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Functional study of classical Protein Tyrosine Phosphatases in mammary epithelial cells

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by

Mathangi Ramesh

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Abstract of the Dissertation

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Reversible protein tyrosine phosphorylation is critical for the dynamic regulation of protein function and is maintained by the synchronized and complementary activity of Protein Tyrosine Kinases (PTKs) and Protein Tyrosine Phosphatases (PTPs). Although considerable progress has been made in understanding the function of PTKs, the study of PTPs has lagged behind. Some PTPs have been implicated as products of oncogenes or tumor suppressors. Particularly in breast cancers driven by the oncogenic receptor tyrosine kinase ERBB2, there is evidence for PTPs playing diverse regulatory roles, suggesting that systematic analysis of PTP function could yield important insights into this field which could,

in turn, aid the search for novel therapeutic strategies against ERBB2-positive breast cancers.

The goal of this thesis project was to investigate the role of classical PTPs in mammary epithelial cell function using organotypic culture as a model system. I carried out an RNAi-based screen to investigate the effects of loss of PTPs in the context of ERBB2 signaling, on morphogenesis in three-dimensional matrigel, using architecture as readout for tumorigenic potential. I found nine candidate PTPs, suppression of which had distinct outcomes with respect to acinus architecture. This included both positive and negative regulators of ERBB2 signaling, thereby highlighting the specificity of PTPs in this signal transduction pathway. The study revealed a novel role for PTPD2 as a positive regulator of the ERBB2 signaling pathway. Suppression of PTPD2 attenuated the multiacinar phenotype of activated ERBB2 in three-dimensional cultures, specifically by inhibiting ERBB2-mediated loss of polarity and lumen filling. In contrast, overexpression of PTPD2 enhanced the ERBB2 phenotype. I also found that the lipid second messenger, phosphatidic acid, bound PTPD2 *in vitro* and enhanced its catalytic activity. Small-molecule inhibitors against Phospholipase D (PLD), an enzyme that produces phosphatidic acid in cells, also attenuated the ERBB2 phenotype. Exogenously added phosphatidic acid was able to rescue the PLD-inhibition phenotype, but only when PTPD2 was present. These findings point towards a novel pathway involving the Protein Tyrosine Phosphatase PTPD2 and the lipid second messenger phosphatidic acid, that acts positively to regulate ERBB2 signaling and may, in the future, be a source of therapeutic targets.

to Keerthi

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Chapter 1

Introduction

Phosphate-containing molecules have been selected as a key building block of life during the course of evolution. Phosphates can readily form esters and anhydrides that are stable at ambient temperatures in an aqueous medium, making them ideal for the generation of biomolecules like nucleic acids and phosphoproteins (1).

Protein phosphorylation has emerged as one of the most prominent types of post-translational modifications on account of its *versatility* and *reversibility*. Phosphorylation can occur on 9 out of the 20 amino acids. Serine, threonine and tyrosine residues are predominantly phosphorylated in eukaryotes. Arginine phosphate is used as an energy storage compound in plants. In prokaryotes, two-component signaling systems use phosphohistidine as an intermediate in phosphate transfer to proteins.

Phosphorylated amino acid residues provide a means of diversifying the chemical nature of protein surfaces. A phosphate group has a large hydrated shell and a high density of negative charge. It can, therefore, form inter- or intramolecular hydrogen bonds that are stronger and more stable than those formed by the negatively charged amino acids, aspartate and glutamate. The charge and steric properties imparted by a covalently attached phosphate group can elicit conformational changes in a protein monomer and cause allosteric transitions within a protein multimer. Phosphate groups can also regulate function, cause a change in subcellular localization, or modulate the stability of proteins. However, the most important function of protein phosphorylation is in cellular signaling cascades, which allow cells to transduce extracellular stimuli on the surface and convey them to the genetic material so as to elicit an appropriate response. Within a specific sequence context, a covalently attached phosphate generates a binding site that allows for specific and inducible recognition of the phosphoprotein by phospho-specific binding domains in other proteins, thus promoting interactions critical to signaling pathways (1).

Reversibility of protein phosphorylation was discovered as an important regulatory mechanism in the 1950s by the seminal work of Edwin Krebs and Edmond Fischer. They found that the phosphorylation status of the glycogen catabolizing enzyme, phosphorylase, regulates its interconversion into 2 functionally distinct forms (2), (3). Phosphorylation converts the enzyme into the catalytically active “phosphorylase a” form whereas removal of the phosphate group converts it to the less active “phosphorylase b” form. These findings introduced for the first time the idea that enzyme activity can be

modulated by the reversible covalent addition and removal of a phosphate group. Fischer and Krebs went on to show that cascades of reversible protein phosphorylation events coupled in series act as a biological amplifier, mediating cellular response to extracellular stimuli. We now know that in response to the stress hormone, adrenaline, cyclic AMP levels are elevated in liver cells. This activates cAMP-dependent protein kinase that phosphorylates the enzyme phosphorylase kinase, thereby activating it. Active phosphorylase kinase phosphorylates the enzyme phosphorylase, converting it to the catalytically active form. Hence glycogen is broken down to glucose, which enters the bloodstream. When the blood glucose rises, the adrenaline level in the blood goes down. The stimulation is turned off and specific enzymes act to dephosphorylate and inactivate the enzymes in the cascade, turning the glucose production down. Thus, the phosphate group serves as a switch that allows an extracellular stimulus, like a hormone, to transmit a transient signal to elicit an appropriate cellular response- in this example, quick mobilization of sugar, giving the muscle and heart energy to combat stress. Reversible protein phosphorylation is now known to regulate a vast array of biological functions including cell growth, differentiation, proliferation and motility (4).

Overall, phosphorylated serines are the most abundant (87%) in eukaryotic proteins, followed by threonine (12%), and tyrosine phosphorylation (1%) (5). The reversibility of protein tyrosine phosphorylation is critical for the dynamic regulation of signal transduction pathways and is regulated by the complementary activity of two families of enzymes: Protein Tyrosine Kinases (PTKs), that transfer the phosphate group from ATP to specific tyrosines in the target protein, and Protein Tyrosine Phosphatases (PTPs), that remove the phosphate group from phosphoprotein substrates by hydrolysis. It has been proposed that PTKs primarily control the amplitude of the signaling response whereas PTPs play a more significant role in controlling the rate and duration of the response (6). Of equal importance in tyrosine phosphorylation-mediated signaling is the role of phosphotyrosine-binding domains like the SRC Homology 2 (SH2) domains, which serve to propagate signaling from activated Receptor Tyrosine Kinases (RTKs) by binding to phosphotyrosine residues, and recruiting downstream signaling complexes. The SH2 domain binding energy for a pTyr residue is higher than that for a pSer or pThr because of the contribution of contacts that can be made with the phenolic ring in addition to interaction with the phosphate. It has been suggested that this could have been an important factor in the selection of

phosphotyrosines in signaling (7). Phosphotyrosine-based signal transduction is, therefore, able to achieve a rich array of diverse regulatory schemes through the interplay of three distinct and specific functional modules: protein tyrosine kinases, the “writers” that generate pTyr, SH2-domains, which act as “readers” of pTyr motifs and facilitate protein-protein interaction, and the protein tyrosine phosphatases which act as “erasers” (8). Although it was originally believed that PTPs serve simply to switch off signaling pathways and clean up after the kinases, it has since become clear, as I will illustrate in this chapter, that PTPs are critical regulators of signaling in their own right, playing an essential role under both normal and pathophysiological conditions. Research in our lab is focused on the role of the Protein Tyrosine Phosphatases (PTPs) as regulators of cellular signal transduction pathways.

Protein Tyrosine Phosphatases

There are 105 genes in humans that encode Protein Tyrosine Phosphatases (PTPs). PTPs are defined by the active site signature motif $HC(X)_5R$ where X is any amino acid, and the invariant cysteine and arginine residues are essential for catalytic activity (9).

PTP-mediated catalysis proceeds via a two-step mechanism. Following substrate binding, the cysteine residue in the signature motif, which typically has a low pKa of 4.8 - 5.4, initiates a nucleophilic attack on the phosphate group of the substrate. This is accompanied by a conformational change in the active site such that an amino acid loop called the WPD loop closes in around the side chain of the pTyr residue of the substrate. This positions an invariant aspartate residue to act as a general acid and protonate the tyrosyl leaving group of the substrate, forming a cysteinyl-phosphate intermediate. In the second step, this intermediate is hydrolyzed by the aspartate residue, now acting as a general base. The aspartate promotes a nucleophilic attack by a water molecule which, leads to hydrolysis of the phospho-cysteine intermediate and release of the phosphate (10) **(Fig. 1-2).**

On the basis of their substrate specificity, PTPs can be broadly divided into two groups: classical PTPs and Dual Specificity Phosphatases or DuSPs. Although both groups of enzymes share the same catalytic mechanism, DUSPs can dephosphorylate protein phosphoserine and phosphothreonine residues in addition to phosphotyrosines whereas

classical PTPs are specific to protein phosphotyrosine residues. The difference in substrate specificity arises on account of the architecture of the catalytic cleft, which contains the active site. In classical PTPs, an invariant tyrosine residue defines the depth of the catalytic cleft, thereby ensuring specificity for phosphotyrosine residues. In contrast, the catalytic cleft of DUSPs is shallower because of the smaller side chains of residues that define its depth. This allows both phosphotyrosine and phosphoserine/phosphothreonine residues to access the catalytic cysteine which lies at the bottom of the cleft (10).

In the human genome, there are 37 genes that encode classical PTPs. This includes 23 transmembrane receptor-like PTPs and 14 non-transmembrane PTPs **(Fig. 1-3). The receptor-like PTPs** (RPTPs) are transmembrane proteins with variable extracellular segments that contain motifs important for cell-cell and cell-matrix adhesion. Many RPTPs contain two intracellular PTP domains: the membrane-proximal D1 domain and the distal D2 domain. With the exception of RPTPα, the phosphatase activity lies in the D1 domain whereas the D2 domain is inactive. In RPTPα, it has also been shown that the catalytic cysteine 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. The crystal structure of RPTPα D1 domains first revealed evidence of dimerization of RPTPs. The observation that a wedge motif N-terminal to the D1 domain occludes the active site of the partner domain suggested that formation of RPTP dimers might serve to inhibit enzymatic function (11). This is in contrast to RTKs where ligand-induced dimerization causes trans-autophosphorylation and activation. It has been observed that transiently expressed RPTPα homodimerizes efficiently on the cell surface via multiple domains, suggesting that dimerization-mediated inhibition of RPTPα biological activity may be physiologically relevant (12). There is evidence in the literature of ligand binding causing functional inactivation of RPTP catalytic activity, as in case of pleiotropin binding to RPTPβ/ζ. It is as yet unclear, however, if this effect is mediated by dimerization (13).

The non-receptor PTPs are cytoplasmic proteins that often contain several non-catalytic regulatory domains in addition to a single catalytic domain. In some cases, like SHP-2, the regulatory domains have been shown to directly control enzyme activity through interactions at the active site. Under basal conditions, the N-terminal SH2 domain of SHP-2 blocks the active site. This is an inhibitory intramolecular interaction that maintains the

enzyme in an inactive state. Proteins that target SHP-2 to signaling complexes engage the SH2 domain by binding through phosphorylated tyrosine residues. This induces a conformational change, relieving the autoinhibitory interaction and exposing the catalytic cleft of the enzyme, thereby activating it for substrate binding and dephosphorylation (14). The regulatory sequences can also act in an indirect manner, controlling substrate specificity (the Kinase Interaction Motif of STEP allows it to interact with its target substrate, ERK) (15) or by modulating subcellular localization and, consequently, access to substrates.

There are 65 genes in the human genome that encode the heterogeneous **Dual Specificity Phosphatases or DUSPs (Fig 1-4)**. They include the VH1-Related Protein Tyrosine Phosphatases, the MAP Kinase Phosphatases (MKPs), which inactivate MAPKs by dephosphorylating both phosphotyrosine and phosphothreonine residues in the activation loop and JNK-stimulatory phosphatase-1 (JSP-1), which specifically increases the phosphorylation and activity of JNK but not p38 and ERK. Some DUSPs show preference for specific phosphorylated residues. For example, VHR (VH1-related DUSP) preferentially dephosphorylates phosphotyrosine residues whereas KAP (cyclin-dependent Kinase Associated Phosphatase) shows preference for phosphoserine/phosphothreonine residues. Some DUSPs target non-protein substrates such as the lipid phosphatase PTEN, the RNA-capping enzymes and the myotubularins that regulate inositol phospholipid-dependent signaling (9).

Some members of the PTP family display the structural features of a DUSP but are catalytically inactive because they lack critical residues important for activity. These PTPs are called **pseudophosphatases**. It has been demonstrated that the pseudophosphatase STYX, can be converted into an active enzyme by mutating a single residue in the active site motif to the consensus sequence of active enzymes (16). In *C. elegans*, several pseudophosphatases (EGG-3, 4 and 5) have been implicated in regulating the activity and subcellular localization of the Dual Specificity Tyrosine Regulated Kinase (DYRK) homolog, MBK-2, that is critical for the oocyte-zygote transition. Although the exact mechanism is unclear, it has been proposed that the “active site” of EGG-4/5 engages the phosphorylated activation loop of the kinase, forcing the latter into an inactive conformation. Alternatively, it is possible that the interaction might position a segment of the pseudophosphatase to

occupy the substrate-binding groove of the kinase like a substrate analog. This would prevent the kinase from binding and phosphorylating its substrates and thus impede downstream signaling (17).

Tyrosine phosphorylation and disease

Disrupting the balance of functions of PTKs and PTPs can alter the normal pattern of protein tyrosine phosphorylation, resulting in the propagation of abnormal responses to extracellular stimuli and the development of disease. Thus, the ability to modulate protein phosphorylation status holds enormous therapeutic potential.

The initial discovery that several viral oncoproteins are constitutively active tyrosine kinases that transform cells through excessive tyrosine phosphorylation, led to an interest in the development of inhibitors that would block the action of these enzymes (7). This interest was fuelled when it was discovered that the oncoprotein that drives chronic myelogenous leukemia (CML) is a fusion protein between BCR and c-ABL, which has constitutive tyrosine kinase activity. The success of Novartis' small-molecule inhibitor, Imatinib (Gleevec), in treating chronic phase CML represented an early success in targeting a tyrosine phosphorylation-mediated pathway.

Nearly 30 other Tyrosine Kinase Inhibitors (TKIs) have since been approved for clinical use, almost all in the treatment of cancer: Iressa (Gefitinib) and Tarceva (Erlotinib), are EGFR inhibitors that have been approved for the treatment of Non-Small Cell Lung Cancer; Sunitinib (Sutent), a split RTK inhibitor and Dasatinib (Sprycel) a SRC/Abl inhibitor have been approved for imatinib-resistant CML patients and Lapatinib (Tykerb), an EGFR/ERBB2 inhibitor has been approved for breast cancer therapy (7). The Janus Kinase inhibitor, Tofacitinib, was approved for the treatment of rheumatoid arthritis in 2012.

Antibody-based approaches have also been developed to counter the activity of PTKs in cancer; Herceptin (Trastuzumab), a humanized monoclonal antibody that targets ERBB2 was the first such drug to be approved. Pertuzumab, which binds to ERBB2 and prevents it from heterodimerizing with ErbB3, has since been approved for combination antibody therapy along with Herceptin. Its effect as a single agent on ERBB2-amplified breast cancers is still being tested. The EGFR–neutralizing monoclonal antibodies

Cetuximab (Erbitux) and Panitumumab (Vectibix) and the anti-VEGF MAb Cevacizumab (Avastin) are other examples of approved drugs that rely on this approach (18).

These drugs have been largely successful in the effective treatment of disease. Yet, several challenges remain with respect to their use in the clinic. On account of the propensity of tumors to generate resistant variants due to their genetic instability, many patients are either resistant to the drug to begin with or acquire resistance during the course of therapy. Secondly, many of the first-generation TKIs are not very selective, often targeting multiple kinases and leading to deleterious side effects. These limitations underscore the importance of gaining a better understanding of protein tyrosine phosphorylation to identify novel therapeutic targets that can be used as standalone therapies or in combination with existing options.

Being critical regulators of tyrosine phosphorylation-mediated signaling, PTPs represent an obvious line of investigation as potential therapeutic targets. There are several examples in the the literature that highlight the importance of PTPs in the etiology of many diseases**.** The role of PTP1B in down-regulating insulin and leptin signaling is well established. Targeted deletion of the gene encoding PTP1B produced healthy mice that displayed increased insulin sensitivity and resistance to obesity induced by a high fat diet, making PTP1B an attractive target for the development of therapies for diabetes and obesity (19), (20)**.** YopH, an essential virulence determinant of the bacterium *Yersinia,* is also a highly active PTP (21). Direct injection of YopH into host cells subverts signaling pathways critical for the host immune response against infection by reversing the phosphorylation state of host PTKs (22). A gain-of-function mutation in the gene encoding PTPN22 is a common risk factor for autoimmune diseases including Grave's disease, rheumatoid arthritis and Systemic Lupus Erythematosus (9).

In the context of human cancers, the identification of the oncogenic capacity of PTKs sparked an interest in the functional role of PTPs as tumor suppressors. PTEN, which is known to have both protein and lipid phosphatase activity, was the first PTP to be identified and characterized as a tumour suppressor. PTEN dephosphorylates phosphatidylinositol 3,4,5-trisphosphate, thereby antagonizing the PI3K/AKT signaling pathway, which is associated with cell survival (23). The gene that encodes PTEN is located on human chromosome 10q23.3, in a region that is frequently deleted in high-grade prostate and brain

tumors. Germline mutations in the *Pten* gene lead to hereditary disorders that predispose the individual to cancer (24), (25). Thanks to large-scale sequencing efforts, several PTP-encoding genes have since been identified as mapping to chromosomal regions that are frequently amplified or deleted in cancer (26). Tumor-derived mutations have also been described in several PTP genes in various tissue-specific cancers. In colorectal cancer, for example, somatic mutations were identified in six PTP genes, some of which have now been functionally characterized to be tumor-suppressive in nature (27). Epigenetic studies involving the analysis of the methylation status of CpG islands in the promoters of PTP genes also suggest a tumor suppressor role for several PTPs as in case of PTPRO and SHP-1 (28).

Beginning with CD45, however, several PTPs have been identified as positive regulators of signaling pathways, making them attractive candidates for targeted therapy in disease states. In B-cells, CD45 regulates the tyrosine phosphorylation and, by extension, the activity of the SRC Family Kinase Lyn. In the absence of CD45, the activating autophosphorylation site of Lyn is hyperphosphorylated, whereas the C-terminal inhibitory site is hypophosphorylated. As a result, the function of Lyn as an inhibitor of BCR signaling is potentiated. This leads to diminished signaling responses to stimulation of the B-Cell Receptor, so much so that CD-45-deficient humans and mice develop a severe combined immunodeficiency phenotype (SCID) (29).

The identification of an oncogenic role for SHP-2 further strengthened the idea that PTPs do not function as passive antagonists of PTKs but could have a direct role in switching on signaling pathways. Gain-of-function mutations of SHP-2 were initially identified in patients of Noonan Syndrome, which is characterized by congenital heart disease and developmental defects. Activating point mutations on the SHP-2 gene have also been identified in 20-25% of patients of sporadic juvenile myelomonocytic leukemia (JMML) (30). As described before, it has been proposed that the SH2 domain of SHP-2 associates with the PTP domain, thereby preventing access of the substrate to the catalytic cleft and maintaining the enzyme in an inactive basal state. Mutations identified in JMML and Noonan Syndrome have been found to cluster at the interface between the N-terminal SH2 domain and the PTP domain, and abolish the association between the two domains, thus generating a constitutively active mutant that promotes signaling through the activation

of RAS/MAPK and SRC (30). SHP-2 can activate RAS by inhibiting RAS-GAP or Sprouty- both negative inhibitors of RAS activity. SHP-2 can also dephosphorylate the transmembrane protein PAG/CBP, thereby blocking the recruitment of Csk, a negative regulator of SRC activity.

Cdc25s are important regulators of the cell cycle during normal eukaryotic cell division and act by dephosphorylating inhibitory phosphorylation sites on cyclin-dependent kinases. In cells with DNA damage, they act as mediators of the checkpoint response. Overexpression of cdc25s has been observed in a wide variety of human cancers and is frequently associated with aggressive disease and poor prognosis. As a result, they have been actively pursued as candidates for the development of inhibitors that can be used to cause cell cycle arrest of proliferating cancer cells (31).

In the context of breast cancer, several potential links have been uncovered between members of the PTP family and disease establishment, maintenance and/or progression. Whole-genome analyses of copy number variations and genomic rearrangements in breast tumor samples from various stages of disease have highlighted focal deletions and amplifications that include several PTP genes (26). For example, it has been found that the gene encoding RPTPα is significantly overexpressed in approximately 30% of primary human breast cancers, a feature that correlates with low tumor grade (32). Expression of PRL-3 (33) and LAR (34) is upregulated in metastatic as compared to benign tumors. In contrast, the expression of PTPRG is lower in breast tumors. Expression of some PTPs is also upregulated in response to specific oncogenes like estrogen and ERBB2 (35), (36). Some of these findings have been validated and further characterized in cell and animal models. However, we have not yet achieved a complete understanding of the functional role of PTPs in mammary epithelial cells and further, in mammary carcinogenesis.

ERBB2: an oncogene in breast cancer

The receptor protein tyrosine kinase (RTK) ERBB2, or HER2, belongs to the EGFR family of RTKs, which also includes ErbB1 (EGFR), ErbB3 and ErbB4. The binding of specific ligands such as EGF, neuregulins and epiregulin activates these RTKs. Upon ligand binding to the extracellular domain, ERBB receptors undergo homodimerization or heterodimerization with other members of this receptor family. This is followed by trans-

phosphorylation of the intracellular domains, either by the dimerization partner or by the intrinsic tyrosine kinase domain, which gets activated as a result of a conformational change. Phosphotyrosine residues, so created, serve as docking sites for the recruitment and activation of signaling molecules such as SH2 domain-containing adapter proteins. These molecules further activate downstream signaling pathways including PI3K/AKT, RAS/MAPK and STAT signaling, ultimately affecting several physiological processes (37).

To date, no ligand has been shown to bind ERBB2. Crystal structure analyses of the ERBB2 extracellular domain suggest that this might be because ERBB2 has a constitutive activated conformation similar to that of EGFR when it is complexed with its ligand but different from that of ErbB3 and ErbB4 in their unactivated conformations. This precludes the need for ligand binding to release an auto-inhibited conformation as in case of other members of this family of RTKs. This makes ERBB2 the preferred heterodimerization partner for the other ligand-activated receptors in this superfamily (38), (39). In addition, ERBB2 can also signal in an autologous manner, often following a mutation or when it is overexpressed. In the latter case, ERBB2 activation has been reported to involve ligand-independent oligomerization and phosphorylation (40), (41), (42). Studies have suggested that dependence on a ligand for activation is a function of concentration / expression level of the receptor; increasing the expression of proto-oncogenic ERBB2 shifted the receptor equilibrium from a monomeric to an aggregated form (40).

ERBB2 was first recognized as an oncogene (*Neu*) in glioblastomas seen in rats treated with the carcinogen ethylnitrosourea (43). Oncogenic activation of *Neu* is on account of a point mutation that causes a single amino acid substitution (valine to glutamine) in the transmembrane domain (44). Normal human ERBB2 acts as a potent oncogene when overexpressed in NIH3T3 cells. However, a critical level of overexpression is essential to achieve transformation (42), (45), (46). Transformation by overexpression of ERBB2 correlated with ligand-independent tyrosine phosphorylation of ERBB2 and the adaptor protein Shc (47). The clinical significance of this observation is underscored by the fact that the ERBB2 gene is overexpressed or amplified in 25% of breast cancer patients (48), (49) and is associated with more aggressive tumor development and poor prognosis in early stages of the disease (50). ERBB2 overexpression is observed in more than 50% cases of Ductal Carcinoma In Situ (DCIS), an early stage of mammary carcinogenesis (51), (52).

This high incidence of gene amplification with accompanying overexpression in non-invasive breast tumors suggests that perturbations of the HER2 gene are among the earliest and most common genetic lesions in human breast cancer. There are two major hypotheses to explain how ERBB2 overexpression acts in an oncogenic capacity. As mentioned before, ERBB2 is the preferred heterodimerization partner for other members of this RTK family **(53)**, particularly the kinase-inactive ErbB3. Consequently, an abundance of ERBB2 results in the formation and ligand-independent activation of more heterodimers, enhancing and prolonging the signal. ERBB2 amplification has also been shown to inhibit downregulation of heterodimers; heterodimerization with ErbB-2 shunts ligand-activated receptors to recycling rather than intracellular degradation (54), (55). Overexpression of ERBB2 results in constitutive activation, driven by ERBB2 self-association (56) to form homodimers (57).

A given ERBB receptor can acquire different signaling properties depending on its dimerization partner (53), (58). Different ERBB dimers recruit or activate different sets of signaling molecules. For example, the p85 subunit of PI3K is thought to associate more efficiently with ErbB3 (59), (60). c-Cbl prefers ERBB1 in a homodimer over ERBB1 in a heterodimer with ERBB2 (61). To add to the complexity, a given EGF-family ligand can cause activation of specific ERBB receptors in a manner that is distinct from other ligands which activate the same set of receptors (62), (63).

ERBB receptor homodimers also exhibit functional differences in their biological signaling abilities. It has been shown, for example, that the EGFR-ERBB2 heterodimer signals differently from the EGFR homodimers**.** In particular, there is evidence for direct binding of the c-SRC SH2 domain to phosphorylated ERBB2 but not phosphorylated EGFR. In established cell lines expressing elevated levels of EGFR, EGF stimulation results in transphosphorylation of ERBB2 and formation of complexes between c-SRC and tyrosine phosphorylated ERBB2 (64). Other studies have uncovered the differences between the cellular response to ligand-independent ERBB2 homodimerization and heregulin-induced signaling, which is thought to involve heterodimerization with HER3.

Many cancer cell lines that overexpress ERBB2 become growth arrested and die by apoptosis if the expression of ERBB2 is suppressed (65), (66), (67), (68). In ovarian cancer cell lines, conditional depletion of ERBB2 prevents tumor growth in nude mice. (69).

Together these results suggest that *ERBB2* gene overexpression is important for the survival and proliferation of these cancer cells that have been selected for *ERBB2* amplification. Results from transgenic mouse models corroborate these findings *in vivo*. Conditional activation of the *Neu* oncogene in mammary epithelium results in invasive carcinomas with pulmonary metastasis. Withdrawal of *Neu* expression in these mice caused regression of the primary tumors and metastases (70). These results strongly suggest that ERBB2 is a relevant therapeutic target in tumors showing overexpression of the gene.

