23 PTP domain were prebound to the Ni-NTA beads. After washing, the Ni-NTA beads were incubated with 100 μg of pervanadate treated MCF10A/ErbB2 lysate at 4 ºC overnight. After co-immunoprecipitation, the Ni-NTA beads were washed by lysis buffer and then were eluted by adding sample buffer. To co-immunoprecipitate purified recombinant PTPN23 PTP domains by Src, 1 μg of the purified recombinant PTPN23 PTP domain was incubated with 100 μg of pervanadate treated MCF10A/ErbB2 cell lysate and 2 μl of anti-Src antibody (Cell Signaling) at 4 ºC overnight. To examine the specificity of the PTP/substrate interaction, 1mM of sodium orthovanadate was added to the co-immunoprecipitation reaction.

Statistics

All statistics were performed using a standard Student's *t*-test.

Construction, Expression and Purification of recombinant His-tagged PTP domain of PTPN23

The construct that expresses the full length wild type PTPN23 (pCDNA3.1-2flag-PTPN23) was obtained from Dr. Pause, McGill University, Canada [125]. Point mutations (E1357A or E1357D) were individually introduced to the full length wild type PTPN23 using QuickChange Site-Directed Mutagenesis Kit (Stratagene) to generate E1357A or E1357D full length PTPN23. Following mutagenesis, the PTP domain (Ile1211 to Val1450) of the wild type, E1357A and E1357D full

length PTPN23 was sub-cloned to bacterial expression vector, pET28a, to generate wild type, E1357A and E1357D PTPN23 PTP domain protein expression constructs. These constructs were then introduced into the bacterial strain BL21-RIL for recombinant protein production. Briefly, 500 µl of the bacteria overnight culture were refreshed in 500 ml of LB medium until OD 600 reached 0.6. The protein expression was induced by the addition of 0.5 mM of IPTG at room temperature overnight. Bacteria harvested from the overnight culture were lysed in 50 ml of 1X PBS by sonication. After sonication, cell debris was removed by centrifugation. The His-tagged recombinant proteins were purified using Ni-NTA column (QIAGEN) according to the manufacturer's instructions. After purification, protein concentration was measure using Bradford Protein Assay (Bio-Rad).

Antibodies and Reagents

Antibodies used in this study were against: laminin V (CHEMICON), PTPN23 (form Dr Maier Lab, University of Milan Medical School), E-cadherin, beta-catenin, caveolin-1, Rab7, Rab11, N-cadherin, Vimentin, Src-pY416, Src, cleaved caspase 3 (Cell Signaling), EEA1(BD Transduction lab), Snail, β-catenin pY142 (Abcam), β-actin and anti-flag M2 bead (Sigma), activated beta-catenin (Millipore), phosphotyrosine 4G10 (Upstate, Millipore), HA tag (COVANCE), Ki-67 (Zymed) and His tag (QIAGEN).In addition Alexa-Fluor-conjugated secondary antibodies (Molecular Probes, Invitrogen) were used for the immunofluorescence

staining. Donkey anti-rabbit or –mouse IgG HRP (GE Healthcare) was used for the western blotting.

Reagents used in this study were: Matrigel and collagen I (BD Bioscience), SU6656 (Sigma), AP1510 (ARIAD).

4.2 Results:

4.2.1 Loss-of-function screening of the PTPome to identify regulators of mammary epithelial cell motility

It has been shown in the Muthuswamy lab that addition of the AP1510 does not affect cell motility of the parental MCF10A cells (unpublished observation). Addition of AP1510 induced the homodimerization and activation of ErbB2, which enhanced cell motility to ~3 fold (data not shown). Therefore, I used the shRNA library that I constructed (Chapter 2) to study systematically the function of PTP family in MCF10A/ErbB2 cells in the absence or presence of AP1510. The library was expressed in MCF10A/ErbB2 cells and the effect on cell motility was examined. Considering the large number of PTP shRNAs in the library, I scaled down the screening by testing shRNAs in pools. In order to study the collaboration of PTPs with ErbB2, I examined the effects of PTP suppression in conjunction with activation of the chimeric ErbB2 with AP1510. I chose four shRNAs per PTP and grouped the shRNAs into 25 pools, according to structural similarity of their target PTPs, and then examined the effect on MCF10A/ErbB2 cell motility in the presence of ErbB2 activation. I identified eight PTP shRNA pools that induced cell motility to ≥ 2.0-fold ($p < 0.01$) and one pool of PTP shRNAs that reduced cell motility to ~50% ($p < 0.01$) (Figure 4-1). For further deconvolution, I focused on six pools of PTP shRNAs (five that induced and one that reduced cells motility) that targeted PTPs that have not been characterized extensively (Figure 4-1B with √).

These six pools targeted 25 PTPs. I tested the effects of suppressing each of these PTPs individually on MCF10A/ErbB2 cell motility, either in the absence or presence of ErbB2 activation. I identified four PTPs that induced and one PTP that reduced MCF10A/ErbB2 cell motility (Figure 4-2A and C; upper) in the absence of ErbB2 activation. On the other hand, five PTPs were identified as inducing, and 10 pools as reducing, cell motility in the presence of ErbB2 activation (Figure 4-2B and C; lower). It is important to note that suppression of these PTPs did not affect cell proliferation (Figure 4-3A) or apoptosis (Figure 4-3B), illustrating that proliferation and death did not contribute to the effects we observed on motility. In summary, these data reveal that the PTPs may function either positively or negatively to regulate mammary epithelial cell motility and that they display specificity in their function rather than exerting pleiotropic effects.

4.2.2 Suppression of PTPRG, PTPN23 and PTPRR induce distinct stimulatory effects on mammary epithelial cell motility

Next, I focused on the three PTPs, PTPN23, PTPRG and PTPRR that enhanced MCF10A/ErbB2 cell motility by more than 2 fold following activation of ErbB2 (Figure 4-2C; underlined). I selected 2 shRNAs for each PTP that efficiently suppressed expression, as analyzed by qPCR (Figure 4-4A), and tested their effect individually on MCF10A/ErbB2 cell motility in the absence or presence of ErbB2 activation (Figure 4-4B). PTPRG shRNAs (shRG) and PTPN23 shRNAs (shN23) induced MCF10A/ErbB2 cell motility either in the absence or presence of the ErbB2 activation. However, in the absence of ErbB2

activation, shN23 had a more pronounced effect than shRG. In contrast, PTPRR shRNAs (shRR) induced cell motility only following the activation of ErbB2. Interestingly, shRG enhanced cell motility preferentially following ErbB2 activation, whereas shN23 had a similar effect in the absence of presence of ErbB2 activation. These results suggest that although PTPRG, PTPRR and PTPN23 are negative regulators of mammary epithelial motility, their effects may be exerted through different mechanisms.

4.2.3 Suppression of PTPN23, but not PTPRG or PTPRR, promotes mammary epithelial cell invasion

In vitro culture of MCF10A cells on extracellular matrix (the Matrigel 3D culture system) permits the formation of sphere-like structures, called acini, that recapitulate the architecture of the mammary epithelium *in vivo* [162]. Activation of ErbB2 through homodimerization in these acini induces the formation of multi-acinar structures that resemble the structure of early stage breast tumor in vivo [166]. However, activation of ErbB2 alone is insufficient to induce cell invasion in the acini [166, 169]. Therefore, I investigated further the ability of shRG, shN23 and shRR to induce MCF10A/ErbB2 cell invasion in the 3D culture system, in the absence or presence of ErbB2 activation. I observed that PTPN23 shRNA induced MCF10A/ErbB2 cell invasion at day 10 in the absence of ErbB2 (Figure 4-5A; Day10). Upon activation of ErbB2, the process was accelerated and invasive structures developed by day 6 (Figure 4-5A; Day 6). In contrast, shRG and shRR did not induce cell invasion, either with or without ErbB2 activation

(Figure 4-5A; Day16). The effect of these shRNAs on cell invasion was quantified in the absence (Figure 4-5B; upper) or presence (Figure 4-5B; lower) of ErbB2 activation. In order to ascertain whether basal ErbB2 activity, in the presence of AP1510, affected the ability of shN23 to induce cell invasion, I examined the parental MCF10A cells. As shown in Figure 4-6, shN23 alone induced cell invasion in MCF10A cells.

In addition, I stained acini that expressed shRG, shN23 and shRR with antibody to laminin V, a marker for the basement membrane that surrounds the acini [268, 269]. As expected, only cells in which PTPN23 was suppressed were able to break through the basement membrane, either in the absence or presence of ErbB2 activation (Figure 4-5C; arrows). Interestingly, I noticed that in the absence of ErbB2 activation, shN23 also induced the mislocalization of laminin V, which phenocopied ErbB2 activation (Figure 4-5C; arrow heads). This suggests that PTPN23 may also be involved in the establishment of cell polarity.

Taken together, these observations reinforce the presence of specificity in PTP function. Suppression of PTPN23, but not PTPRG or PTPRR, augmented mammary epithelial cell invasion, an effect that was further enhanced by activation of ErbB2.

4.2.4 Suppression of PTPN23 induces mammary epithelial cell scattering and impairs cell-cell adhesion

MCF10A/ErbB2 cells normally form cell-cell contacts and cell clusters (Figure 4B; arrow head) when grown in subconfluent culture [162]. Suppression

of PTPN23 protein expression by shRNA (Figure 4-7A) induced ~5 fold more single cells that do not form cell-cell contacts with others in shN23 cells compared to control (Figure 4-7B and C). Moreover, cell scattering was observed only in MCF10A/ErbB2 cells that expressed shN23, but not shRG or shRR (Figure 4-7D). This is consistent with the effect of shN23 on the induction of cell invasion.

Cadherin mediated cell-cell adherens junctions are the major mechanical force that tethers cells together. Therefore, I analyzed further the integrity of the adherens junctions in shN23 cells. Because MCF10A cells undergo spontaneous morphological changes in responses to different cell density [270], I grew the cells in sparse, subconfluent, confluent or super-confluent cultures and then examined the effect of shN23 on the formation of cell-cell adherens junctions. As shown in Figure 4-8A, cell scattering was observed in sparse and subconfluent cultured shN23 cells. However, the cell scattering phenotype was lost when shN23 cells were grown to confluence (Figure 4-8A). Moreover, E-cadherin and β-catenin staining suggested that the formation of the cell-cell adherens junctions was impaired in sparse and subconfluent shN23 cells (Figure 4-8B and C), but not, when these cells were grown to confluence (Figure 4-8B and C). I tested further the integrity of the cell-cell adherens junctions of the confluent shN23 cells by challenging them with increasing concentrations of EGTA, which depleted Ca^{2+} and thus disrupted cadherin-based adhesion. Interestingly, I found that the adherens junctions of the shN23 cells were more sensitive to EGTA than the

control cells (Figure 4-9), indicating impairment of the function of adherens junctions even in confluent shN23 cells.

4.2.5 Suppression of PTPN23 induces caveolin-1 mediated endocytosis of E-cadherin and causes accumulation of E-cadherin in early endosomes

E-cadherin is the major cadherin protein of the adherens junctions. I observed that ectopic expression of E-cadherin in shN23 cells (Figure 4-10A) restored the formation of the adherens junctions (Figure 4-11; arrow heads) and inhibited cell scattering (Figure 4-10B). These results suggest that E-cadherin is an important target for the effects of PTPN23 on adherens junctions and cell scattering in mammary epithelial cells.

It has been shown that the BRO1 domain of PTPN23 can associate with proteins in the ESCRT pathway and regulate endosome trafficking [271, 272]. Suppression of PTPN23 in Hela cells inhibits early endosomal cargo traffics to late endosomes and lysosomes or recycling endosomes and causes the accumulation of internalized cell surface proteins in the early endosomes [124]. In Figure 4-8B, I observed the disruption of E-cadherin localized to the cell-cell adhesion of the shN23 cells, suggesting that PTPN23 suppression may cause mislocalization of cadherin to the endosomes. To pursue this further, I traced the subcellular localization of E-cadherin in control or shN23 cells. Consistent with the observation in Hela cells, I also observed accumulation of EEA1 positive vesicles, an early endosome marker, in the shN23 cells (Figure 4-12A; middle). Interestingly, the colocalization of E-cadherin with EEA1 was also increased in

shN23 cells (Figure 4-12A; right). This suggests that E-cadherin accumulated in the early endosome in the shN23 cells. In addition, the formation of both Rab7- and Rab11-positive vesicles was reduced in shN23 cells (Figure 4-12D and E; middle), suggesting that the forward trafficking of early endosome to late endosomes and recycling endosomes was impaired in shN23 cells.

Depending on cell type and stimulus, it has been shown that E-cadherin could be internalized via either clathrin-dependent endocytosis or caveolin-1-mediated endocytosis [235-237]. Interestingly, I observed co-localization of E-cadherin with caveolin-1 (Figure 4-12B; right), but not with clathrin (Figure 4-12C; right), suggesting that E-cadherin was internalized by the caveolin-1-mediated endocytosis pathway. Interestingly, there is a similar number of the caveolin-1 positive vesicles in the control and shN23 cells (Figure 4-12B; middle), suggesting that PTPN23 does not control the formation of the caveolin-1 vesicles. However, the colocalization of E-cadherin with caveolin-1 positive vesicles was increased in shN23 cells (Figure 4-12B; right), suggesting that E-cadherin internalization through caveolin-1 mediated endocytosis was promoted in the shN23 cells. Furthermore, the internalization of E-cadherin was blocked when cells became confluent (Figure 4-13). This was consistent with my observations that the formation of the cell-cell adherens junctions was partially rescued in confluent shN23 cells.

4.2.6 Suppression of PTPN23 induces expression of the mesenchymal proteins

Cell scattering and impairment of cell-cell adhesion is connected with the epithelial-mesenchymal transition (EMT), which is characterized by the down-regulation of epithelial proteins such as E-cadherin, and the up regulation of mesenchymal proteins such as N-cadherin, vimentin, snail and matrix metalloproteases (MMPs). In light of the effects I observed on E-cadherin localization, I examined the expression of epithelial and mesenchymal markers. Neither the expression of E-cadherin, nor that of its binding partner β-catenin, was suppressed in shN23 cells (Figure 4-14). Nevertheless, the expression of mesenchymal proteins including, N-cadherin, vimentin and snail, was up regulated in the shN23 cells (Figure 4-15A). Moreover, the nuclear localization of Snail was also enhanced in the shN23 cells (Figure 4-15B).

I also analyzed the secretion of matrix metalloproteases (MMPs) in shN23 cells that were grown in the 3D system. I detected an MMP activity at ~92kDa, the expected molecular weight of MMP9, following suppression of PTPN23 (Figure 4-15C). Interestingly, MMP9 activity was detected in Day 10 acini that were grown both with and without ErbB2 activation (Figure 4-15C). However, the Day 6 acini secreted MMP9 only when they were grown with ErbB2 activation (Figure 4-15C), consistent with a function of ErbB2 to accelerate shN23 induced cell invasion. These data suggest that suppression of PTPN23 induced the expression of mesenchymal proteins, but is not sufficient to down-regulate the expression of epithelial proteins or generate significant changes in epithelial cell morphology.

4.2.7 Suppression of PTPN23 enhances downstream signaling through activation of Src Family Kinases

MCF10A/ErbB2 cells are sensitive to serum starvation and produced a high basal protein tyrosine phosphorylation that interfered with characterizing of the effect of PTPN23 on cellular signaling. Therefore, I generated 293 cells that ectopically expressed the chimeric ErbB2 receptor (293-B2 cells) to analyze the effects of PTPN23. As shown in Figure 4-16, the addition of the dimerizer, AP1510, led to the activation of the chimeric ErbB2 in serum-starved 293b2 cells (Figure 4-16; panel 2). However, there was no significant difference on ErbB2 phosphorylation in the control or shN23 cells (Figure 4-16; panel 2), suggesting that PTPN23 did not control the phosphorylation status of ErbB2 directly. I further dissected the phosphorylation of 42 different receptor tyrosine kinases (RTKs) in control and shN23 cells, by using a phospho-RTK antibody microarray kit (commercially available from R&D Systems). Again, there was no change on the phosphorylation of these RTKs (data not shown). This suggests that the effect of PTPN23 is downstream of the RTKs.

Next, I examined the phosphorylation of the Src family of kinases (SFKs), as well as the MAPKs, ERK1/2, p38 and JNK, which have been implicated in the regulation of cell motility and invasiveness. I observed that only the phosphorylation of the SFKs, at the equivalent of Tyr416, the autophosphorylation site in Src in mouse, was up-regulated in the shN23 cells (Figure 4-16; panel 4). This indicates that PTPN23 is a potential negative regulator of SFKs. Interestingly, ErbB2 activation did not affect the

phosphorylation of SFKs Tyr416 (Figure 4-16; panel 4), suggesting that the phosphorylation of SFKs is regulated by shN23 independently of ErbB2 activation. This is consistent with our observation that PTPN23 shRNA alone induced cell migration and invasion independently of ErbB2 signaling.

In Figure 4-8C, I observed mislocalization of β-catenin in shN23 cells. Therefore, I tested whether suppression of PTPN23 induced the activation of β-catenin. It has been shown that β-catenin is activated through the dephosphorylation of Ser37/Thr41 [273]. In addition, phosphorylation of β-catenin on Tyr142 has also been reported to promote activation of β-catenin [274, 275]. Therefore, I examined the phosphorylation status of β-catenin at these sites. Interestingly, I found that the non-phsophoSer37/Thr41 on β-catenin was increased in shN23 cells (Figure 4-16; panel 7). In addition, phosphorylation of Tyr142 on β-catenin was also increased (Figure 4-16; panel 6). Taken together, these data indicated that β-catenin is activated in the shN23 cells.

