Investigating the Role of PHIP1 in Breast Cancer

by

Chan Mi Lee

A thesis submitted in conformity with the requirements
for the degree of Master of Science

Department of Laboratory Medicine & Pathobiology
University of Toronto, Canada

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Chan Mi Lee

Master of Science

Laboratory Medicine & Pathobiology
University of Toronto

2011

Abstract

PHIP1 is a novel downstream transcriptional co-regulator of insulin-like growth factor-I receptor (IGF-IR), a tyrosine kinase receptor that is often elevated and autophosphorylated in breast cancer. In this study, I show that PHIP1 is upregulated in MCF10A cells stably overexpressing IGF-IR signaling components and that knock-down of PHIP1 significantly inhibits breast cancer cell proliferation by inducing transcriptional upregulation of p21 and downregulation of cyclin D2. I also show that stable overexpression of PHIP1 in MCF10A cells can lead to its proteasomal degradation. Together, our data indicate that PHIP1 is implicated in breast cancer cell growth and suggest a number of avenues that await exciting discovery.
Acknowledgments

My time here at the University of Toronto has been an enormous learning experience and one of the most memorable moments of my life. However, despite the ups and downs I had, it was a journey worth undergoing, and it made me stronger and more knowledgeable. As I present this fruit of the labor, I would like to express appreciation to all those who stood by me on this journey.

My first thanks goes to my supervisor, Dr. Maria Rozakis-Adcock, for her patience throughout the years, and the opportunity to be part of the exciting research team. I am also grateful all the members of the Rozakis lab, for sharing their scientific knowledge and for making my time in the laboratory stimulating and enjoyable.

I would like to sincerely thank my committee members, Dr. Eldad Zacksenhaus and Dr. Stephane Angers, for their generous time and expertise to advise and guide me through my research education.

I also express my gratitude to all my friends on the sixth floor MSB and the LMP staffs, for their friendships and all the fun times we had together.

Finally, I specially thank my family for the limitless support, understanding and love. And also to God, who is my friend for life, for His eternal presence within me.
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<th>Full Form</th>
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<tbody>
<tr>
<td>7-AAD</td>
<td>7-aminoactinomycin D</td>
</tr>
<tr>
<td>aa</td>
<td>amino acid</td>
</tr>
<tr>
<td>3’UTR</td>
<td>3’untranslated region</td>
</tr>
<tr>
<td>BD</td>
<td>bromodomain (domain)</td>
</tr>
<tr>
<td>bHLH</td>
<td>basic helix loop helix</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CAF</td>
<td>chromatin assembly factor-1</td>
</tr>
<tr>
<td>CBP</td>
<td>CREB binding protein</td>
</tr>
<tr>
<td>CDK</td>
<td>cyclin dependent kinase</td>
</tr>
<tr>
<td>CKI</td>
<td>cyclin dependent kinase inhibitor</td>
</tr>
<tr>
<td>CIS</td>
<td>carcinoma in situ</td>
</tr>
<tr>
<td>CRL</td>
<td>cullin-RING finger ligase</td>
</tr>
<tr>
<td>CUL</td>
<td>cullin (protein)</td>
</tr>
<tr>
<td>DCAF</td>
<td>DDB1 and CUL4-associated factor</td>
</tr>
<tr>
<td>DDB1</td>
<td>damaged DNA binding protein</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified eagle medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>ECL</td>
<td>enhanced chemiluminescent</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
</tr>
<tr>
<td>EGFP</td>
<td>enhanced green fluorescent protein</td>
</tr>
<tr>
<td>EMT</td>
<td>epithelial-to-mesenchymal transition</td>
</tr>
<tr>
<td>ErbB</td>
<td>avian erythroblastosis oncogene B</td>
</tr>
<tr>
<td>ER</td>
<td>estrogen receptor</td>
</tr>
<tr>
<td>ERK1/2</td>
<td>extracellular signal-regulated kinase 1/2</td>
</tr>
<tr>
<td>ETD</td>
<td>extralobular terminal duct</td>
</tr>
<tr>
<td>FAK</td>
<td>focal adhesion kinase</td>
</tr>
<tr>
<td>FACS</td>
<td>fluorescence activated cell sorting</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>FISH</td>
<td>fluorescent in situ hybridization</td>
</tr>
<tr>
<td>G1 phase</td>
<td>cell cycle gap 1</td>
</tr>
<tr>
<td>G2 phase</td>
<td>cell cycle gap 2</td>
</tr>
<tr>
<td>GAP</td>
<td>GTPase activating protein</td>
</tr>
<tr>
<td>GAPDH</td>
<td>glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>GSK-3β</td>
<td>glycogen synthase kinase-3β</td>
</tr>
<tr>
<td>GST</td>
<td>glutathione S-transferase (tag)</td>
</tr>
<tr>
<td>HA</td>
<td>hemagglutinin (tag)</td>
</tr>
<tr>
<td>HAT</td>
<td>histone acetyltransferase</td>
</tr>
<tr>
<td>HDAC</td>
<td>histone deacetylase</td>
</tr>
<tr>
<td>HEK293</td>
<td>human embryonic kidney cells</td>
</tr>
<tr>
<td>HEPES</td>
<td>N-2-hydroxyethyl piperazine-N-ethanesulphonic acid</td>
</tr>
<tr>
<td>HER2</td>
<td>human epidermal growth factor receptor 2</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>TDLU</td>
<td>terminal ductal-lobular unit</td>
</tr>
<tr>
<td>TF</td>
<td>transcription factor</td>
</tr>
<tr>
<td>TGF-β</td>
<td>transforming growth factor β</td>
</tr>
<tr>
<td>TMA</td>
<td>tissue microarray</td>
</tr>
<tr>
<td>UPP</td>
<td>ubiquitin-proteasome pathway</td>
</tr>
<tr>
<td>WAP</td>
<td>whey acidic protein</td>
</tr>
<tr>
<td>WDR</td>
<td>tryptophan-aspartic acid repeat (domain)</td>
</tr>
<tr>
<td>WT</td>
<td>wild-type</td>
</tr>
</tbody>
</table>
Chapter 1. Introduction