Therapeutic strategies that target ERBB2 are based on small-molecule inhibition of its tyrosine kinase function, as in case of the dual EGFR-ERBB2 inhibitor Lapatinib, or targeting of the extracellular domain with specific antibodies. The recombinant humanized monoclonal antibody, Trastuzumab (Herceptin) represents the most widely employed therapy against ERBB2-positive breast cancers (71). In metastatic breast cancers, Trastuzumab has proven to be active in combination with chemotherapy, resulting in improved overall survival, longer time to progression, higher response rate and a longer duration of response compared to chemotherapy alone (72), (73). The drug is primarily thought to exert its effect by downregulating the level of ERBB2 at the cell surface by stimulating endocytosis. Some studies also attribute the anti-proliferative effect of Herceptin on its ability to block receptor aggregation and effect antibody-mediated cytotoxicity. However, 2-3% of the patients who receive trastuzumab treatment are at a risk for congestive heart failure (74). Even in the selective subset of patients designated "ERBB2-positive" the response rate is <40% as a single agent in first line treatment of metastatic breast cancer (75). The median duration of response to Trastuzumab monotherapy is between 9 and 12 months (76). In patients who received Trastuzumab and chemotherapy the median time to disease progression was 7.4 months, i.e. the majority of patients who achieve an initial response to Trastuzumab-based regimens develop resistance within one year (77). These lines of evidence for both primary *de novo* and acquired (treatment-induced) resistance to Trastuzumab suggest that factors besides ERBB2 overexpression may be involved in response to this agent.

This necessitates a thorough characterization of signaling events initiated by ERBB2 and their regulation in the context of Trastuzumab treatment. There is also an urgent need to

identify biomarkers to guide personalized therapy in patients who develop progressive ERBB2-positive metastatic breast cancer, particularly while receiving trastuzumab, and to identify novel “druggable” targets in the ERBB2 signaling pathway for combination therapy.

The importance of PTPs as regulators of ERBB2 signaling

Given that ERBB2 signaling initiates via protein tyrosine phosphorylation, it is intuitive that PTPs will play an important regulatory role in this pathway. There are several lines of evidence in the literature that support this line of reasoning. Several different PTPs have been found to act at distinct levels in the ERBB2 signaling pathway. Certain PTPs such as PTPRO, PTPBAS and PTPN9 directly dephosphorylate the C-terminal phosphotyrosines on activated ERBB2, thus negatively modulating ERBB2 signaling in various different models of mammary carcinogenesis (78), (79), (80). Recently, our lab used an RNAi-mediated loss-of-function screen to identify three PTPs, PTPN23, PTPRG, and PTPRR, which inhibited ERBB2-induced mammary epithelial cell motility, and of which PTPN23 also inhibited ERBB2-induced mammary epithelial cell invasion. PTPN23 was shown to exert its effects by recognizing E-cadherin and β-catenin directly as the substrates, as well as dephosphorylating the autophosphorylation site in SRC, thereby inactivating the kinase (81). BDP1/ PTPN18 inhibits ERBB2 but not EGFR signaling by inhibiting the phosphorylation of ERBB2 and the adaptor protein Gab1, ultimately affecting signaling from the MAPK cascade (82). Phosphatases have also been implicated in resistance to the anti-ERBB2 drug, Herceptin. It has been reported that PTEN activation underlies the antitumor activity of Herceptin and that PTEN-deficiency correlates strongly with Herceptin resistance. (83),(84). Most recently, our lab has shown that PTPα regulates the phosphorylation of FAK and that suppression of this phosphatase leads to a Grb7-dependent increase in migration of human mammary epithelial cells in response to ERBB2 activation (85).

Some PTPs have been identified to act as positive regulators of ERBB2 signaling, supporting tumor establishment and progression. PTP1B is overexpressed in breast cancer, coincident with the overexpression of ERBB2 (86). Inhibition of PTP1B in a mouse model of ERBB2-driven breast cancer delayed the onset of mammary tumors and reduced the incidence of metastasis; PTP1B potentiates ERBB2-induced signaling by dephosphorylation of p62DOK and activation of mitogen- activated protein (MAP) kinase (87). Consistent with

an oncogenic role for PTP1B, transgenic mice overexpressing PTP1B in the mammary gland displayed increased tumorigenesis (88). In an *in vitro* model of breast cancer, activation of ERBB2 leads to the formation of atypia-like structures only when PTP1B is present (89). SHP2 is another PTP that has also been shown to be required for ERK activation in ERBB2- mediated carcinogenesis. (90). Finally, a receptor PTP, PTPα, has been shown to play an important role in ERBB2-mediated transformation, exerting its effects by dephosphorylating the inhibitory C-terminal site in SRC, thereby activating the kinase (91).

These results highlight the diverse roles played by some PTPs in ERBB2 signaling and suggest that a systematic analysis of PTP function should yield important new insights into the regulation of ERBB2 signaling. Accordingly, the goal of this dissertation is to study the functional role of individual classical (phosphotyrosine-specific) PTPs in mammary epithelial cell-based models of breast cancer both in the presence and absence of ERBB2 signaling. The findings from such an investigation, together with the existing body of knowledge can be applied towards the novel therapeutic strategies that can complement current anti-ERBB2 therapies for the treatment of breast cancer.

Models to study mammary epithelial cell function

Epithelial tissue forms the surface of the body as well as glands such as breast and prostate. Epithelial cells are characterized by a polarized morphology, specialized cell-cell contacts and attachment to an underlying basement membrane. This well-ordered architecture is essential for tissue function and is disrupted during the pathogenesis of epithelial tumors. Classification of epithelial tumors and estimation of disease prognosis relies even today on the architectural changes resulting from the diseased condition.

Several model systems have been used widely to address how cancer genes and pathways affect normal tissue structure and function (92). **Primary human tumor tissue** represents the most clinically relevant option to get a snapshot of a stage of the disease, particularly for genetic, epigenetic and histological analyses. However, there are limitations with respect to access and often, the tumor isolate is cross-contaminated with surrounding normal tissue, which complicates clean analysis of the proteome. Another drawback of this system is that it cannot be used to study biochemical and cell biological events leading to

tumor establishment as cell lines derived from tumors do not exhibit any growth control properties.

Genetically engineered mouse models of disease, afford several advantages to understanding epithelial tumors. This includes the power to manipulate gene function *in vivo*, to study the effect of the immune component and stroma on tumor establishment/ progression and to view processes that contribute to oncogenesis in real-time (for example, by intravital imaging). However, the extent to which individual GEMs can recapitulate human disease depends significantly on the initiating oncogene and the strategy used to drive its expression. Further, large-scale cellular analysis to investigate biochemical changes can be challenging.

Xenografts of genetically engineered cell lines allow for the examination of human breast cancer cells in the context of complex multicellular and cell-ECM interactions *in vivo.* Tumor formation usually occurs rapidly and reproducibly and large cohorts of tumor-bearing mice can be generated. However, there are several technical limitations of this approach also. First of all, this method makes use of use of immunocompromised mice; the absence of an intact immune system may profoundly affect tumor development and progression. Secondly, the microenvironment of the site of injection into the mouse may not be physiologically comparable to human tissue stroma. Finally, metastatic cells in mouse models primarily colonize the lung and not other sites observed frequently in human disease. Xenograft models of clinical isolates suffer from limited transplantation efficiency.

Cell culture models are the most tractable model system to study the cellular processes and signaling pathways involved in oncogenic transformation, albeit *in vitro*. Gene expression profiling studies suggest that no single cell line is truly representative of the heterogeneity of the disease. However, individual **breast cancer cell lines** share some genetic and genomic features of individual breast cancer subtypes. Therefore, when considered as a system, they can provide preliminary insights into the molecular mechanisms of disease onset and progression. They are also easily propagated and amenable to genetic manipulation. However, over the course of several years in culture, cell lines acquire genetic changes, resulting in phenotypic variations that might preclude comparison between different studies.

Traditionally, cell culture models make use of cells that have been cultured on a non-physiological substratum like tissue culture plastic as monolayers or in soft agar as colonies. Due to the absence of a relevant microenvironment, such a system cannot recapitulate the architectural organization or functional aspects of mammary epithelial tissue. **Three-dimensional organotypic cell culture** overcomes this limitation by allowing epithelial cells to organize into structures that reliably emulate various aspects of breast architecture *in vivo*. A recent study comparing the gene expression profile, genomic alterations and morphology of breast cancer cell lines cultured in 2D vs. 3D conditions revealed that 3D cultures more closely mimic the *in vivo* environment (93). In other words, the morphology of breast cancer cell lines in three-dimensional assays correlates with their profiles of gene expression.

The mammary epithelium of an adult breast is organized into ducts and lobules. Ducts terminate in the highly branched Terminal Ductal Lobular Unit (TDLU), which is comprised of multiple individual units called acini. Plating single mammary epithelial cells on a bed of Matrigel™ (matrix comprising tumor-derived, reconstituted basement membrane and components like laminin, collagen IV and entactin derived from the Engelbreth-Holm Swarm tumor) initiates a well-characterized morphogenetic program that culminates in the formation of spheroids surrounded by a basement membrane with a single outer layer of polarized, growth-arrested cells and a central hollow lumen, much like a mammary acinus. These structures are readily amenable to experimental manipulations as well as detailed microscopic analyses, which allows investigators to dissect complex signaling interactions that are difficult to study *in vivo*. The physicochemical properties of the matrix itself can be manipulated in order to study the effects on morphogenesis. Finally, the system can potentially be scaled up in order to perform large-scale biochemical analyses including proteomics studies.

There are several highly regulated, sequential steps in the developmental program that are critical to the establishment and maintenance of the structural integrity of mammary acini modeled thus in 3D. Breast epithelial cells initially divide to form a cluster of cells. At Day 5-8, two distinct populations of cells become evident within an acinus: an outer layer of cells in direct contact with the matrix and an inner subset of cells lacking matrix contact. A distinct signaling dichotomy emerges between these cell populations that determines the

subsequent architectural development of the acinus. The outer cell layer undergoes apico-basal polarization, a feature that is maintained during the entire morphogenetic process. These cells also receive survival and proliferation cues. The inner cell population, on the other hand does not receive survival signals, and undergoes apoptosis, regulated by Bim. This creates and maintains a hollow lumen inside the acini. This is followed by suppression of proliferation in the outer cell layer resulting in a steady state cell number in the mature acinus (94) **(Fig. 1-5).**

One caveat of three-dimensional organotypic culture is that it is homotypic in nature, i.e., it only allows for the study of interactions between cells of the same type. Examination of paracrine interactions between tumor cells and stromal cells, a crucial component of tumor progression, is precluded. Consequently, insights from this system would have to be corroborated in animal models of disease in order to establish their function in mammary carcinogenesis.

MCF-10A is a spontaneously immortalized but non-transformed human mammary epithelial cell line derived from the breast tissue of a premenopausal patient with fibrocystic changes (95). These cells exhibit several features of normal breast epithelium including non-tumorigenicity in nude mice, lack of anchorage-independent growth and dependence on hormones and growth factors for proliferation and survival (95). However, there are certain caveats: In spite of having a stable, near-diploid karyotype, MCF-10A cells harbor cytogenetic and epigenetic abnormalities associated with long-term *in vitro* culture, key among them being the expression of basal cell markers. Despite these changes, morphogenesis of MCF-10A cells on reconstituted basement membrane results in growth-arrested structures similar to those obtained with other normal human mammary epithelial cells (96). Therefore, I decided to use three-dimensional culture of this cell line as the model system for my proposed loss-of-function study.

Application of 3D culture in modeling activity of the oncogene ERBB2

The three-dimensional organotypic cell culture system provides the appropriate structural and functional context fundamental for examining the biological activities of cancer genes and for modeling early events in carcinoma formation. Whereas only subtle differences in morphology were observed between normal and tumor cells grown in two-

dimensional culture, three-dimensional basement membrane culture revealed a stark contrast in architectural phenotype (97).

Oncogenes differ in their ability to transform mammary epithelial cells. 3D cultures can effectively recapitulate these differences through the resulting morphogenetic phenotype. Ectopic expression of Cyclin D1 or inactivation of the Retinoblastoma protein (Rb) with HPV16 E7 (oncoprotein from human papilloma virus 16) results in hyperproliferation in acinar structures cultured in 3D. Yet, these structures retain a hollow lumen; proliferating cells lacking contact with the basement membrane undergo apoptosis. This leads to the formation of larger acini (98). Expression of anti-apoptotic Bcl-family proteins alone only delays lumen formation, suggesting that glandular architecture is resistant to isolated oncogenic insults. The lumen is filled when oncogenes that enhance proliferation are co-expressed with those that inhibit apoptosis, or when ERBB2, which induces both activities, is activated by homodimerization. Constitutive activation of Akt (a Serine/threonine kinase that is usually activated only in the outer cells of mature acini) results in activation of mTOR, which causes hyperproliferation and an increase in cell size (99). Activated Akt also significantly amplifies the proliferation provoked by cyclin D1 or HPV E7 during morphogenesis in an EGF-independent manner. Co-expression of CSF-1R and its ligand in MCF-10A acini results in a robust invasive phenotype including the progressive disruption of junctional integrity due to relocalization of E-cadherin from the plasma membrane (100). In spite of its ability to induce SRC activity to a level comparable to that induced by CSF-1R, c-MET does not have the same effect on acinus architecture.

The three-dimensional organotypic culture system also serves to distinguish qualitatively between signaling initiated by homodimers of the ERBB2 family as opposed to heterodimers. Activation of ERBB2 homodimers in MCF-10A cells results in the formation of non-invasive, multiacinar structures that resemble the phenotype of DCIS lesions. Importantly, this phenotype is only observed in 3D cultures but not in 2D cultures, illustrating the advantage of using the 3D culture system to study signaling in early breast cancer development. Activation of ErbB1 homodimers does not induce multiacinar structures (101). However, heterodimerization of ErbB1 and ERBB2 results in the formation of multiacinar structures, with individual cells capable of invading through the extracellular matrix (102). These results underscore the functional diversity between various receptor dimers and

highlight that the importance of the dimerizing partner in deciding the phenotypic outcome, possibly by exerting distinct effects on the various signaling pathways (101).

Chimeric ERBB2

As the preferred partner for heterodimerization, ERBB2 occupies a central role among all the receptors of the ERBB family. ERBB2 is amplified and overexpressed in more than 50% of the cases of Ductal Carcinoma In Situ, a feature that is correlated with poor clinical prognosis (51), (52). Overexpression of ERBB2 in cultured cells causes ligand-independent receptor phosphorylation and transformation. Under such conditions, receptor activation is thought to involve ligand-independent homodimerization (42), (46), (40). It is, therefore, crucial to be able to isolate the biological function of ERBB2 homodimers from the complex network of combinatorial interactions involving homodimers and heterodimers of ERBB receptors and their ligands.

Studies aimed at investigating signaling and biological outcomes downstream of ERBB receptors originally used hematopoietic cells that lacked expression of endogenous ERBB receptors or fibroblasts that express very low levels of these receptors. However, these cell systems are not representative of mammary epithelium. Mammary epithelial cells express more than one member of the ERBB receptor family, making it difficult to distinguish between the signaling and biological specificities of different ERBB receptor homo- or heterodimers with natural peptide ligands. The use of single-chain antibodies to inhibit ERBB2 function provided significant insight into the role of ERBB2-containing heterodimers in breast-tumor-derived cells. However, there were no forward approaches to delineate the specific effects of ERBB2 homodimers in mammary epithelial cell signaling and breast cancer.

A synthetic ligand-mediated controlled dimerization strategy circumvents this problem (103). This approach can be used to selectively activate a chimeric ERBB2 receptor, using a cell-permeable, bivalent ligand called AP1510. This will preclude contributions from endogenous ERBB receptors or EGF ligands secreted in an autocrine manner. The chimera consists of the extracellular and transmembrane domains of the p75 low affinity nerve growth factor (NGF) receptor, p75NGFR, ERBB2 cytoplasmic domain and the AP1510-binding domain from FK-506 binding protein. The chimeric ERBB receptors can

be dimerized with the bivalent FKBP ligand, AP1510 **(Fig. 1-6).** The ensuing downstream signaling mimics that initiated by the full- length molecule.

Activation of ERBB2 in preformed MCF-10A 3D acini induces re-initiation of proliferation and the generation of non-invasive multiacinar structures (103). The luminal space is filled with proliferating cells that have been protected from apoptosis. This suggests that ERBB2 activation overcomes the growth inhibitory effect in polarized epithelial cells and allows cell survival in the lumen of acinar structures. The cells in the middle of the filled acinus also deposit collagen IV around their surface, suggesting a disruption of tight junctions and apical polarity. However, they remain contained within the basement membrane.

ERBB2 homodimers but not ERBB1 homodimers (103) or ERBB1-ERBB2 heterodimers (102) are uniquely able to disrupt normal regulation of proliferation and organization of MECs to form non-invasive multiacinar structures. These atypia-like structures show properties similar to ERBB2-overexpressing pre-malignant comedo-type DCIS tumors *in vivo*. This observation suggests that the activity of ERBB2 homodimers might contribute to the phenotype of comedo-DCIS. It also highlights the reliability and effectiveness of inducible chimeric forms of ERBB2 in 3D cell culture as a model system for elucidating the mechanisms involved in early stages of carcinogenesis *in vitro.*

Significance of proposed research:

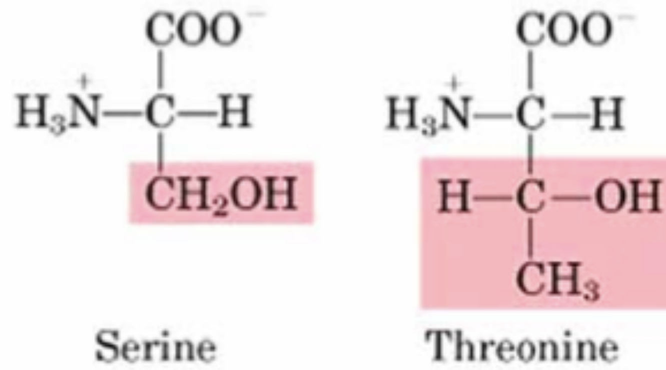
The aim of this dissertation project is to carry out a loss-of-function study of classical (phosphotyrosine-specific) PTPs in mammary epithelial cells by employing the MCF-10A three-dimensional organotypic cell culture system.

Although there is evidence that PTPs make diverse contributions to signal transduction pathways, the physiological roles and substrates of many of these enzymes remain largely uncharacterized to date. A systematic RNAi-based study of PTPs is expected to reveal novel functions of these molecules in the physiological pathways that regulate mammary tissue architecture and, by extension, function. Such a study also has the potential to generate candidate PTPs for investigation as tumor suppressors or oncogenes in breast cancer. Elucidating functional links between individual PTPs and specific signaling pathways in breast cancer could potentially reveal novel therapeutic

targets and biomarkers from among the PTPs themselves or the signaling pathways that they regulate.

Given the challenges underlying the use of anti-ERBB2 therapies in the clinic, the need for a thorough understanding of signaling events initiated by ERBB2 is pressing. Signal transduction pathways driven by ERBB2 form a complex network with multiple levels of crosstalk and redundancy between effectors. Characterizing the mechanisms that regulate this network will provide avenues for the development of novel therapeutic strategies against ERBB2-positive tumors. PTPs represent a crucial aspect of regulation of ERBB2 signaling and can potentially be exploited for therapeutic purposes. A strategy for treatment of ERBB2-positive breast cancers could be to combine the use of pharmacological inhibitors against PTPs (shown to function positively in the context of ERBB2 signaling) with anti-ERBB2 molecules like Herceptin. Inhibition of specific receptors known to activate a certain pathway may not be sufficient to result in a significant inhibition of signaling. It has been suggested that simultaneous blockage of multiple effectors of a signaling pathway might provide a more potent alternative for treatment than inhibition of a single component (104). A deeper understanding of PTP-mediated regulation of ERBB2 signaling might also help us understand the mechanisms of primary or acquired resistance to the anti-ERBB2 drug, Herceptin and devise strategies to overcome them.

Figure 1-1: Structure of the amino acids most commonly phosphorylated in eukaryotic proteins (Lehninger Principles of Biochemistry, David Nelson and Michael Cox, 5th edition)



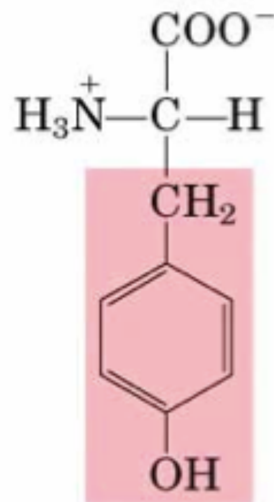


Figure 1-2: General catalytic mechanism of PTPs (Tonks, N.K., FEBS Letts., 2003)

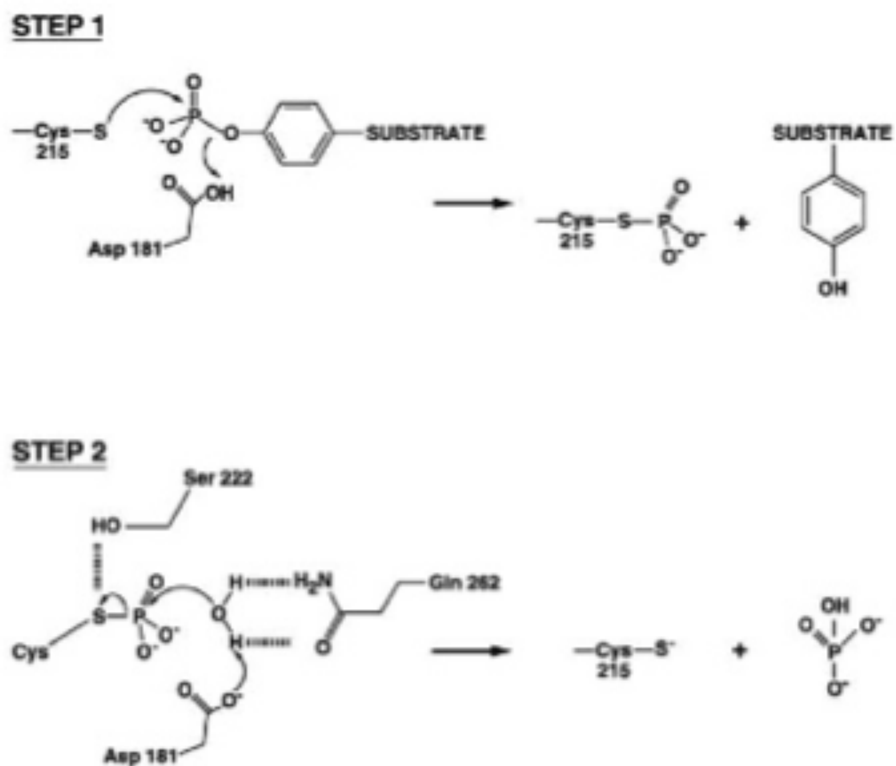


Figure 1-3: The classical Protein Tyrosine Phosphatases (Tonks, N.K., Nat. Rev. Mol. Cell Biol., 2006)

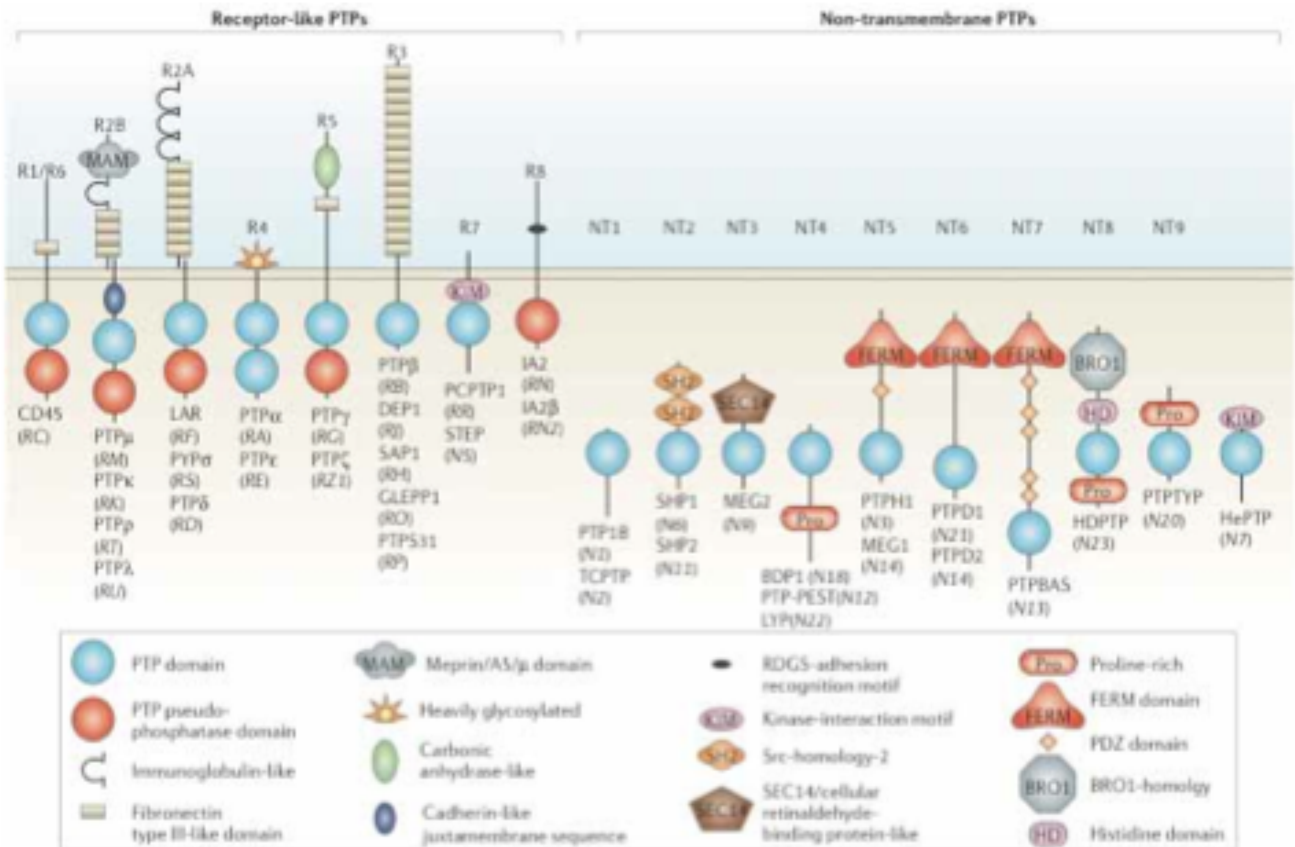


Figure 1-4: The Dual Specificity Phosphatases (Tonks, N.K., Nat. Rev. Mol. Cell Biol., 2006)

Figure 1-5: Morphogenesis of MCF-10A acini in three-dimensional culture (Debnath, J. and Brugge, J.S., Nat. Revs. Cancer, 2005)