4.2.8 Src is a direct substrate of PTPN23

The conserved Asp181 residue of PTP1B plays a crucial role as a general acid in the first step of catalysis [152]. Our lab has shown that a mutation of this site (D181A) impairs the catalytic activity of PTP1B but maintains its affinity for substrates, thus generating a “substrate trapping” mutant form of the enzyme [26]. Interestingly, the residue in PTPN23 that is equivalent to D181 of PTP1B is Glu rather than Asp. Therefore, in order to identify the potential physiological substrates of PTPN23, we generated the recombinant PTPN23 PTP domains in

the format of wild type (wt) or the substrate trapping mutant, E1357A or EA mutant. In addition, we also mutated this Glu residue back to Asp, E1357D or ED mutant, to examine the importance of the Glu residue at this position. As shown in Figure 7B, the indicated recombinant PTPN23 PTP domain was used to pull down potential substrates from pervanadate-treated MCF10A/ErbB2 cell lysate. We observed that several proteins coprecipitated with EA mutant, as detected by blotting with an anti-phosphotyrosine antibody (Figure 4-17; left). Addition of vanadate reduced the pTyr signal to that associated with the wild type (Figure 4-17; left), consistent with these proteins being substrates of PTPN23. Interestingly, there were three proteins that migrated to the expected size for E-cadherin, β-catenin and Src. Therefore, we characterized further the potential interaction between PTPN23 PTP domain and these three proteins. It was striking that the PTPN23 EA mutant displayed a strong interaction with these three proteins, whereas, the wt or ED mutant did not (Figure 4-17; right; upper and middle, respectively). Addition of vanadate disrupted the interaction between PTPN23 EA mutant and these three proteins, consistent with a direct enzyme-substrate interaction. Overall, the data suggest that E-cadherin, β-catenin and Src may be potential physiological substrates of PTPN23 in mammary epithelial cells.

4.2.9 Inhibition of SFKs activity impairs shN23-induced phenotypes

I have shown that SFK activity was elevated in 293-B2 cells that expressed shN23 and that Src was a direct substrate of PTPN23. To understand whether SFK activity is essential for the function of PTPN23, I tested the effect of the SFK inhibitor, SU6656 [276], on shN23-induced cell motility, cell invasion and

the formation of cell-cell adherens junctions. Interestingly, the SFK inhibitor suppressed shN23 induced cell motility (Figure 4-18) and shN23-induced cell invasion (Figure 4-19). Moreover, inhibition of SFK activity promoted cell-cell adhesion (Figure 4-20). Taken together, these results suggest SFKs play an important role in mediating the function of PTPN23 in the control of mammary epithelial cell motility, invasion and the formation of cell-cell adherens junctions.

4.2.10 Characterization of PTP1B as a negative regulator of mammary epithelial cell motility:

In an experiment independent of the screening, I characterized the role of PTP1B in MCF10A/ErbB2 cell motility. PTP1B is the prototypic member of the PTP family. The most astonishing finding regarding this enzyme is that PTP1B knockout mice are resistant to high fat diet induced obesity and diabetes [156, 157]. This finding make PTP1B a potential target for the treatment of obesity and diabetes [26, 158]. Recently, studies from the groups of Tremblay and Neel pointed out the important role of PTP1B in ErbB2 signaling [159, 160]. In their studies, they crossed transgenic mice that expressed an activated form of ErbB2 with PTP1B null mice. They found that the breast tumor development was delayed in the absence of PTP1B. Moreover, the development of lung metastasis was also attenuated. This suggests that PTP1B is a positive regulator that promotes ErbB2 induced tumor growth and metastasis. However, the mechanism by which loss of PTP1B attenuated breast tumor formation and metastasis is unclear.

To examine the function of PTP1B in mammary epithelial cell motility, I suppressed PTP1B in MCF10A/ErbB2 cells and then tested for its effect on cell motility. Simultaneously, I also tested the effect of TCPTP on ErbB2 induced MCF10A/ErbB2 cell motility. TCPTP is the PTP most closely-related to PTP1B. It shows 72% sequence identity and 86% similarity to PTP1B in the catalytic domain. As shown in Figure 4-21, I identified two shRNAs that suppressed PTP1B protein expression to ~60%. In addition, two shRNAs that suppressed TCPCP protein expression to ~90% was also identified. The PTP1B shRNAs significantly reduced MCF10A/ErbB2 cell motility either in the absence or presence of ErbB2 activation (Figure 4-22). In contrast, the TCPTP shRNAs did not affect MCF10A/ErbB2 cell motility (Figure 4-22). This suggests that PTP1B but not TCPTP is a positive regulator of mammary epithelial cell motility. Interestingly, inhibition of PTP1B activity in MCF10A/ErbB2 cells using a PTP1B specific inhibitor, also suppressed MCF10A/ErbB2 cell motility (Figure 4-23). This confirms that PTP1B is a positive regulator of mammary epithelial cell motility. Taken together, these findings provide an example for PTPs to function as positive regulator in mammary epithelial motility. In addition, it also establishes an *in vitro* model system to study the role of PTP1B as positive regulator in mammary epithelial cells.

4.3 Discussion:

In this study, I systematically examined the role of PTPs in the regulation of mammary epithelial cell motility, in the absence or presence of ErbB2 signaling. I observed that the majority of the PTP shRNAs had little or no effect on mammary epithelial cell motility (Figure 4-1), consistent with specificity in the effects of PTPs on cell function. In addition, I observed that in some cases suppression of particular PTPs enhanced migration, whereas suppression of others was inhibitory, consistent with roles as negative or positive regulators of signaling, respectively. The specificity, and subtlety, of regulation by PTPs is also seen in the differential effects of suppressing PTPN23, PTPRG and PTPRR on signaling in the absence or presence of the ErbB2 activation, including the fact that suppression of PTPN23, but not PTPRG and PTPRR, induced cell invasion. Taken together, these data highlight the functions of PTPs as specific regulators of signaling pathways, rather than as pleiotropic suppressors of tyrosine phosphorylation-dependent signaling that are linked to a housekeeping function.

I have shown for the first time that suppression of PTPRG and PTPRR enhanced mammary epithelial cell motility (Figure 4-4). The *PTPRG* gene is localized at human chromosome 3p21, a hot spot for deletion in breast cancer, and expression of PTPRG is reduced in lung, ovarian and breast tumors, suggesting its potential to function as a tumor suppressor [128, 133-135]. In addition, *PTPRG* gene has been shown to play a role as an estrogen-regulated tumor suppressor in breast cancer [101, 138]. These findings are consistent with a role for PTPRG in the development of breast cancer. PTPRR contains a KIM

(Kinase Interaction Motif) sequence, which is known to be important in recognition of MAPKs [146]. In *PTPRR*-deficient mice, the phosphorylation of p42/p44 MAPKs has been shown to be enhanced in the brain [147]. Moreover, a splicing isoform of PTPRR, PTP-SL, has been shown to regulate the phosphorylation and nuclear translocation of ERK5 [148]. These results suggest PTPRR is a negative regulator of the MAPK signaling pathways, which is consistent with our finding that suppression of PTPRR induced cell motility only following ErbB2 activation.

I am aware that screening PTP shRNAs in pools may lead to masking of some effects that could be revealed if the shRNAs were tested individually. For example, in a particular pool, if some shRNAs promote motility whereas others suppress it, I probably will not observe a phenotype in this pool. In addition, the effect of a functional shRNA may be diluted by other non-functional shRNAs. However, as the first experiment to study systematically the function of PTPs by using the PTP shRNA library, I felt that it was important to keep the screen concise. In order to reduce the possible masking effects, I pooled the PTP shRNAs according to the structural similarity of their target PTPs. From the screen, I found that shRNA pools that target the MKPs promoted cell motility (Figure 4-1; mix8 and 9). This is consistent with the role of MKPs as negative regulators of MAPK signaling pathways [41]. In addition, a pool that contains shRNAs that target slingshots (SSH1, SSH2 and SSH3) also promotes cell motility (Figure 4-1; mix12). This is consistent with their role to dephosphorylate Cofilin and LIMK1, which promote F-actin depolymerization and severing, to

inhibit cell motility [277]. Moreover, the pool that contains shRNAs that target PTEN and PTEN-like phosphoinositide phosphatases, TPIP and TPTE, also induced cell motility (Figure 4-1; mix13), consistent with the function of PTEN to inhibit the PI3K/AKT signaling pathway. Taken together, these observations validate the feasibility to screen the shRNAs pools.

I have focused my attention on PTPN23 because of the potential links I observed between this enzyme and cell invasion. The *PTPN23 gene* is also mapped at human chromosome 3p21 [115]; however, the function of PTPN23 in the regulation of the development of breast cancer has not been characterized. It has been shown that suppression of PTPN23 induces endothelial and bladder carcinoma cell motility [127, 278]. In addition, it has been shown that the phosphatase activity of the Rat ortholog of PTPN23 is important for suppression of RAS-mediated transformation of fibroblasts [116]. As I have introduced in Chapter 1, two amino acids of the active site signature sequences of PTPN23 deviate from the consensus of other active members of the PTP family. Therefore, there has been debate about whether PTPN23 possesses catalytic activity [125]. Here, I observed that PTPN23 plays a role in the regulation of mammary epithelial cell motility and invasion (Figure 4-4 and 4-5), suggesting that PTPN23 has the potential to be an important regulator of the development of breast cancer. I found that the phosphorylation of β-catenin and Src was elevated when PTPN23 was suppressed. Using the substrate trapping mutants, I further identified E-cadherin, β-catenin and Src as direct substrates of PTPN23. Inhibition of Src blocked PTPN23 depletion induced cell motility, invasion and

scattering. Therefore, my data suggest that PTPN23 is a catalytically active enzyme.

Src is an important regulator of a variety of signaling pathways that control cell proliferation, migration, adhesion and invasion. Some PTPs, including PTPRA, PTP1B and PTPRE, have been reported to function as activators of Src through dephosphorylation of the C-terminal inhibitory tyrosine residue (Try527) [279]. In contrast, PTP-BAS has been shown to regulate Src activity negatively through directly dephosphorylating the autophosphorylation site of Src (Tyr416) [280]. Here I showed that suppression of PTPN23 increased Src activity. I also demonstrated a direct enzyme-substrate interaction between PTPN23 and Src (Figure 4-16). These results suggest PTPN23 is a direct negative regulator of Src activity. Overexpression or hyperactivation of Src is observed in many human cancers including breast cancer [281]. However, it has been shown in transgenic mouse models that overexpression or hyperactivation of Src is not sufficient to induce a higher grade of breast tumor and its metastasis [282, 283]. Interestingly, transgenic overexpression of Src in the p21-/- background dramatically induces tumor growth and metastasis [284], suggesting the inactivation of tumor suppressors augments Src transforming activity. Given the negative effect of PTPN23 on Src activity, loss of PTPN23 may promote tumor growth and metastasis in breast tumors for which Src is overexpressed.

Src is a broad function signaling regulator. In addition to the role I reported in this dissertation, it also influences cell proliferation, survival and motility through the transmission of signals from RTKs and the integrins (reviewed in

[285]). Activation of RTKs leads to the autophosphorylation of tyrosine residues in their cytosolic segments, which creates docking sites to recruit and activate signaling molecules including Src. In turn, thus further phosphorylates and activates RTKs to create additional phosphotyrosine binding sites on the RTKs to recruit adaptor proteins that mediate the activation of downstream signaling such as Ras/MAPK and PI3K/AKT signaling pathways and promotes cell proliferation, survival as well as motility. Integrins are transmembrane proteins that form the cell-ECM adhesions. They link ECM components to the actin cytoskeleton, mediated by multiple structural and signaling proteins including talin, paxillin, vinculin, α-actinin, FAK and Src [286]. Integrin-ECM interaction promotes the autophosphorylation and activation of FAK. Tyrosine phosphorylation of FAK on Tyr397 provides a docking site for Src thereby leading to the recruitment and activation of Src. Activation of Src further phosphorylates FAK on multiple tyrosines, which enhances FAK activity and creates additional binding sites for proteins, including p130CAS, Paxillin and p190RhoGAP, which play a central role in the regulation of the rearrangement of the actin cytoskeleton and thereby contributes to the control of cell motility [287]. I found that suppression of PTPN23 did not affect the activity of MAPKs, FAK and Paxillin, suggesting that Src-mediated RTK and integrin signalings are not affected upon PTPN23 depletion. This is consistent with the effect of PTPN23 being restricted to regulation of the functions of Src that control endocytosis.

In this dissertation, I used an SFK inhibitor, SU6656, to confirm the involvement of Src in PTPN23-regulated cell motility, invasion and scattering. I

found that inhibition of SFK activity rescued PTPN23 shRNA-induced changes in cell motility, invasion and scattering. SU6656 was first identified in the Courtneidge lab as a SFK inhibitor [276]. It shows selective inhibition, with specificity towards purified recombinant SFK, but not PDGFR or a panel of PTKs including, FGFR, IGF1R, Met, Frk, Csk, Abl and Cdk2 at <2μM of concentration *in vitro* [276]. Routinely, we have to use higher concentration of a small molecule inhibitor for experiments in cells compared to studies with purified proteins *in vitro*. This is because the cell membrane represents a barrier to uptake of the inhibitor and the metabolic machinery of the cell may also affect its potency. I observed that the stimulation of cell motility by PTPN23 shRNA was inhibited by low μM concentration of SU6656 (Figure 4-18), which is consistent with activation of Src being a primary component of the effects of suppressing PTPN23. As shown in Figure 4-19, SFK inhibitor displayed similar inhibitory effects in suppressing cell invasion, consistent also with the role for Src in the function of PTPN23 in control of cell invasion.

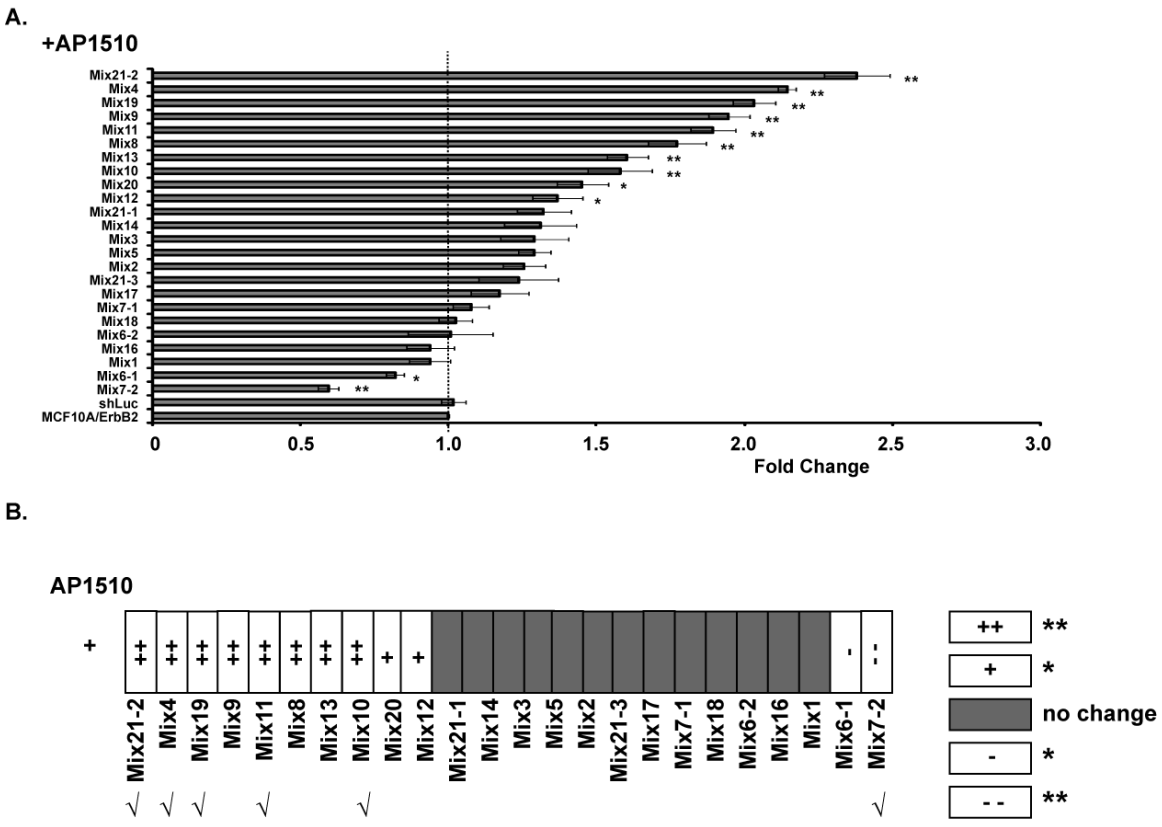

Figure 4-1: Loss-of-function screening of the PTPome to identify regulators of mammary epithelial cell motility. (A) The effect of the indicated pools of the PTP shRNAs on MCF10A/ErbB2 cell motility in the presence of ErbB2 activation. The MCF10A/ErbB2 cells were infected by indicated pools of shRNAs that target structurally similar PTPs. Data are presented as change in cell motility. Error bars represent S.E.M. (n=3; "*" P<0.05; "**" P<0.01). (B) Summary of the results in (A). "+" indicates an increase in cell motility. "-" indicates a decrease in cell motility. "√" represents the pools selected for further deconvolution.