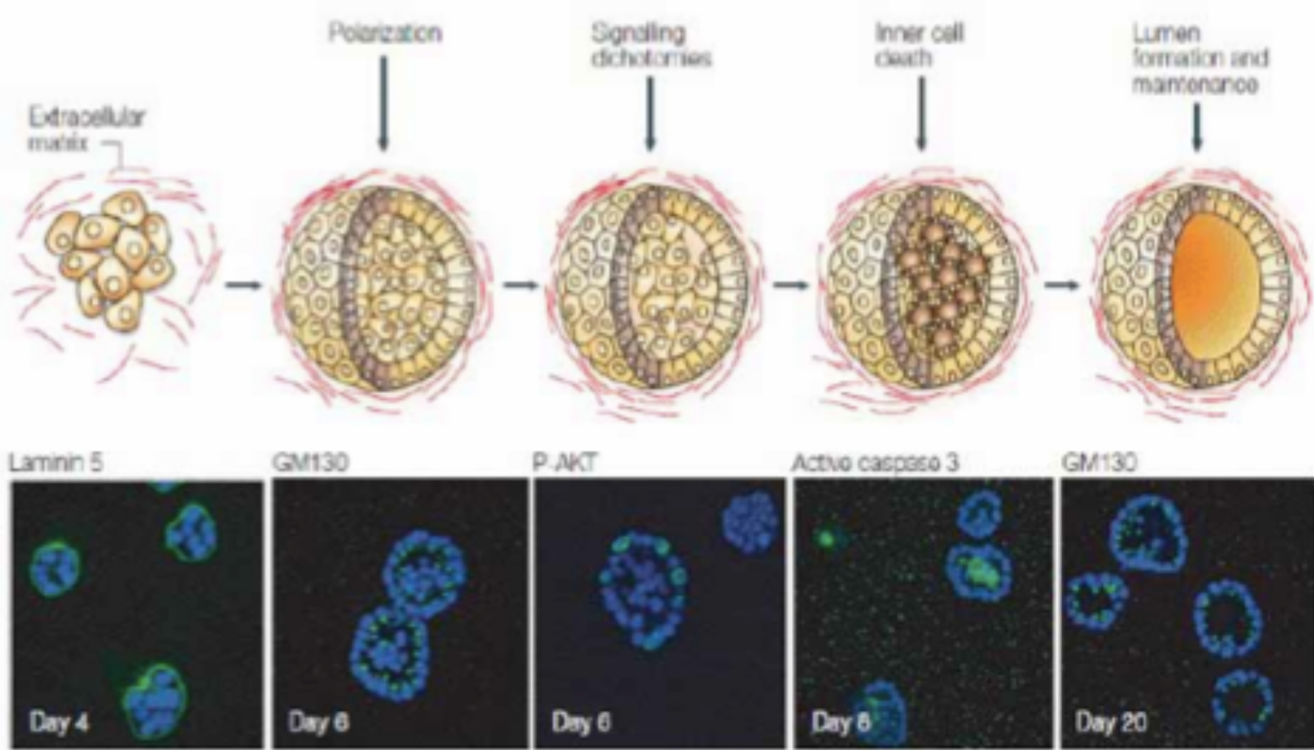
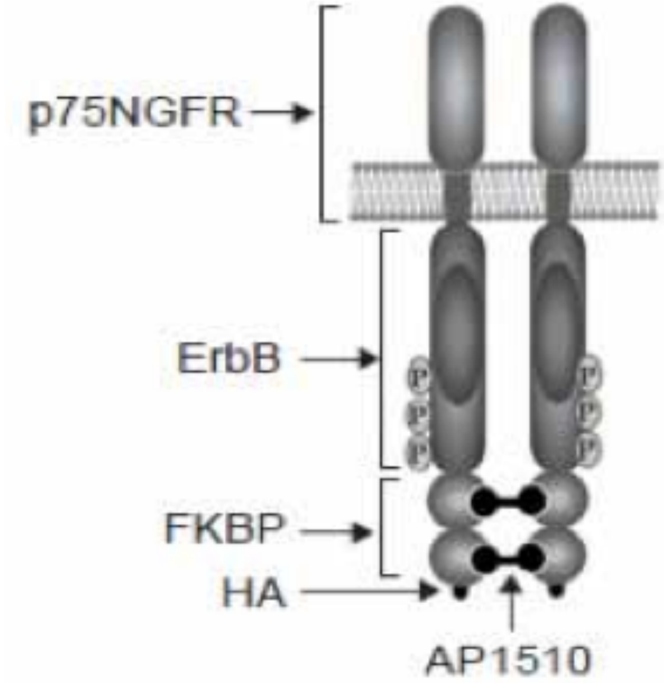


Figure 1-6: Structure of chimeric ERBB2 (Muthuswamy, S.K., Nat Cell Biol., 2001)



Chapter 2

Loss-of-function screen of classical PTPs in three- dimensional 10A.B2 cell culture system

Introduction

In Chapter 1, I introduced the importance of PTPs as critical regulators of cellular signal transduction pathways, with a focus on ERBB2 signaling in breast cancer. There have been many significant discoveries in the recent past that have provided insight into PTP function. However, many members of this family are, to date, poorly characterized with respect to their role in mammary epithelial cells and in the onset/progression of breast cancer. Therefore, I undertook an RNAi-mediated loss-of-function study of the 37 classical PTPs in the human genome, using architecture of 10A.B2 mammary epithelial cells in three-dimensional matrigel as readout for tumorigenic potential. These cells overexpressed a chimeric ERBB2 molecule, thereby allowing us to assess the contribution made by individual PTPs to ERBB2 signaling.

RNA interference (RNAi) is an evolutionarily conserved form of post-transcriptional gene silencing in which double-stranded RNA (dsRNA) induces the degradation of the homologous mRNA, mimicking the effect of reduction or loss of gene activity. RNAi was first identified as a defense mechanism triggered by double-stranded RNAs to protect cells from parasitic nucleic acids in lower organisms (105), (106). Eventually, a conserved RNAi mechanism was also found in higher eukaryotes (107).

The RNAi machinery can be triggered by exogenous or endogenous sources of double-stranded RNA. MicroRNAs (miRNAs) are a well-characterized source of endogenous triggers. They comprise a family of 22 nucleotide-long single stranded, non-protein coding RNAs that regulate gene expression in a sequence -specific manner. Although originally discovered in *C. elegans,* we now know that miRNAs regulate a wide variety of physiological processes in mammalian cells and have diverse expression patterns (108).

In general, miRNAs are derived from larger, Pol II-transcribed, polyadenylated precursors that form imperfect stem-loop structures (109). These are called primary miRNAs (pri-miRNAs). The mature miRNA is most often derived from one arm of this precursor hairpin, and is released from the primary transcript through stepwise processing by two RNase III enzymes. First, Drosha processes the nascent transcript into 70-90

nucleotide-long pre-miRNAs in the nucleus, generating 2-nucleotide long 3' overhangs at the cleavage site. The overhangs are recognized by the nuclear export factor, Exportin 5, which then transports the pre-miRNAs into the cytoplasm. Pre-miRNAs are processed further by Dicer into small miRNA duplexes that contain both the mature miRNA and its complementary strand. Only one strand of the duplex is preferentially incorporated into the ribonucleoprotein complex called the RNA-Induced Silencing Complex (RISC). The extent of complementarity between the miRNA and its target determines the mechanism of silencing. In animals, most miRNAs bind to the target 3'-UTR with imperfect complementarity at multiple sites and function as translation repressors (108).

The analysis of loss-of-function phenotypes is an important approach to investigate the biological function of genes. RNAi technology can now be used for large-scale loss-of-function genetic screens in mammalian systems by systematic manipulation of the endogenous miRNA biogenesis pathway. There are three methods to trigger the RNAi machinery to suppress the expression of a gene of interest:

- Use of double stranded RNA duplexes **(siRNA or short interfering RNA)**, complementary to the mRNA of the target gene and generated *in situ* by cleavage of long dsRNAs
- Use of artificial short hairpin RNAs **(shRNAs),** which are structurally similar to the stem-loop structured endogenous pre-miRNA
- Use of homologous artificial **pri-miRNAs** under the control of the Pol II or Pol III transcription machinery (110).

In this study, I have used shRNAs in order to suppress endogenous expression of PTPs in MCF-10A human mammary epithelial cells.

An understanding of the structure and biology of human miRNAs has led to their use as a scaffold for shRNAs that can efficiently suppress the expression of several target mammalian genes. Cullen and colleagues found that miR30, a mammalian miRNA, can inhibit the translation of a reporter gene that bears an artificial 3' UTR with miR30 complementary sites (111). Using the miR30 precursor RNA as a template, they substituted the miR30 stem sequences with artificial sequences of similar length and used it to achieve effective and regulated target gene inhibition. RNA Polymerase II-mediated transcription 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. miR30-based shRNAs have since been shown to inhibit gene expression more potently than traditional stem-loop RNAs (112).

Several groups have used the miR30 design to develop artificial shRNA expression systems. Scott Lowe's group developed the pMLP vector based on the backbone of Clonetech's pMSCV (Murine Stem Cell Virus) vector design. The shRNAs are incorporated into the sequence of the mir30 cassette and are expressed under the control of the viral LTR (Pol II promoter). Downstream of the shRNA expression cassette, there is a GFP marker expressed from an Internal Ribosome Entry Site (IRES) to track delivery and expression in cells, and a puromycin marker for selection of cells that stably express the construct. Dickins et al. have shown successful suppression of p53 expression in mammalian cells using the pMLP shRNA expression system (113).

My proposed functional study makes use of a library of short hairpin RNA constructs against all the PTPs in the human genome. A former graduate student generated this library in our lab, Guang Lin based on the aforementioned pMLP vector design (81). Guang used the RNAi Codex program developed at CSHL, to design 5 shRNAs targeting each PTP in the human genome. In order to choose 5 out of the many possible shRNAs suggested by the program, priority was given to hits that satisfied the following criteria:

1. Regions that unique to a PTP but conserved among different transcription or splicing variants of the gene.
2. Regions conserved with mouse homologs of the gene
3. Regions equally distributed along the gene.
4. Short hairpins that contain G/C at the 5' end and A/T at the 3' end on the sense strand.

The PTP shRNAs were obtained from Open Biosystems as 97-mers consisting of a common 5' miR30 flanking sequence, a sense strand targeting sequence, a common miR30 loop sequence, an antisense strand targeting sequence and a common 3' miR30 flanking sequence. This oligo would encode an RNA transcript that can form a stem-loop structure based on the complementary sequence of the sense and antisense siRNA sequence. The common 5' and 3' miR30 flanking sequence are a part of the naturally existing miR30 and

can be recognized and digested by Drosha. Guang cloned the PTP shRNA-containing miR30 cassette from the commercially synthesized oligo into the retroviral shRNA expression vector, pMLP.

PTPs included in this study

In my study I propose to focus on the 37 classical PTPs. As they are specific for protein phosphotyrosine residues, I would be analyzing a smaller subset of the phosphoproteome for substrate identification than if I were to also consider phosphoserine and phosphothreonine residues. Secondly, many of the tools available for identification of PTP substrates are more readily applicable to classical PTPs. This includes the "substrate-trapping mutant" strategy (114) and anti-phosphotyrosine antibodies which preclude the need to label cells and tissues.

Results from pilot experiments: proof-of-concept

At the time of proposing the screen, preliminary experiments were being conducted in our lab to study the function of some PTPs in MCF-10A acinar morphogenesis in three-dimensional cultures:

- **PTPRO** emerged as a candidate for investigation following a microarray experiment performed in the Muthuswamy lab on MCF-10A cells undergoing 3D morphogenesis. It was observed that although the level of PTPRO was low in the early stages of morphogenesis, as the structures differentiated into growth-arrested, mature acini, there was a marked increase in PTPRO expression (80). This suggested that PTPRO might have an important role in the establishment of the structure of the mature acinus. Several studies have suggested growth-inhibitory properties for PTPRO and some have identified it as a candidate tumor suppressor in human cancers (115) including hepatocellular carcinoma, where its expression is silenced by methylation (116). Follow-up experiments revealed that suppression of PTPRO with multiple shRNAs led to a significant and reproducible increase in the size of MCF-10A acini (80). Ki67-staining indicated that these structures showed delayed proliferation arrest, whereas staining with antibodies to cleaved caspase-3 revealed that there was no effect on apoptosis (80). In 10A.B2 cells, suppression of PTPRO combined with ERBB2 activation enhanced the formation of hyperplastic, multiacinar

structures. It was subsequently shown that ERBB2 is a direct physiological substrate of PTPRO; suppression of PTPRO increased the tyrosine phosphorylation of ERBB2, (80) indicating that PTPRO directly regulates ERBB2 activation by modulating the phosphorylation status of ERBB2.

- Density Enhanced Phosphatase-1 or **DEP-1** was first cloned in our lab from cells grown to confluence, suggesting that DEP-1 was potentially involved in density-dependent regulation of cell growth and proliferation (117). *PTPRJ*, the gene that encodes DEP-1, maps to the mouse colon cancer susceptibility locus *Scc1,* which is deleted in a variety of human cancers, including breast cancer (118). Re-expression of DEP-1 in cancer cell lines defective in the protein, rescued cell transformation phenotypes (119). Consistent with these observations, our lab showed that shRNA-mediated suppression of DEP-1 in MCF-10A cells was sufficient to induce a multiacinar phenotype in 3D culture (Lin, G. and Tonks, N.K., unpublished observations). It is worth noting that despite them being structurally related, the suppression of PTPRO and DEP-1 results in different three-dimensional phenotypes, suggesting regulation of distinct signaling pathways.
- **RPTPα** has been implicated in a variety of biological and pathological processes including tumorigenesis (120). It is known to act as a positive regulator of signaling pathways and shows oncogenic potential in some human tumors (26). This PTP can transform rat embryonic fibroblasts by activating SRC family kinases (121). Consistent with this observation, RPTPα is overexpressed in several human cancers. In breast cancer however, expression of RPTPα varies widely and there are conflicting reports regarding its role in ERBB2-driven tumors (32), (122). In three-dimensional culture studies performed in our lab, suppression of RPTPα alone did not have a significant effect on MCF-10A acini. However, whereas in ERBB2-induced multiple-acini like structures, the central cells fail to undergo apoptosis, suppression of RPTPα rescued this apoptosis defect in the context of AP1510-induced ERBB2 signaling (Boivin B and Tonks NK, unpublished observations).

The suppression of 3 different PTPs showed 3 distinct phenotypes in three-dimensional culture. These results established proof-of-concept for the proposed PTP loss-of-function study and highlighted the potential of the 3D morphology screen to reveal novel roles of PTPs in mammary epithelial cell function. In view of these precedents, I used RNAi to study

systematically the effects of suppression of classical PTPs in 10A.B2 cells cultured in Matrigel^{TM}.

Unlike cells grown on plastic dishes, the use of the 3D culture system in my proposed study would allow me to evaluate simultaneously the effect of PTP loss by RNAi on signaling pathways that regulate proliferation, apoptosis, and disruption of cell polarity and organization – critical processes that affect the ability of MCF-10A mammary epithelial cells to develop into mature acini. The use of the 10A.B2 cell line would allow me to test for co-operation between ERBB2 and specific PTPs in the regulation of acinus morphology. Knockdown of PTPs that co-operate with ERBB2 and function positively in this signaling context would attenuate the multiacinar phenotype characteristic of ERBB2 whereas ablation of PTPs that function to inhibit ERBB2 signaling would enhance the multiacinar phenotype. This study, therefore, has the potential to reveal novel roles for PTPs in regulating physiological pathways that influence the form and function of mammary epithelium both in the normal state and during early tumor formation driven by ERBB2.

10A.B2 cells expressing a short hairpin against Luciferase serve as control in the proposed screen. Although RNAi is invoked in these cells, the short hairpin is unable to cause gene suppression in the absence of a relevant target. Consequently, these cells undergo normal morphogenesis into polarized, growth-arrested, hollow spheroids over a period of 10-12 days in 3D culture. The effect of PTP suppression alone on acinus morphology, if any, will be visible in acini stably expressing functional hairpin(s) against the target PTP and can be compared to those expressing Luciferase hairpin at each time point. Activation of ERBB2 signaling by addition of AP1510 during development in 3D culture disrupts polarization, abolishes luminal clearing by protecting the central cells from apoptosis and prevents proliferative suppression, leading to a multiacinar phenotype. In this context, Luciferase hairpin-expressing cells will serve as a positive control. RNAi-mediated suppression of PTPs that co-operate with ERBB2 and function positively in this context is expected to attenuate this phenotype. On the other hand, shRNA-mediated suppression of PTPs that function to inhibit ERBB2 signaling would enhance the multiacinar phenotype as has been shown in case of PTPRO.

For each of the 37 classical PTPs, our library contains 5 individually designed shRNAs. Testing each of these hairpins individually would consume a lot of time and reagents,

particularly Matrigel™. In the interest of conserving these resources and for logistical ease, we decided to employ a pooled hairpin strategy, combining multiple hairpins against a single PTP in one pool. In order to determine how much we could dilute a functional hairpin with a non-functional one and still be able to score a 3D phenotype, a hairpin dilution experiment was performed in our lab using shRNAs against PTPRO. Guang Lin, a former graduate student had observed that individual functional hairpins against PTPRO induced a small but significant increase in the size of MCF-10A acini in 3D culture. He made pools with fixed amount of a functional PTPRO short hairpin diluted with increasing amounts of a non-functional one and cultured MCF-10A cells stably expressing these hairpin ratios in 3D. He found that only at a 1:1 ratio, and no higher, could the size increase phenotype be observed and scored successfully. In light of this result, I selected 4 shRNAs against each PTP and combined them into 2 pools of 2 shRNAs each for screening so that even if only one out of the 2 hairpins in a pool is effective at suppression of the target PTP, a 3D phenotype will not be missed **(Fig. 2-1).**

Materials and Methods

Cell culture

10A.B2 cell line (MCF-10A mammary epithelial cell line stably overexpressing chimeric ERBB2) and the virus packaging cell line 293 Phoenix were obtained from the Muthuswamy lab, CSHL and maintained in culture as described in (96).

Reagents

Growth factor-reduced Matrigel^{TM} was from BD Biosciences (Catalog No. 354230, Lot Number 84140-01), AP1510 was from ARIAD Pharmaceuticals

Production of retrovirus carrying shRNA constructs (pooled)

293-Phoenix, a VSVG-pseudotyped retrovirus packaging cell line was transfected with a 1:1 pool of 2 distinct hairpin-expressing constructs against one PTP using Lipofectamine 2000 as per manufacturer's instructions (Day 0). Virus was collected by harvesting the overlying growth medium on Day 3 and was used for infection after filtration through a 0.45μm syringe-fitted filter.

Retroviral infection of 10A.B2 cells and selection of stable pools

24 hours prior to infection, 10A.B2 cells were seeded at a density of 130,000-150,000 cells per 6 cm dish. The following day, the old medium was aspirated and 1ml of freshly collected virus was added (MOI=3 to 5) along with 1ml of MCF-10A growth medium (96) containing 8 μg/ml polybrene. A "no-virus control" plate was also included. The plates were then sealed and centrifuged at 1000 rpm for 1 hour at room temperature. The cells were incubated at 37 degrees for 4-6 hours after which 2 ml of MCF-10A growth medium was added. The cells were incubated overnight at 37°C. The following morning (Day1) the old medium was aspirated and replaced by fresh medium. On Day 2, the appropriate antibiotic was added (puromycin at 2 μg/ml for PTP short hairpin constructs or hygromycin at 150 μg/ml) to select stable cell lines. Following the death of all cells in the control plate, the medium in the infection plates was replaced by MCF-10A growth medium without puromycin. 24 hours later, the drug was added again and maintained as such until the plate became fully confluent. The stable cells were maintained for one additional passage with

the drug. Thereafter, the cells were either cultured without puromycin for one passage before use in three-dimensional cell culture or frozen in MCF-10A growth medium containing 20% FBS for later use.

Three-dimensional morphogenesis assay

Eight-well chamber slides (BD Biosciences) were used for three-dimensional morphogenesis assays. Each well was first coated with 70 µl of a freshly thawed aliquot of Growth factor-reduced Matrigel™ on ice. Thereafter, the slides were incubated at 37°C to allow solidification of the gel. 10A.B2 cells were first washed with PBS, then treated with trypsin and collected in DMEM/F12 supplemented with 20% horse serum. The cells were spun down and resuspended in Assay medium (96) at a concentration of 100,000 cells per 4ml. The cells were mixed 1:1 with assay medium containing 5% Matrigel and 10ng/ml EGF. 400 µl of this mix was added to each chamber of the Matrigel-coated slide (5,000 cells per well). shLuc hairpin-expressing cells served as control. The experiment was conducted in duplicates for each cell line and each condition. Assay medium containing 5ng/ml EGF and 2.5% Matrigel was used to replace overlay medium every 4 days with or without 1µM AP1510 to stimulate chimeric ERBB2. The morphology of the acini was followed by phase microscopy until Day 16.

Microscopy

Phase images from at least 32 random fields per condition were taken every 4 days at 20X objective using Zeiss Axiovert 200M and the mosaic function of the AxioVison 4.4 software.

Acinus size quantitation

The surface area of each acinus at endpoint (Day 16) was measured using Axiovision 4.4 software (Zeiss) at the relevant scaling. At least 150 acini were quantified per condition. Structures having the appearance of multiple partially or fully filled acini within the same basement membrane and beyond a predetermined size cutoff (determined individually for each experiment) were designated “multiacinar structures”. For each condition, the number of multiacinar structures formed was calculated as a percentage of the total number of structures and the average of two wells was used to calculate the fold

difference as compared to shLuc hairpin-expressing control.

Results

In order to investigate the functional role of classical PTPs in mammary epithelial cell function, we employed a loss-of-function approach in the three-dimensional organotypic culture model system. A library of shRNAs (81) against phosphotyrosine-specific PTPs was expressed in 10A.B2 cells (MCF-10A cells that ectopically express a chimeric form of ERBB2, which can be selectively activated using a small molecule dimerizer, AP1510) to study the effect of loss of PTPs, either by themselves, or in combination with ERBB2 activation. The architecture of mammary acini-like structures formed in three-dimensional cultures was used as the phenotypic readout of PTP suppression and was compared to Luciferase short hairpin-expressing control cells.

In order to make the screen more manageable, we tested shRNAs in pools. We chose 4 shRNAs per PTP and grouped them into 2 pools of 2 hairpins each. One of the first PTPs I tested in the screen was PTPRO. It has been previously shown that suppressing PTPRO enhances the formation of hyperplastic, multiacinar structures when ERBB2 signaling is activated using AP1510 (80). The 10A.B2 cell line expressing the functional hairpin against PTPRO (diluted 1:1 with a non-functional hairpin) reproduced the ERBB2-dependent phenotype. This served as a positive control for the screen **(Fig. 2-2c).**

The screen generated 9 novel candidate PTPs, suppression of which had distinct effects on MCF-10A acinar architecture **(Fig. 2-2a)**. In the absence of AP1510 stimulation, we found that shRNA pools against 3 PTPs- PTPRK, PTP-BAS and PTPRU, disrupted acinus morphology, resulting in the formation of partially filled, disorganized structures. **(Fig. 2-2b)** This suggests that these 3 PTPs are important regulators of mammary epithelial architecture in their own right. In the context of AP1510-induced ERBB2 signaling, we found that shRNA pools against 7 PTPs had distinct effects on acinus architecture. shRNA pools against PTPRS, PTPD2, PTPTYP and PTPHe attenuated the multiacinar phenotype characteristic of ERBB2 activation in 3D culture, suggesting that they were potential positive regulators of ERBB2 signaling. On the other hand, shRNA pools against PTPRU, PTPRM and PTPRZ1 enhanced the ERBB2 phenotype, with suppression of PTPRU causing ERBB2-dependent invasion in Matrigel, indicating that this group of PTPs are putative negative regulators of ERBB2 signaling **(Fig.2-3c).** The distinct phenotypic outcomes of expressing shRNAs against some PTPs suggests that they have distinct roles in MCF-10A

acinar morphogenesis which could be ERBB2 dependent or independent. We may, therefore, infer that these PTPs act as specific regulators of signal transduction pathways that regulate mammary acinus architecture.

In order to prioritize the hits for further investigation, we applied the following criteria:

1. PTPs that act as positive regulators of ERBB2 signaling were given the highest priority as they might provide an avenue for therapeutic intervention in ERBB2-positive tumors.
2. PTPs that show the same phenotype with both short hairpin pools were preferred. This suggests that there exists more than one shRNA that can suppress the PTP to an extent sufficient to observe a phenotype in 3D culture. Consequently, the hit is less likely to be a false positive.
3. In view of their potential ability to regulate signaling pathways directly in response to ligand binding, receptor-like or transmembrane PTPs were given priority if they satisfied the aforementioned criteria. This is because of the possibility of manipulating their functions *via* their extracellular domain for therapeutic purposes.
4. Relatively poorly characterized PTPs

Of the 4 PTPs that acted as putative positive regulators of ERBB2 signaling, both shRNA pools against PTPD2 showed a significant decrease in the AP1510-induced multiacinar phenotype **(Fig. 2-3)**, suggesting that the phenotype was produced by at least 2 distinct short hairpins. Further, although there are some references to PTPD2 in the context of cellular transformation, not a lot is known about the functional role of PTPD2 in the context of different oncogenic insults, particularly ERBB2. This suggested that our preliminary findings in the context of ERBB2 activation could reveal novel insights into the function of this PTP in mammary epithelial cancers driven by ERBB2. Consequently, we focused our efforts on PTPD2.

Discussion

The results obtained from the first round of screening underscore the specificity in the function of PTPs in mammary epithelial cells and the potential of a three-dimensional morphogenesis-based screen in revealing the roles of signaling molecules in mammary epithelial cells.

We observed that the majority of PTP shRNAs had little or no effect on mammary acinus architecture in 3D culture. I acknowledge that without validating PTP suppression by the shRNAs used, we cannot be certain that these represent "true negatives". Inefficient hairpins or long half-life of the target protein might preclude significant suppression by RNAi, as a result of which there is a possibility of missing potential 3D phenotypes. However, the primary aim of this project was to identify candidate PTPs that played a role in MCF-10A morphogenesis and characterize their function. Therefore, our strategy going forward was to prioritize the positive hits from the screen and select one for follow-up studies.

Verification of protein knockdown also faces several challenges in this system. First of all, efficient antibodies do not exist against all the classical PTPs. An alternative would be to test for suppression of mRNA levels by quantitative PCR. However, it is known that some PTPs, like PTP1B, have a very long half-life at the protein level. As a result, in spite of a significant level of knockdown of mRNA levels, the residual protein might still be able to exert its biological effects, generating an erroneous interpretation of its functional role. The second limitation is that, at this time, it is not known if all the classical PTPs are expressed in 10A.B2 cells. Considering that activation of the oncogene ERBB2 is accompanied by changes in gene expression (37), it is possible that the levels of specific PTPs may be up- or downregulated, depending on whether they act as positive or negative regulators of ERBB2 signaling. In fact, it has been shown that in the same cell type, expression levels of genes vary not only with the cell culture context i.e, two-dimensional plastic as opposed to three-dimensional Matrigel (93), but also with the developmental stage, i.e. in a temporal manner. For example, at early stages in MCF-10A acinar morphogenesis, the mRNA level of PTPRO was undetectable whereas there was a sudden spike in its levels at later stages of morphogenesis (80). Given that the readout of our screen is morphogenetic changes in 3D culture, validation of short hairpins would also have to be carried out by harvesting cells

at various different time points, necessitating an exponential increase in time and resources, particularly Matrigel. Finally, western blots and QPCR-based analyses represent an average protein or mRNA level in all the cells in a given population whereas individual acini are formed from single cells seeded on a bed of matrigel. As a result, the two results might not be in agreement.

We observed that 9 out of the 37 PTPs tested in the screen registered as positive hits. However, it is important to note that suppression of individual PTPs did not have the same phenotypic outcome with respect to acinus morphology in three-dimensional cultures. Some PTPs disrupted acinus morphology in an ERBB2-independent manner. On the other hand, suppression of 7 PTPs had an ERBB2-dependent effect on acinus architecture, with some PTPs acting as positive regulators of ERBB2 signaling and others acting as negative regulators. These results underscore the fact that PTPs do not serve a housekeeping role as pleiotropic regulators of signaling pathways. Rather, they serve specific functions in physiological processes that regulate three-dimensional morphogenesis of mammary epithelial cells.

Figure 2-1: Hairpin dilution experiment using shRNAs against PTPRO (Guang Lin) MCF-10A cells expressing control (Luc), a functional short hairpin against PTPRO (shRO#4), non-functional short hairpin against PTPRO (shRO#5), or functional short hairpin diluted with non-functional short hairpin at a ratio of 1:1 (1:1 mix) or 1:4 (1:4 mix) were cultured in three-dimensional matrigel. At day 12, acinus size was measured and percentage of acini in each size bracket was plotted.

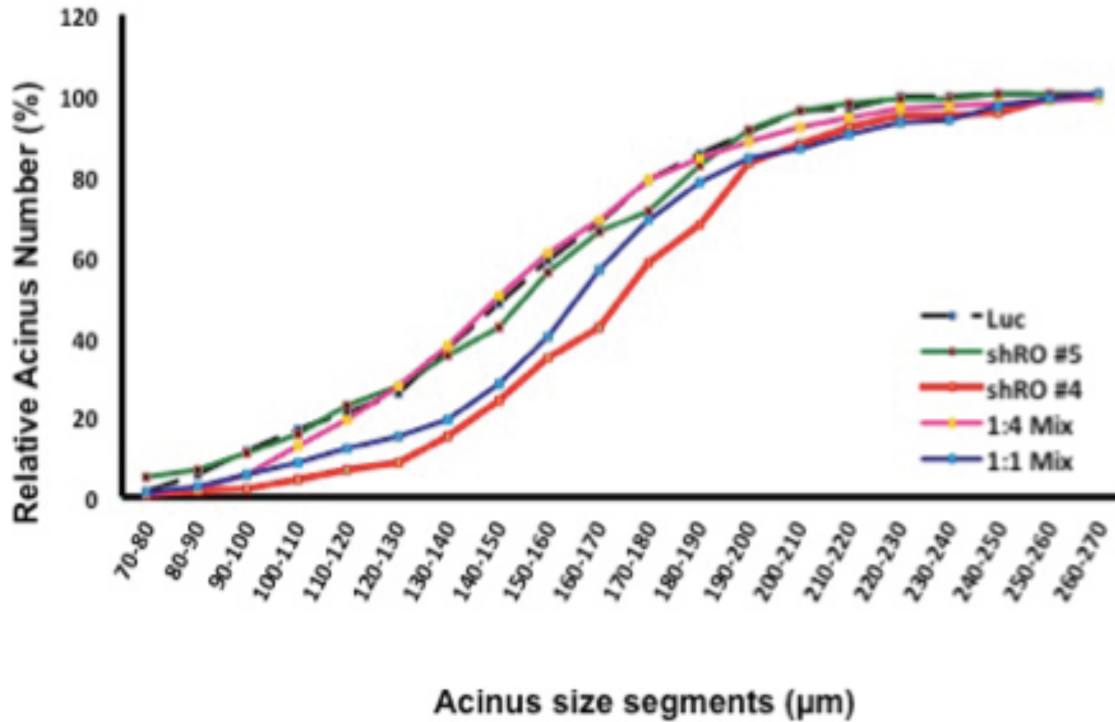
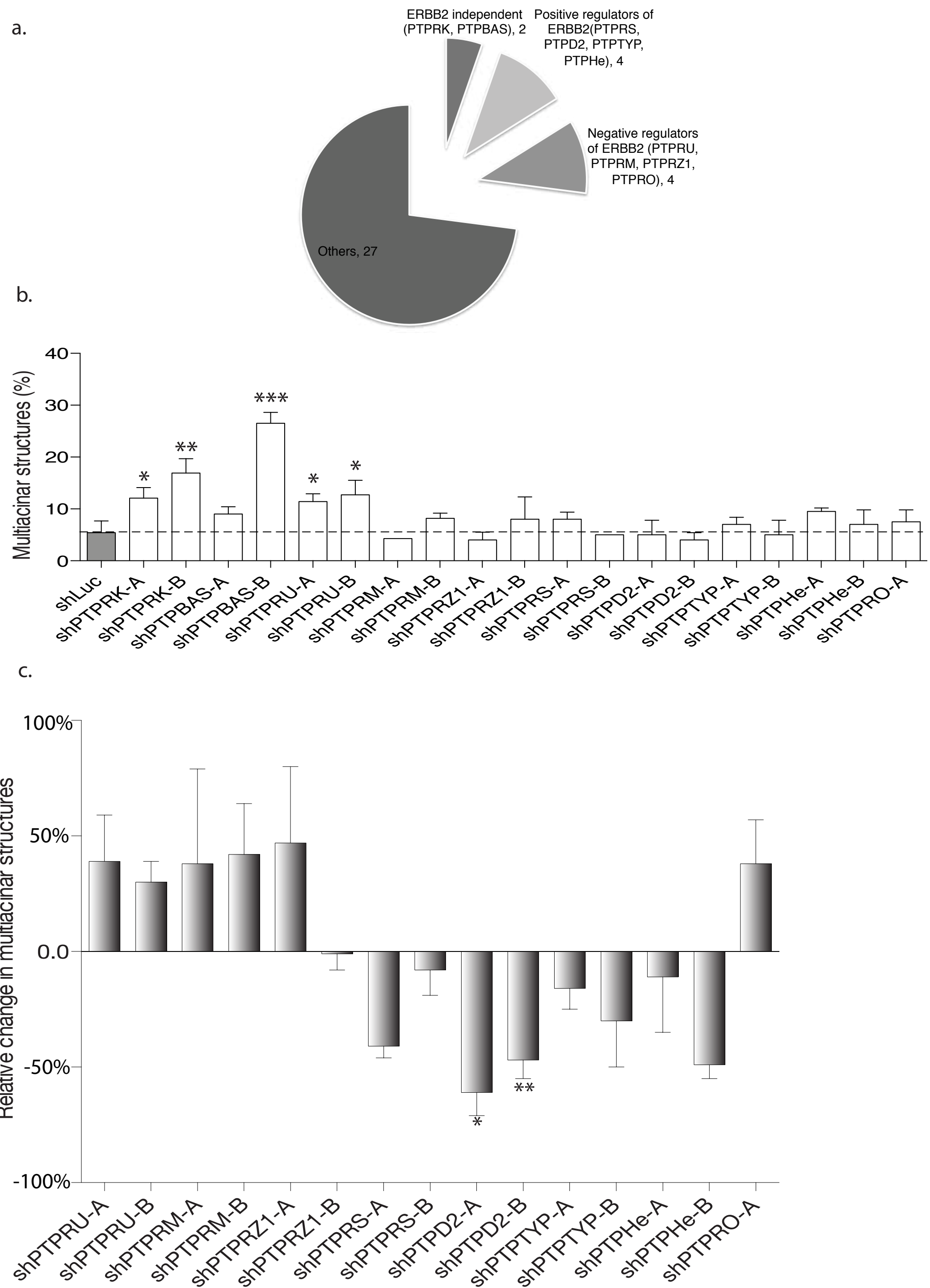
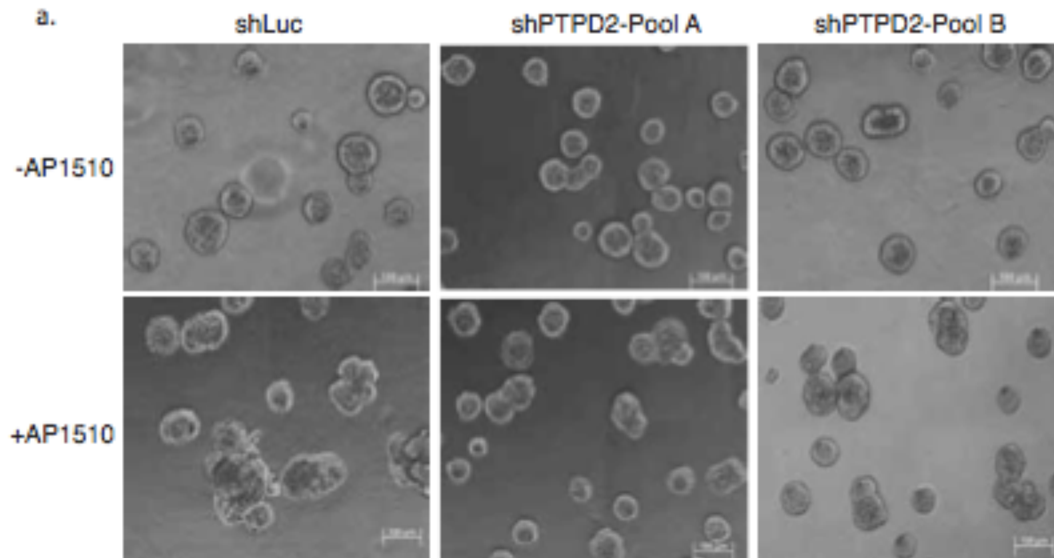


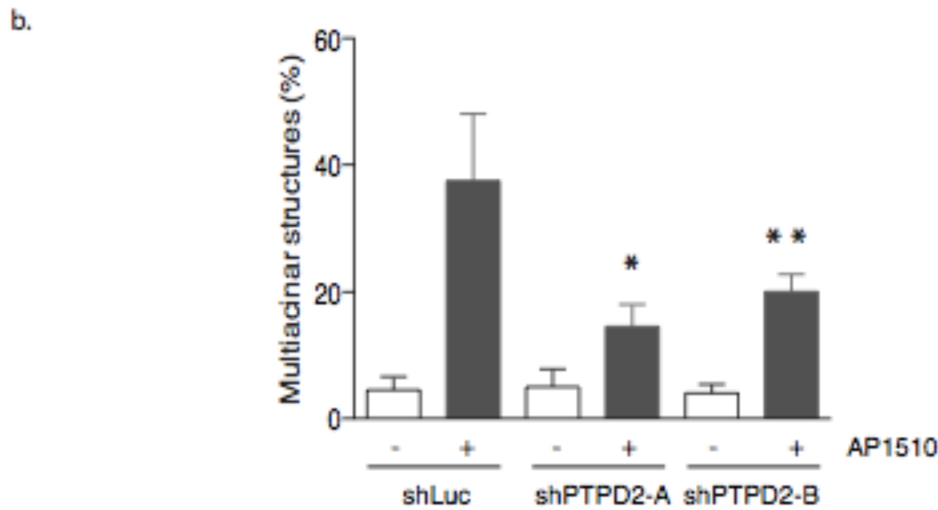
Figure 2-2: Loss-of-function screen of classical PTPs to identify regulators of mammary epithelial morphogenesis. a) Schematic representation of results of shPTP screen by three-dimensional culture in Matrigel in the absence (b) or presence (c) of AP1510. 10A.B2 cells were infected with pools of short hairpins (designated A or B) or control short hairpin targeting firefly luciferase (shLuc). Data are represented as percentage of acini that formed multacinar structures, mean ± S.D. (b) or change in percentage multiacinar strcutures formed relative to AP1510-stimulated shLuc controls (c).



a.
ERBB2 independent (PTPRK, PTPBAS), 2
Positive regulators of ERBB2(PTPRS, PTPD2, PTPTYP, PTPHe), 4
Negative regulators of ERBB2 (PTPRU, PTPRM, PTPRZ1, PTPRO), 4
Others, 27
b.
Multiacinar structures (%)
40
30
20
10
0
*
**
***
*
*
shLuc
shPTPRK-A
shPTPRK-B
shPTPBAS-A
shPTPBAS-B
shPTPRU-A
shPTPRU-B
shPTPRM-A
shPTPRM-B
shPTPRZ1-A
shPTPRZ1-B
shPTPRS-A
shPTPRS-B
shPTPD2-A
shPTPD2-B
shPTPTYP-A
shPTPTYP-B
shPTPHe-A
shPTPHe-B
shPTPRO-A
c.
Relative change in multiacinar structures
100%
50%
0.0
-50%
-100%
*
**
shPTPRU-A
shPTPRU-B
shPTPRM-A
shPTPRM-B
shPTPRZ1-A
shPTPRZ1-B
shPTPRS-A
shPTPRS-B
shPTPD2-A
shPTPD2-B
shPTPTYP-A
shPTPTYP-B
shPTPHe-A
shPTPHe-B
shPTPRO-A

Figure 2-3: Two shRNA pools against PTPD2 suppressed the AP1510-induced ERBB2 phenotype. (a) Representative phase-contrast images of acinar structures expressing control shLuc or short hairpin pools against PTPD2, treated with AP1510 or vehicle for 12 days. (b) For quantitation of the multiacinar phenotype, data are represented as percentage of acini that form multiacinar structures, mean ± S.D. * $p<0.05$, ** $p<0.005$





Chapter 3

PTPD2 acts a positive regulator of ERBB2 signaling

Introduction

PTPD2 cDNA was first identified from human skeletal muscle and described as a non-transmembrane protein tyrosine phosphatase (123). Soon afterwards, another group independently isolated the cDNA from normal human breast tissue and breast tumor cell lines (124). This 1187-amino acid PTP possesses N-terminal sequence homology to the band 4.1-ezrin-radixin-moesin protein family (FERM domain) and a single C-terminal PTP domain. It is, therefore, also called "PEZ" (Phosphatase with Ezrin domain). A linker, rich in proline and acidic amino acids, connects the two domains. Northern blot analysis indicates that PTPD2 is expressed in a variety of human tissues including kidney, lung and placenta (125).

PTPD2 has been found to localize to multiple subcellular locations, depending on factors such as cell type, cell-matrix adhesion, serine phosphorylation and cell confluence, suggesting differential regulation of PTPD2 function in different cellular contexts. Consistent with the presence of a FERM domain (responsible for targeting proteins to the cytoskeleton-plasma membrane interface), in transiently transfected COS7 AND HEK293 cells, murine PTPD2 is distributed in the Triton X-100 insoluble, membrane/cytoskeleton-associated fractions (126). Some studies suggest that cell-substrate adhesion regulates the intracellular localization of PTPD2 through serine phosphorylation; cell detachment induces dephosphorylation and translocation of PTPD2 from the cytosol to the membrane-associated cytoskeleton. This suggests a role for this PTP in adhesion or ECM-driven signaling pathways (127). In endothelial cells, the subcellular localization of PTPD2 is regulated by cell density and serum concentration; PTPD2 is nuclear in sparsely plated, actively proliferating cells in the presence of serum whereas it is cytosolic, concentrated at intercellular junctions, in confluent, quiescent monolayers. TGF-β, which inhibits cell proliferation but not migration also inhibits translocation of PTPD2 from the cytosol to the nucleus (125).

Variations in the observed subcellular localization of PTPD2 suggest that this enzyme could have multiple roles, involving the dephosphorylation of different substrates. Accordingly, there are several reports that suggest diverse functional roles for PTPD2 in various cell types. Overexpression of murine PTPD2 inhibits cell growth and adhesion to extracellular matrix proteins like type IV collagen and laminin. It also causes a decrease in

the actin stress fibers and the number of focal adhesions (127). Consistent with its localization at adherens junctions, a truncation mutant of PTPD2 lacking the catalytic domain acts as a dominant negative, enhancing overall tyrosine phosphorylation at adherens junctions and cell motility (128). This suggests that PTPD2 could play an important role in regulating the assembly or disassembly of adhesion complexes. Specifically, β-catenin is one of the substrates of PTPD2 in this context. During zebrafish development, expression of PTPD2 is highly upregulated in specific tissues and contributes to organogenesis through the regulation of TGF-β signaling (129). Overexpression of PTPD2 in Madin-Darby Canine Kidney (MDCK) cells, a well-established model for epithelial polarization, causes Epithelial to Mesenchymal-Transition (EMT), also by activating canonical TGF-β signaling. This causes increased cell motility and changes in cellular morphology and gene expression patterns typical of cells undergoing EMT (129), a process associated with epithelial cells breaking the constraints of cell-cell adhesion and taking on migratory properties. The *Drosophila* ortholog of PTPD2 (Pez) acts in the Hippo signaling pathway to restrict intestinal stem cell (ISC) proliferation. Under conditions of intestinal stress, ISC proliferation requires deactivation of the Hippo signaling pathway and activation of the transcriptional coactivator *Yorkie*. Pez binds an upstream component, *Kibra*, and acts as a negative regulator of *Yorkie* (130) in a manner independent of its catalytic activity.

The most widely characterized function of PTPD2 is as a negative regulator of the oncogenic transcription factor, Yes-Associated Protein1 or YAP1 (131). The exact mechanism, however, is unclear. One study reports that in MCF-10A cells, PTPD2 interacts with YAP1 and promotes nucleus to cytoplasm translocation during contact inhibition, thus inhibiting the transactivation of YAP1 (132). Another study, however, reports that PTPD2 negatively regulates YAP by directly dephosphorylating it at Y357 (133), a site implicated in the regulation of YAP's tumor suppressor function in response to DNA damage (134).

There is evidence in the literature suggesting a role for PTPD2 in human disease. *PTPN14*, the gene that encodes PTPD2, has been identified as a modifier of angiogenesis and hereditary hemorrhagic telangiectasia via EphrinB2 and ACVRL1/ Activin Receptor-like Kinase. Human polymorphic variants of *PTPN14* are associated with clinical severity of the disease (135). Another study reports a causal role for *PTPN14* deficiency or loss-of-function

in lymphatic hyperplasia and interaction between PTPN14 and VEGF-R3, known to be important in angiogenesis (136).

In the context of human cancer, PTPD2 is frequently mutated in colorectal cancers (27). The somatic mutations reported in this study were all missense mutations, primarily clustered around the linker region. However, the functional significance of these mutations is yet to be determined. It has been reported that PTPD2 directly dephosphorylates p130Cas at Y128 in colorectal cancer cells. Consistent with a tumor suppressor role for PTPD2, colorectal cancer cells homozygous for the Y128F (non-phosphorylatable) knock-in mutation, exhibit significantly reduced migration and colony formation, impaired anchorage-independent growth and slower xenograft tumor growth in nude mice (137). In a mutational study of human breast cancer, it was observed that the *PTPN14* gene is mutated at a frequency higher than “background” mutation rate, suggesting a possible role for this gene in disease pathogenesis (138).

To summarize, multiple reports in the literature suggest that PTPD2 is an interesting candidate to investigate, particularly in the context of human cancer. However, the functional role of this enzyme is yet to be defined. Our observations suggest that PTPD2 acts positively to regulate ERBB2 signaling in 10A.B2 cells cultured in three-dimensional Matrigel. This chapter describes the characterization of this phenotype.

Materials and Methods

Reagents

Antibodies:

PTPD2 (R and D Systems MAB4458) Ki67 (Zymed), GM130 (BD Transduction Labs), cleaved caspase-3 (Cell Signaling), phosphotyrosine antibody 4G10 (Millipore), HA.11 (Covance), phospho-Akt p-T308, Total Akt, phospho-ERK1/2 and Total ERK (Cell Signaling)

Preparation of lysates and Immunoblotting

To harvest total cellular lysates from 10A.B2 stable cells, a fully confluent plate of cells was washed with 5 ml 1X phosphate-buffered saline (PBS) and extracted in 500µl of RIPA Buffer (50mM Tris-HCl, 150mM NaCl, 1% NP-40, 0.5% Sodium deoxycholate, 0.1% SDS, pH 8.0 containing 2mM sodium orthovanadate and Roche protease inhibitor cocktail) Cells were lysed on a rotating wheel at 4°C for 30 minutes after which the lysate was cleared by centrifugation. The supernatant was quantitated using Bradford Reagent and equal amounts of lysate resolved by SDS PAGE. The resolved proteins were transferred onto a nitrocellulose membrane. The membrane was blocked for 30 minutes at room temperature and then incubated overnight in primary antibody as per manufacturer's instructions. The following morning, the membrane was washed thrice with IX TBST after which it was incubated in the appropriate horseradish peroxidase-conjugated secondary antibody at room temperature for 1 hour. The membrane was then washed again using 1X TBST and finally developed using chemiluminescent developing agents from GE Healthcare.

To check the activation status of effectors downstream of ERBB2, 70-90% confluent 10A.B2 cells were washed twice with 1X PBS and starved in DMEM/F12 medium (without any additives) overnight (14-16 hours). The following morning, the cells were stimulated in DMEM/F-12 containing 1µM AP1510 for the desired duration and lysed for immunoblotting.

Immunofluorescence

For Ki67, GM130, and cleaved caspase-3 staining of three-dimensional cultures, acini were fixed and stained at Day 16, Day 18 and Day 8 respectively as described in (96).

Briefly, acini were fixed with 5% formalin (diluted fresh using 1X PBS) for 30 minutes at room temperature. After rinsing with 1X PBS: glycine, the acini were permeabilized with 0.5% Triton X-100 in 1X PBS for 10 minutes at room temperature. They were then rinsed with 1X I.F. wash buffer and blocked with freshly prepared and filtered primary block (1X I.F. wash buffer containing 10% goat serum) for 1-1.5 hours at room temperature. This was followed by incubation in secondary block (primary block containing 1:100 dilution of $F(ab)_2$) for 30-40 minutes at room temperature. The acini were incubated with primary antibody diluted in secondary block overnight at room temperature. The following morning they were rinsed with 1X IF wash and incubated in the desired Alexa-conjugated secondary antibody (diluted 1:100 in primary block) for 1 hour at room temperature. Hereon, the experiment was carried out with the slides wrapped in aluminium foil. The acini were rinsed again in 1X IF wash buffer, the nuclei counterstained with DAPI (diluted in 1X PBS) for 10 minutes at room temperature and after a final wash, the structures were mounted with Prolong Gold Antifade Reagent (Molecular Probes) and allowed to dry before visualization.

Detachment Culture

Tissue culture plates were coated with 12 mg/ml poly-HEMA in 95% ethanol and incubated at 37°C until dry. 10A.B2 cells were plated in complete growth medium with or without 1µM AP1510 in suspension at a density of 400,000 cells/ml for 48 hours as described in (139). At the end of 48 hours, cells were collected and washed in 1X PBS before lysis for immunoblotting.

Results

Individual shRNAs against PTPD2 recapitulated the ERBB2-dependent phenotype

We deconvoluted the pools of short hairpins against PTPD2 into individual shRNAs and selected two that efficiently suppressed expression, verified by immunoblotting **(Fig. 3-1a).** To test whether these individual short hairpins recapitulated the phenotype observed with the pools used in the screen, we treated cells expressing control (shLuc) or PTPD2 short hairpins with the dimerizer, AP1510. Activation of ERBB2 induced the formation of multiacinar structures in control acini; however, the number of these structures was significantly reduced in cells expressing the PTPD2 short hairpins **(Fig. 3-1b, top panel)**. In order to analyze differences in the size of these three-dimensional acini, we measured the area of individual structures from two-dimensional phase images. Activation of ERBB2 in Luciferase short hairpin-expressing cells resulted in the formation of large multiacinar structures with a significant variation in the size distribution whereas activation of ERBB2 in cells expressing shRNAs against PTPD2 formed significantly smaller multiacinar structures without a marked variation in the size distribution **(Fig. 3-1b, bottom panel).**

Suppression of PTPD2 did not affect ERBB2-induced hyperproliferation

Activation of ERBB2 in mammary acini induces the formation of multiacinar structures by causing hyperproliferation, lumen filling and disruption of apico-basal polarity (101). We tested which of these phenotypic outcomes was affected by loss of PTPD2. ERBB2-induced changes in proliferation were measured in control and PTPD2 short hairpin-expressing acini by staining AP1510- treated structures with an antibody against Ki67 (a marker of proliferating cells) Interestingly, ERBB2-induced proliferation in shPTPD2 acini was similar to that of control acini. **(Fig. 3-2)**

Suppression of PTPD2 inhibited ERBB2-induced lumen filling and loss of polarity

Activation of ERBB2 causes survival of matrix-detached cells, resulting in lumen filling (101). When stimulated with AP1510, acini derived from control 10A.B2 cells showed luminal filling whereas those that were derived from shPTPD2 cells did not **(Fig. 3-3).**

During morphogenesis, the outermost cells in a mammary acinus that are in contact with the extracellular matrix undergo apico-basal polarization. Polarized cells can be

distinguished by the localization of the *cis*-Golgi marker, GM130, towards the side of the cells facing the prospective lumen (98). Activation of ERBB2 disrupts this axis of polarity as a result of which GM130 is localized to face the lateral or basal surface of the cells. AP1510-induced stimulation of ERBB2 signaling in control (shLuc) acini resulted in the expected mislocalization of GM130. However, in PTPD2 short hairpin-expressing acini, GM130 was localized to the apical surface of the cells in spite of ERBB2 activation with AP1510 **(Fig. 3-3)** Together, these data suggest that PTPD2 is required specifically for ERBB2-mediated lumen filling and disruption of apico-basal polarity but not for ERBB2-mediated hyperproliferation.

Suppression of PTPD2 enhanced apoptosis in acini with activated ERBB2

ECM-detached cells in a developing acinus do not receive survival cues and die by apoptosis. However, ERBB2 activation can override these apoptotic signals, causing lumen filling. We tested whether suppression of PTPD2 inhibited ERBB2-induced lumen filling by causing apoptotic cell death. Upon staining with an antibody against cleaved caspase-3, a marker for apoptosis, we observed little staining in ERBB2-induced multiacinar structures. However, acini derived from PTPD2 knockdown cells showed increased caspase-3 staining in the context of ERBB2 activation **(Fig 3-4).** This suggests that PTPD2 is required for ERBB2 to override apoptotic signals.

Overexpression of PTPD2 enhanced the ERBB2-induced multiacinar phenotype

In order to validate further the role of PTPD2 in ERBB2 signaling, we tested the effect of PTPD2 overexpression in the context of ERBB2 activation. Acini derived from cells stably overexpressing PTPD2 or vector control **(Fig 3-5a)** were cultured in three-dimensional Matrigel™ and stimulated with the dimerizer AP1510 to activate ERBB2 signaling. We observed that PTPD2-overexpressing cells showed an enhanced multiacinar phenotype in the context of ERBB2 activation as compared to controls. **(Fig 3-5b)** This suggests that PTPD2 co-operates with ERBB2 to transform mammary epithelial cells - a threshold amount of the protein is critically required for ERBB2 to induce a transformed phenotype whereas an excess of the protein enhances the ERBB2 phenotype.

PTPD2 is amplified or overexpressed in breast cancers

To investigate whether there were alterations in the copy number or mRNA expression level of PTPD2 in human breast cancer, we analyzed 463 human tumor samples from the TCGA data set using cBioPortal (www.cbioportal.org) (140), (141). The results indicated that PTPD2 is altered in 14% of the tumors analyzed, a majority of the alterations being amplification or mRNA upregulation events (**Fig. 3-6**). This is consistent with a pro-oncogenic role for this enzyme and is comparable to the frequency of such alterations observed in the gene encoding PTP1B, which has also been shown to act as a positive regulator of ERBB2 signaling (87), (88).