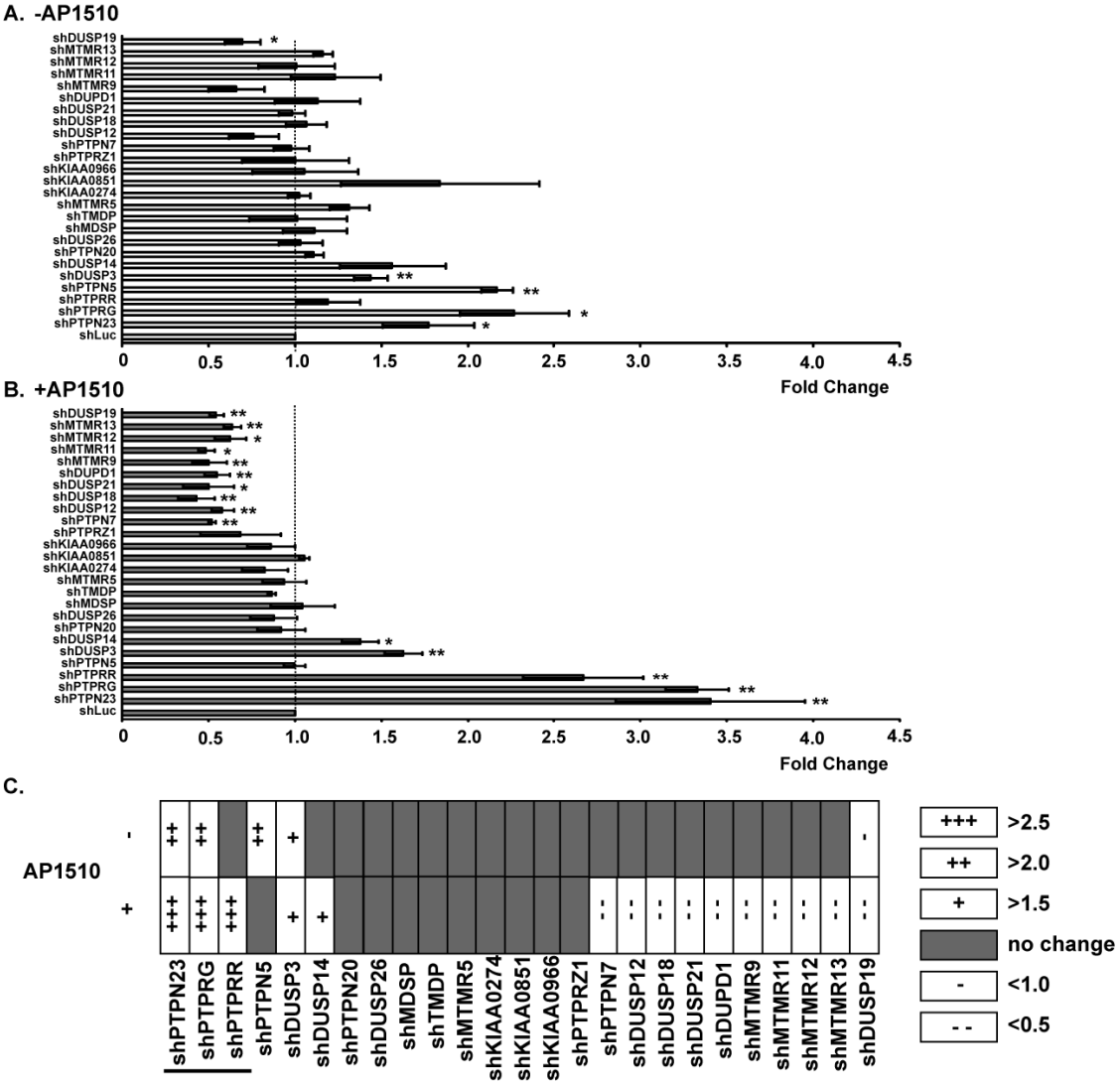

Figure 4-2: Loss-of-function screening of the PTPome to identify regulators of mammary epithelial cell motility. (A) The effect of the indicated PTPs on MCF10A/ErbB2 cell motility in the absence of ErbB2 activation. The MCF10A/ErbB2 cells were infected by the indicated PTP shRNA (pool of four shRNAs that targets one PTP). Cell motility was measured in the absence of ErbB2 activation. Data are presented as change in cell motility. Error bars represent S.E.M. (n=3; "*" P<0.05; "**" P<0.01). shLuc: MCF10A/ErbB2 cells that express shRNA targets to firefly luciferase. (B) The effect of the indicated PTPs on MCF10A/ErbB2 cell motility in the presence of ErbB2 activation. Cells were manipulated as described in (A) in the presence of ErbB2 activation. Data are presented as change in cell motility. Error bars represent S.E.M. (n=3; "*" P<0.05; "**" P<0.01). (C) Summary of the results presented in (A) and (B). "+" indicates an increase in cell motility. "-" indicates a decrease in cell motility. Underline marks the PTPs selected for further characterization.

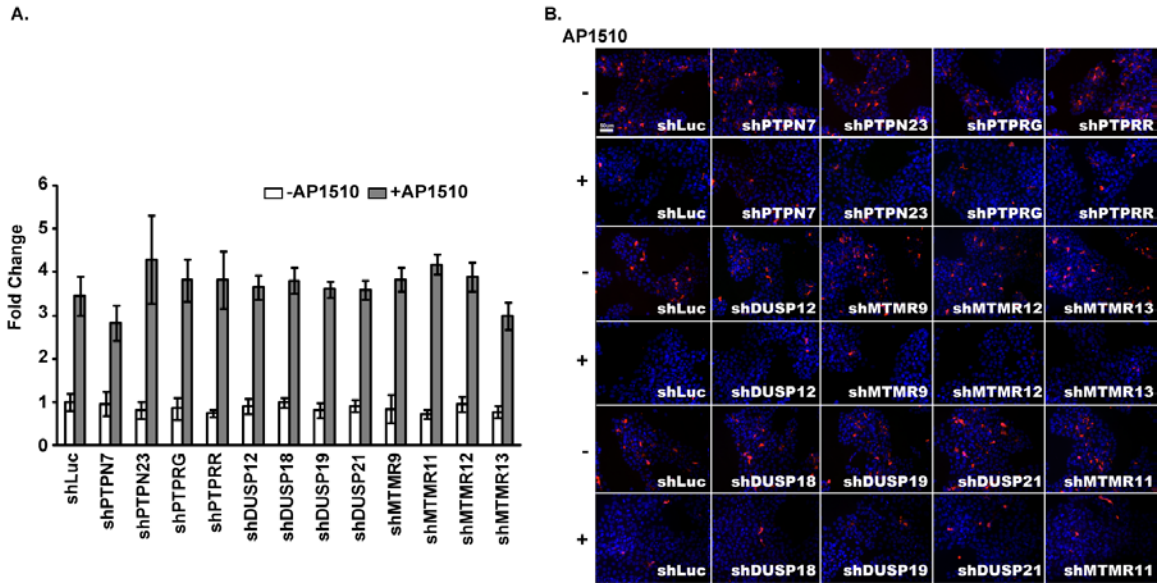

Figure 4-3: PTP shRNA does not affect MCF10A/ErbB2 cell proliferation and apoptosis. (A) PTP shRNA does not affect MCF10A/ErbB2 cell proliferation. Proliferation of MCF10A/ErbB2 cells that expressed the indicated shRNA was examined by Ki-67 staining. The percentage of Ki-67 positive cells was measured. At least 6 random fields were counted. The experiment was repeated for 3 times and the average was measured. Data are presented as change in cell proliferation. Error bars represent S.E.M. (n=3). (B) PTP shRNA does not affect MCF10A/ErbB2 cell death. Caspase 3 activity of MCF10A/ErbB2 cells that expressed indicated shRNA was measured by activated caspase 3 immunofluorescence staining.

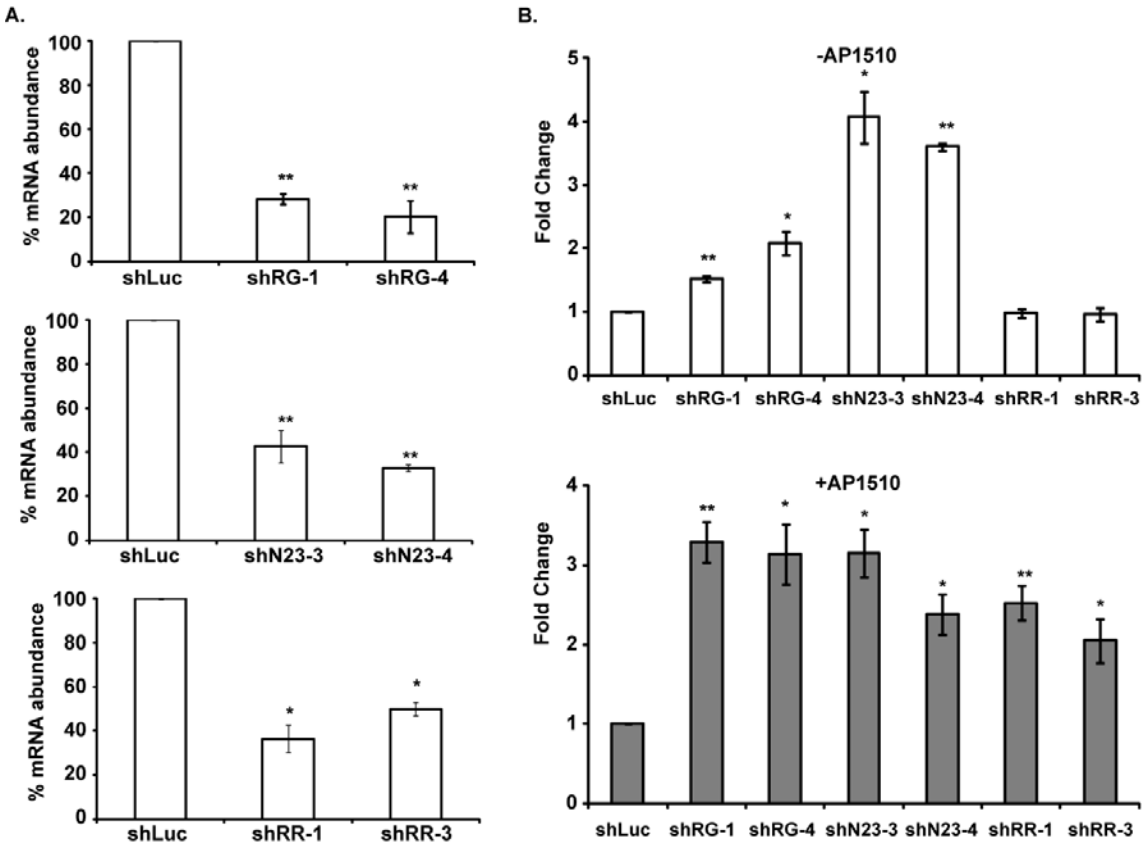

Figure 4-4: Suppression of PTPN23, PTPRG and PTPRR induce distinct stimulatory effects on mammary epithelial cell motility. (A) Suppression of PTPN23, PTPRG and PTPRR by shRNA. The mRNA level of the indicated PTPs was measured by qRT-PCR. Data are presented as change in mRNA level upon the expression of the indicated shRNA. Error bars represent S.E.M. (n=3; "*" P<0.05; "**" P<0.01). (B) PTPN23, PTPRG, and PTPRR are negative regulators of MCF10A/ErbB2 cell motility. The motility of MCF10A/ErbB2 cell that expressed the indicated PTP shRNA was measured in the absence (upper) or presence (lower) of ErbB2 activation. The motility of the control cells was normalized to 1. Error bars represent S.E.M. (n=3; "*" P<0.05; "**" P<0.01).

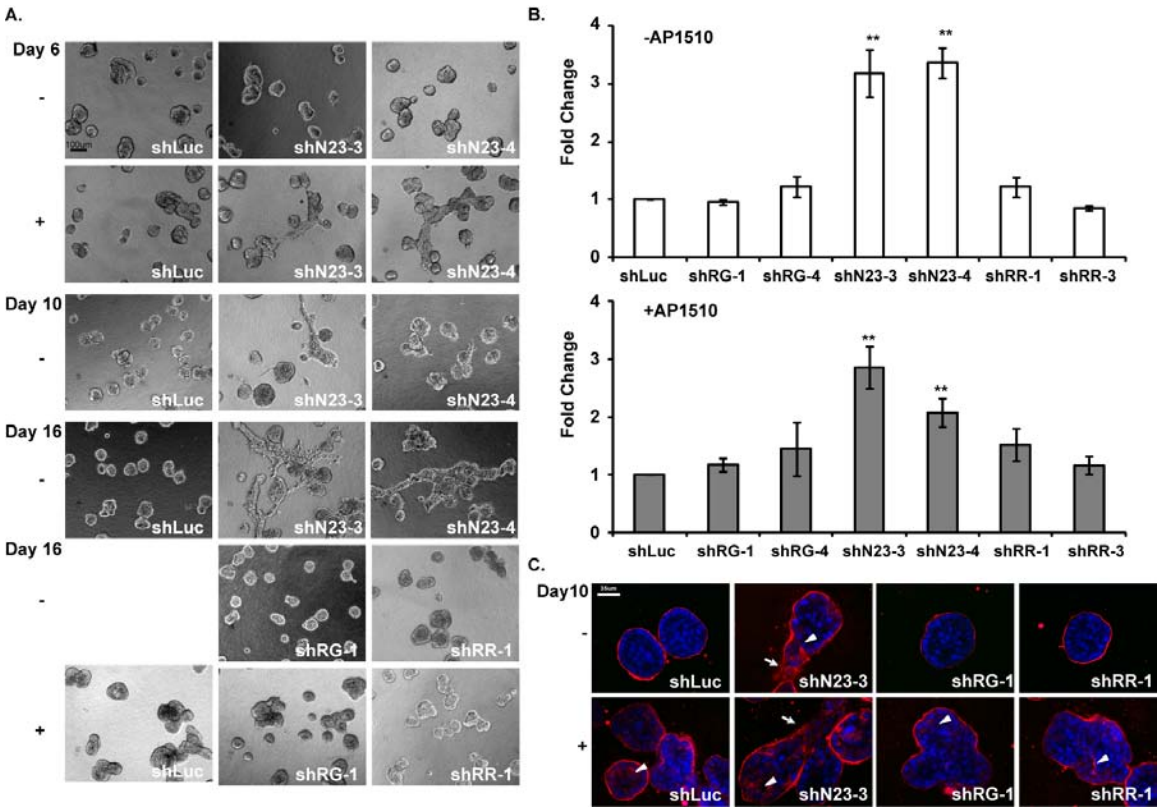

Figure 4-5: Suppression of PTPN23, but not PTPRG or PTPRR, promotes mammary epithelial cell invasion. (A) Suppression of PTPN23, but not PTPRG or PTPRR, induces MCF10A/ErbB2 cell invasion. Cell invasion of the indicated cell line was measured using the 3D invasion assay. Scale bar represents 100μm. (B) Quantification of the cell invasion. The invasion ability of the MCF10A/ErbB2 cell that expressed the indicated shRNA was quantified. Data are presented as change in cell invasion. Error bars represent S.E.M. (n=3; "*" P<0.05; "**" P<0.01). (C) Suppression of PTPN23 promotes MCF10A/ErbB2 cell to break through the basal membrane. MCF10A/ErbB2 acini that expressed the indicated shRNAs were stained with laminin V antibody in the absence or presence of ErbB2 activation. Arrows indicate cell invasion. Arrow heads indicate laminin V mislocalization. Scale bar represents 35μm.

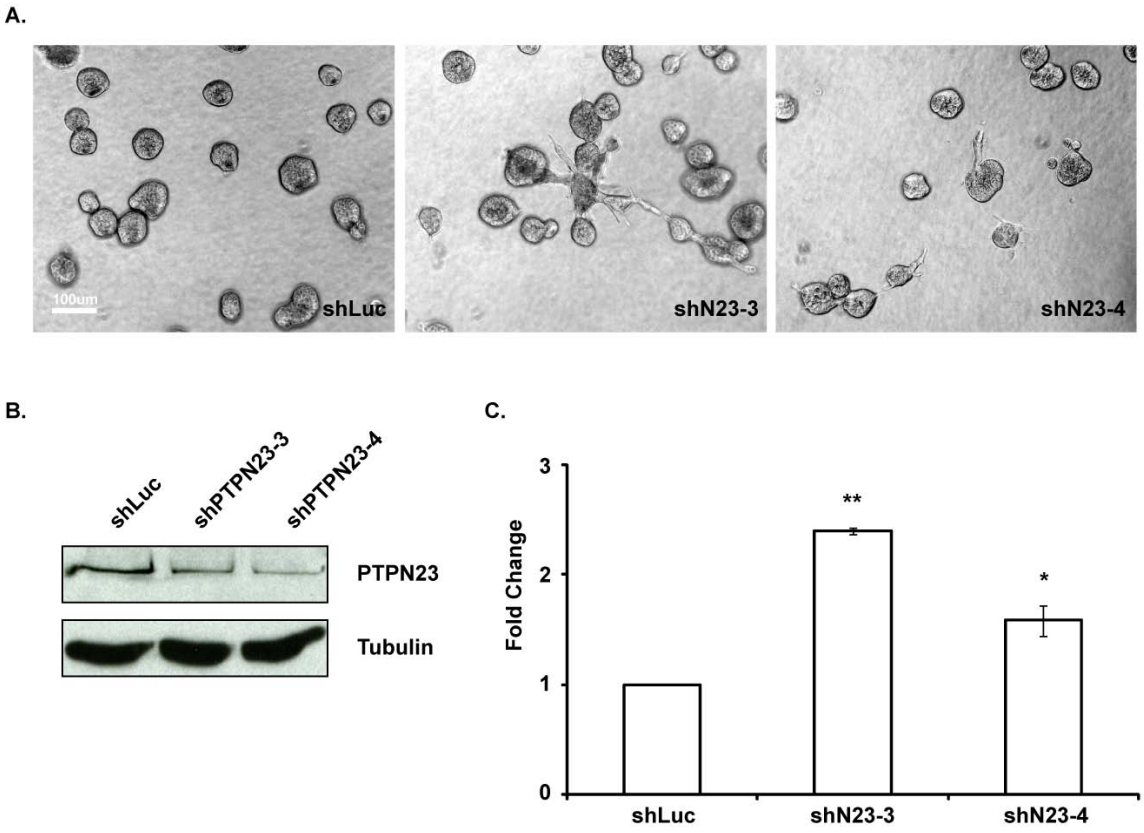

Figure 4-6: Suppression of PTPN23 induces MCF10A cell invasion. (A) Suppression of PTPN23 induces MCF10A cell invasion. The ability of shN23 to induce cell invasion was examined in 3D invasion assay. Morphology of the representative acini was photographed. Scale bar represents 100μm. (B) PTPN23 shRNA suppresses the expression of PTPN23 at protein level. Expression of PTPN23 was examined in MCF10A cells that expressed the indicated shRNA. The expression of α-tubulin was used as the loading control. (C) Quantification of cell invasion induced by shN23. The ability of shN23 to induce cell invasion was quantified. Data are presented as change in cell invasion. Error bars represent S.E.M. (n=3; "*" P<0.05; "**" P<0.01).