Suppression of PTPD2 attenuated effector pathways under conditions of acute ERBB2 stimulation in attached cells

Following dimerization, ERBB2 undergoes trans-phosphorylation, creating phosphotyrosine sites that recruit and activate signaling complexes. This results in the activation of effector pathways, chiefly, RAS/MAPK and PI3K/AKT (37). We evaluated the effects of PTPD2 suppression on the activation of these pathways following acute stimulation with AP1510. Suppression of PTPD2 did not affect the overall tyrosine phosphorylation status of ERBB2 under these conditions. However, we observed a dramatic reduction in AKT activation in PTPD2 knockdown cells as compared to controls **(Fig 3-7a).** Any effect on ERK signaling was less pronounced. This suggests that PTPD2 is essential for optimal activation of effector pathways downstream of ERBB2.

Suppression of PTPD2 attenuated ERBB2 phosphorylation specifically in suspension cultures

We had observed previously that loss of PTPD2 prevented ERBB2-mediated survival of cells in the lumen. Cells cultured in suspension (detachment conditions) mimic several aspects of signaling observed in ECM-detached luminal cells, which ultimately undergo apoptosis (142). Therefore, we tested the effect of PTPD2 loss on ERBB2 signaling in cells cultured in suspension. We observed that there was no appreciable difference in AP1510-induced ERBB2 phosphorylation between control cells and PTPD2 knockdown cells cultured under attached conditions. However, following 48 hours in suspension culture, PTPD2 knockdown cells showed lesser AP1510-induced ERBB2

phosphorylation as compared to controls. These results suggest that PTPD2 has a context-dependent function in the regulation of ERBB2 signaling- loss of PTPD2 negatively affects ERBB2 phosphorylation specifically in cells cultured in suspension, which model the ECM-detached luminal cells **(Fig. 3-7b).**

Discussion

The results presented in this chapter point towards a novel, positive role for PTPD2 in ERBB2 signaling. Suppression of the PTP attenuated the multiacinar phenotype characteristic of ERBB2 activation, specifically by inhibiting ERBB2-mediated lumen filling and loss of polarity but not hyperproliferation. It is worth noting that this is distinct from the mechanism of action of PTP1B, another classical PTP that also acts as a positive regulator of ERBB2 signaling, but by regulating both ERBB2-induced proliferation and cell survival (89). Our observation emphasizes the need for exquisite control of effector pathways that regulate mammary acinus architecture, and highlights the specific functional roles of PTP family members in receptor tyrosine kinase signaling.

Previous studies of PTPD2 function in various tissues and cancer models have suggested diverse roles for this phosphatase in oncogenic signaling. Overexpression of PTPD2 in epithelial MDCK cells has been shown to cause changes in cell morphology, increase in cell motility and changes in gene expression (129) observed in epithelial cancer cell lines that have undergone Epithelial-Mesenchymal Transition (EMT), a process associated with metastasis. A high frequency of mutations has been described in *PTPN14* (the gene that encodes PTPD2) in breast cancers (138). However, these mutations have not been functionally characterized.

It has been shown that suppression of PTPD2 in MCF-10A cells results in the disruption of acinus morphology by activating the oncogenic transcription factor, YAP1 (132), (133). However, we did not observe a significant effect of PTPD2 knockdown on MCF-10A acinus morphology. In contrast, our observations support a pro-oncogenic role for PTPD2 in the context of ERBB2 signaling. This difference between the two observations can be attributed to the cell lines used in the two studies. The study by Chen *et al.* uses parental MCF-10A cells, which, in general, are dependent on EGF signaling. In our study, we used 10A.B2 cells, which overexpress a chimeric ERBB2 molecule and are dependent on the ERBB2 signaling pathway. The observation that PTPD2 has functionally distinct roles under these two conditions underscores the fact that some PTPs may function negatively or positively depending on the signaling context. There are precedents for context-dependent function among the members of the PTP family. As explained before, the gene that encodes PTP1B is co-amplified with *ERBB2;* PTP1B is known to play a pro-

oncogenic role in the context of ERBB2-driven mammary carcinogenesis (88), (87). However, in ovarian cancer cells, PTP1B inhibits pro-survival signals induced by IGF-1, suggesting a tumor suppressive role (143). SHP2 plays a well-characterized oncogenic role in several juvenile epithelial and hematopoietic tumors; activating point mutations in *PTPN11* (30), the gene that encodes SHP-2, generate a constitutively active mutant that promotes signaling through the activation of RAS/MAPK and SRC. However, heterozygous inactivating mutations have recently been found in the SHP-2 gene in metachondromatosis, a rare inherited disorder featuring cartilage tumors. SHP-2 acts as a tumor suppressor in cartilaginous tissues, acting through an FGFR/MEK/ERK-dependent pathway to prevent excessive proliferation downstream of the Hedgehog signaling pathway (144).

The report by Chen *et al*. also shows that the tumor suppressor function of PTPD2 is specifically through the regulation of YAP1; loss of PTPD2 has no effect when YAP1 is also knocked down (132). YAP1 has been shown to regulate p53 family members positively and itself be negatively regulated by Akt in promoting apoptosis. The YAP1 gene is localized on 11q22.2, a site of frequent loss-of-heterozygosity (LOH) in breast cancers. Immunohistochemistry analysis of human breast tumors shows significant loss of YAP protein, consistent with specific deletion of the YAP gene locus (145). It has been suggested that the phosphorylation status of YAP1 can influence the specificity of target gene activation (134). It is, therefore, conceivable that the functional role of PTPD2 in ERBB2 signaling could either be independent of YAP1 or by regulating the phosphorylation of YAP1 at tyrosine residues that might affect its transcriptional co-activator function to positively regulate ERBB2 signaling.

We report that suppression of PTPD2 attenuated the multiacinar phenotype characteristic of ERBB2 in three-dimensional cultures, suggesting that a threshold amount of this enzyme is critical for the ERBB2 signaling pathway to function optimally. Overexpression of PTPD2, on the other hand, enhanced the ERBB2 phenotype. These results are consistent with data from human tumor samples in which PTPD2 is amplified/overexpressed at a frequency comparable to PTP1B- an established positive regulator of ERBB2-induced oncogenic signaling. Interestingly, out of the 463 tumors analyzed in the data set, both ERBB2 and PTPD2 are co-amplified and/or simultaneously overexpressed only in a small subset of these tumors. It is possible, that in tumors in which

ERBB2 but not PTPD2 is amplified, the specific effectors regulated by PTPD2 are activated by amplification/overexpression/gain-of-function mutations. The result of such a change would be comparable to PTPD2 upregulation.

Suppression of PTPD2 specifically affected ERBB2-induced lumen filling and loss of epithelial polarity but not ERBB2-induced hyperproliferation. There are precedents in the literature of these physiological processes being independently regulated downstream of ERBB2. For example, expression of a mutant form of the polarity protein Par6, which is unable to bind to aPKC, reverses the multiacinar and cell survival phenotypes characteristic of ERBB2 without affecting hyperproliferation (146). Another study provides evidence that hyperproliferation and loss of polarity are phenotypes resulting from two independent effector pathways downstream of a β4 integrin-ERBB2 complex (147). It remains to be tested whether PTPD2 functions in ERBB2 signaling by regulating one or more of these downstream effector pathways or an as yet uncharacterized pathway.

Both apoptosis and autophagy have been shown to contribute towards lumen formation (98),(148). We observed a small increase in caspase-3 activation in PTPD2 knockdown acini in the context of ERBB2 activation. This enhanced luminal apoptosis presumably counters AP1510-evoked hyperproliferation, resulting in normal acinus architecture. While it does not rule out autophagy, this observation suggests that PTPD2 is *at least* required for ERBB2 to override apoptotic signals. One possibility is that ERBB2 could be signaling to anti-apoptotic pathways *via* PTPD2. However, it has also been suggested that polarity proteins themselves can directly regulate cell survival pathways (146) raising the possibility that PTPD2 could be regulating these pathways indirectly by acting on polarity proteins. In 10A.B2 cells cultured in suspension, (a condition that mimics the ECM-detached cells of the prospective lumen), loss of PTPD2 prevented robust ERBB2 phosphorylation upon AP1510 stimulation. It is conceivable that this attenuation of ERBB2 activation adversely affects downstream signaling to anti-apoptotic pathways.

Figure 3-1: Suppression of PTPD2 with two distinct short hairpins suppresses the AP1510-induced ERBB2 phenotype in three-dimensional cultures. a) Immunoblot showing PTPD2 protein level in 10A.B2 cells expressing control or individual shRNAs targeting PTPD2. The expression of β-actin was used as loading control. b) Representative phase- contrast images of acinar structures, expressing indicated shRNAs and treated with AP1510 or vehicle control for 12 days. c) For quantitation of the multiacinar phenotype, data are expressed as percentage of acini that form multiacinar structures ± S.E.M. (***$p<0.0005$) (left). Size distribution of multiacinar structures expressed as area in μm^2 (right)

Figure 3-2: Suppression of PTPD2 did not affect ERBB2-mediated proliferation. a) Representative images of acinar structures stimulated with AP1510 or vehicle for 12 days and immunostained for proliferation marker Ki67 (red) Nuclei were costained with DAPI in blue. b) Quantitation of Ki67-positive acini.

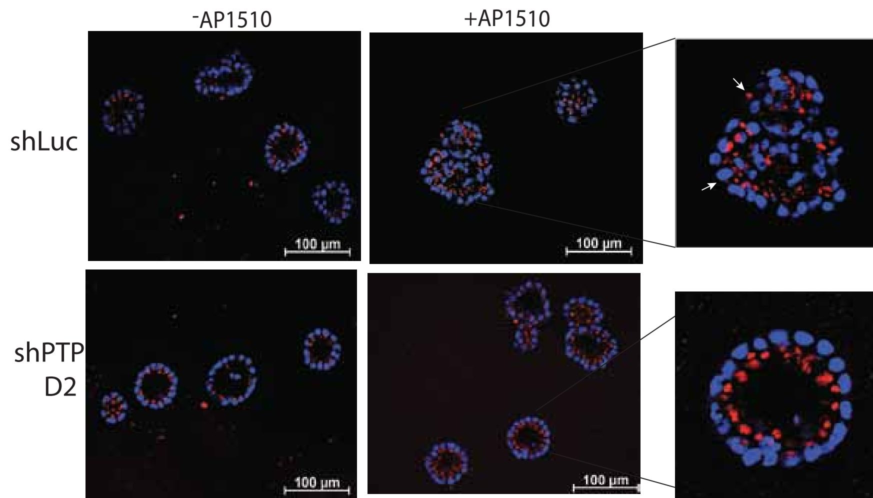


Fig. 3-3 : Suppression of PTPD2 inhibits ERBB2-mediated lumen filling and loss of polarity
Representative images of cross-sections of acinar structures stimulated with AP1510 or vehicle control and immunostained for cis-Golgi marker GM130 (red). Nuclei were costained with DAPI in blue. Insert shows representative AP1510-stimulated structures with arrows indicating mislocalization of GM130 to lateral or basal surfaces.

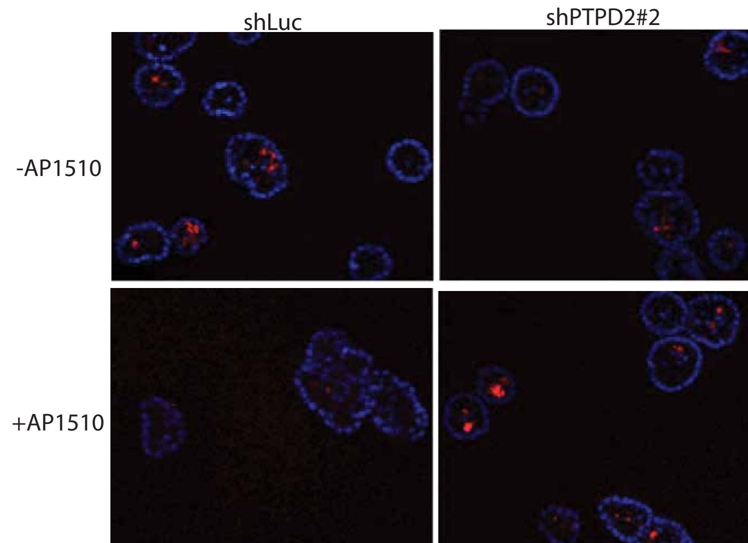


Fig. 3-4 : Suppression of PTPD2 enhanced apoptosis in AP1510-induced 3D cultures
Representative images of cross-sections of acinar structures stimulated with AP1510 or vehicle control and immunostained for apoptosis marker, cleaved caspase-3 (red). Nuclei were costained with DAPI in blue

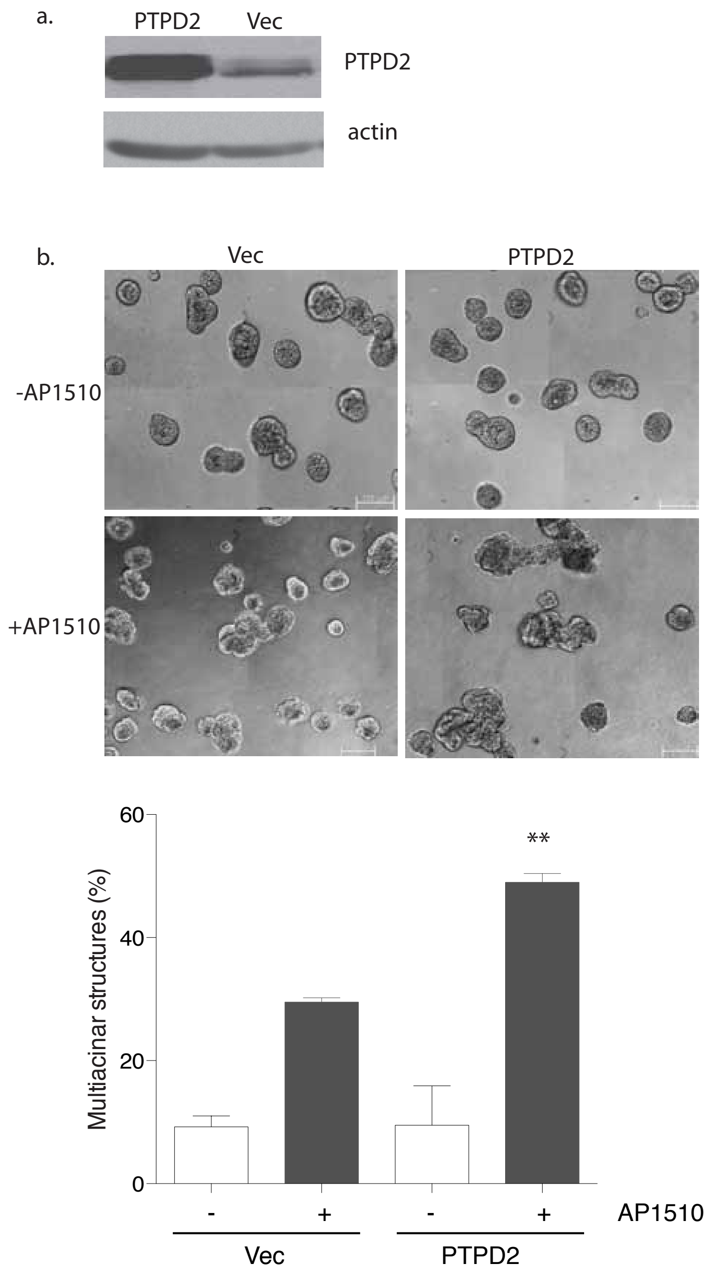


Fig. 3-5. Overexpression of PTPD2 enhanced the AP1510-induced phenotype
a) Immunoblot showing PTPD2 protein level in 10A.B2 cells expressing vector control or PTPD2 cDNA. The expression of β-actin was used as the loading control. b) Representative phase-contrast images of acini expressing empty vector or PTPD2 cDNA and treated with AP1510 or vehicle control for 12 days (top) Quantitation of the multiacinar phenotype, data are expressed as percentage of acini that form multiacinar structures, mean ± S.E.M. (** $p<0.005$) (bottom)

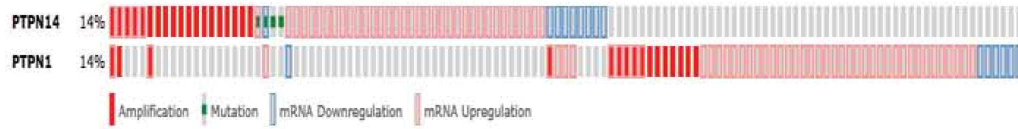
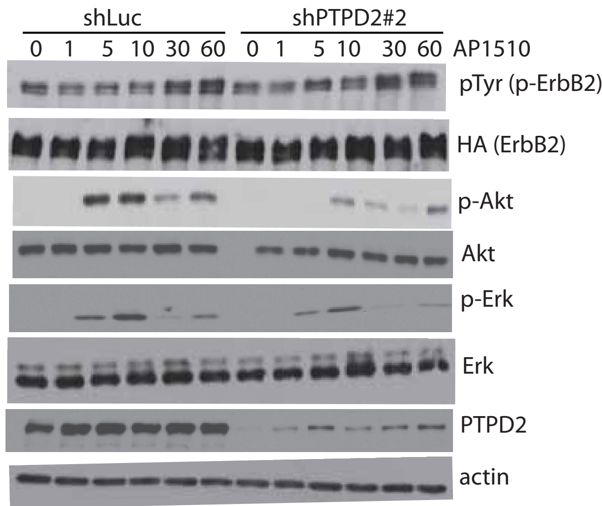


Fig. 3-6 : Alterations in PTPN14 and PTPN1 in human breast tumor samples
Data generated by the TCGA Research Network (http://cancergenome.nih.gov/) and analyzed using cBioPortal.com. Individual genes are represented as rows and individual tumor samples are represented as columns

A.



B.

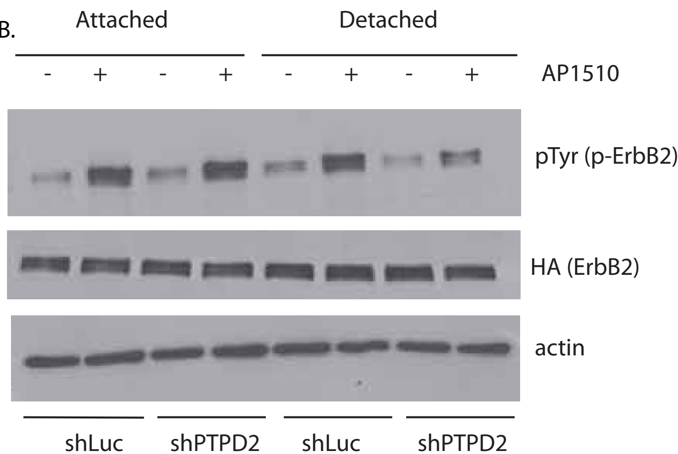


Fig. 3-7 : Suppression of PTPD2 attenuated ERBB2 effector pathways

a) Time course of phosphorylation-induced activation of the indicated mediators of ERBB2 signaling was measured by immunoblotting. 10A.B2 cells that expressed either control shLuc or shPTPD2 hairpins were serum starved overnight and subjected to ERBB2 activation with AP1510 for the indicated time (min) before cell lysis. β-actin was used as the loading control. b) 10A.B2 cells expressing control shLuc or shPTPD2 hairpins were plated under ECM-attached or ECM-detached conditions for 48 hours with or without AP1510. Phosphorylation status of ERBB2 was measured by immunoblotting

Chapter 4

In pursuit of PTPD2 substrates

Introduction

To understand fully the functional role of PTPD2 in ERBB2 signaling, we attempted to identify the physiological substrates of this PTP in 10A.B2 cells stimulated with AP1510. In the previous chapter, I reported that suppression of PTPD2 in suspension cultures, (surrogate model for investigating signaling changes in ECM-detached cells) prevented robust tyrosine-phosphorylation of chimeric ERBB2 as compared to control cells following AP1510 stimulation. Given this difference in tyrosine phosphorylation, we tested the hypothesis that PTPD2 acts by dephosphorylating specific phosphotyrosine substrates under these conditions. This chapter details my efforts to identify the substrate(s) of PTPD2 in the context of ERBB2 signaling.

Materials and Methods

Reagents

YAP1 antibody (Sigma). SRC Inhibitor SU6656 (Calbiochem)

Constructs

Full length flag-tagged PTPD2 constructs were kindly provided by Yeesim Khew-Goodall (Centre for Cancer Biology, SA Pathology, Adelaide/ University of Adelaide) In order to purify full-length PTPD2 from bacteria for *in vitro* trapping experiments, the cDNA was subcloned into the bacterial expression vector, pET28(a).

PTPD2 catalytic domain (886-1187) in pNIC28Bsa4 vector was a gift from Stefan Knapp (University of Oxford). Active site mutants D1079A (substrate-trapping mutant) and R1127M were generated using Quick-Change mutagenesis (reagents from Agilent Technologies) as per manufacturer's instructions.

YAP1shRNA constructs were obtained from Addgene; shYAP1#1 and shYAP1#2 in pLKO1 (lentiviral) vector are respectively Addgene plasmids 42540 and 42541 as described in (149).

Purification of recombinant His-tagged PTP domain mutants of PTPD2

6XHis-tagged PTPD2 catalytic domain (Stefan Knapp lab) or mutants thereof were introduced into the bacterial strain BL21-RIL for recombinant protein production. Briefly, 5ml of overnight bacterial culture was added to 500ml LB medium until OD_{600} reached 0.6. Cultures were induced with 1mM IPTG and allowed to grow for 4 hours at 18°C. Cells were harvested and lysed in 25ml of Lysis Buffer (50mM HEPES, 100mM NaCl, 1%Triton X-100, 0.5% DMSO, pH7.0 containing protease inhibitors) by sonication. The sonicated sample was subjected to ultracentrifugation for 1 hour at 4°C. The supernatant was filtered through a 0.45μm filter and loaded onto a Ni^{2+}-NTA column. The column was washed with Wash buffer (50mM HEPES, 250mM NaCl, 1mM DTT, pH7.0), containing 50mM Imidazole. Bound 6xHis-tagged protein was eluted using wash buffer containing 300mM Imidazole. Purity of the protein preparation was checked by running an aliquot on an SDS-PAGE gel followed by Coomassie staining.

***In vitro* substrate trapping**

48-hour detachment cultures (with or without AP1510 treatment) were collected and washed with 1X PBS. The cells were lysed in 20mM HEPES, 150mM NaCl, 1% NP-40, 10% glycerol, pH 7.5 containing protease inhibitors and 3mM IAA), on a rotating wheel for 30 minutes at 4°C. The lysates were cleared by centrifugation for 10 minutes at 4°C and the supernatant quantified using Bradford reagent. 1mg lysate was used for IP and the volume made up to 1ml using lysis buffer without IAA. 5% was set aside as “input”. 5mM DTT was then added to inactivate any unreacted IAA. The lysate was then incubated with Ni^{2+}-NTA bound 6XHis-tagged recombinant wild type or mutant PTP at 4°C. In parallel, bead-bound D-A mutant was also treated with 2mM pervanadate (freshly prepared by mixing equimolar amount of sodium orthovanadate and hydrogen peroxide) for 30 minutes at room temperature and used for IP. Thereafter, the beads were washed thrice with lysis buffer (without IAA), resuspended in Laemmli sample buffer, boiled for 5 minutes and resolved by SDS-PAGE. The co-immunoprecipitated tyrosine-phosphorylated proteins were detected by western blot using anti-phosphotyrosine 4G10 antibody.

Results

Use of "substrate-trapping mutant" strategy to identify potential substrates of PTPD2 in the context of AP1510-stimulated ERBB2 signaling

We first employed the "substrate trapping mutant" strategy, developed in our lab (114) as a tool to identify potential substrates of PTPD2 from among the proteins that are tyrosine phosphorylated following ERBB2 activation. In order to carry out the *in vitro* substrate-trapping experiment, I first attempted to generate recombinant, full-length PTPD2. However, I had difficulty in solubilizing the 140kDa protein **(Fig 4-1).**

In the interest of time, I altered my strategy to use only the PTP domain of PTPD2 **(Fig. 4-2)**. We first tested the bacterially expressed, purified PTP domain in an *in vitro* enzymatic assay using an artificial substrate, DiFMUP. As expected, we found that the recombinant PTP domain was catalytically active whereas the D-A trapping mutant showed a dramatic reduction in activity **(Fig. 4-3).** When Ni^{2+}-NTA affinity-purified PTPD2 catalytic domain was mixed with lysates from suspension cultures (expected to contain potential substrates) and subjected to anti-phosphotyrosine immunoblotting, I observed that two bands bound more strongly to the substrate-trapping mutant form rather than wild-type enzyme (molecular weight 250-150kDa and 75-50kDa respectively) suggesting that they could be putative substrates **(Fig. 4-4)**. Although these tyrosine phosphorylated proteins bound to the trapping mutant, the relative increase in binding over wild type PTP was only about 2-fold. This is much lower than what we would expect of a classical substrate.

Treatment with pervanadate irreversibly oxidizes the catalytic cysteine that attacks the phosphotyrosine substrate (150). If the enzyme-substrate interaction is active site mediated, it is expected that pervanadate treatment would prevent the D-A mutant from binding to the putative substrate. However, we observed that phosphoproteins bound equally well to untreated and pervanavadate-treated D-A mutant **(Fig. 4-5),** suggesting that the phosphotyrosine-PTP interaction might not be mediated only by the active site.

Whereas the D-A mutation affects only substrate turnover (V_{max}), mutation of an active site arginine residue to methionine (R-M mutation), inhibits both substrate affinity (K_m) as well as turnover (V_{max}). I found that the R1127M mutant of PTPD2 also trapped the same set of pTyr proteins **(Fig. 4-6)** as the D-A mutant**.** This suggests that the primary interaction

of these phosphoproteins with PTPD2 is not mediated by the enzyme's active site alone and that other residues in the catalytic domain might be involved. Although these phosphoproteins might still be substrates of PTPD2, this result made it more challenging to prove. Therefore, I resorted to a "candidate-approach" to substrate identification, guided by the literature.

A "candidate approach" to substrate identification

There is evidence in the literature that PTPD2 regulates the oncogenic transcription factor, Yes-Associated Protein 1 (YAP1) (133)**,** (132). The functional role of YAP1 in breast carcinogenesis is not entirely clear and it has been suggested that the phosphorylation status of YAP1 can influence the specificity of target gene activation (134). We tested whether, under conditions of ERBB2 activation, PTPD2 regulates YAP1 tyrosine phosphorylation, thereby affecting its function as a transcription factor. However, we did not observe tyrosine phosphorylation of YAP1 in 10A.B2 cells, both under basal conditions and following AP1510 stimulation**.** Furthermore, we found that suppression of YAP1 with 2 different functional shRNAs **(Fig. 4-7a)** did not affect the multiacinar phenotype of ERBB2 activation **(Fig.4-7b),** suggesting that YAP1 was not a critical substrate of PTPD2 in ERBB2 signaling.

SRC family kinases are also known to be critical downstream regulators of ERBB2 signaling (151), (152) and are regulated by tyrosine phosphorylation at both activating and inhibitory sites (153). We hypothesized that PTPD2 co-operates with ERBB2 signaling by modulating the activation of SRC. We tested whether a small molecule inhibitor against SRC (SU6656) could affect the enhanced ERBB2 phenotype observed upon PTPD2 overexpression. We observed that whereas SU6656 showed a marked inhibition of the multiacinar phenotype in control 10A.B2 cells, it did not affect the more aggressive multiacinar phenotype of PTPD2-overexpressing cells **(Fig. 4-8)**. Suppression of PTPD2 also did not affect SRC phosphorylation status. This suggests that overexpressed PTPD2 did not act through the regulation of SRC in ERBB2 signaling.

Discussion

The observation that suppression of PTPD2 prevented robust phosphorylation of ERBB2 under conditions of detachment culture **(Fig. 3-7B)** was consistent with a positive role for PTPD2 in ERBB2-mediated cell survival. However, it suggested that the tested sites on ERBB2 were unlikely to be direct targets of the phosphatase. To better understand the mechanism by which PTPD2 regulates the ERBB2 pathway, we wanted to identify substrates of the phosphatase. As an unbiased approach to substrate identification, we first used the "substrate-trapping mutant" strategy, developed in our lab based on mutational and structural analysis of PTP1B (114) The principle behind this method is that mutating the conserved aspartic acid residue in the WPD loop to alanine (D-A), allows the mutant PTP to bind its physiological substrates but not to dephosphorylate the target efficiently. As a result, the D-A mutant and the tyrosine-phosphorylated substrate become locked in a stable, dead-end complex. In contrast, wild-type enzyme dephosphorylates the phosphotyrosine substrate efficiently, resulting in rapid turnover and release of the substrate. Consequently, following immunoprecipitation, the trapping mutant but not the wild type enzyme, is expected to bring down the associated phosphotyrosine substrate. Guided by the phenotype being investigated and the signaling pathways the PTP is known to participate in, antibodies against proteins that correspond to the apparent molecular mass of the trapped protein(s) can be used to determine the identity of the substrate. Although low abundance and lack of efficient, commercially available antibodies can prevent the detection of some substrates, this technique has been applied widely to identify novel tyrosine-phosphorylated substrates which can eventually be validated in a cellular setting with full-length PTP (154).

In this chapter, I described our efforts to use this strategy *in vitro* to identify potential substrates of PTPD2 in the context of ERBB2 signaling. There are some caveats to this approach. Recombinant, purified PTP protein mixed with cell lysate is not subject to the same spatio-temporal localization or regulation. Secondly, the isolated catalytic domain of the PTP in question might not be under the same physiological constraints as the full-length molecule in an *in vivo* setting. These two factors may influence subcellular access and/or binding to the potential substrate. Furthermore, it is often difficult to distinguish selective dephosphorylation of specific residues in a multi-phosphorylated protein. Nevertheless, we

proceeded with this line of preliminary investigation in order to identify candidates that could subsequently be validated by more physiologically relevant approaches.

The results of our *in vitro* substrate-trapping experiment are not consistent with what is expected of classical pTyr substrates of PTPs that have been identified in the past using the same approach. We did not observe a significant enrichment in the binding of specific tyrosine-phosphorylated proteins to the substrate-trapping mutant form of PTPD2 as compared to wild-type enzyme.

In *in vitro* enzymatic assays, the observed activity of wild type PTPD2 was much lower than what is observed with other active members of the PTP family. Consistent with our observation, Stefan Knapp's lab has reported that when tested against a panel of tyrosine -phosphorylated peptide substrates, PTPD2 shows a poor rate of dephosphorylation as compared to other classical PTPs (155). Another recent study (156) reports that PTPD2 binds strongly to acidic peptide substrates (K_m comparable to highly active phosphatases like PTP1B and TC-PTP) but shows a very low rate of turnover (k_{cat}=0.073±0.003 s^{-1}). Steady state and pre-steady state kinetic data suggest that the low k_{cat} value can be attributed to the slow breakdown of the covalent intermediate. The authors, therefore, suggest that PTPD2 might function as a single turnover enzyme (156). Analysis of the primary sequence and crystal structure of the catalytic domain (Glu895-Ser1184) of PTPD2 (157) reveals the presence of an atypical residue at a crucial position in the active site. In PTP1B, (the prototype for phosphotyrosine-specific PTPs) the active site signature motif lies at the base of a pocket, the depth of which is crucial for restricting the specificity of the enzyme to the hydrolysis of phosphotyrosine-containing peptide substrates. The depth of this cleft is defined by the aromatic phenyl side chain of the tyrosine residue at position 46 (158). Mutation of this residue to a smaller, aliphatic residue like serine or leucine but not the aromatic residue, phenylalanine, significantly weakens the affinity of the enzyme for pTyr-RCML substrate *in vitro* and reduces the rate of turnover (114), (159). The crystal structure of substrate-bound PTP1B shows that the phenyl side chain of Tyr46 mediates a (π-π) stacking interaction with the phenyl ring of the incoming phosphotyrosine (158), possibly stabilizing the substrate in the catalytic cleft and orienting it for subsequent steps in catalysis. PTPD2 contains an atypical isoleucine residue at the corresponding position. The change to a relatively smaller, aliphatic side chain at this

position alters the depth of the catalytic cleft and might preclude stabilization of the incoming phosphotyrosine substrate. It has, therefore, been suggested as an explanation for the poor turnover of phosphotyrosine substrates observed with PTPD2. However, physiological tyrosine-phosphorylated substrates of PTPD2 have been identified (128), (137) suggesting that in the context of ERBB2 signaling in cells, there might be mechanisms in play to potentiate the tyrosine phosphatase function of PTPD2, including regulatory proteins and allosteric modifications.

It is also possible that the D1079A mutant of PTPD2 does not represent an efficient trapping mutant for all tyrosine phosphorylated protein substrates. Further structure-function and mutational analysis of the catalytic domain might be required in order to be able to design a robust substrate-trapping mutant that permits formation and isolation of a more stable PTP-substrate complex. The observation that pervanadate-treated D-A mutant and the R-M mutant also co-immunoprecipitated the putative phosphotyrosine substrates to the same extent as the trapping mutant underscores the possibility that other residues in the active site are either involved in substrate binding in the first place or stabilize substrate binding during the course of catalysis.

In light of these challenges, we changed our strategy to test candidate tyrosine phosphorylated proteins as potential substrates of PTPD2 by using our knowledge of the ERBB2 signaling pathway and clues in the literature. One of the first substrates we tested was the oncogenic transcription factor YAP1 (Yes Associated Protein 1), which is regulated by PTPD2. Whereas some reports suggest that PTPD2 dephosphorylates a specific tyrosine residue on YAP1, (133) others suggest that YAP1 is bound and sequestered by PTPD2 in the cytoplasm independent of its catalytic function (132). YAP1 has been implicated as a potent oncogene in breast cancer in some cases (160) whereas other reports suggest that YAP1 acts as a tumor suppressor by positively regulating members of the p53 family under conditions of DNA damage (161). It has also been suggested that the phosphorylation status of YAP1 can influence the specificity of target gene activation (134). It is, therefore, conceivable that under conditions of ERBB2 activation, YAP1 phosphorylation at specific tyrosine residues is finely regulated in order to enable it to activate pro-ERBB2 target genes. The shRNA-mediated knockdown of YAP1 in 10A.B2 cells did not affect the AP1510-induced multiacinar phenotype, suggesting that it might not

be required for ERBB2 signaling. Further, we did not find any evidence of change in YAP1 localization in AP1510-stimulated cells, irrespective of PTPD2 status.

We also tested whether PTPD2 acts as a positive regulator of ERBB2 signaling by regulating the phosphorylation status of SRC Family Kinases (SFKs). SRC shows elevated kinase activity downstream of ERBB2 signaling (151), (152). It phosphorylates ERBB2 at Y877, thereby activating it further, but also phosphorylates several downstream effectors that are critical for the various oncogenic phenotypes characteristic of ERBB2 signaling. The activity of SRC is regulated by phosphorylation on two different sites; the activating site Y416, and the inhibitory site Y527 (153). Several PTPs are known to regulate SRC function by acting on either of these sites. For example, PTPN23 dephosphorylates SRC at Y416 and loss of PTPN23 increases ERBB2-mediated cell motility and invasion in three-dimensional matrigel (81). In contrast, PTP1B is reported to act as a co-operating oncogene in ERBB2 signaling by dephosphorylating SRC on the inhibitory phosphorylation site, Y527 (89). RPTPα is also known to act as a SRC Y527 phosphatase. We therefore tested whether PTPD2 also regulates ERBB2 signaling positively through the phosphorylation-mediated regulation of SRC activity. We had observed previously that overexpression of PTPD2 enhanced the AP1510-induced multiacinar phenotype of ERBB2. However, inhibition of SRC in this context did not attenuate the phenotype, suggesting that PTPD2 did not act through SRC in the context of ERBB2 signaling and that overexpression of PTPD2 could bypass the requirement for SRC. This result highlights the specificity of PTPs in ERBB2 signaling; although many PTPs might ultimately act as positive regulators of ERBB2, they may act at different levels of the signaling pathway, on various distinct downstream effectors.

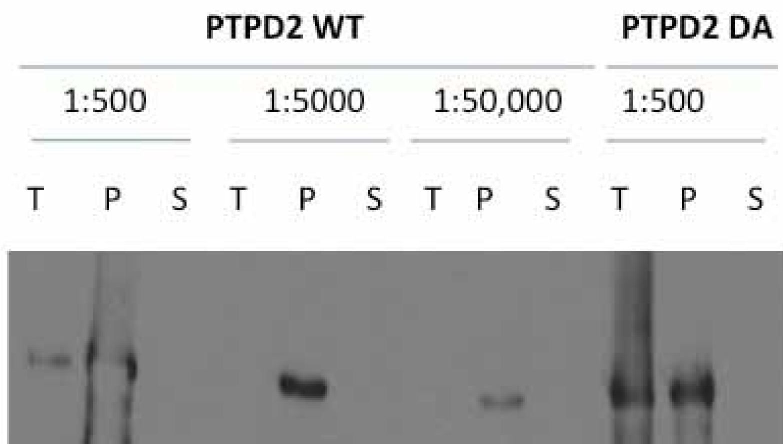


Fig. 4-1 : Full length PTPD2 could not be solubilized
6XHis-tagged full -length wild type (WT) and D-A trapping mutant of PTPD2 were expressed in bacteria and cells were lysed by sonication. Following ultracentrifugation, aliquots of soluble and insoluble fraction were resolved by SDS-PAGE and probed with anti-His antibody. (T- Total sonicated lysate, P- insoluble pellet fraction, S- soluble fraction or supernatant, numbers indicate dilution of anti-His antibody)

Fig. 4-2 : Purification of PTPD2 catalytic domain
Following affinity purification using a Ni2+-NTA column, the purity of recombinant, 6xHis-tagged wild type (WT) or substrate strapping mutant (DA) cataytic domain of PTPD2 was analyzed by SDS-PAGE followed by Coomassie staining.

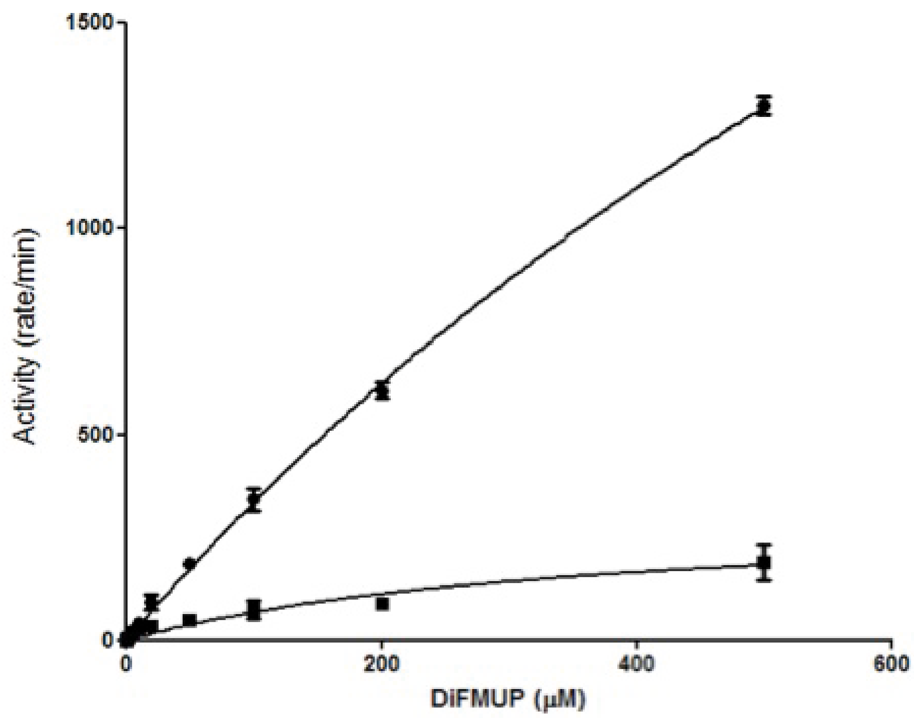


Fig. 4-3 : In vitro activity assay using wild type and mutant PTPD2 catalytic domain
Bacterially-expressed, wild type (circle) and D-A substrate trapping mutant (square) form of PTPD2 catalytic domain were incubated with increasing concentrations of DiFMUP and phosphatase activity measured after 30 minutes at 30 deg.

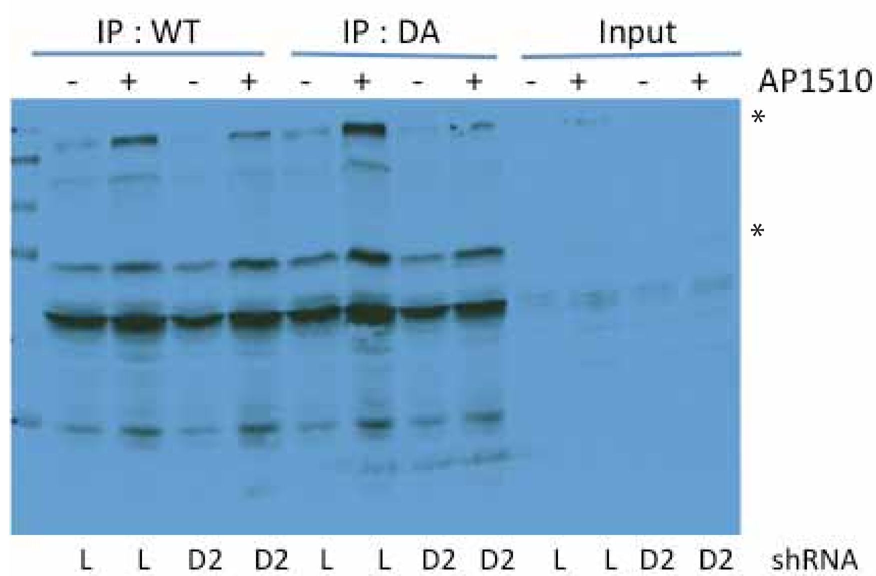


Fig. 4-4 : Phosphotyrosine immunoblot following ***in vitro*** substrate trapping does not show significant enrichment of pTyr proteins with the D-A trapping mutant
Equal amounts of lysate from 10A.B2 cells cultured in suspension for 48 hours with (+) or without (-) AP1510, were incubated with Ni2+-NTA bead bound wild type (WT) or substrate trapping mutant form (DA) of PTPD2 catalytic domain. The immunoprecipitated proteins were resolved by SDS-PAGE and probed with anti-phosphotyrosine antibody (L- Luciferase short hairpin, D2- shPTPD2 short hairpin)

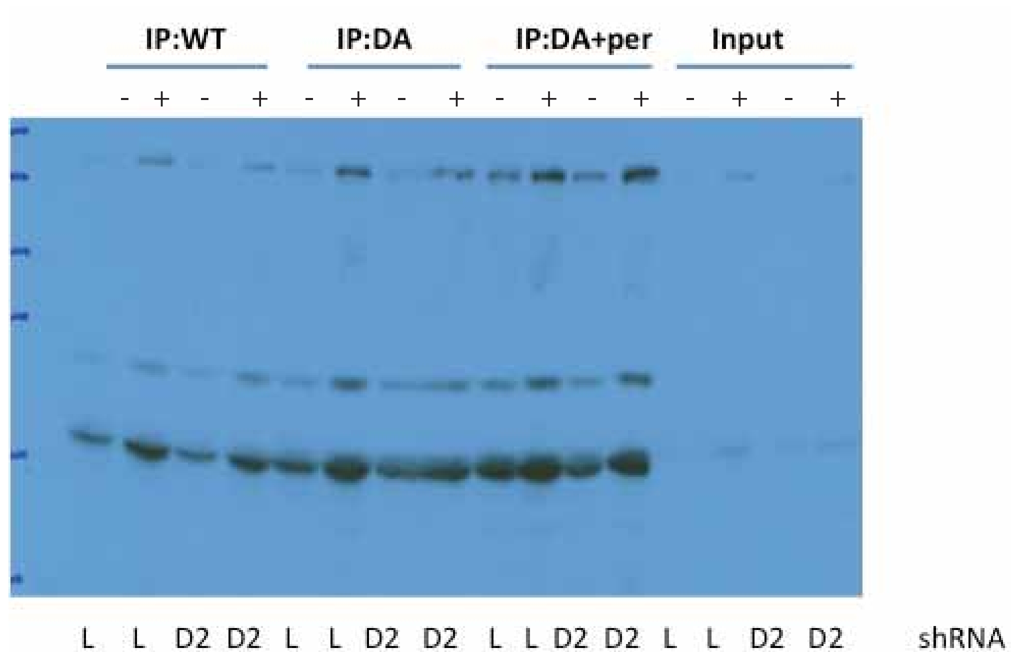


Fig. 4-5 : Pervanadate treatment did not prevent phopshoprotein binding to D-A mutant

Equal amounts of lysate from 10A.B2 cells cultured in suspension for 48 hours with (+) or without (-) AP1510, were incubated with Ni2+-NTA bead bound PTPD2 wild type (WT) , substrate trapping mutant form (DA), or substrate-trapping mutant treated with pervanadate (DA+per). The immunoprecipitated proteins were resolved by SDS-PAGE and probed with anti-phosphotyrosine antibody (L- Luciferase short hairpin,D2- shPTPD2 short hairpin)

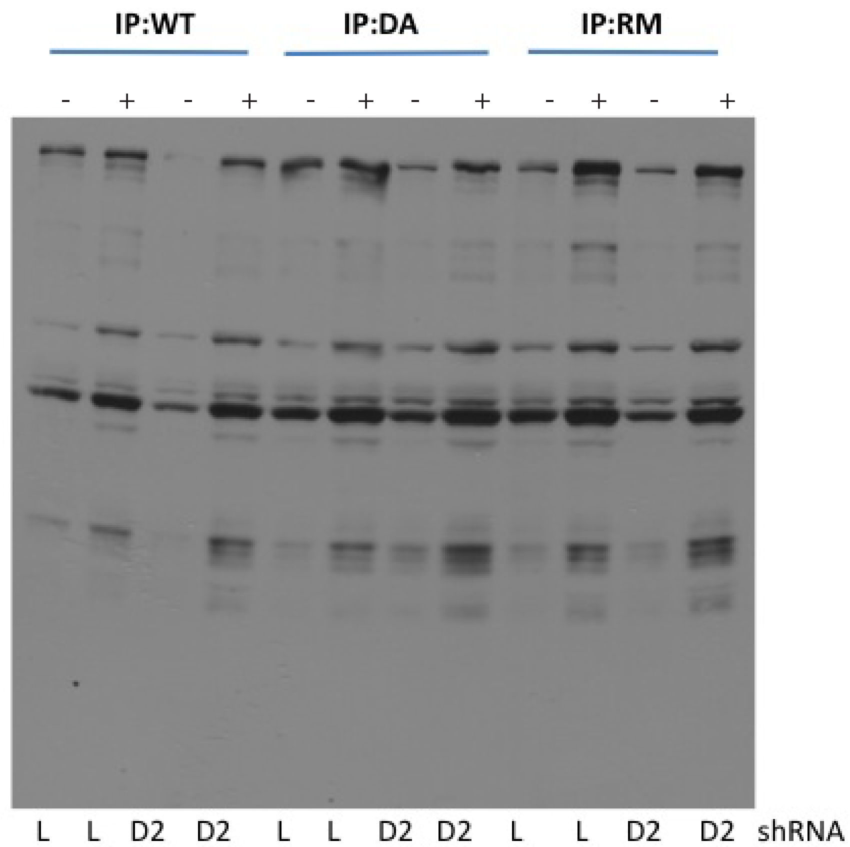


Fig. 4-6 : Phosphotyrosine protein binding to D-A mutant and R-M mutant was comparable
Equal amounts of lysate from 10A.B2 cells cultured in suspension for 48 hours with (+) or without (-) AP1510, were incubated with Ni2+-NTA bead bound wild type (WT), D-A or R-M mutant of PTPD2 catalytic domain. The immunoprecipitated proteins were resolved by SDS-PAGE and probed with anti-phosphotyrosine antibody (L- Luciferase short hairpin, D2- shPTPD2 short hairpin)

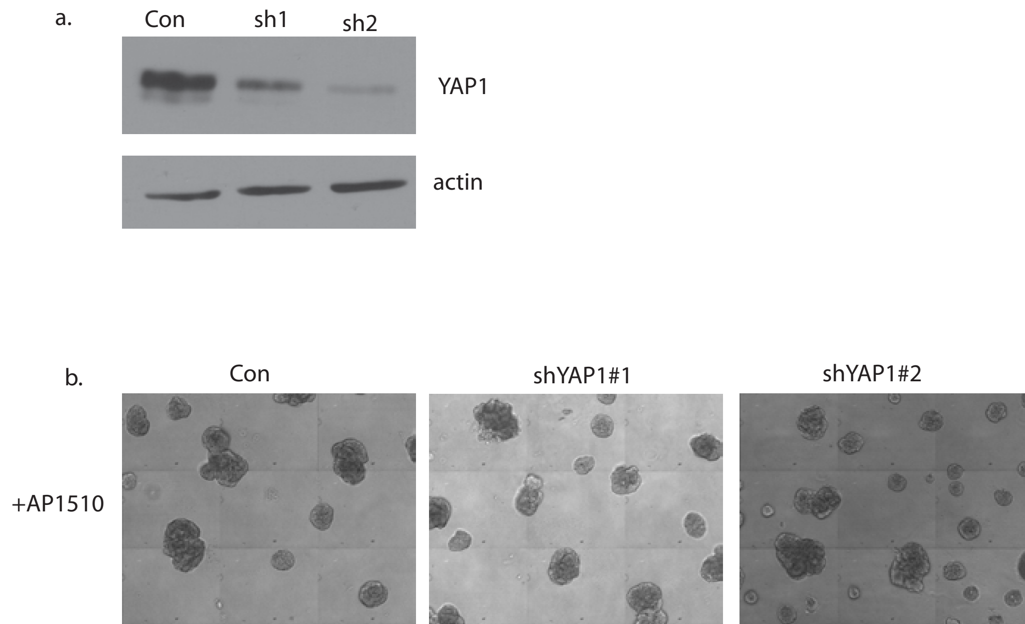


Fig. 4-7 : Suppression of YAP1 did not affect the AP1510-induced phenotype of ERBB2 in three-dimensional cultures

a) Immunoblot showing YAP1 protein level in 10A.B2 cells expressing control shLuc or shRNAs targeting YAP1. The expression of β-actin was used as the loading control. b) Representative phase-contrast images of acinar structures expressing indicated shRNAs and treated with AP1510 for 12 days

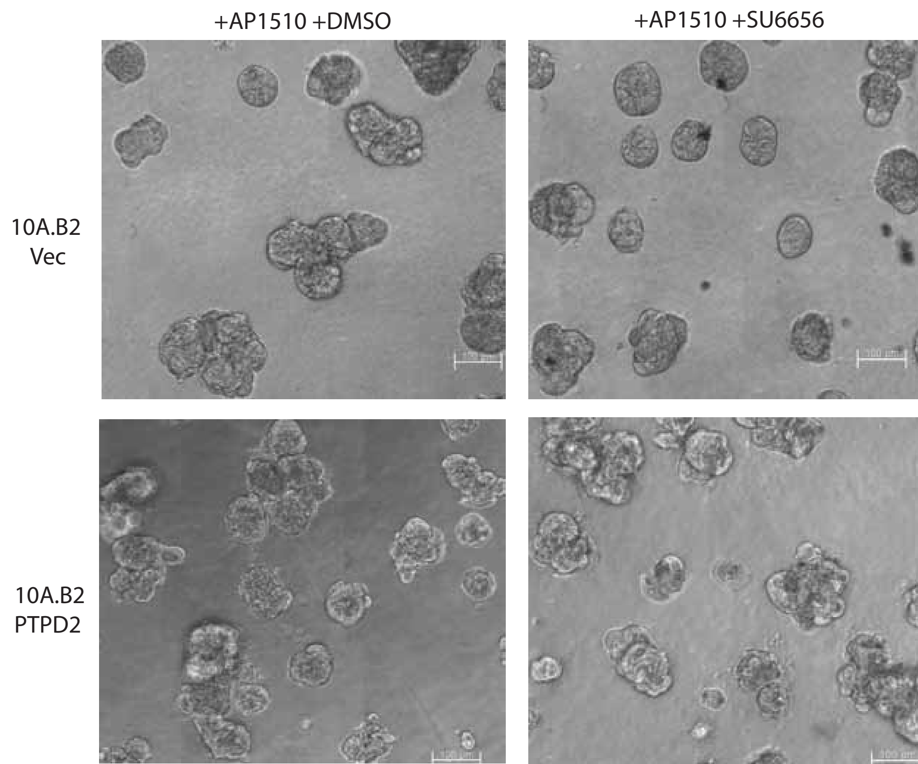


Fig. 4-8 : SRC inhibitor SU6656 did not affect the PTPD2 overexpression phenotype in the context of ERBB2 activation
Representative phase-contrast images of AP1510-stimulated acinar structures expressing vector control or PTPD2 and treated with vehicle (DMSO) or SRC inhibitor SU6656 (5μM) for 12 days.

Chapter 5

Phospholipase D2-generated phosphatidic acid acts as a positive regulator of ERBB2 signaling by regulating PTPD2 activity

Introduction

In chapter 4, I described the challenges we faced in identifying tyrosine-phosphorylated substrates of PTPD2 in the context of ERBB2 signaling. I also discussed the potential impact of the presence of an atypical active site residue on the specificity of PTPD2 towards phosphotyrosines. Briefly, PTPD2 possesses an isoleucine residue in place of the consensus tyrosine at a critical position in the phosphotyrosine recognition loop. It has been suggested that the resulting change in the depth of the catalytic cleft might influence substrate preference, and the aliphatic nature of the isoleucine side chain might preclude stabilization of an incoming phosphotyrosine substrate, thereby accounting for the lower activity of PTPD2 towards phosphotyrosine substrates compared to other PTPs (155).

The tyrosine residue in question is also absent in Dual Specificity Phosphatases, including the lipid phosphatase PTEN, in which it is often replaced by an aliphatic amino acid. We, therefore, considered the possibility that the shallow catalytic cleft of PTPD2 could bind and turnover phosphoserine and phosphothreonine substrates better than phosphotyrosines. However, there are limitations to finding phosphoserine/phosphothreonine substrates from a large pool of phosphorylated proteins in the cell. Firstly, phosphorylated serine and threonine residues account for the bulk of the phosphoproteome as a result of which, finding specific substrates of a given PTP is a far bigger challenge. Secondly, commercially available anti-phosphoserine and anti-phosphothreonine antibodies are not very efficient at detecting phosphorylation levels of proteins in total cell lysates or even following immunoprecipitation with a trapping mutant. Finally, the D-A mutant might not act as an effective trapping mutant for phosphoserines/phosphothreonines as it is expected to for phosphotyrosine substrates. Indeed, it was for these reasons that the original shPTP screen in 3D culture was limited to classical PTPs. Nevertheless, we tested whether PTPD2 could dephosphorylate phosphoserine and phosphothreonine-containing peptides. However, we did not observe a significant turnover of these phosphorylated peptide substrates *in vitro* with recombinant catalytic domain of PTPD2. Some PTPs are known to recognize phosphorylated lipids as substrates. We, therefore, turned our attention towards phospholipids. My findings on the interaction between PTPD2 and the lipid, phosphatidic acid and its functional role in ERBB2 signaling constitute the following chapter.