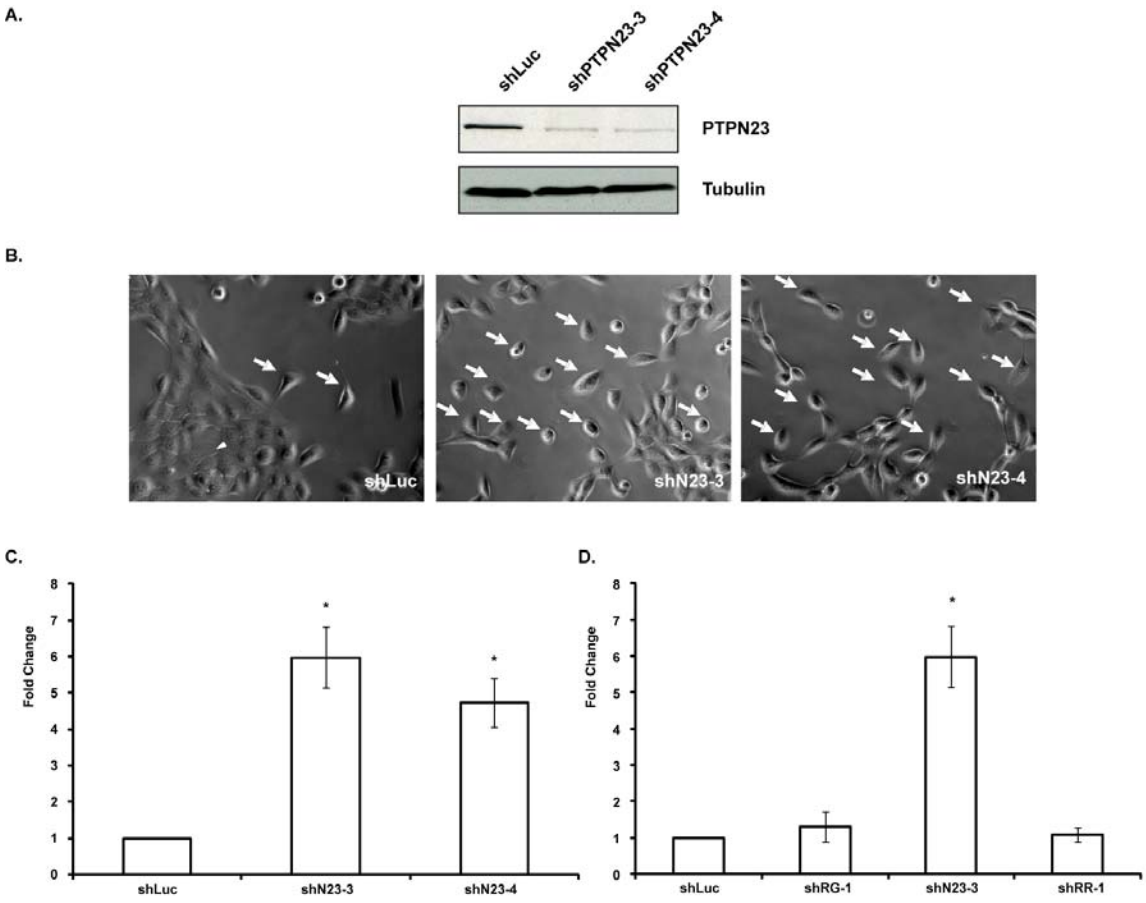

Figure 4-7: Suppression of PTPN23 induces mammary epithelial cell scattering. (A) PTPN23 shRNA suppresses the expression of PTPN23 at protein level. The expression of PTPN23 was examined in confluent MCF10A/ErbB2 cells that expressed the indicated shRNA. The expression of α-Tubulin was used as the loading control. (B) Suppression of PTPN23 induces MCF10A/ErbB2 cell scattering. Morphology of the MCF10A/ErbB2 cell that expressed the indicated shRNA was photographed in sub-confluent culture. Arrows indicate cell scattering. Arrowhead indicates cell cluster. Scale bar represents 100 μm. (C) Quantification of cell scattering. Cell scattering was quantified by counting the number of the single cells per filed. At least 5 random fields were measured in each condition. Data are presented as change in cell scattering. Error bars represent S.E.M. (n=3; "*" P<0.05). (D) Suppression of PTPN23, but not PTPRG or PTPRR, induced cell scattering. Cell scattering was quantified as in (C). Error bars represent S.E.M. (n=3; "*" P<0.05).

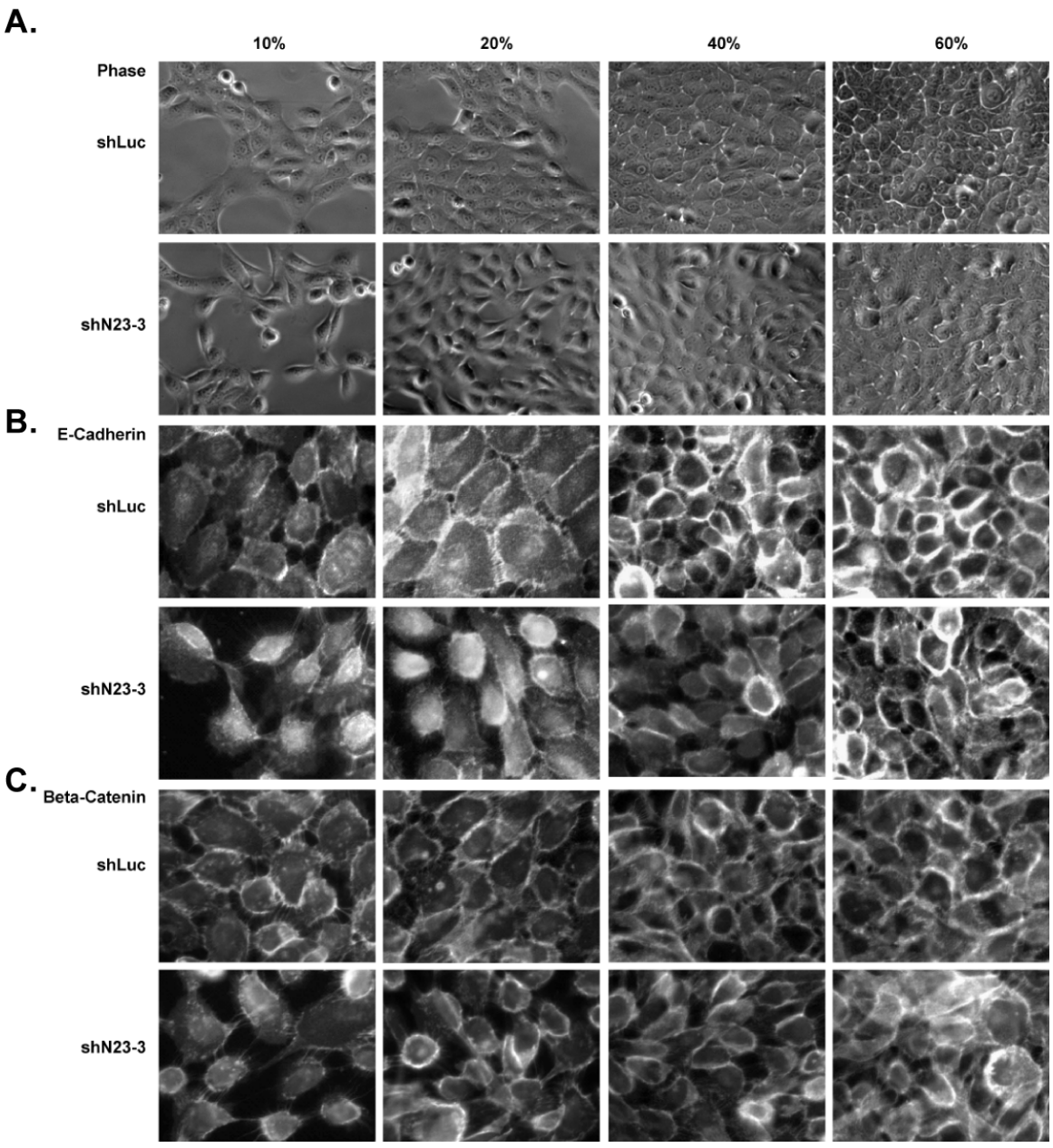

Figure 4-8: Suppression of PTPN23 delays the formation of adherens junctions. MCF10A/ErbB2 cells that expressed the indicated shRNAs were as indicated. Images of the phase or E-cadherin and β-catenin immunofluorescence staining were taken. Scale bars represent 100μm or 20μm, respectively.

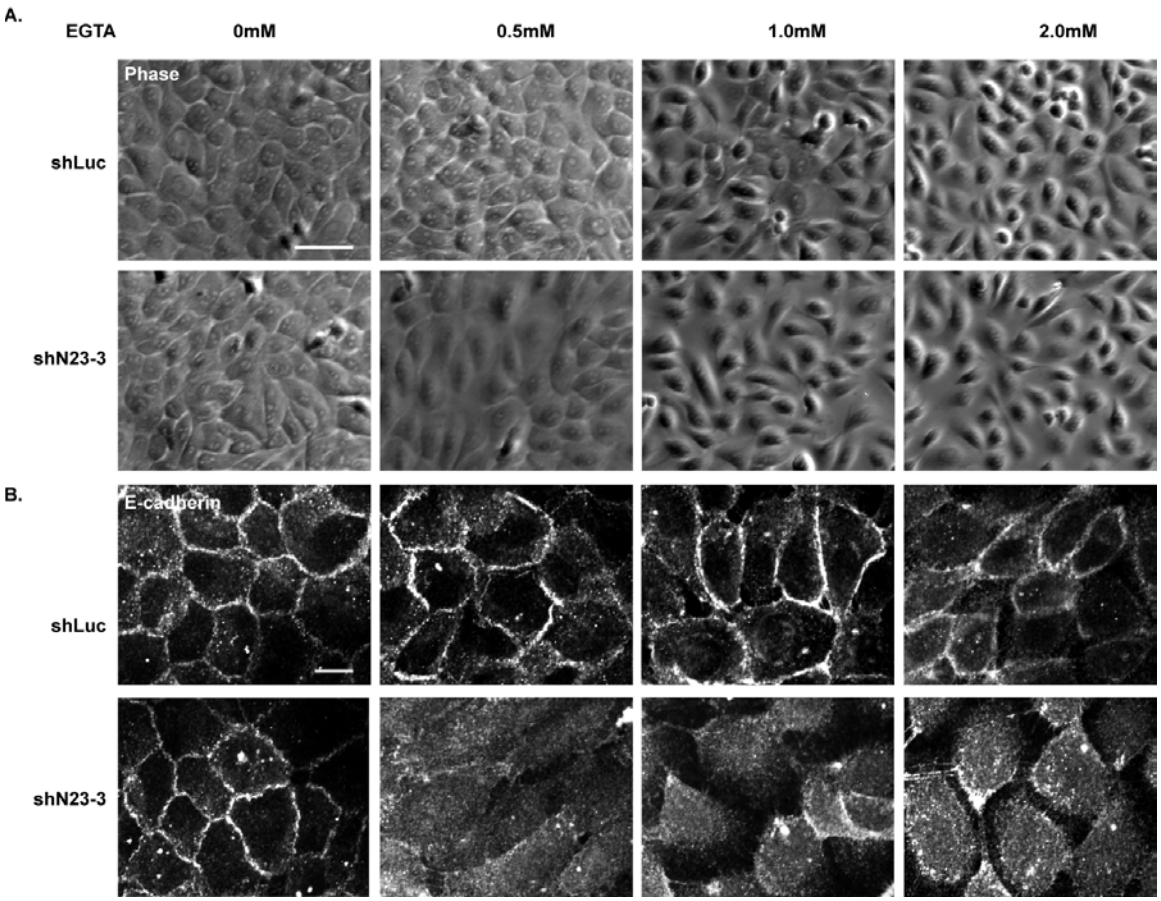

Figure 4-9: Suppression of PTPN23 sensitizes adherens junctions to EGTA treatment. MCF10A/ErbB2 cells that expressed the indicated shRNAs were grown in confluent culture. The indicated concentration of EGTA was added to the cells for 1 hour. Images of the phase or E-cadherin immunofluorescence staining were taken. Scale bars represent 100μm or 20μm, respectively.

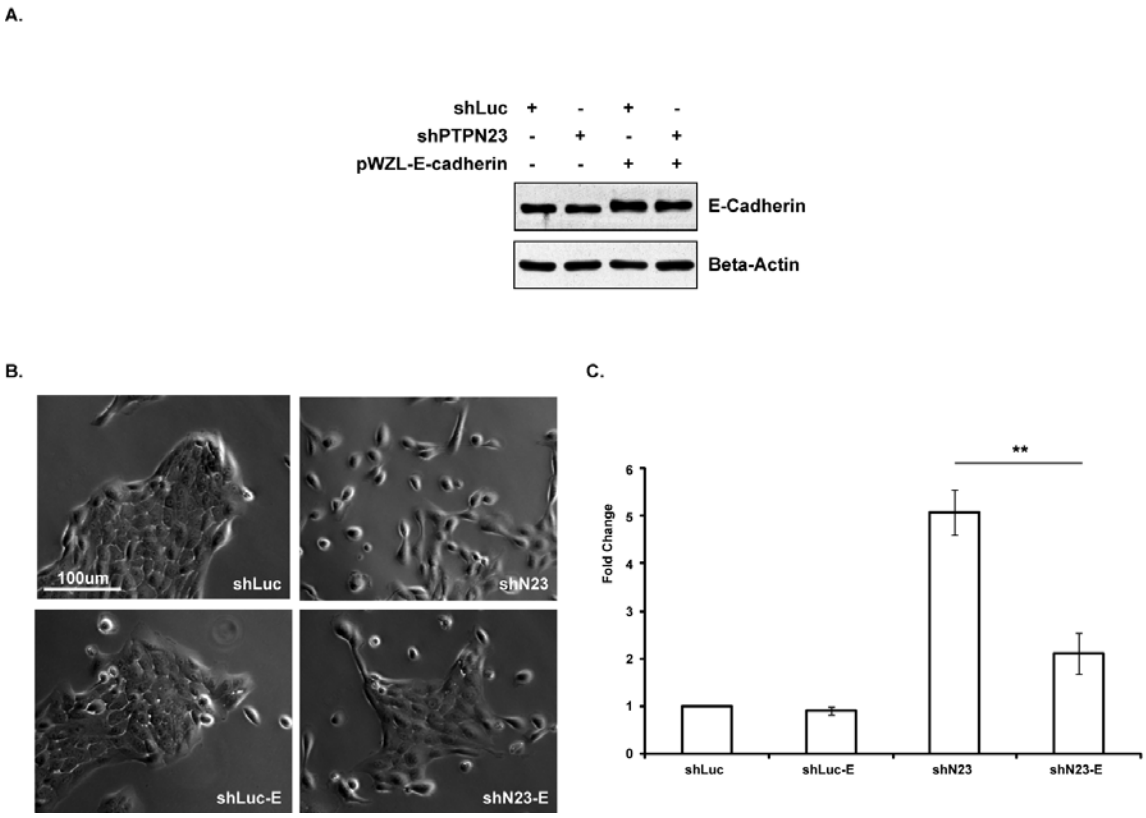

Figure 4-10: Ectopic expression of E-cadherin blocks cell scattering induced by PTPN23 depletion (A) Ectopic expression of E-cadherin in MCF10A/ErbB2 cells that expressed the indicated shRNA. The expression of β-actin was used as the loading control. Relative signal intensity was quantified using image J software. (B, C) Ectopic expression of E-cadherin rescued shN23-induced cell scattering. The morphology of the MCF10A/ErbB2 cells that expressed the indicated constructs was imaged (B) and quantified (C). Error bars represent S.E.M. (n=3; "**" P<0.01).

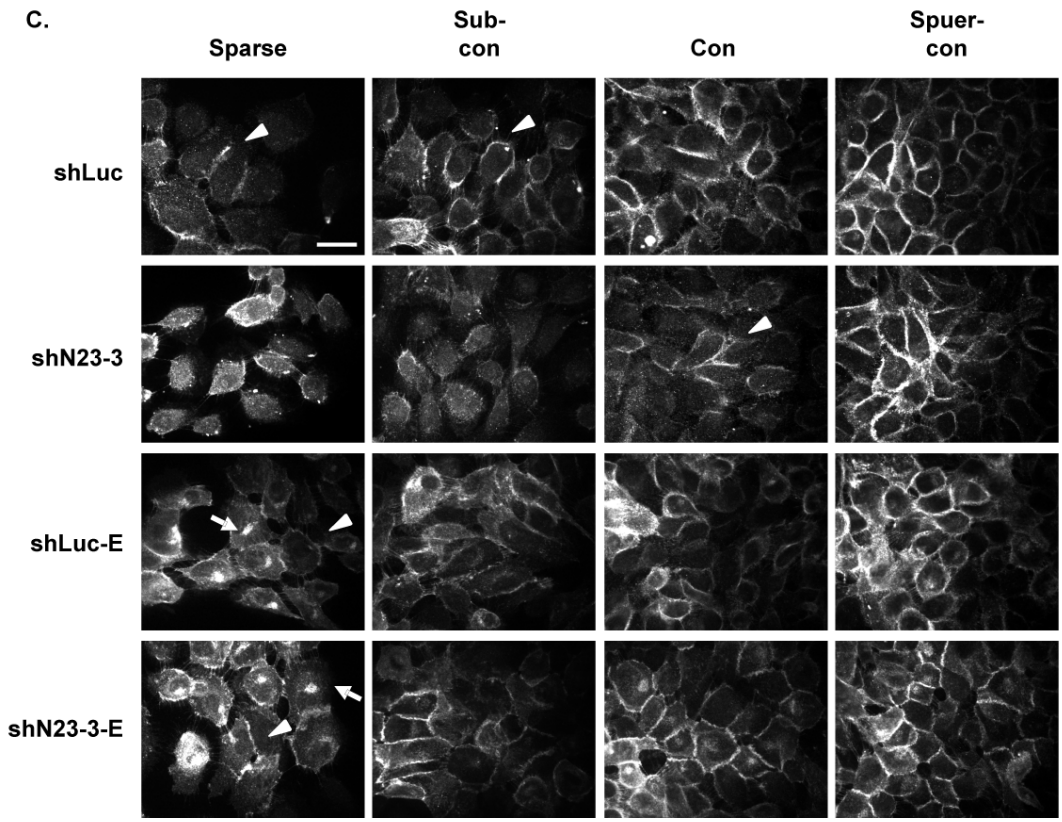

Figure 4-11: Ectopic expression of E-cadherin rescues shN23-induced impairment of the adherens junctions. The indicated MCF10A/ErbB2 cell lines were grown as indicated. Images of E-cadherin immunofluorescence staining were taken. Arrowheads indicate adherens junctions. Arrows indicate the accumulation of ectopically expressed E-cadherin. Scale bar represents 20 μm.