Materials and Methods

Reagents

Lipid strips (Echelon Biosciences), L-α-Dipalmitoyl-[glycerol-^{14}C(U)]-Phosphatidic Acid (Perkin Elmer), unlabeled L-α-Phosphatidic Acid and Phosphatidylserine (Avanti Polar Lipids), DiFMUP (Molecular Probes), 5-Fluro-2-Indolyl des-chlorohalopemide or FIPI (Sigma-Aldrich), PLD1 inhibitor VUO359595 and PLD2 inhibitor VUO364739 (Alex Brown, Vanderbilt University), anti-Flag antibody (Sigma, clone M2)

Constructs

PTP1B (1-321) is described in (162). Full length flag-tagged PTPD2 construct was kindly provided by Yeesim Khew-Goodall (Centre for Cancer Biology, SA Pathology, Adelaide/ University of Adelaide)

Protein-lipid overlay assay

As per manufacturer's instructions (Echelon)

Lipid Binding Assays

[^{14}C]-DPPA was resuspended in 20mM Imidazole, 1mM EDTA, 1mM DTT, pH7.0 and vesicles were prepared by sonication until the solution became clear. Recombinant catalytic domain of PTPD2 or PTP1B bound to Ni^{2+}-NTA beads was incubated with various concentrations of [^{14}C]-DPPA for 30 minutes at room temperature. The PTP-[^{14}C]-DPPA complex was separated from unbound [^{14}C]-DPPA by centrifugation, and bound radioactivity was measured by liquid scintillation counting.

Phosphatase assays

For phosphatase assays, varying concentrations of DiFMUP (0-500 μM) was added to assay buffer (50mM HEPES, 100mM NaCl, 0.01% Tween, 0.1% DMSO, 2mM DTT, 2mM EDTA, pH 6.5) containing 0.1μM purified PTPD2 in a final volume of 100μl. The fluorescence emitted at 450nm was monitored continuously for 20 min using a Gemini XPS fluorescence plate reader. For assays using radiolabeled substrate, reduced carboxamidomethylated and maleylated lysozyme (RCML) was phosphorylated

on tyrosine to a stoichiometry of 0.8 mole ^{32}P per mole of protein using recombinant GST-FER kinase and [$\gamma^{32}P$] ATP, and activity was measured as described in (163), (164).

Three-dimensional morphogenesis assays

To inhibit PLD in mammary acini cultured in three-dimensional Matrigel, small-molecule inhibitors were added at the following concentrations to the overlay medium once in 2 days:

FIPI (dual inhibitor) - 1μM

VUO359595 (PLD1-specific inhibitor)- 2.5 μM

VUO364739 (PLD2- specific inhibitor)- 2.5 μM

Results

Phosphatidic acid binds to PTPD2 *in vitro* and enhances its catalytic activity

To test for the binding of PTPD2 to known phospholipids, we employed a commercially available protein-lipid overlay assay in which binding of a recombinant protein to an array of immobilized lipids can be detected immunologically. Recombinant PTPD2 specifically bound only to phosphatidic acid **(Fig. 5-1a).** Interestingly, the substrate-trapping (DA) and phosphatase-dead (CS) mutants of PTPD2 also bound to PA equally well, suggesting that PA is not a substrate of PTPD2, but a binding partner **(Fig. 5-1b,c).** Consistent with this observation, *in vitro* dephosphorylation assays, wild type PTPD2 catalytic domain was unable to dephosphorylate PA.

The PTPD2-PA interaction was verified by performing binding assays with liposomes of [C^{14}]-labeled Dipalmitoyl Phosphatidic Acid (DPPA). We found that recombinant PTPD2 (catalytic domain) bound strongly to the radiolabelled lipid **(Fig 5-2a).** Recombinant PTP1B (1-321), on the other hand, bound very poorly in comparison, indicating specificity in the binding of PTPD2 to DPPA **(Fig. 5-2a).** Full length PTPD2, ectopically expressed in cells, also bound efficiently to [C^{14}]-labeled DPPA **(Fig. 5-2b)** *in vitro.*

We then tested the functional significance of PA binding to PTPD2 function. When recombinant PTPD2 was treated with PA, we observed an increase in the activity of the enzyme towards DiFMUP, an artificial substrate **(Fig. 5-3a).** This suggests that phosphatidic acid acts as a positive regulator of the catalytic activity of PTPD2. This effect was not seen when PTPD2 was treated with phosphatidylserine **(Fig.5-3b).**

We also tested whether PA had a similar effect on the catalytic activity of full-length PTPD2 towards phosphorylated RCM Lysozyme. We found that PA binding resulted in approximately three-fold increase in the catalytic activity of the enzyme towards the phosphoprotein substrate **(Fig 5-4a)** without altering substrate affinity. This suggests that the effect of PA binding to PTPD2 is specifically at the level of increased substrate turnover. Full length PTP1B, derived from cells served as a negative control in this experiment **(Fig. 5-4b).** Taken together, these data suggest that PA binds to PTPD2 *in vitro* and augments its catalytic activity.

Small molecule inhibitors of Phospholipase D (PLD) suppressed the multiacinar phenotype by inhibiting ERBB2-mediated lumen filling

We hypothesized that phosphatidic acid-mediated regulation of PTPD2 is crucial for the function of PTPD2 in cells as a positive regulator of ERBB2 signaling. Therefore, we tested whether manipulation of the levels of phosphatidic acid in cells would attenuate the multiacinar phenotype of ERBB2 in 3D cultures. FIPI (5-Fluro-2-Indolyl des-chlorohalopemide), is a well-characterized small molecule that has been used widely to inhibit Phospholipase D (PLD), an enzyme that generates phosphatidic acid from phosphatidylcholine (165). In the absence of signaling from chimeric ERBB2, FIPI had no effect on the morphogenesis of 10A.B2 cells. However, FIPI treatment inhibited the AP1510-induced multiacinar phenotype of 10A.B2 cells in 3D culture **(Fig.5-5a).** FIPI treatment reduced the percentage of multiacinar structures formed upon AP1510 stimulation and also inhibited the increase in size of the structures formed **(Fig 5-5b).** This suggests that the function of PLD enzyme and, by extension, a critical level of phosphatidic acid is required for ERBB2 signaling.

Two isoforms of mammalian PLD have been identified: PLD1 and PLD2, which differ in basal activity, localization and regulatory functions (166)**.** To understand better the relative importance of each isoform in the regulation of ERBB2 signaling, we treated 10A.B2 acini with previously reported isoform-selective inhibitors (167), (168) in the context of AP1510-stimulated ErbB2 activation. We found that the PLD2-specific inhibitor, VUO364739, but not the PLD1-specific inhibitor, VUO359595, significantly suppressed the AP1510-induced multiacinar phenotype of ERBB2 as compared to vehicle control **(Fig. 5-6a, b).** The PLD1-specific inhibitor shows a small decrease in the size distribution of multiacinar structures as compared to control, whereas the PLD2-specific inhibitor causes a drastic reduction in the size distribution of multiacinar structures resulting from AP1510 stimulation **(Fig. 5-6c).** This result implicates the PLD2 isoform as a positive regulator of ERBB2 signaling.

We had observed that RNAi-mediated suppression of PTPD2 attenuated the formation of multiacinar structures by inhibiting specifically ERBB2-mediated lumen filling and loss of polarity but not ERBB2-induced hyperproliferation. We tested whether inhibition of PLD2 also affected the formation of multiacinar structures in the same manner. The

results of PLD inhibition with the dual inhibitor, FIPI, and the PLD2 isoform-preferring inhibitor, VUO364739, were strikingly similar to those observed with loss of PTPD2 in the context of ERBB2 activation. ERBB2-induced proliferation in acini treated with either inhibitor was similar to that of DMSO-treated acini **(Fig 5-7a).** However, ERBB2 activation failed to cause lumen filling in structures treated with PLD inhibitors **(Fig 5-7b)**. Small-molecule inhibition of PLD2 phenocopied shRNA-mediated suppression of PTPD2 in the context of ERBB2 signaling, suggesting that PLD2 and PTPD2 might be acting in the same effector pathway that regulates ERBB2-mediated lumen filling and loss of polarity.

Suppression of PTPD2 inhibits phosphatidic acid-mediated rescue of the PLD-inhibition phenotype

We investigated whether PTPD2 and PLD2 functioned in the same pathway downstream of ERBB2 by way of an epistasis experiment. It has been shown that exogenously added phosphatidic acid is incorporated into cell membranes and can elicit cellular responses (169). We tested whether rescue of the PLD-inhibition phenotype by exogenously added PA is dependent on PTPD2. In FIPI-treated control acini, we observed that the addition of phosphatidic acid to the 3D overlay medium rescued the effect of the PLD inhibitor. In shPTPD2 acini, however, phosphatidic acid did not rescue the FIPI-treatment phenotype, indicating that PTPD2 was required for signaling downstream of the lipid. **(Fig. 5-8a, b)** We infer that PTPD2 is required for the functional role played by PLD and, by extension, phosphatidic acid in ERBB2 signaling.

Discussion

Lipid species have been widely shown to function as critical second messengers in signal transduction pathways. Among the earliest examples is the hydrolysis of PIP_2 in pathways involving Ca^{2+}-mobilizing extracellular signal molecules (170). Lipid second messengers can modulate enzyme catalytic activity as in case of PKC enzymes (170) or act by recruiting signaling molecules to specific compartments in cellular membranes, thereby regulating access to potential substrates and interacting partners (171), (169). Negatively charged phospholipids can also exert biophysical effects on membranes following a local increase in concentration, thereby playing a role in secretory and endocytic pathways.

Phosphatidic acid (PA) is a bioactive phospholipid, the cellular levels of which are tightly regulated in the cell by convergent synthetic and degradative enzymes (166). The tight regulation of these pathways results in low basal cellular concentrations of PA that can be rapidly and transiently induced upon stimulation of cells with agonists. The primary source of PA in cells is the hydrolysis of phosphatidylcholine (PC) by the enzyme Phospholipase D (PLD) to choline and phosphatidic acid (PA). Whereas PA itself is a biologically active, free choline is not thought to fulfill any intracellular signaling roles (172).

PA generated *via* PLD activity can serve several functions in cellular signaling. As a binding partner, PA can effect protein recruitment to specific membrane compartments. For example, the interaction between Sphingosine Kinase 1 and PA is needed for the translocation of SK1 to plasma membrane rich in PA (173). PA recruitment of proteins has also been suggested in the formation and trafficking of secretory vesicles from the trans-Golgi network (174). Thus, PA can play a biophysical role as a fusogenic lipid. It also serves as an intermediate for the production of other bioactive lipids like diacylglycerol, which activates PKC enzymes and lysophosphatidic acid, a potent mitogen. A key function of PA is its ability to modulate the activity of several enzymes involved in signal transduction including isoforms of cAMP-specific phosphodiesterases (175) and the protein kinase Raf-1 (176), which was also shown to translocate to PA-enriched membranes (177). PA has been implicated in cellular survival pathways that prevent apoptosis through the activation of mTOR and S6K in a manner parallel to the PI3K survival pathway. Of particular relevance to our findings, it has been reported that PA can bind to and augment the catalytic activity of the phosphotyrosine-specific PTP, SHP-1 (178). Both *in vitro* as well as in intact cells, SHP-

1 is activated towards the EGF receptor by PA, suggesting that lipid modulation of SHP-1 activity may be physiologically relevant (179). In contrast, protein phosphatase-1γ (PP1γ) is inhibited noncompetitively by direct binding with PA (180). In line with these observations, we found that PA binding to PTPD2 resulted in enhancement of the phosphatase activity of PTPD2 towards phosphorylated RCM Lysozyme substrate *in vitro*. It is possible that PA binding to the catalytic domain leads to conformational changes, which enhance the stabilization of an incoming phosphotyrosine substrate and turnover.

The two best-characterized mammalian isoforms of PLD are PLD1 and PLD2. Though homologous, the two PLD isoforms differ in several respects. PLD1 is predominantly perinuclear, suggestive of Golgi, ER or late endosomal distribution whereas PLD2 is most often reported to localize to the plasma membrane. Mammalian PLD1 is stimulated by PI4, $5P_2$ and GTPγS- loaded ARF (181). Mammalian PLD2 exhibits significantly higher basal activity and is not activated by ARF or Rho GTPases. It does, however, require PI4, $5P_2$ (182).

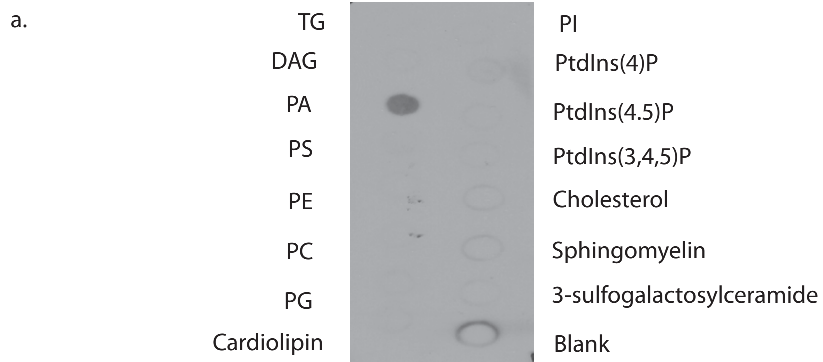
From a functional standpoint in human cancer, there appears to be some specificity in the activity of the two isoforms. Mice lacking PLD1, but not PLD2, incurred fewer lung metastases than wild-type mice on account of changes in the tumor microenvironment (183). Infection of the gastric epithelium by the bacterium *Helicobacter pylori* leads to selective induction of PLD1 expression by via activation of NF-κB (184). PLD2 overexpression leads to elevated adhesion, invasion and metastasis in a lymphoma cell line (185). In colorectal cancer, a clear correlation has been observed between PLD2 expression and tumor size and patient survival (186). Cross talk between PLD2 and PDGF-mediated signaling (187) and the oncogenic kinase RET (188) are also reported in association with cancer progression in sarcomas and thyroid cancer cells respectively. The role of Phospholipase D isoforms in cancer progression has been shown to be a function of catalytic activity. It has been demonstrated that active PLD enhances lymphoma cell metastasis whereas catalytically inactive PLD inhibits metastasis, MMP-2 expression and glioma cell invasion (189), (190).

In the context of breast cancer, results from xenograft mouse models suggest a pro-tumorigenic role for PLD2. PLD2 mutations have been identified in breast cancer (191). Further, a high level of PLD activity has been observed in breast cancers (192), (193),

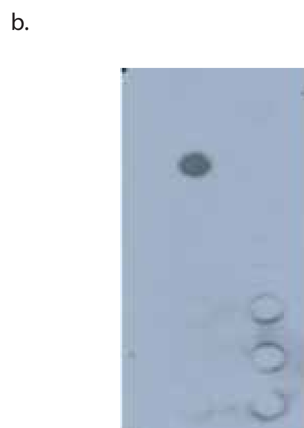
(194). Overexpression of PLD2 in low invasive breast cancer cells induces a highly aggressive phenotype; primary tumors formed in SCID mice xenotransplanted with PLD2-overexpressing cell lines were larger, grew faster and developed more lung metastases. Delivery of PLD-specific small-molecule inhibitors attenuated primary tumor growth and lung metastases (195). Suppression or inhibition of PLD2 in metastatic breast cancers decreased both tumor size and formation of metastases *in vivo* (195). Consistent with a pro-oncogenic role for PLD2 in breast cancer, we found that inhibition of the PLD2 isoform suppressed the multiacinar phenotype of activated ERBB2 in 3D cultures by preventing ERBB2-mediated lumen filling.

According to one report, PLD2 is also known to act as a Guanine Nucleotide Exchange Factor. We also found that exogenously added phosphatidic acid can rescue the PLD-inhibition phenotype, but only when PTPD2 is present. This result supports a central role for PTPD2 downstream of PLD2 activity in ERBB2 signaling. We speculate that the enhancement of the catalytic function of PTPD2 by PLD2-generated phosphatidic acid is important to sufficiently augment the activity of PTPD2 for the dephosphorylation of critical downstream mediators of ERBB2 signaling.

a.



b.



c.

Fig. 5-1 : Catalytic domain of PTPD2 bound specifically to Phosphatidic acid from among a panel of immobilized phospholipids
Strips containing the indicated phospholipids were probed with 0.5 µg/ml of bacterially expressed, 6xHis-tagged catalytic domain of PTPD2 or mutants thereof. Bound protein was detected by immumoblotting with anti-His antibody.

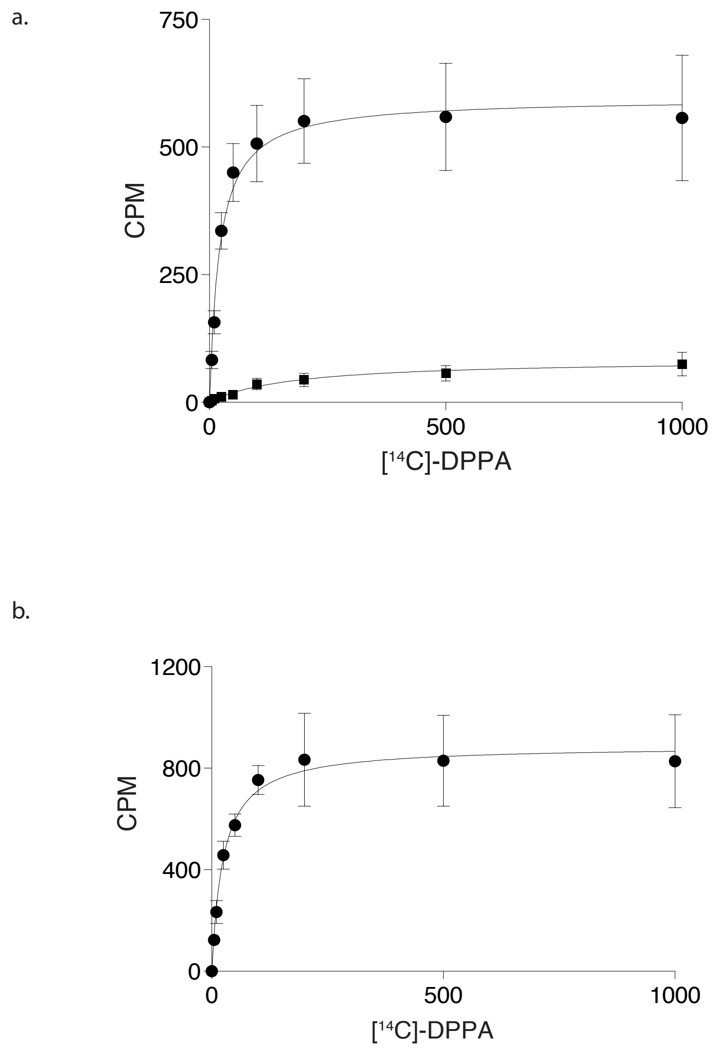


Fig. 5-2 : Phosphatidic acid bound to PTPD2 but not PTP1B
Recombinant PTPD2 catalytic domain (circle) or PTP1B (square) (a), or full length PTPD2 (b) was incubated with various concentrations of [14C]DPPA, the PTP-[14C]DPPA complex was separated from unbound [14C]DPPA by centrifugation and bound radioactivity was measured by scintillation counting.

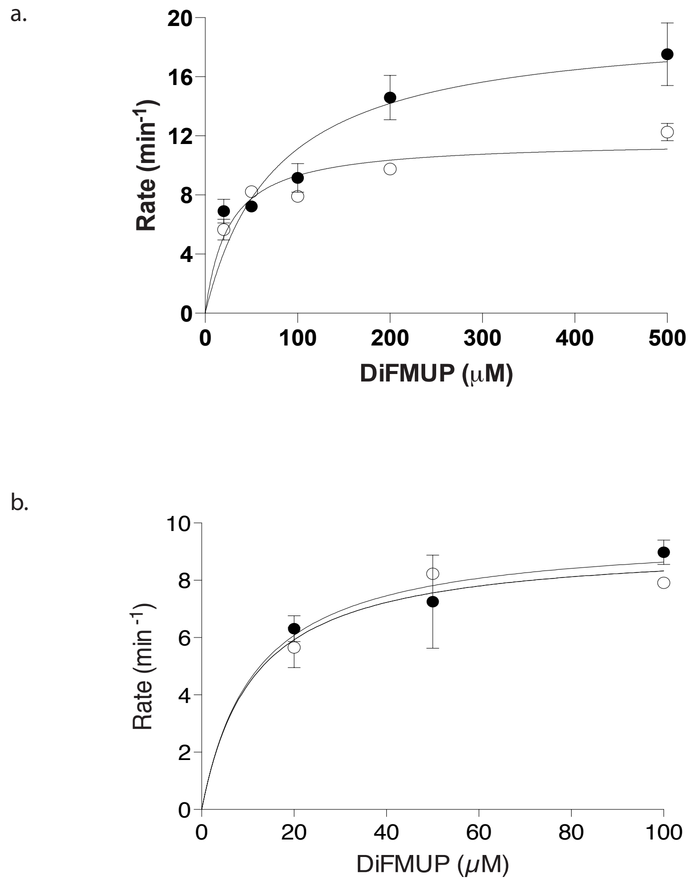
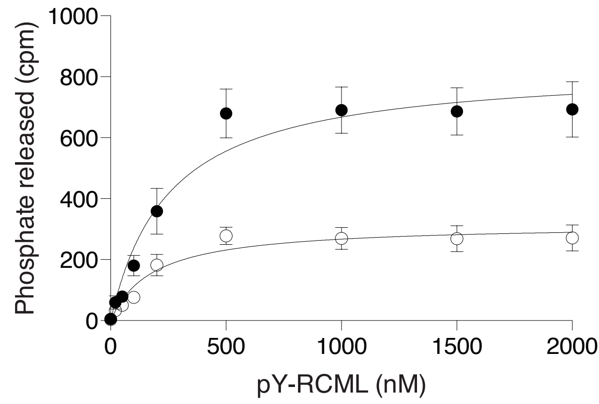


Fig. 5-3 : Phosphatidic acid enhances the phosphatase activity of PTPD2 towards DiFMUP *in vitro.*
Recombinant PTPD2 catalytic domain was left untreated (open circle) or incubated (solid circle) with phosphatidic acid (a) or phosphatidylserine (b) for 30 minutes and the phosphatase activity was measured using DiFMUP as substrate.

a.



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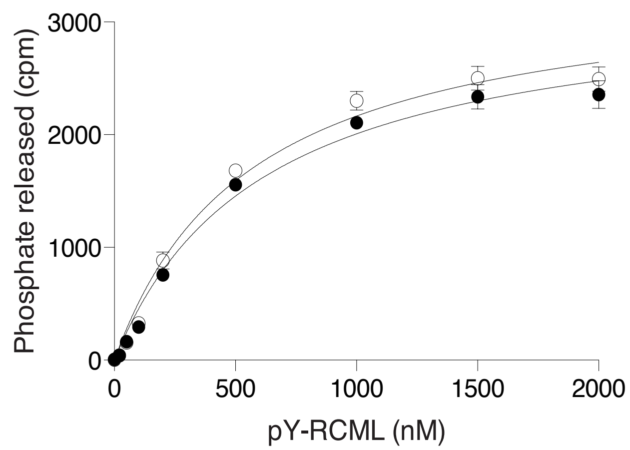


Fig. 5-4 : The catalytic activity of full length PTPD2 but not PTP1B, was enhanced following treatment with phosphatidic acid

PTPD2 (a) or PTP1B (b), immunoprecipitated from cells,were left untreated (open circle) or incubated with [14C] DPPA (solid circle) for 30 minutes and the phosphatase activity was measured using 32P-RCML as substrate.

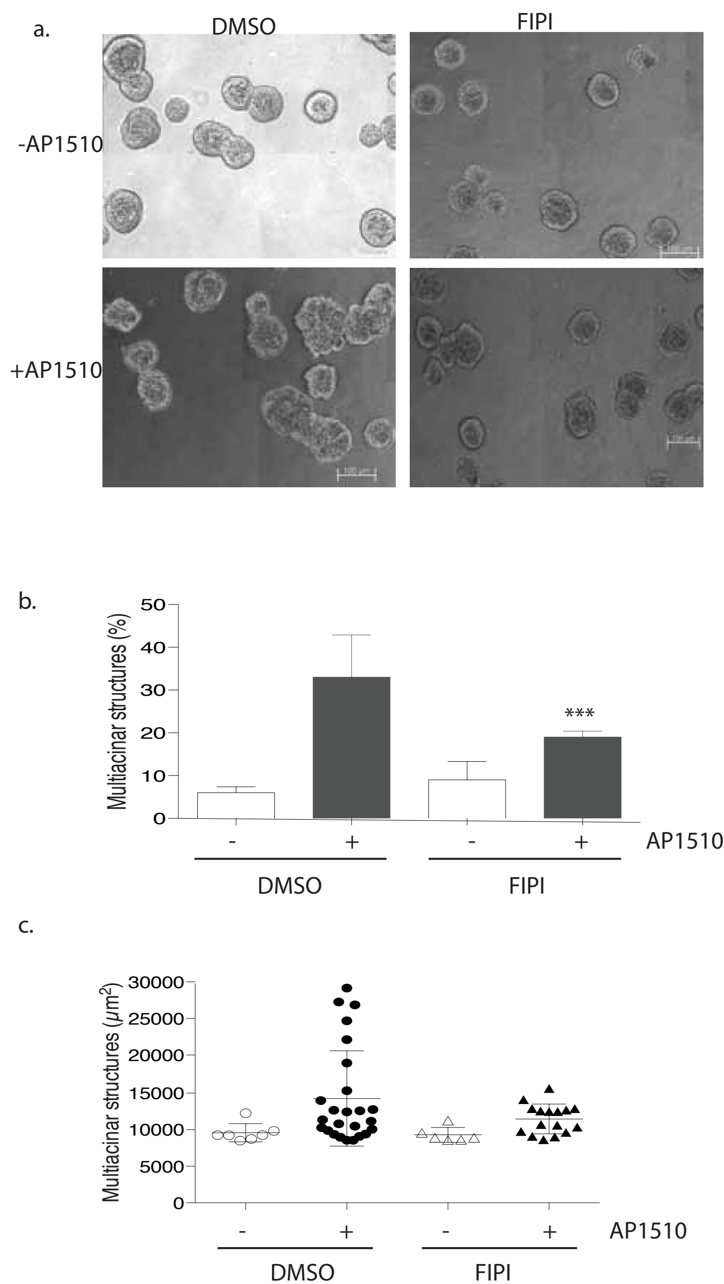


Fig. 5-5 : FIPI, a small-molecule inhibitor of PLD, inhibited the ERBB2 phenotype in three dimensional cultures

a) Representative phase-contrast images of 10A.B2 acinar structures treated with vehicle (DMSO) or dual PLD inhibitor (FIPI), with or without AP1510 stimulation. b) For quantitation of the multiacinar phenotype, data are expressed as percentage of acini that formed multiacinar structures, mean ± S.E.M. (*** $p<0.0005$). c) Size distribution of multiacinar structures expressed as area in µm2.