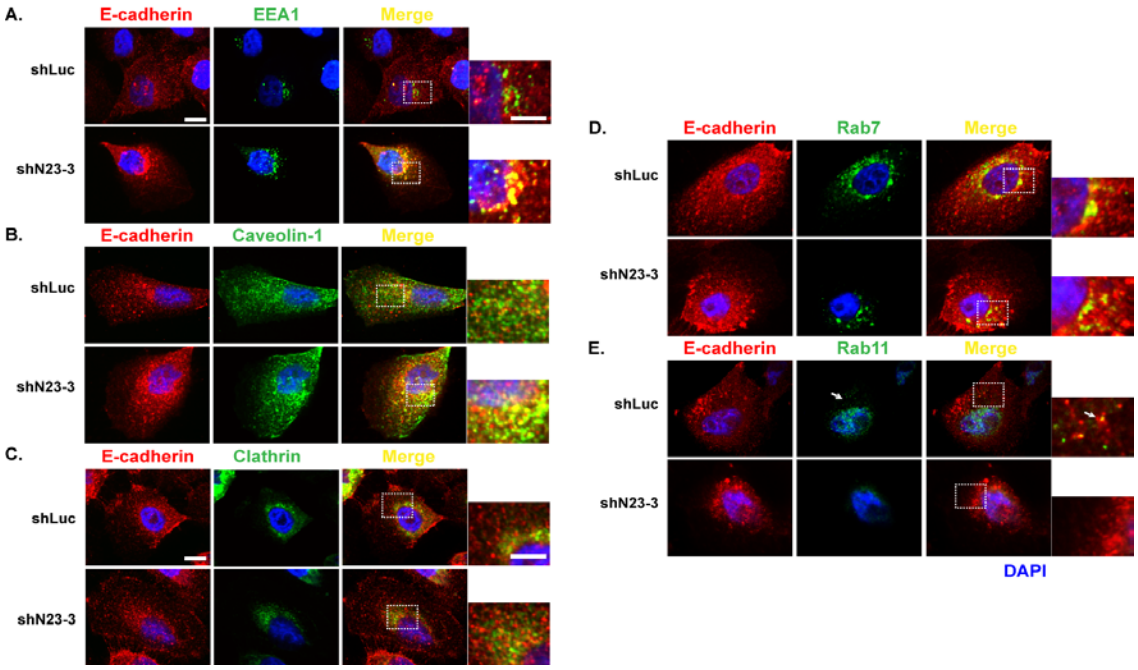

Figure 4-12: Colocalization of E-Cadherin with EEA1(A), Caveolin-1(B), Clathrin (C), Rab7(D) and Rab11(E) in sparse MCF10A/ErbB2 cells that expressed the indicated shRNA. Arrow indicates Rab11 vesicles. Scale bar represent 10 μm. The selected regions were enlarged in the right corner. Scale bar represents 5.5 μm.

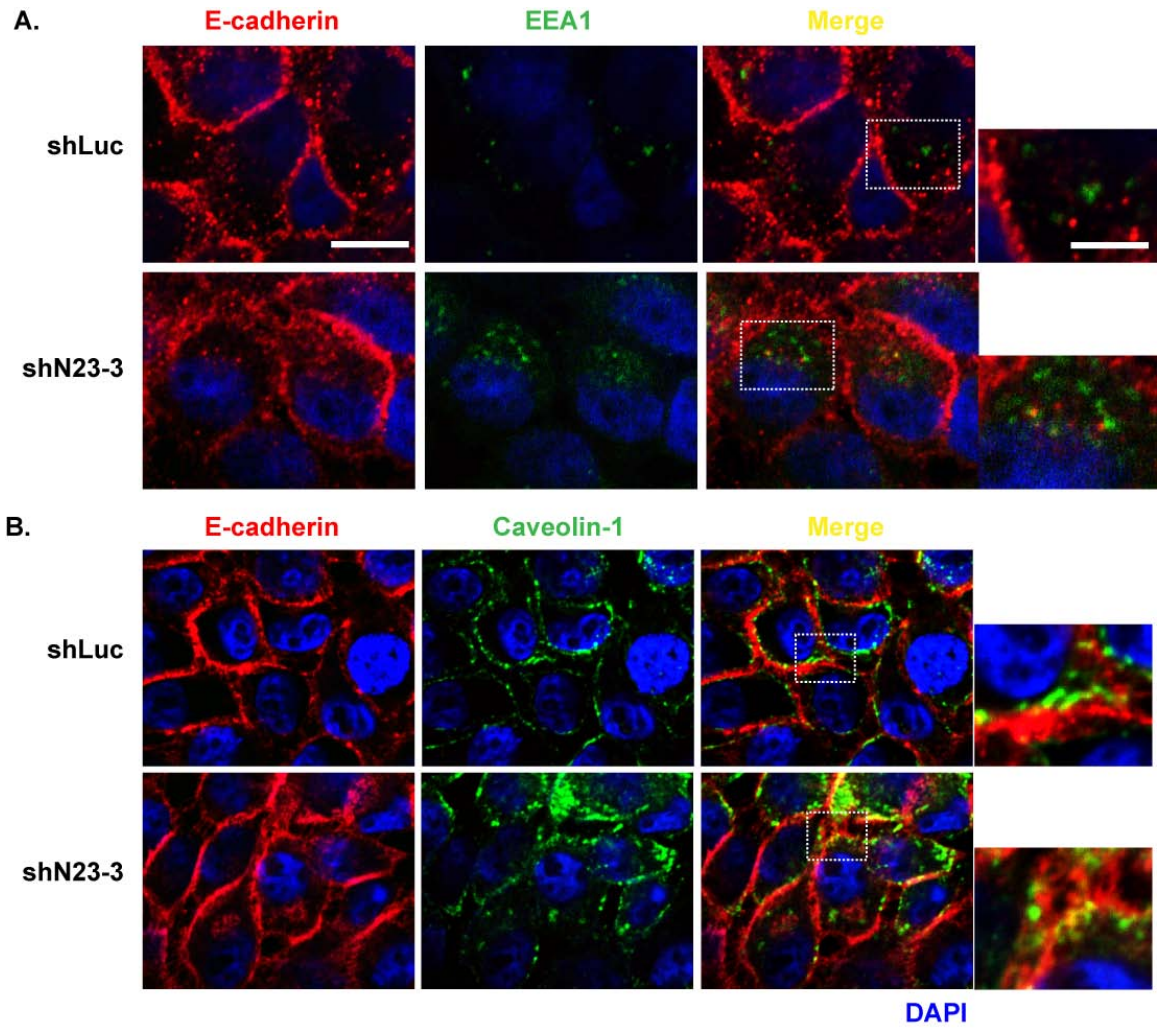

Figure 4-13: Colocalization of E-Cadherin with EEA1(A) and Caveolin-1(B), in confluent MCF10A/ErbB2 cells that expressed the indicated shRNA. Scale bar represent 10 µm. The selected regions were enlarged in the right corner. Scale bar represents 5.5 µm.

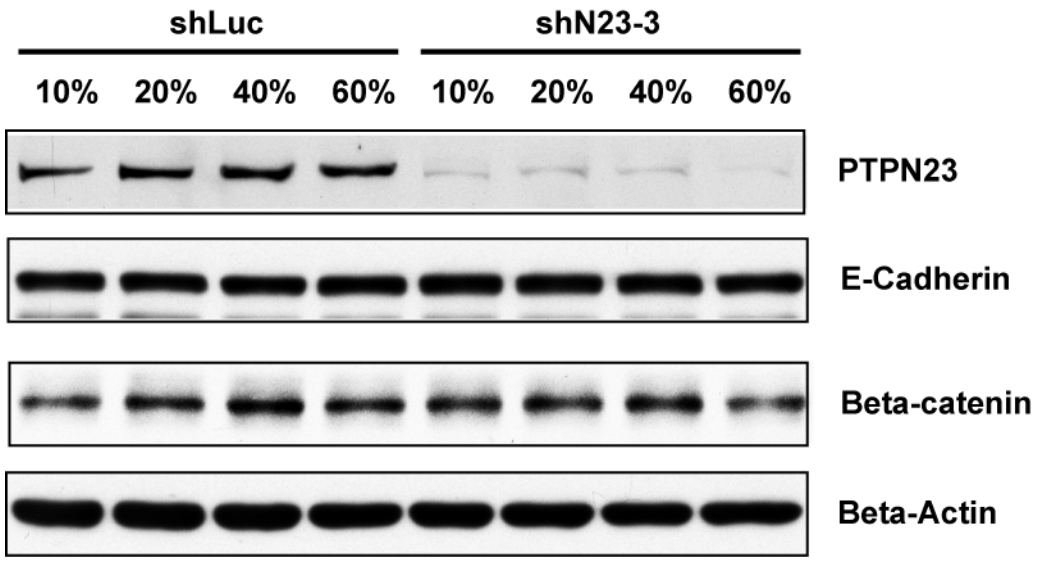

Figure 4-14: Suppression of PTPN23 does not affect E-cadherin or β-catenin expression. MCF10A/ErbB2 cells that expressed the indicated shRNAs were grown as indicated. The expression of E-cadherin and β-catenin were examined. The expression of β-actin was used as the loading control.

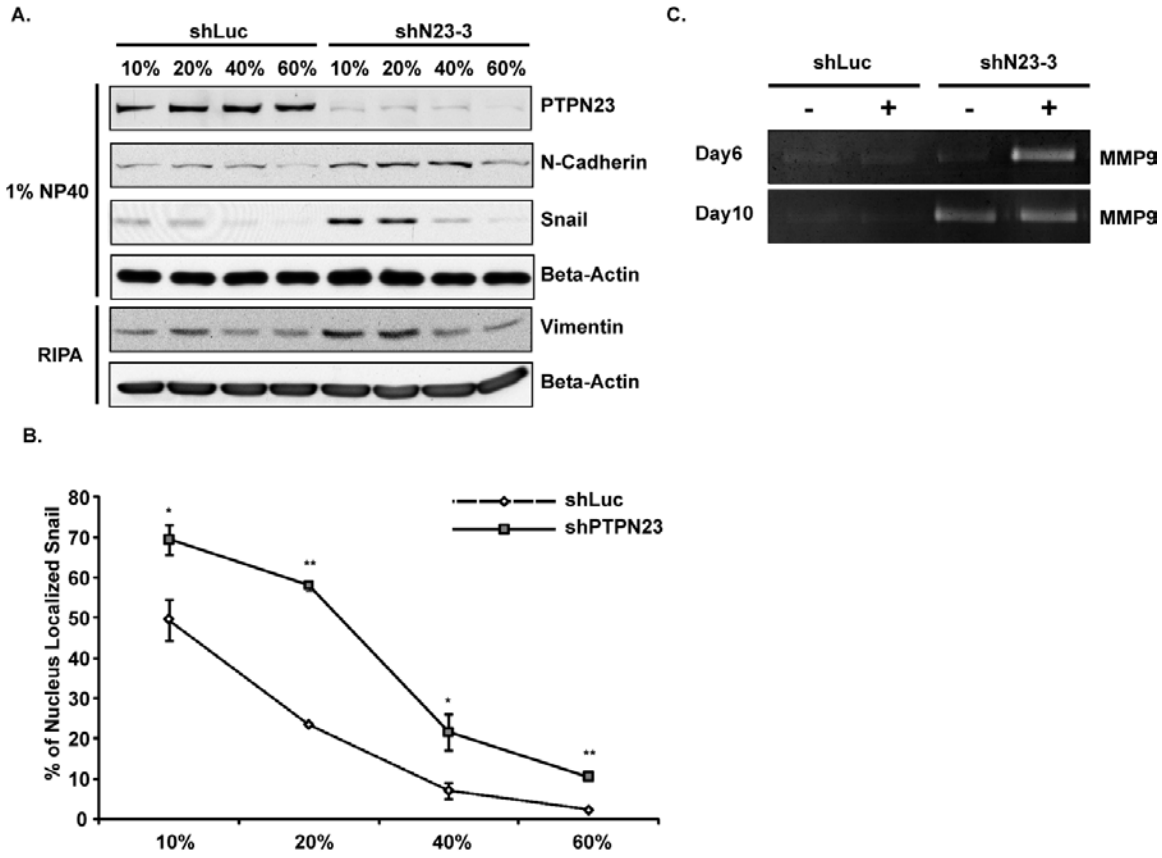

Figure 4-15: Suppression of PTPN23 induces the expression of mesenchymal proteins. (A) Suppression of PTPN23 induces the expression of mesenchymal proteins. The expression of mesenchymal protein was examined in MCF10A/ErbB2 cells that expressed indicated shRNA. Cells were grown as described. The expression of β-actin was used as the loading control. (B) Suppression of PTPN23 enhances nucleus localization of Snail. Nuclear localization of Snail in MCF10A/ErbB2 cells that expressed indicated shRNA was measured. Cells were grown as indicated. The percentage of nuclear localized Snail was quantified as the average of 8 random fields. Data were presented as percentage of nucleus localized Snail. Error bars represent S.E.M. (n=3; "*" P<0.05; "**" P<0.01). (C) Suppression of PTPN23 induces the expression of MMP9. The MMP activity of the MCF10A/ErbB2 cells that were grown in the indicated condition was measured.

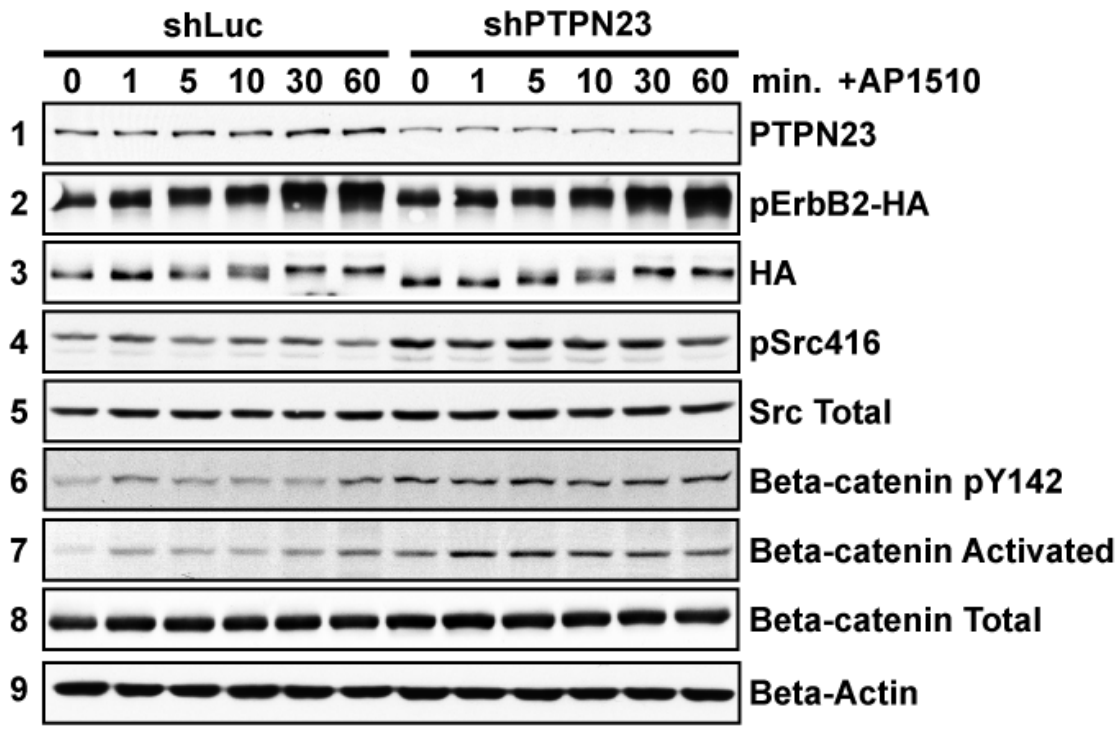

Figure 4-16: Suppression of PTPN23 activates SFK and β-catenin. The tyrosine phosphorylation of the indicated proteins was measured in 293-B2 cells that expressed shLuc or shN23 upon ErbB2 activation. The expression of β-actin was used as the loading control.

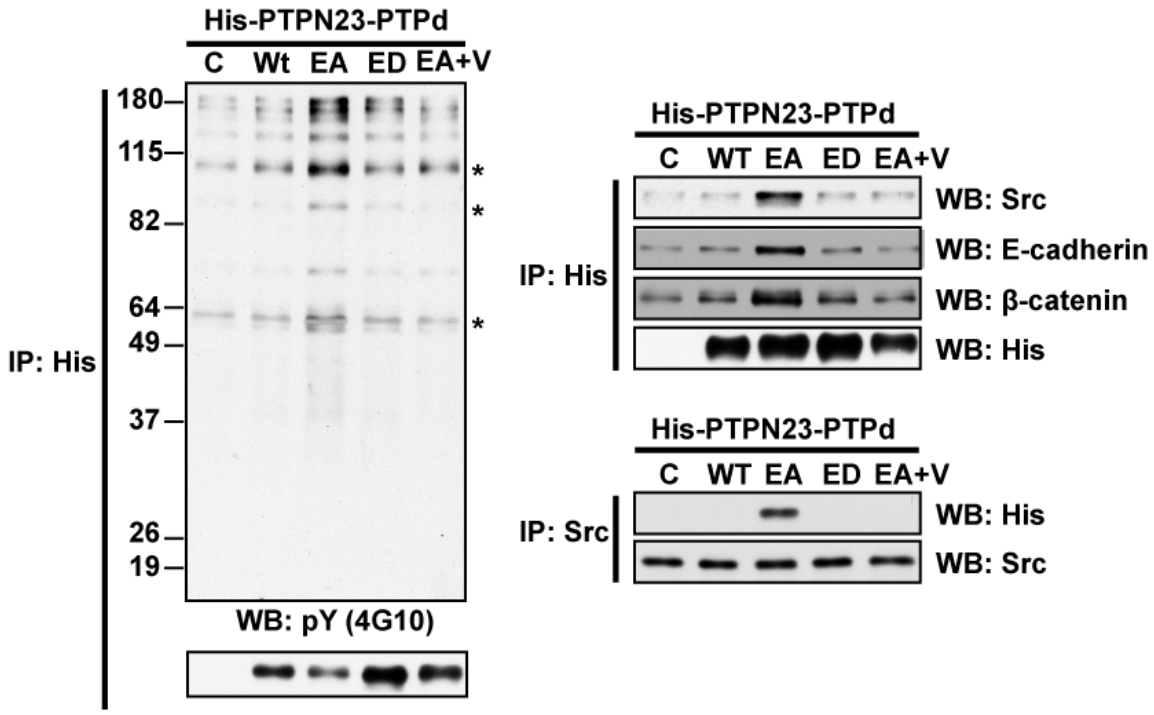

Figure 4-17: E-cadherin, β-catenin and Src are direct substrate of PTPN23. The indicated purified recombinant His-tagged proteins were used to pull down their potential substrates from pervanadate treated MCF10A/ErbB2 lysate and were then detected by anti-phosphotyrosine antibody (Left) or anti-E-cadherin, anti-β-catenin and anti-Src antibody (Right; upper); empty vector control (EV), wild type (Wt), E1357A (EA), E1357D (ED) or E1357A plus vanadate (EA+V). In reverse, endogenous Src was precipitated. Recombinant PTPN23 PTP domains coprecipitated with Src were detected by anti-His tag antibody (Right; lower). Stars mark the position of E-cadherin, β-catenin and Src.

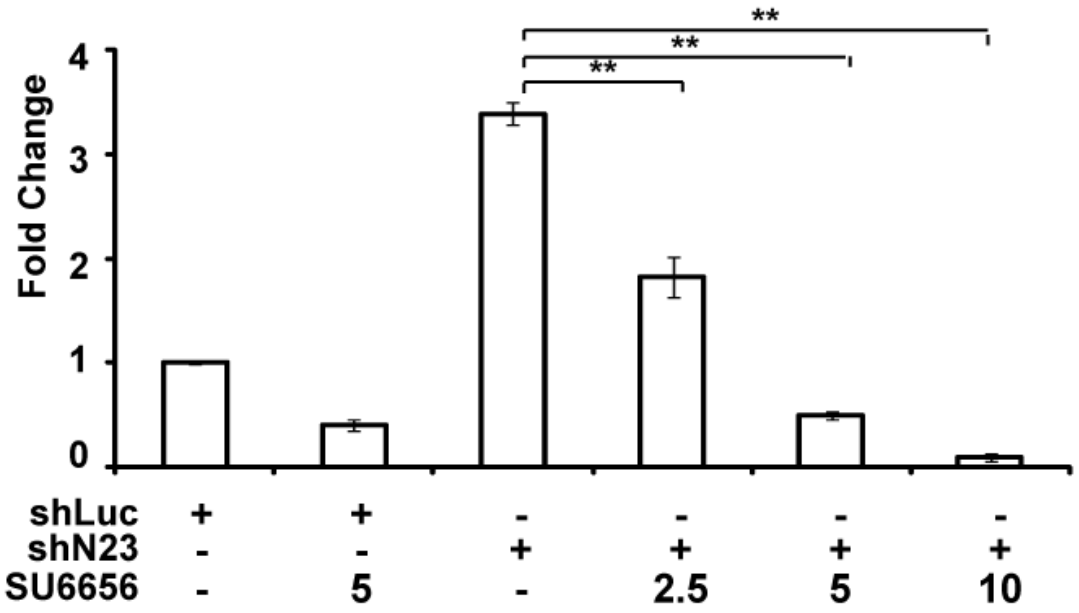

Figure 4-18: Suppression of SFK activity inhibits shN23-induced MCF10A/ErbB2 cell motility. The motility of the MCF10A/ErbB2 cell that expressed the indicated shRNAs was measured in the absence or presence of indicated amount of SU6656. Data are presented as change in cell motility. Error bars represent S.E.M. (n=3; “**” P<0.01).

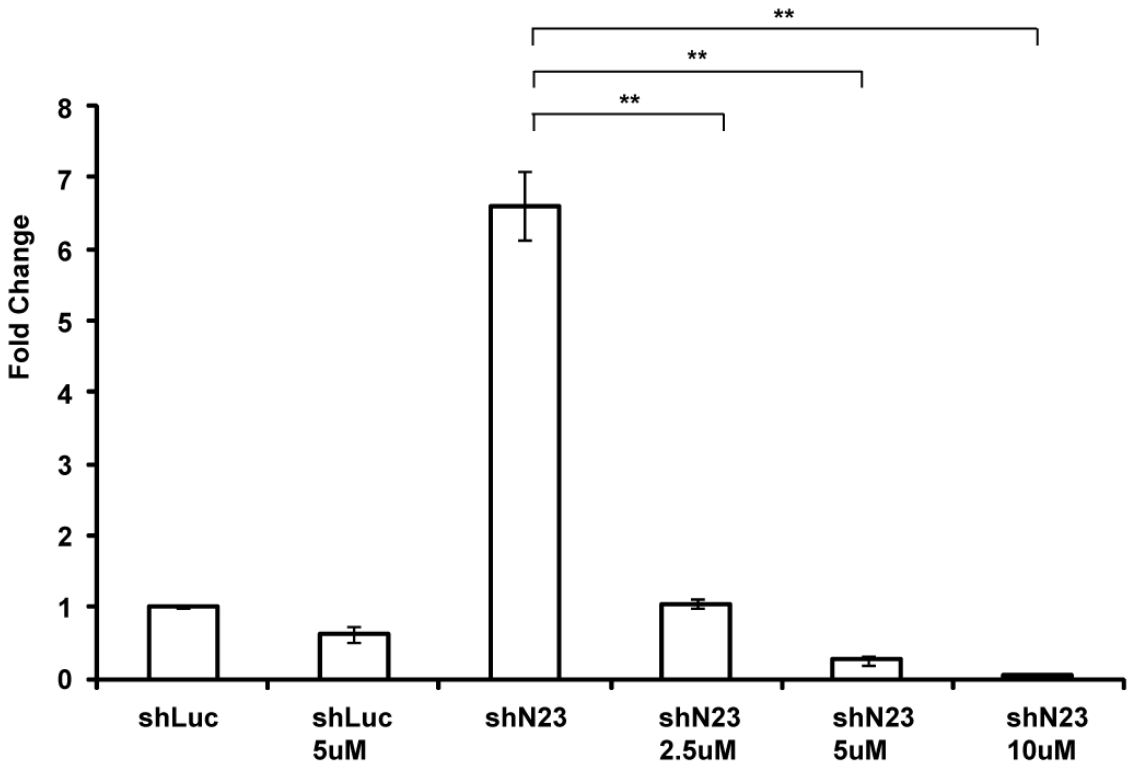

Figure 4-19: Suppression of SFK activity inhibits shN23-induced MCF10A/ErbB2 cell invasion. The invasion of the MCF10A/ErbB2 cell that expressed the indicated shRNAs was quantified in the absence or presence of indicated amount of SU6656. Data are presented as change in cell invasion. Error bars represent S.E.M. (n=3; “**” P<0.01).

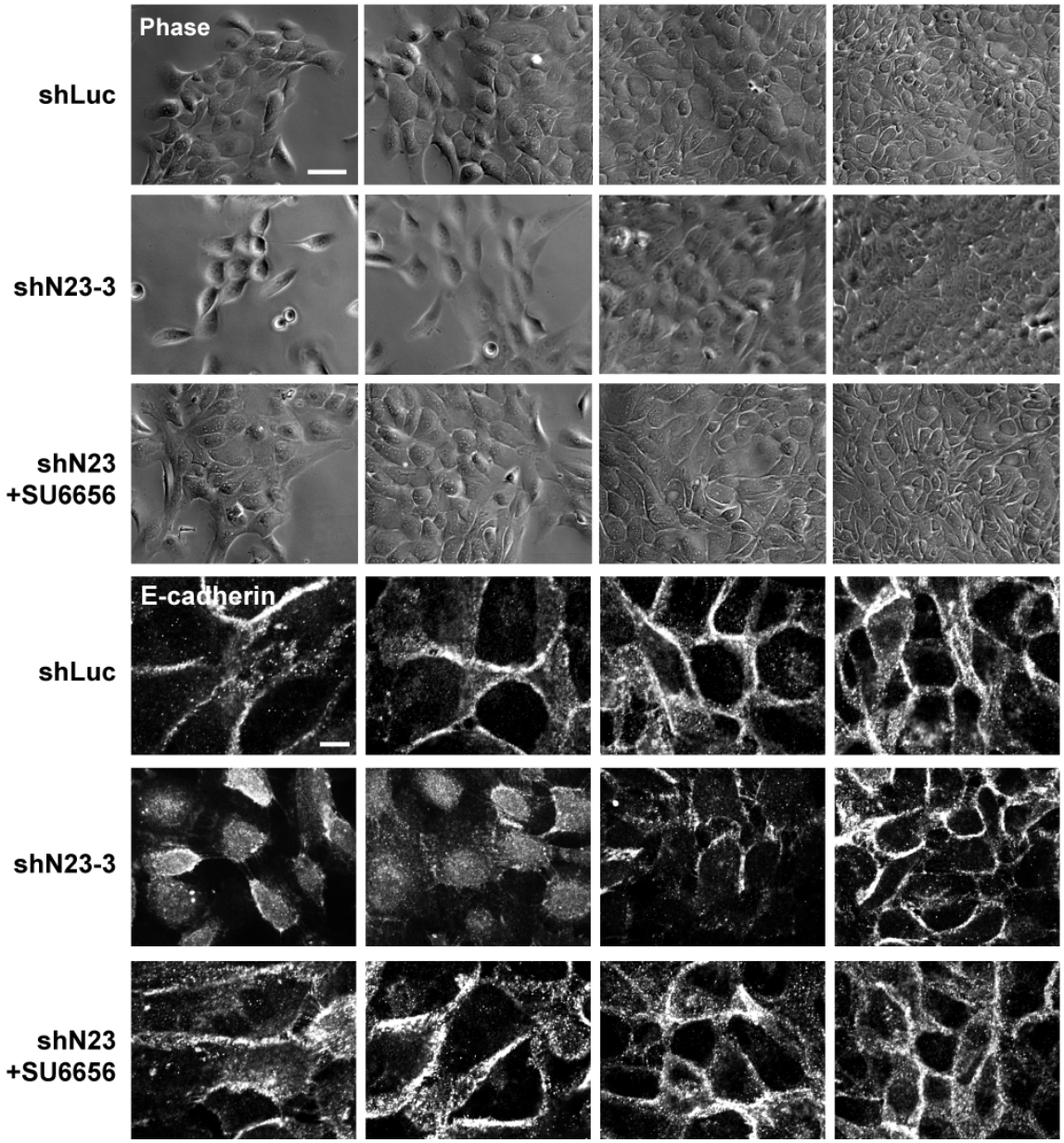

Figure 4-20: Suppression of SFK activity inhibits shN23-induced cell scattering and disruption of adherens junctions. MCF10A/ErbB2 cells that expressed the indicated shRNAs were grown as indicated in the absence or presence of 5μM of SU6656. Images of the phase (A) or E-cadherin immunofluorescence staining (B) were taken. Scale bars represent 100μm or 20μm, respectively.

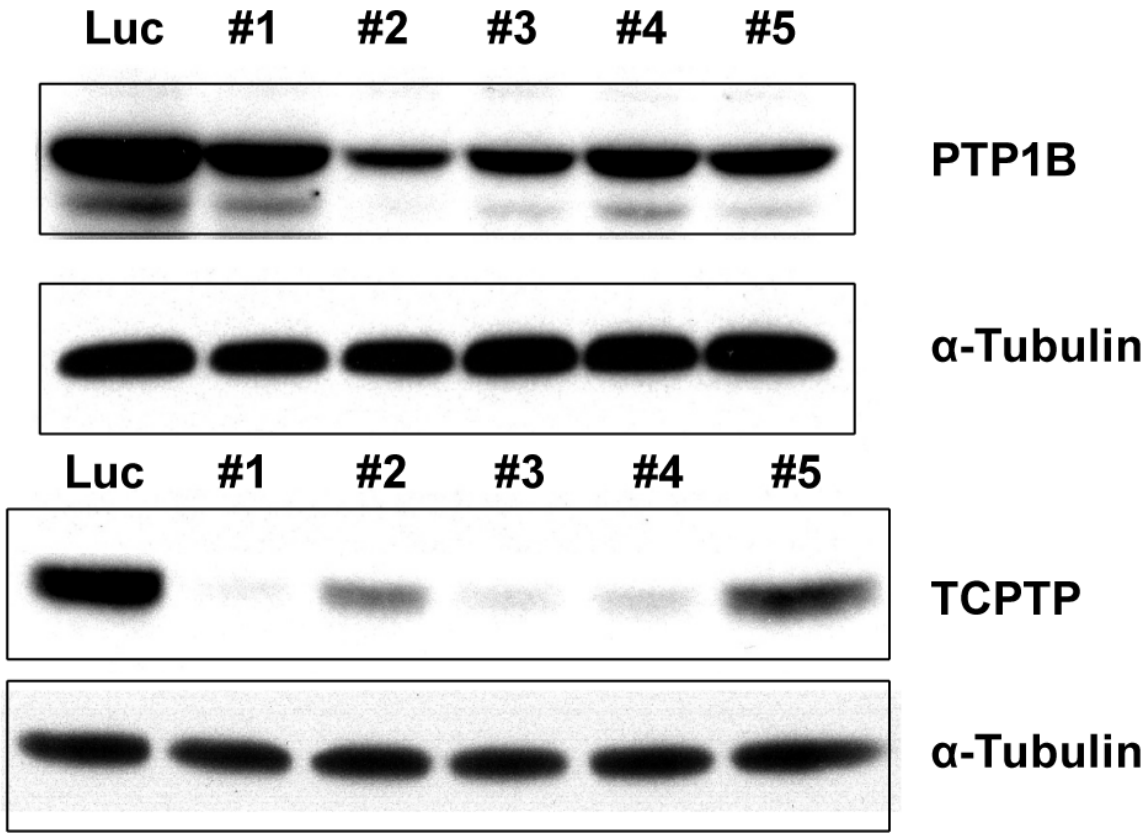

Figure 4-21: Suppression of PTP1B and TCPTP by shRNA. Total cell lysate from MCF10A/ErbB2 cells that expressed the indicated shRNA was used for analyzing the expression of PTP1B or TCPTP. The expression of α-tubulin was used a loading control.

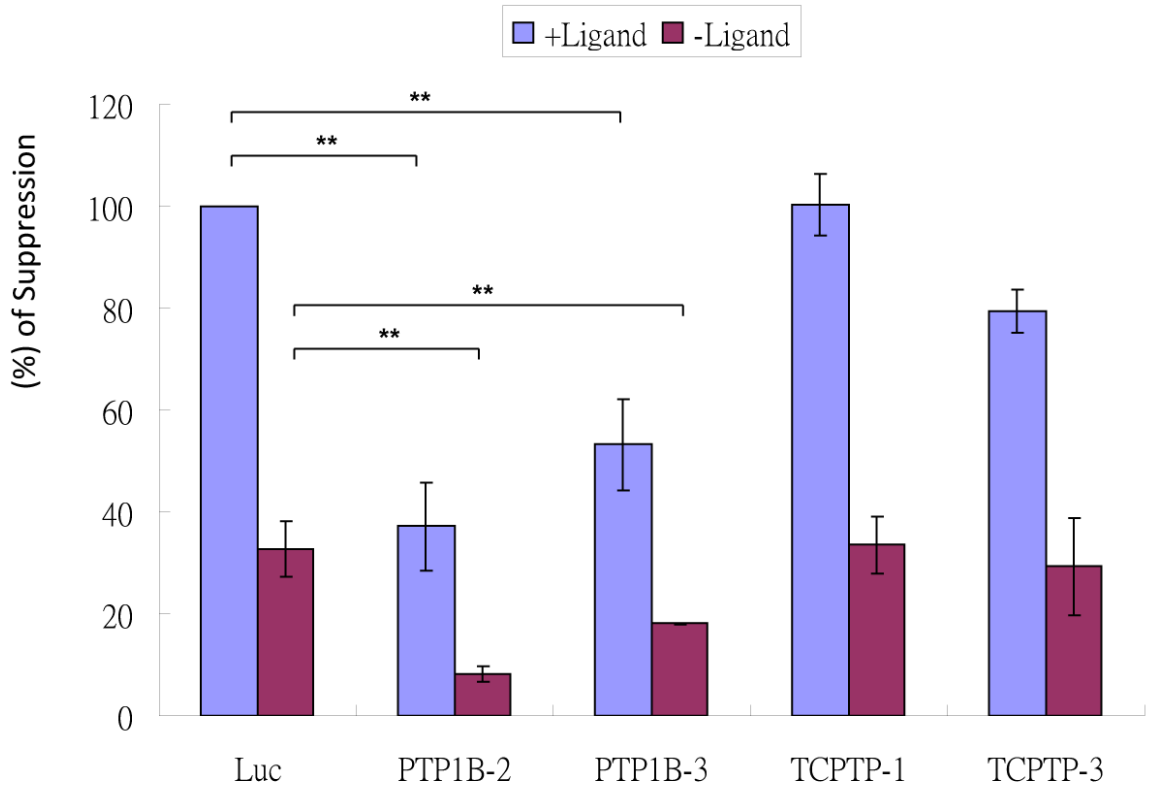

Figure 4-22: Suppression of PTP1B, but not TCPTP, inhibits cell motility. The cell motility of MCF10A/ErbB2 cell that expressed the indicated shRNA was examined in the absence (purple) or presence (blue) of ErbB2 activation. Data are presented as percentage to the control cells with ErbB2 activation. Error bars represent S.E.M. (n=3; "**" P<0.01).

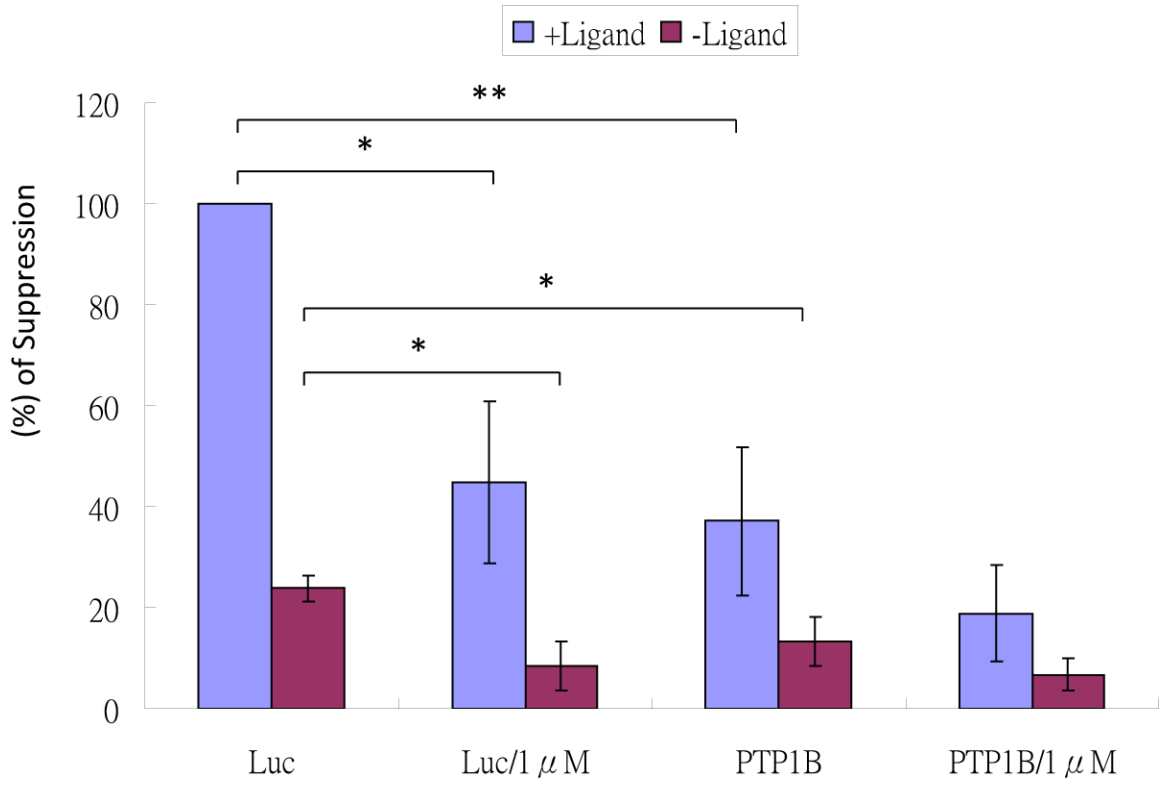

Figure 4-23: PTP1B inhibitor, MSI-1873, inhibits cell motility. The motility of MCF10A/ErbB2 cell that expressed the indicated PTP shRNA were examined in the absence (purple) or presence (blue) of 1μM of AP1510 and 1μM of MSI-1873. Data are presented as percentage to the control cells with ErbB2 activation. Error bars represent S.E.M. (n=3; “*”<0.05; “**” P<0.01).