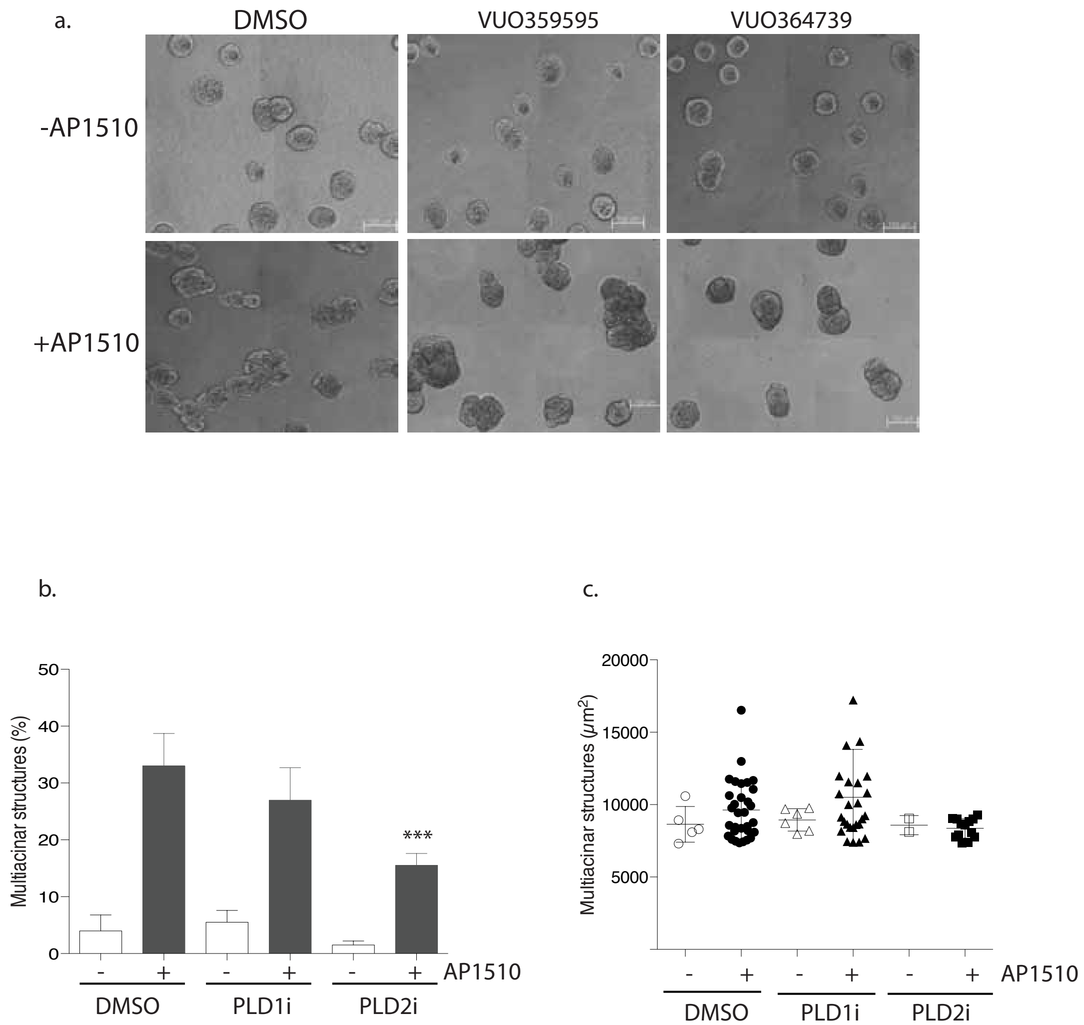


Fig. 5-6 : Small molecule inhibitor of Phospholipase D2 (PLD2), not PLD1 suppressed the ERBB2 phenotype in three-dimensional cultures

a) Representative phase-contrast images of 10A.B2 acinar structures treated with vehicle (DMSO), PLD1-specific inhibitor (VUO359595), or PLD2-specific inhibitor (VUO364739), with or without AP1510 stimulation. b) For quantitation of the multiacinar phenotype, data are expressed as percentage of acini that formed multiacinar structures, mean ± S.E.M. (*** $p<0.0005$). c) Size distribution of multiacinar structures expressed as area in μm2.

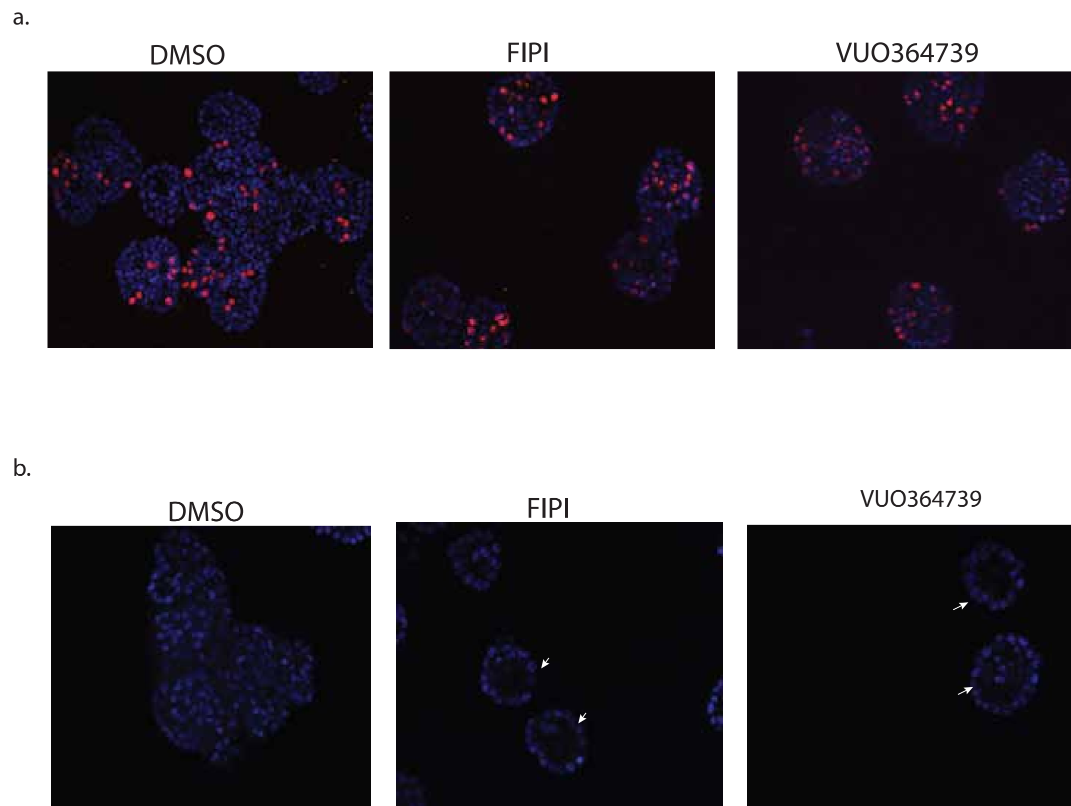
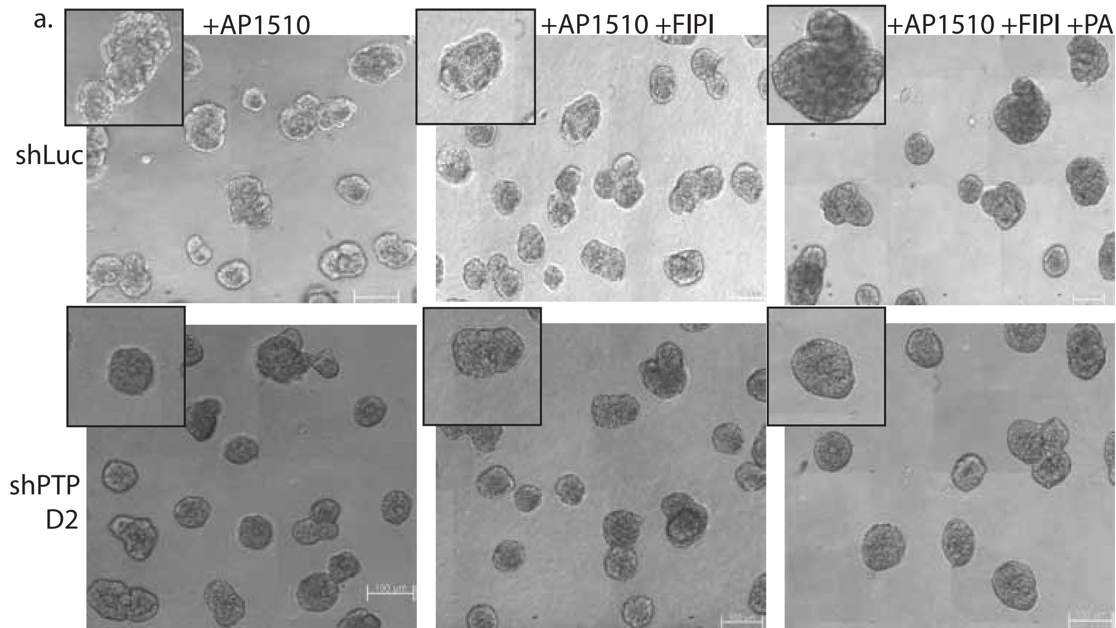


Fig. 5-7 : Small-molecule inhibition of PLD inhibited ERBB2-induced lumen filling but not hyperproliferation
a) AP1510-stimulated acinar structures treated with DMSO, FIPI or PLD2-specific inhibitor were immunostained for the proliferation marker Ki67 (red). Nuclei were costained with DAPI in blue.
b) Cross-sectional view of acini in a) with DAPI-stained nuclei in blue. White arrows indicate acini with clear lumen



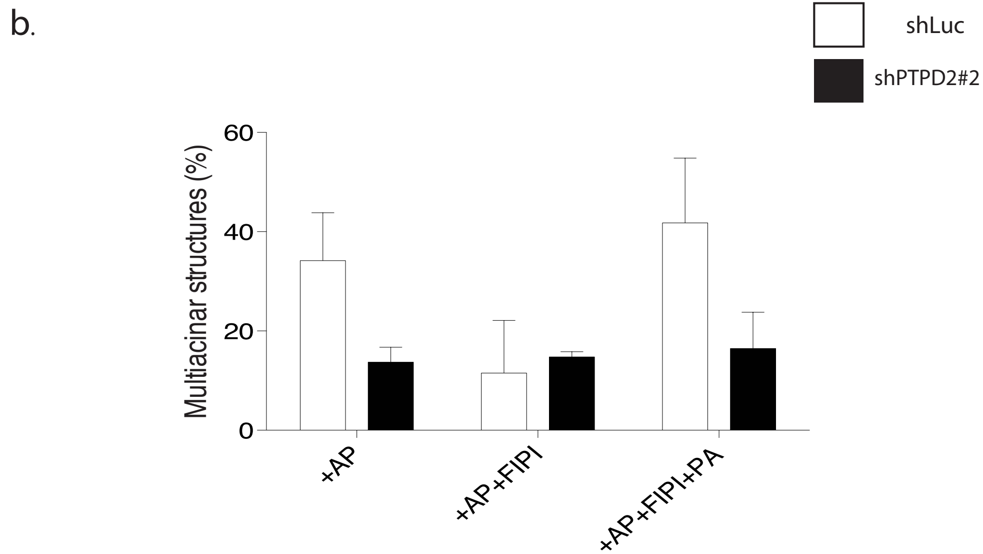


Fig. 5-8 : Suppression of PTPD2 inhibited phosphatidic acid-mediated rescue of the PLD- inhibition phenotype

a) Phase-contrast images of AP1510-stimulated acinar structures expressing the indicated short hairpins, and treated with vehicle (DMSO), dual PLD inhibitor, FIPI, or FIPI and phosphatidic acid. Inserts show representative acini. b) For quantitation of the multiacinar phenotype, data are expressed percentage of acini that form multiacinar structures, mean ± S.E.M

Chapter 6

Conclusions and Future Perspectives

Conclusions

The study of PTK function in disease has led to the identification of several candidates for targeted inhibition in disease. The resulting anti-PTK drugs have achieved significant success in the treatment of several human cancers. However, drug-resistant patient populations have been identified in almost all cancer subtypes, highlighting the importance of pursuing new therapeutic targets to complement current anti-PTK strategies.

Given our understanding of reversible tyrosine phosphorylation in signal transduction, PTPs represent an avenue with enormous potential for therapeutic intervention. Although they are known to make diverse contributions to signal transduction pathways, their characterization has lagged behind those of the PTKs; the functions and physiological substrates of most members of this family remain unknown to this day.

I conducted an shRNA-based loss-of-function PTP screen in 10A.B2 mammary epithelial cells cultured in three-dimensional Matrigel^{TM} to identify PTPs that played a role in mammary epithelial cell function, independent of or in the context of hyperactivation of the oncogenic receptor tyrosine kinase, ERBB2. I identified nine candidate PTPs, suppression of which had distinct outcomes with respect to acinus morphology. This included both positive and negative regulators of ERBB2 signaling, thereby highlighting the specificity of PTPs in this signal transduction pathway.

The goal of my thesis project has been to characterize the function of one of the targets emerging from this screen. I chose PTPD2 for further study, as it acted as a positive regulator of ERBB2 signaling, and could potentially be a druggable target in ERBB2-driven breast cancers. Moreover, besides its role in the Hippo signaling pathway, this PTP has not been well characterized to date. I found that suppression of PTPD2 with 2 different functional hairpins attenuated the AP1510-induced multiacinar phenotype, specifically by inhibiting ERBB2–mediated lumen filling and loss of polarity but not ERBB2-induced hyperproliferation. In contrast, overexpression of PTPD2 enhanced the number and size of multiacinar structures formed following ERBB2 stimulation with AP1510. This result suggested that the ERBB2 signaling pathway is sensitive to the levels of PTPD2; a threshold level of the phosphatase is essential for optimal functioning of the pathway. This

is the first description of a pro-oncogenic role for this enzyme in breast cancer and is consistent with data from the Cancer Genome Atlas.

We also show for the first time that PTPD2 bound specifically to phosphatidic acid *in vitro*. Phosphatidic acid enhanced the catalytic activity of PTPD2, but not PTP1B, towards tyrosine-phosphorylated RCM Lysozyme. Inhibition of Phospholipase D (PLD), the enzyme that produces phosphatidic acid in cells, attenuated the AP1510-induced multiacinar phenotype. The use of isoform-specific inhibitors revealed that the PLD2 isoform was required for ERBB2 function. Furthermore, PLD2 inhibition acted by inhibiting lumen filling and not hyperproliferation, comparable to the observation in PTPD2 knockdown acini.

Epistasis experiments revealed that PLD2 and PTPD2 function in the same pathway downstream of ERBB2. Exogenously added phosphatidic acid was able to rescue the PLD-inhibition phenotype, but not when PTPD2 was suppressed. These data can be integrated into a model of PTPD2 function centered on the regulation of its catalytic function by the lipid second messenger phosphatidic acid, to act as a positive regulator of ERBB2 signaling. PLD2, acting downstream of activated ERBB2, produces PA. PA binds to PTPD2 and augments its catalytic function, allowing the enzyme to dephosphorylate as yet uncharacterized substrates in the ERBB2 signaling pathway. This dephosphorylation event is critical for ERBB2-mediated lumen filling and loss of epithelial polarity **(Fig 6-1).** In the event of inhibition of PLD2, (and by extension, reduced levels of PA) or suppression of PTPD2, these critical substrates remain in the phosphorylated state, which impedes the formation of multiacinar structures characteristic of ERBB2.

Future Directions

The data presented in this thesis point towards a novel signaling axis involving the Protein Tyrosine Phosphatase, PTPD2 and the lipid second messenger, phosphatidic acid, that acts to regulate the ERBB2 pathway in a positive manner in mammary epithelial cells. This axis can, therefore, be pursued as a potential source of prognostic and therapeutic targets in ERBB2-positive breast cancer. The data directly implicate PTPD2 and PLD2 as two potential "druggable" candidates. A deeper biochemical understanding of the function and regulation of these molecules could reveal mechanisms for pharmaceutical inhibition. These efforts would be supplemented by the elucidation of other effectors of this pathway, which themselves might serve as targets for inhibition. Following are some areas for immediate investigation aimed at achieving these objectives:

1. **To test whether PLD2 is activated in response to ERBB2 activation**

My data show that PLD2 is required for optimal ERBB2 signaling in three-dimensional cultures. It would be interesting to test whether PLD2 is activated downstream of ERBB2 in mammary epithelial cells. ERBB2 signaling propagates via a cascade of phosphorylation events and several positive regulators downstream of ERBB2 are activated by tyrosine phosphorylation, for example, the adapter protein GRB2 and the tyrosine kinase SRC (37). In the context of EGF signaling, PLD2 is known to associate physically with activated EGFR and subsequently be tyrosine phosphorylated (196), resulting in an increase in lipase activity. PLD is also known to be a substrate for receptor and non-receptor kinases. In mast cells, for example, tyrosine phosphorylation of PLD2 at specific tyrosine residues by Fyn and Fgr is required for cell activation (197)**.** *In vitro* phosphorylation of PLD2 at distinct tyrosine residues by EGFR, SRC and JAK3 causes positive or negative effects on lipase activity (198). In light of these observations, we tested whether PLD2 is activated by tyrosine phosphorylation following ERBB2 activation. However, I did not observe tyrosine phosphorylation of ectopically expressed PLD2 following acute activation of chimeric ERBB2.

We cannot rule out that PLD2 activation might take place by other mechanisms in the context of ERBB2 signaling. One possibility is that ERBB2 signaling could regulate the cellular levels or distribution of lipids like PIP_2 which are known to modulate PLD2 activity

and, by extension, levels of PA in cells. There is also evidence of activation of PLD2 through serine phosphorylation; Cdk5-mediated phosphorylation and activation of PLD2 is responsible for EGF-dependent insulin secretion. Given that several Ser/Thr Kinases act as effectors of ERBB2 signaling, it is possible that they directly phosphorylate and activate PLD2 following ERBB2 activation.

2. To identify the PA binding site on PTPD2

Although several PA-binding proteins have been identified, the precise PA binding site has not been defined. PA binds to both positively charged amino acid residues and surface-exposed hydrophobic residues in different proteins. We have observed that PA not only bound to the catalytic domain of PTPD2 but also the FERM domain, which is known to localize proteins to the plasma membrane. Mutational analysis of these domains can be used to identify the PA binding site. This will allow us to determine the relative importance of PA-binding to the two domains in the function of PTPD2 in cells as a positive regulator of ERBB2 signaling. If a PA-binding deficient mutant of PTPD2 is unable to recue the effect of suppression of PTPD2, we can design inhibitors to the PA-binding site of PTPD2 as a way of attenuating the ERBB2 signaling pathway in breast cancer.

3. To carry out structure-function analysis of phosphatidic acid in the regulation of PTPD2

It will be interesting to identify the critical structural elements of PA that are required for interaction with PTPD2 and regulation of its catalytic activity. PA analogs that differ in the phosphate head group, side chains or the lipid acyl linkage have previously been used to study the interaction between PA and the phosphatase, PP-1. It was found that the presence of the phosphate as well as both fatty acid side chains but not the specific composition of the side chains (180) is important for the ability of PA to bind PP-1 and inhibit its activity.

4. **To test additional mechanisms for regulation of PTPD2 by PA**

Our data show that *in vitro,* phosphatidic acid binds to and activates PLD2. As described previously, local enrichment of PA in compartments of the plasma membrane plays an important role in the recruitment of signaling molecules. It will be interesting to test whether

PA also regulates PTPD2 localization in cells. We have observed that the FERM domain of PTPD2 also binds to PA. It is conceivable that following acute stimulation of chimeric ERBB2, localized production of PA by PLD2 would result in translocation of PTPD2 to a specific plasma membrane compartment via the FERM domain. This could be accompanied by interaction of the lipid with the catalytic domain, enhancing the phosphatase activity of PTPD2 towards tyrosine-phosphorylated substrates, also in the same compartment. Should we observe localization of PTPD2 to the plasma membrane, we might be able to better focus our efforts to identify relevant substrates of PTPD2 in the context of ERBB2 signaling.

Commercially available antibodies against PTPD2 efficiently detect endogenous levels of the protein in MCF-10A cells by immunoblotting but are ineffective for immunofluorescence staining. This has posed a significant challenge to conducting localization studies. We have initiated a collaboration with Bryce Nelson at the Antibody Core Facility at CSHL to generate antibodies against PTPD2 that can address this limitation.

5. To identify specific substrates of PTPD2 in the context of ERBB2 signaling

It is critical to identify the specific substrates of PTPD2 in the ERBB2 signaling pathway. Quantitative phosphoproteomics approaches, which can compare overall changes in phosphorylation in control v. shPTPD2 cells, provide the best opportunity to identify putative substrates in an unbiased manner and without the limitations imposed by antibody availability. However, these changes may be caused directly or indirectly by the PTP under study. To implicate the phosphoprotein as a direct substrate, it is necessary to determine binding with a trapping mutant form of the PTP. As discussed before, this will require a thorough understanding of the catalytic mechanism of PTPD2 and mutational analysis to identify the optimal substrate-trapping mutant. Identification of the substrate may provide insight into a novel effector pathway essential for the functioning of the ERBB2 pathway and may be a source of therapeutic targets.

6. To validate our findings from cell-based models in disease-relevant mouse models

Transgenic mouse models, developed by interbreeding PTPD2 deficient mice with those that express wild-type or activated rat ERBB2 *(ptpd2-/- x Neu),* will be a valuable tool to establish the role of PTPD2 in ERBB2-driven breast cancer *in vivo.*

In mice expressing activated ERBB2, the promoter used to drive oncogene expression is an important consideration, as it would affect expression levels, developmental timing and the cell types in which the oncogene is expressed, thereby affecting the tumor phenotype. Mice bearing the wild-type ERBB2 allele under the control of the mouse mammary tumor virus (MMTV) promoter exhibit spontaneous deletions in the transgene, leading to constitutive activation (199). Knock-in mice that express the activated ERBB2 under the control of the endogenous promoter show amplification of this genomic region, much like that observed in human breast cancer (200). However, they exhibit significantly decreased tumor latency and low metastasis rate (200), which does not recapitulate aggressive ERBB2+ human breast cancers. For this reason, models in which the ERBB2 oncogene, or a mutant thereof, is driven by MMTV are preferred to examine how tumor suppressors and oncogenes modulate ERBB2-induced mammary carcinogenesis. Studies that have implicated PTP1B as a positive regulator in ERBB2-driven mammary carcinogenesis have previously made use of MMTV-NDL2 mice (which overexpress, in the mammary gland, an altered ERBB2 transgene containing an in-frame deletion in the extracellular domain) or MMTV-NeuNT mice (87,88).

At the moment, there are no reports of PTPD2 -deficient mouse models. Studies in zebrafish suggest that PTPD2 is required for early embryonic development (129). Suppression of PTPD2 using antisense morpholino oligonucleotides results in severe architectural defects in the organs where PTPD2 is expressed (129). It is possible that knocking out PTPD2 in mice could have similar effects in organogenesis that might result in embryonic lethality. This would pose a technical limitation in generating transgenics with ERBB2 mouse models.

Mosaic mouse models represent an alternative strategy to establish the role of PTPD2 in mammary tumorigenesis *in vivo*. This model exploits the fact that mammary gland

development essentially takes place after birth. Surgical removal of mammary epithelium from the fat pad of a 3 week-old mouse generates an orthotopic site for transplantation of mammary progenitor cells that have been engineered in culture to harbor desired genetic changes, in our case, PTPD2 suppression, in the context of hyperactive ERBB2. In the absence of interference from endogenous mammary epithelium, this will allow us to study the ability of transplanted cells to form tumors in the reconstituted breast tissue. This model has been successfully used in the Muthuswamy lab, to verify the oncogenic function of the tyrosine kinase BRK, in ERBB2-driven breast cancer (201). Our data from three-dimensional organotypic culture suggests that loss of PTPD2 attenuates the ERBB2-driven multiacinar phenotype. We could test for the effect of PTPD2 suppression on tumor latency, primary tumor burden and incidence of metastasis in the context of ERBB2 activation. Further, tumors formed in the transplanted mice could provide valuable material for biochemical analyses to complement those carried out in cell-based models.

In order to validate the role of PLD2 in ERBB2-driven tumorigenesis *in vivo*, it would also be interesting to evaluate the phenotype of PLD2-deficient mice in an ERBB2 background. PLD2 knockout mice lend themselves to these studies as they are viable and do not show an overt phenotype (202). Characterization of the PA-binding site on PTPD2 will allow us to generate PA binding-deficient PTPD2 knock-in mice that can subsequently be interbred with ERBB2 mice. Evaluation of the phenotypes of the resulting mice would allow us to validate the importance of PA-binding on the role of PTPD2 as a positive regulator of ERBB2-mediated mammary carcinogenesis *in vivo.* Together with biochemical characterization of the PLD2-PTPD2 signaling pathway, these studies could provide a basis for prognostic and therapeutic interventions targeting PTPD2 in ERBB2-positive breast cancers.

7. To examine the role of PTPD2 in signaling driven by ERBB2 heterodimers

Our results suggest a positive role for PTPD2 in signaling driven by ERBB2 homodimers in human mammary epithelial cells. However, it is known that ERBB2 also undergoes heterodimerization with other members of the same family (37). It will be important to determine if the PLD2-PTPD2 axis functions in a positive manner in signaling driven by ERBB2 heterodimers- particularly with EGFR and ERBB3. Similar to the model used in this study, the Muthuswamy lab has developed a system to study selectively signaling from

ERBB2-EGFR heterodimers using chimeras that can be activated using a small-molecule cell-permeable ligand (103). This model can also be adapted for the study of ERBB2-ERBB3 hetreodimers in human breast tissue.

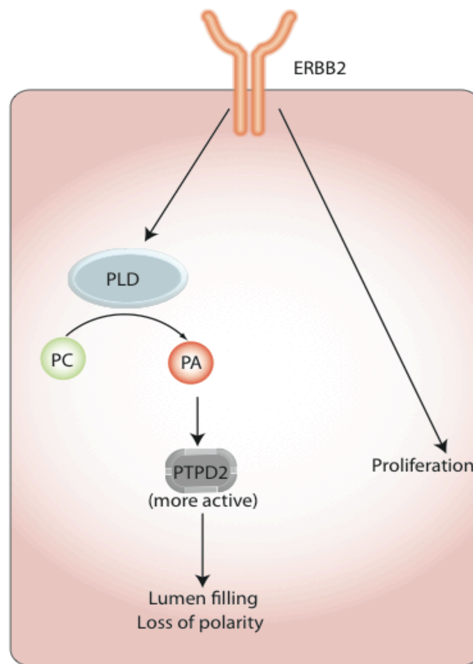


Fig. 6-1 : Model of phosphatidic acid-mediated regulation of PTPD2 function in ERBB2 signaling in mammary epithelial cells

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