Chapter 5: Conclusion and Future Directions.

I have constructed a retroviral-based PTP shRNA library which contains ~5 shRNAs that target each member of the PTP superfamily of enzymes (~500 shRNAs in total). I have illustrated an application of this shRNA library in the systematic study of the function of the PTP family in mammary epithelial motility and invasion. In the screen, I identified PTPs that can either function as positive or negative regulators of mammary epithelial motility. I have characterized further three PTPs, PTPN23, PTPRG and PTPRR, which enhanced cell motility when their expression was suppressed. I have found that suppression of PTPN23, but not PTPRG or PTPRR induced cell invasion, demonstrating that PTPN23 is a novel regulator of mammary epithelial cell motility and invasion. During the last part of my study, I have characterized thoroughly the function of PTPN23 in mammary epithelial cell motility and invasion. All in all, my study highlights the potential to examine systematically the function of PTP family by using RNAi-mediated loss-of-function screens.

In general, the approach involves several steps (Figure 5-1), the construction of the PTP shRNA library, the RNAi-mediated loss-of-function screen in a particular human-cell-line-based system, the validation of the identified PTP candidates and the detailed characterization of the identified PTP candidates.

5.1 Construction of the PTP shRNA library and the design of RNAi-mediated loss-of-function screen:

I have constructed a PTP shRNA library that can be utilized in any human cell-line-based system to study systematically the function of the PTP family. The first step of the screen is to generate the stable cell lines that express the shRNAs. In order to speed up the screen, I sub-divided the shRNA library into pools, according to the structural similarity of their target PTPs. The advantage of this strategy is that it speeds up and reduces the work load of the screen. However, as I have mentioned earlier, the disadvantage of this strategy is the potential for masking effects. To reduce the potential masking effect introduced by combining the shRNAs, I pooled the shRNA according to the structural similarity of their target PTPs. In addition, smaller pools of shRNAs that contain four shRNAs to target the same PTP can be used in the future.

The proper use of external and internal controls is another important issue in conducting the screen. I used shRNA that targets firefly luciferase, which is not expressed in MCF10A cells, as my external negative control. Because it had yet to be determined whether most of the PTPs have a function in mammary epithelial cell motility, I decided to use shRNA that targets a non-PTP protein, AF6, as my external positive control. AF6 is a multi-domain scaffold protein that is involved in signaling that controls the organization of cell junctions. It can bind to Ras and is also an effector of Rap1, a Ras-like small GTPase that regulates cell junction formation in response to extracellular stimuli that are sensed by RTKs or GPCRs. AF6 also regulates cadherin function and the actin cytoskeleton

and, therefore, modulates cell migration and the formation of cell junctions. The Muthuswamy Lab has observed that suppression of AF6 can induce MCF10A cell motility. Therefore, at the beginning of the screen I included MCF10A/ErbB2 cells that express AF6 shRNA as a positive control to ensure that the assay was conducted properly. In my hands suppression of AF6 enhanced cell motility by ~2 folds.

In addition to the external controls, the shRNA pools that contain shRNAs targeting MKPs, PTEN and slingshots served as internal controls at the time of screen. Although the precise function of these PTP in mammary epithelial cell motility is still unknown, they have been shown to suppress signaling pathways that are known to promote cell motility. As expected, pools that contain shRNAs targeting these PTPs enhanced cell motility. Taken together, these findings validate the specificity of the screen.

It is important to note that the amount of the virus used to infect the cell, to generate the shRNA-expressing stable cell line, has to be carefully adjusted. I found that MCF10A cells yielded abnormal acini in 3D culture when they were infected by high amount of virus. This suggests that over-infection of these cells may cause off-target effects of the shRNAs, which interferes with the readout of the screen. In my screen, I control the multiplicity of infection (MOI) of my virus infection to one. This means that each cell was infected by only one virus. Therefore, I could regulate the expression of the shRNA to a reasonable level to avoid off-target effects.

5.2 Validation of the identified PTPs:

Following the screen, the identified PTPs were prioritized according to the following three criteria.

1. The significance of the change caused by the shRNA.
2. Analyzing the classical PTPs first: this is because the classical PTPs specifically regulate the phosphorylation status of tyrosine residues in proteins, which can be studied globally by using anti-pTyr antibodies, a panel of which has been generated in the lab. Furthermore, the application of substrate trapping mutants to characterize substrate specificity is ideally suited to the further characterization of the classical PTPs.
3. Analyzing the DSPs after the classical PTPs: Since DSPs are heterogeneous in structure, it is more difficult to design an effective substrate trapping mutant. Moreover, there are technical limitations to detection of phosphoserine or phosphothreonine directly by antibodies.

After the PTP candidates were selected for further characterization, the suppression efficiency of the shRNAs that target these PTPs was verified. I examined this by qRT-PCR or western blot depending on the availability of an antibody that recognizes the PTP. In addition, the phenotype caused by the selected shRNA was confirmed by another shRNA that targets the same PTP. In my screen, I focused on three PTPs, PTPN23, PTPRG and PTPRR; because they induced > 2.0-fold of changes and they are all classical PTPs. After

choosing these three PTPs, I further confirmed the suppression efficiency of the shRNAs that target them. In addition, I also confirmed the phenotype with a second shRNA that target the same PTP.

5.3 Characterization of the Identified PTPs individually

Because suppression of PTPN23 induced cell invasion, I decided to characterize the function of this PTP individually. I found that suppression of PTPN23 induced cell scattering and disrupted cell adherens junctions in 2D culture. Ectopic expression of E-cadherin rescued these phenotypes, suggesting that E-cadherin-mediated cell adhesion is disrupted in PTPN23-depleted cells. Furthermore, I demonstrated that suppression of PTPN23 induced E-cadherin internalization and caused accumulation of the internalized E-cadherin in early endosomes. These data suggest that PTPN23 can regulate the intracellular transportation of E-cadherin between cell plasma membrane and the endosomes. In addition, I identified E-cadherin, β-catenin and Src as direct physiological substrates of PTPN23. Suppression of PTPN23 induced the activation of β-catenin and Src. Inhibition of Src disrupted cell motility, invasion and scattering induced by PTPN23 depletion. Therefore, my data suggest that PTPN23 is a catalytically active enzyme that exerts its effect through modulating the phosphorylation of Src. It has been shown that Src phosphorylates E-cadherin on its cytoplasmic segment. Phosphorylation of E-cadherin disrupts its association with β-catenin, which leads to the release and activation of β-catenin [279]. In

addition, phosphorylation of E-cadherin also promotes its internalization [234, 280]. Taken together, I have proposed a model (Figure 5-2) for the function of PTPN23 in regulation of cell motility, invasion and scattering. In mammary epithelial cells, E-cadherin is localized to the adherens junctions where it forms a complex with catenins to maintain cell-cell contact. In the absence of PTPN23, Src becomes activated, which in turn increases the phosphorylation of E-cadherin, the release and activation of β-catenin and the internalization of E-cadherin to endosomes. In addition, suppression of PTPN23 may also directly regulate the phosphorylation of E-cadherin and β-catenin and thus disrupt their association. Activation of β-catenin and the internalization of E-cadherin both contribute to the increase of the expression in the mesenchymal proteins that promote mammary epithelial cell motility, invasion and scattering.

5.4 Future directions

This study reveals functional insights to the members of the PTP family. I have shown that PTPRO controls the morphogenesis of the MCF10A acini and the ErbB2 signaling. I demonstrated that PTPN23 regulates cell motility and invasion. I identified E-cadherin, β-catenin and Src as direct substrates of PTPN23. PTPN23 affected the internalization and intracellular transportation of E-cadherin possibly by modulating the activity or phosphorylation of E-cadherin, β-catenin and Src. In addition, I observed that the suppression of DEP1 induce cell transformation independent of ErbB2 activation. Suppression of PTPRG and

PTPRR as well as some other members of the DSPs induced or reduced mammary epithelial cell motility in the absence or presence of ErbB2 activation. In the future, the downstream signaling of these PTPs *in vitro* in mammary epithelial cell lines could be further examined. In addition, it would be interesting to study the effect of PTPN23 depletion in mammary epithelial cell invasion *in vivo* in a mosaic mouse model.

5.4.1 PTPRO

In this study I found that suppression of PTPRO induced the formation of bigger acini, suggesting that PTPRO play a role in the regulation of morphogenesis of MCF10A acini in 3D culture. On the other hand, I observed that suppression of PTPRO augments the effect of ErbB2 activation. In addition, I found that ErbB2 is a direct substrate of PTPRO, suggesting that PTPRO can modulate ErbB2 signaling directly through controlling the phosphorylation status of the PTK. In addition to the formation of homodimers, ErbB2 can heterodimerize with other ErbB family RTKs, such as ErbB1. The Muthuswamy lab has generated an MCF10A cell line that expresses the chimeric ErbB1 and ErbB2. Addition of a small chemical dimerizer, rapalog, induces the formation of the ErbB1/ErbB2 heterodimers and causes cell invasion [169]. Because suppression of PTPRO promotes ErbB2 phosphorylation, I could examine the effect of PTPRO depletion on the ability of the ErbB1/ErbB2 heterodimers to induce cell invasion in MCF10A cell line that expresses the chimeric receptor PTKs.

It has been report that proteins such as the Eph family of tyrosine kinases [281], SYK [282] and the oncogenic fusion protein BCR/ABL [283] are direct substrates of PTPRO. Eph are receptor tyrosine kinases that have been shown to mediate the development and tumorigenesis of the mammary gland [284]. Chicken PTPRO can dephosphorylate the second tyrosine residue in the conserved juxtamembrane region of the Eph tyrosine kinases to control their sensitivity in response to ephrins *in vivo* [281]. Interestingly, ephrin-B2 is highly expressed in the MCF10A cells. Disruption of the interaction between ephrin-B2 and EphB4 impairs the formation of cell-cell contact in MCF10A cells [285]. More importantly, Min Yu has found that the expression of EphA2 mRNA is higher in MCF10A cells that were grown in 3D than those were grown in 2D. Activating ErbB2 induces the expression of EphA2. These data suggest that the expression of EphA2 is important for the development of MCF10A acini in the 3D culture. In addition, it also suggests that PTPRO could regulate EphA2 phosphorylation to modulate the morphogenesis of MCF10A acini. Therefore, it would be interesting to define the possible functional links between PTPRO and EphA2 in the future.

Recently, it has been shown in mouse fibroblasts that expression of Dvl1 or a constitutively active β-catenin (β-catenin S37A) induces the expression of PTPRO [286]. In addition, PTPRO interacts with Wnt through its extracellular domain and down regulates Wnt signaling [286]. This suggests a negative feedback loop of the Wnt signaling pathway through controlling the expression of PTPRO. However, the role of the phosphatase activity of PTPRO in this process, if any, remains to be determined. Since Wnt signaling is an important morphogen

in the morphogenesis of many tissue and organs, it would be interesting to further characterize the role of PTPRO in the regulation of the Wnt signaling pathway in the morphogenesis of MCF10A acini. In addition, the substrate trapping mutant form of PTPRO can be used in an unbiased screen to pull down novel substrates of PTPRO, the identity of which would be determined by mass spectrometry [287]. The identification and characterization of such novel substrates of PTPRO may help us to understand further the function of the enzyme.

5.4.2 PTPN23

I have revealed that suppression of PTPN23 induced the internalization of E-cadherin through caveolin-1-mediated endocytosis, impaired the endosomal transportation of E-cadherin and caused the accumulation of E-cadherin in early endosomes. These changes led to the disruption of the adherens junctions and the increase of cell scattering. It has been shown that the BRO domain and the proline-rich domain of PTPN23 can interact with components of the ESCRT complex, such as CHMP4 and TSG101 [120, 124]. The major function of the ESCRT complex is to control the formation of the multivesicular bodies (MVBs), which regulate the transportation of the early endosomal proteins to late endosomes/lysosomes or recycling endosomes [121-123]. The association of PTPN23 BRO1 domain with components of the ESCRT complex suggests that PTPN23 may regulate the function of the ESCRT complex to modulate endosomal transportation. Therefore, I would examine further the interaction

between PTPN23 and proteins in the ESCRT complex in MCF10A cells. I would also study the effect of these interactions on E-cadherin intracellular transportation and mammary epithelial cell invasion.

In addition to the ESCRT complex, cell polarity complexes may also be downstream effectors of PTPN23. It has been shown that the Par6-aPKC complex is required for activated ErbB2 to disrupt cell polarity in MCF10A 3D acini [166, 288]. The cell polarity complexes, including Par, Scribble and Crumbs, have been shown to regulate the endocytosis and endosomal transportation of E-cadherin [232, 239, 289-293]. I have found that suppression of PTPN23 not only induces cell motility and invasion, but also disrupts cell polarity in MCF10A 3D acini (Figure 4-5). In addition, suppression of PTPN23 disrupted endosomal transportation of E-cadherin. Taken together, these data suggest that PTPN23 may regulate the function of the cell polarity complexes. Therefore, I would investigate the subcellular localization of PTPN23 in the MCF10A acini. Because of the lack of an anti-PTPN23 antibody that recognizes PTPN23 in immunofluorescence staining, I would express Flag-tagged PTPN23 in the MCF10A acini and then stain the acini with anti-Flag antibody. In addition, I would generate antibodies for PTPN23, to detect the localization of the endogenous protein. Moreover, the co-localization of the cell polarity complexes and PTPN23 could also be addressed. Biochemically, I would examine the protein-protein or enzyme-substrate interaction between PTPN23 and the cell polarity complex.

In this study, I have observed an increase in Src and β-catenin phosphorylation upon the suppression of PTPN23. In addition, I have identified E-cadherin, β-catenin and Src as direct physiological substrates of PTPN23 using a substrate trapping mutant. These data suggest that PTPN23 is an active enzyme. PTPN23 could regulate the function of the SFKs and E-cadherin/β-catenin complex by directly regulating the phosphorylation of these proteins. In the future, I would analyze further this signaling pathway, including the identification of the tyrosine residues on E-cadherin that are dephosphorylated by PTPN23.

5.4.3 DEP1

I have observed that suppression of DEP1 induced the formation of multi-acinar structures independent of the activation of ErbB2. In has been shown that Met, which is aberrantly up-regulated in several human tumors, is a substrate of DEP1 [111]. In addition, overexpression of Met in MCF10A 3D acini disrupts the localization of E-cadherin to the cell-cell contact region, and thus causes the EMT phenotype and cell invasion [170]. Taken together, these data suggest that suppression of DEP1 may lead to hyperactivation of Met and disruption of the morphogenesis program of the MCF10A 3D acini. In the future, the DEP1 phenotype needs to be further validated in particular by detecting the changes in tyrosine phosphorylation. Once the phenotype is validated, any functional link between DEP1 and Met in the morphogenesis of the MCF10A acini can be addressed.

5.4.4 PTPRG and PTPRR

PTPRG and PTPRR were both identified in the screen as PTPs that promote mammary epithelial cell motility when their expression is suppressed. However, the function of these two PTPs has not been carefully characterized, although PTPRR has been shown to regulate the MAPKs signaling pathways. The restrictions for the study of these enzymes are the lack of proper tools to analyze them, such as the antibodies that recognize these enzymes and the constructs that enable the expression of these enzymes *in vitro*. To overcome these obstacles, I have been trying to analyze the downstream signaling of a particular PTP by using the quantitative phosphoproteomic approach-SILAC (stable isotope labeling with amino acids in cell culture), which does not depend on the use of such antibodies or expression constructs. I expect that based on the application of this approach I can analyze the downstream signaling of PTPRG and PTPRR. Importantly, this method is also applicable to study other members of the PTP family.

5.4.5 Systematic characterization of downstream signaling of PTPs by quantitative phosphoproteomics-SILAC:

The classical way of studying downstream signaling of a PTP is through the use of anti-phosphotyrosine antibodies and/or site specific anti-phosphotyrosine motif antibodies to detect cell-wide and/or specific tyrosine phosphorylation changes in control or PTP shRNA expressing cells. By the

identification and characterization of such changes we can uncover the downstream effectors of the target PTP. Indeed this is the method I have used to study the downstream signaling of PTPRO and PTPN23 in this dissertation. However, this method depends on the availability of such antibodies that could determine the identity of the target proteins. Therefore, it limits the study of downstream signaling to only the field of known proteins. In addition, one protein usually contains more than one phosphorylation site. It is hard to distinguish the site that has changed in phosphorylation from the others that may have no change unless there are phosphorylation site specific antibodies available.

In order to study the downstream signaling of PTPs systematically and impartially, without the limitation of antibody availability, I have been using the quantitative proteomic method-SILAC to determine the overall protein phosphorylation changes in PTP shRNA expressing MCF10A/ErbB2 cells. These studies have been conducted in the collaboration with Dr. Tzu-Ching Meng, Dr. Kay-Hooi Khoo and Dr. Suh-Yuen Liang at the Academia Sinica, in Taiwan. In SILAC experiments, proteins are metabolically labeled within growing cells in medium containing light or heavy isotope amino acids (Figure 5-3) [294]. Peptides generated from proteins that are harvested from these cells become distinguishable in mass spectrometry (MS) spectra (Figure 5-3a). Peptide pairs that consist of two peptides identical in sequence but different in isotope labeling are called SILAC pairs. On the spectra, the difference in peptide abundance of a particular SILAC pair reflects the difference in the protein it represents (Figure 5-3b). By combining this method with methods used to enrich phosphopeptides,

such as TiO2 purification or anti-p-Y immunoprecipitation of phosphotyrosine containing peptides, I can measure the changes in overall protein phosphorylation in two stimulation conditions. By using such a method I can uncover the ultimate effects of the expressed PTP shRNA on protein phosphorylation. The changes in protein phosphorylation can be further confirmed and validated through the use of site specific antibodies if available. It is important to note that the phosphorylation changes identified using SILAC analysis may be caused directly or indirectly by the target PTP. Proteins with phosphorylation sites directly affected by the target PTP are potential substrates of the PTP. This can be further confirmed by testing the existence of a direct enzyme-substrate interaction between PTP and the identified proteins using substrate trapping mutant immunoprecipitation.

In a pilot experiment, I chose PTPN23 shRNA expressing MCF10A/ErbB2 cells as the first target to be analyzed because I have demonstrated that suppression of PTPN23 induced the phosphorylation of β-catenin and Src. Therefore, the phosphorylation status of these two proteins can serve as internal control of the assay. Briefly, I grew shLuc expressing MCF10A/ErbB2 cells in light isotope- and shN23 expressing MCF10A/ErbB2 cells in heavy isotope-labeled amino acid containing medium, for 6 doubling times to completely label the total cellular proteins. Proline was added to the cell culture medium to prevent proline to arginine conversion, which can cause confusion when interpreting the MS spectra. Cell lysate recovered from these two conditions was mixed in the ratio of 1:1 and then was digested by trypsin. After purifying the trypsin-digested peptides through reverse-

phase columns, the peptides were enriched by using SCX-TiO2 treatment to enrich phosphopeptides (phosphotyrosine, phosphoserine and phosphothreonine) or anti-phosphotyrosine antibody to enrich specifically the phosphotyrosine peptides. Afterwards, the enriched phosphotyrosine peptides were analyzed by LC/MS-MS. The changes in protein phosphorylation are interpreted in terms of signaling networks that were affected by the suppression of PTPN23 using bioinformatics approaches. The data were encouraging, and suggested that cytoskeleton-remodeling and cell-cell junction-remodeling were affected, consistent with the function of PTPN23 that I have characterized.

However, we have encountered some technical limitations in identifying the membrane proteins, which are important regulators of cell motility and invasion. In general, the use of detergent in mass spectrometry is problematic because of detergent interference with the readout. However, detergents are essential to extract proteins from membranes. In the future, I will modify the SILAC protocol to increase the sensitivity of this method to detect membrane proteins. I will extract the proteins by using detergent-containing extraction buffer. After protein extraction, the detergent will be removed through buffer exchange. Then the extracted-proteins will be used in the SILAC analysis.

5.4.6 Study the function of PTPs in vivo by using mosaic mouse model

In addition to the *in vitro* approaches I have proposed above, the function of PTPN23 in mammary epithelial cell invasion will be studied *in vivo* in transgenic mouse models. Recently, Gingras et. al. generated a transgenic

mouse that expresses a truncated PTPN23, which only contains the Bro1domain. [295]. They found that mice that express the truncated *Ptpn23* is lethal around embryonic day 9.5, suggesting that *Ptpn23* is an essential gene for the early development of mouse. This causes a technical limitation to study the function of PTPN23 using the knockout mouse technique.

To overcome this technical limitation, I will study the function of PTPN23 using mosaic mouse models. The mosaic mouse model allows us to suppress the expression of genes using RNAi. There is no genomic deletion of the genes in the process. Therefore, it avoids lethality caused by gene deletion. The mosaic mouse model involves several steps; the generation of progenitor cells, the genetic modification of the progenitor cells, the transplantation of the progenitor cells to the mammary tissue of the mice, and the detection of tumor development and metastasis. In the Muthuswamy lab, they have verified the oncogenic function of a tyrosine kinase, Brk, in breast cancer by using this model [296]. In collaboration with the Muthuswamy lab, I will express shRNAs that targeted mouse homologues of PTPN23 in Comma-1D cells and transplant these modified Comma-1D cells as my progenitor cells in the mice and then monitor the development and metastasis of the tumor. Comma-1D cells are mouse mammary epithelial cells that are derived from mammary tissue of BALB/c mice. They are characterized by only a few genomic alternations, including a mutant allele of p53 and an amplification of the distal end of chromosome 11, and are known to have pluripotent properties following transplantation. Therefore, I will study the function of PTPN23 in mammary epithelial cell development and

tumorigenesis *in vivo* by using the mosaic mouse model. I expect that because PTPN23 shRNAs induced mammary epithelial cells invasion, expressing PTPN23 shRNAs in Comma-1D cells may promote tumor formation and metastasis in mice.

To examine the effect of suppression of PTPN23 in Src overexpressing mammary epithelial cell, Src will be overexpressed in Comma-1D cells that express PTPN23 shRNA. The mammary tumor development and metastasis in these mice will be monitored. In addition, the combine effects of suppression of PTPN23 and ErbB2 overexpression in mammary epithelial cells will also be analyzed. I will overexpress ErbB2, or the oncogenic mutant form of ErbB2, Neu, in Comma-1D cells that express PTPN23 shRNA. The mammary tumor development and metastasis will be monitored. Because Src is a downstream substrate of PTPN23, I expect that overexpression of Src will accelerate the development and metastasis of the breast tumors. On the other hand, I have shown that activation of ErbB2 accelerates PTPN23 depletion-induced cell invasion. Therefore, I would expect that overexpression of ErbB2 can promote the growth of the breast tumor and its metastasis.

5.5 Perspectives:

Many PTKs have been demonstrated to be involved in the development of cancer. Recent studies on the PTKs have led to the development of small molecular inhibitors, as well as antibody-based inhibitors, that target them for the treatment of cancer, including breast cancer. Clinically, the therapeutic success rate of these drugs is limited. In particular, patients with drug resistant tumors

have been identified, suggesting the importance of identifying new therapeutic targets or strategies that complement current anti-PTK therapies. PTPs, as the enzymes that cooperate with the PTKs to regulate protein tyrosine phosphorylation-related signaling transduction, hold enormous therapeutic potential in the treatment of cancer as well as other diseases. However, for most of the PTPs, their function has remained uncharacterized. In this study I illustrate an approach to globally analyze the function of the PTP family. I characterize the function of some members of the PTP family including PTPN23, PTPRG and PTPRR in mammary epithelial cell motility. In addition, I further identify PTPN23 as a novel regulator in mammary epithelial cell invasion. Hopefully, further applications of this approach to study systematically the function of the PTP superfamily of enzymes, the role of the PTPs in the regulation of cell signaling can be characterized. In the long run, new therapeutic targets and strategies that target the PTPs themselves, or the signaling pathways that are controlled by the PTPs, can be identified for the treatment of human diseases including breast cancer.

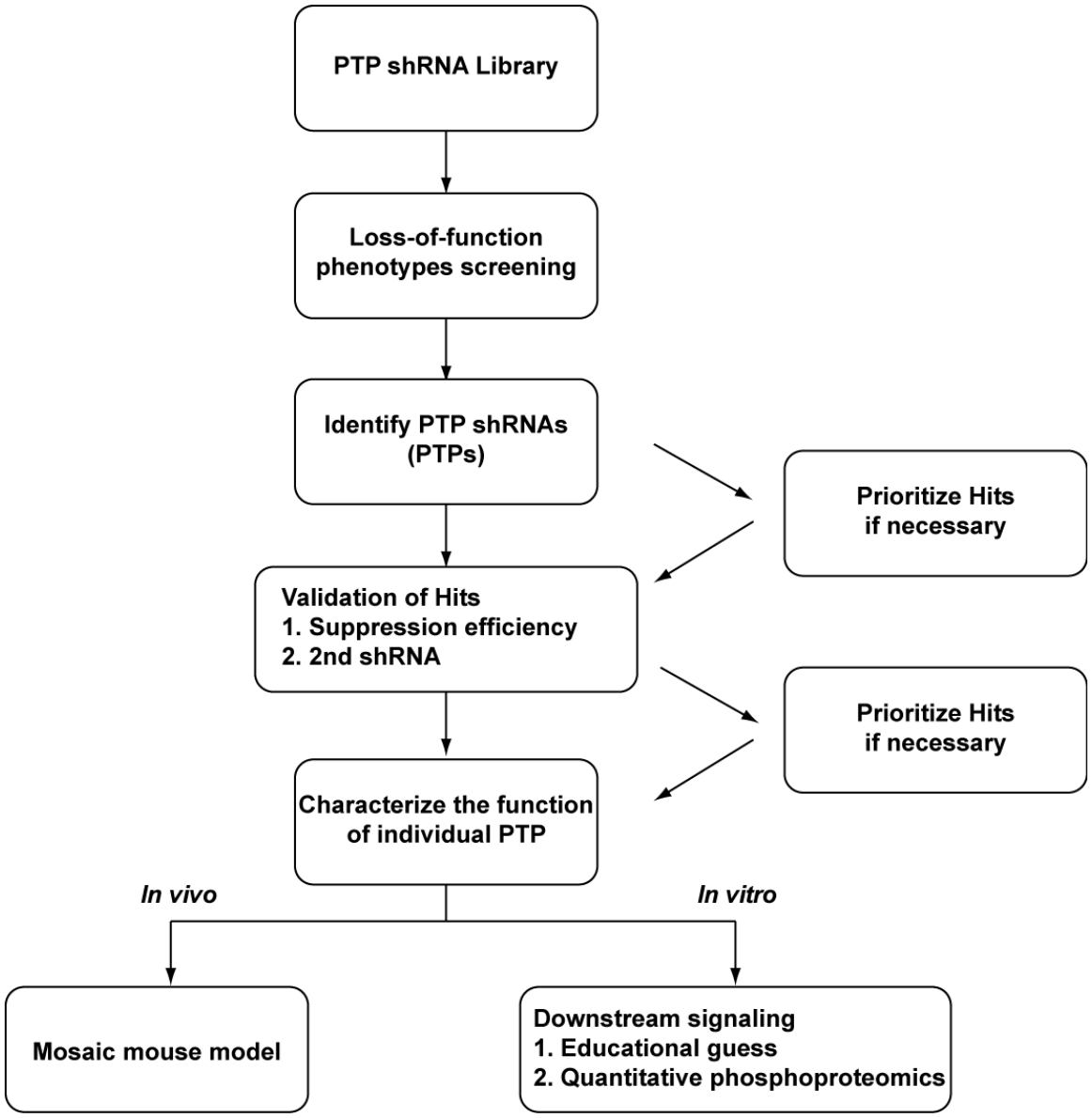

Figure 5-1: Flowchart for study systematically the function of PTP family by using RNAi-mediated screens.

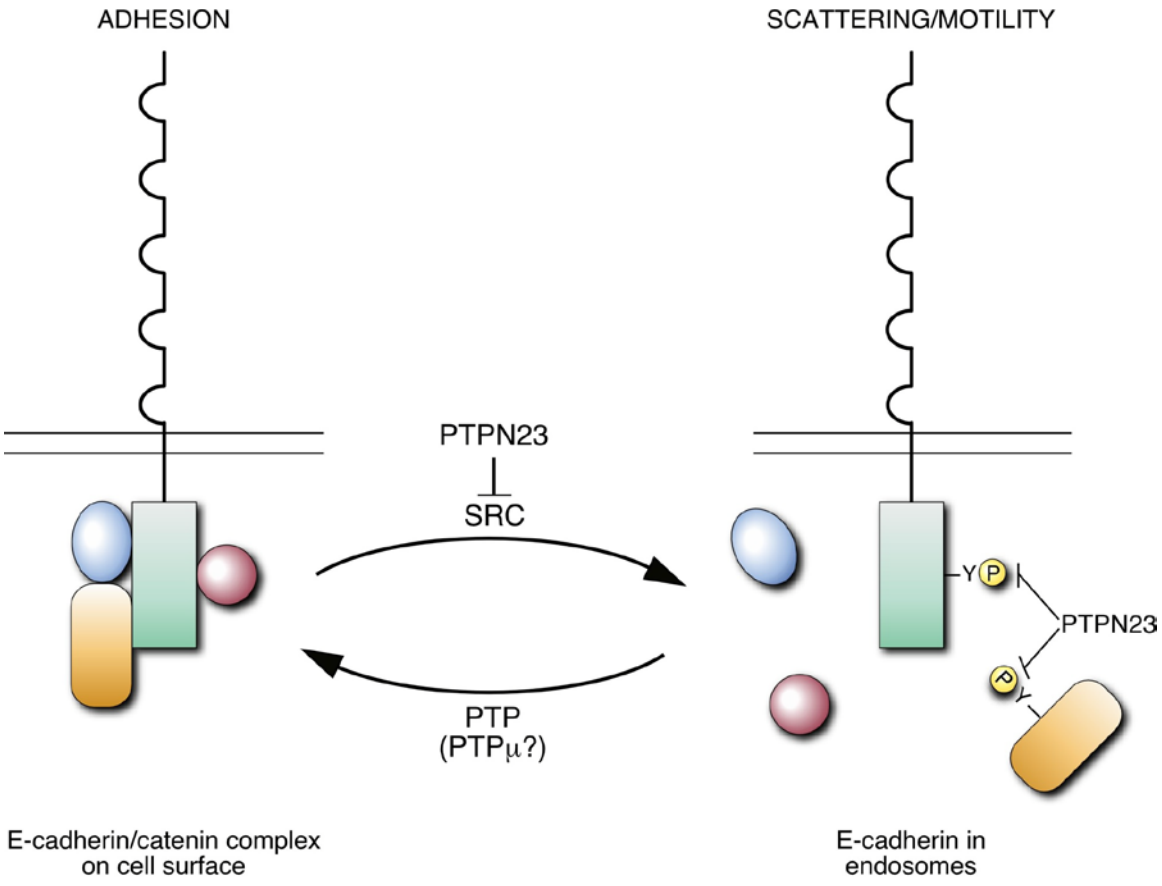

Figure 5-2: Model for the function of PTPN23

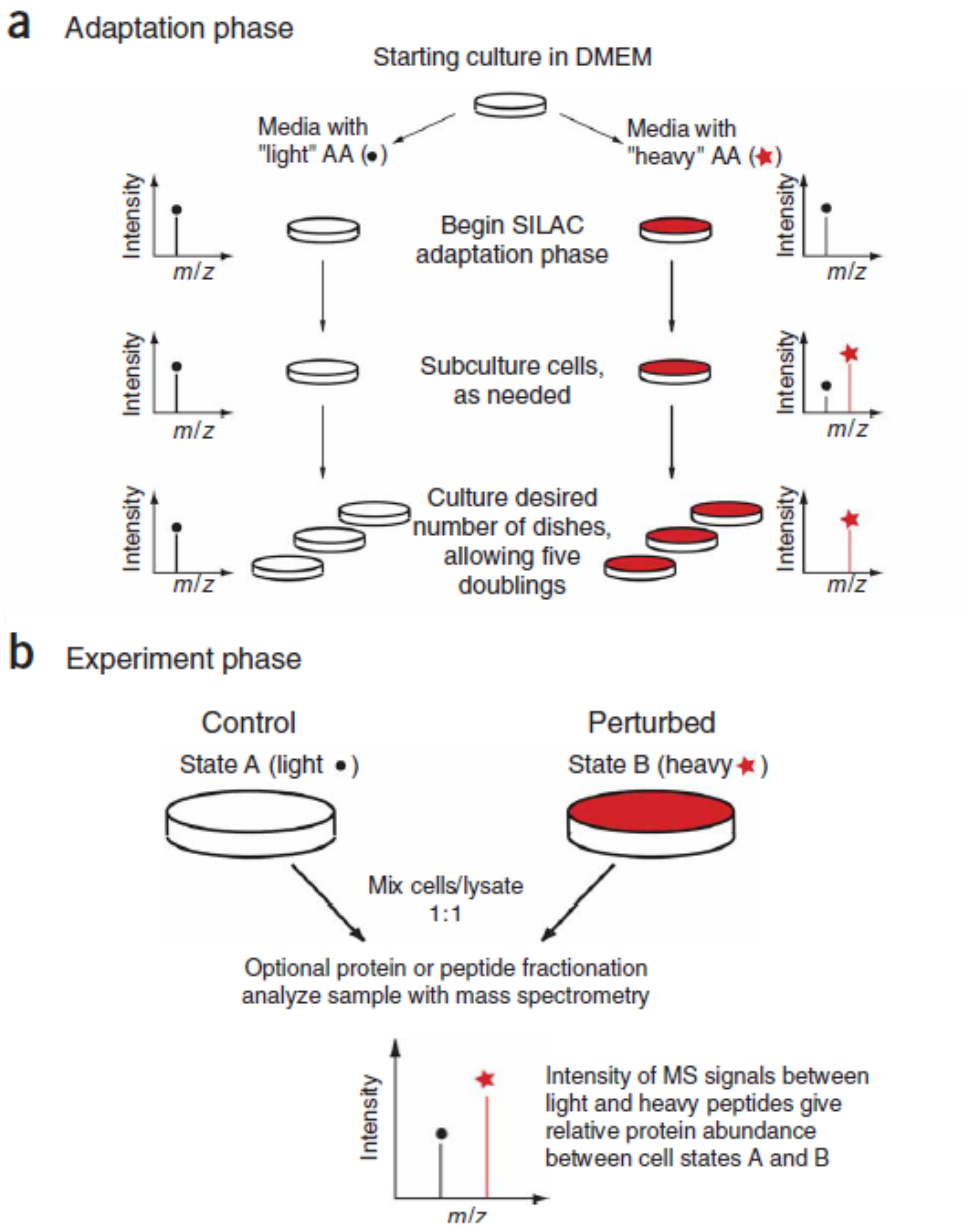

Figure 5-3: Overview of SILAC protocol. The SILAC experiment consists of two distinct phases—an adaptation (a) and an experimental (b) phase. (a) During the adaptation phase, cells are grown in light and heavy SILAC media for at least 6 doubling until the heavy cells have fully labeled by the heavy amino acids (red star). (b) In the second phase, the two cell populations are differentially treated, inducing changes in the proteome. Total protein harvested from these two cell populations are mixed in the ratio of 1:1. The protein mixture then is digested to peptides and then is analyzed by MS for protein identification and quantification. (From Ong S.E. and Mann M. Nat Protoc. 2006, 1(6):2650-60)

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