1.1 Breast Cancer

Breast cancer is the most common cause of cancer among women globally (Bray et al, 2004) as well as in Canada (Canadian Cancer Society, 2010). It imposes a significant burden to public health with high morbidity and mortality rates, and in 2010 alone, 23,200 women in Canada are estimated to be diagnosed with breast cancer while resulting in 5,300 deaths. In addition, approximately 1 in 9 women will develop breast cancer during their lifetime while 1 out of 28 will die of the disease.

The probability of being diagnosed with breast cancer in women increases dramatically with age. As an example, when compared to women in their 20s of age, the risk is more than 10-fold for women in their 30s, more than 100-fold for women in 50s and larger than 1,000-fold for women in their 80s (Schedin, 2006). Given the fact that more women are giving birth to first child in their 30s in modern society and the average age of child-bearing is increasing steadily (Kroman et al, 2003), the morbidity and mortality due to or associated with breast cancer are also likely to be augmented in the future. However, despite the huge research efforts to elucidate the mechanism of breast tumorigenesis over the past decades, our understanding of the communication networks is yet incomplete. There is thus a notable need for more comprehensive knowledge of the complex cellular events that are involved in the transformation, development and metastasis of breast cancer cells.

1.1.1 Mammary gland structure

Each breast harbors the mammary gland, which is comprised of 15 to 20 lobes, or compartments. Each lobe, in turn, consists of smaller lobules that contain milk-secreting glands called alveoli in grapelike clusters surrounded by the connective tissue (Tortora and Derrickson, 2006). The functional unit of the mammary gland is called the terminal ductal lobular unit (TDLU), which consists of extralobular terminal duct (ETD) and intralobular terminal duct (ITD) (Allred et al, 2001; Ohuchi et al, 1984; Figure 1A). The ITD refers to the central space of the lobule and have outpouchings called acini, or ductules. (Ohuchi et al, 1984). The acini, in turn, contain a hollow lumen surrounded by a single polarized layer of luminal epithelial cells (Allred et al, 2001; Bissell et al, 2002).

There are two major different tissue structures that define each mammary gland: the epithelium and the stroma. The adenocarcinoma of the breast is thought to originate from the epithelial cells of the TDLU (Bissell et al, 2002). The epithelium forms the ducts and alveoli, which
are collectively connected as the lactiferous duct and open to the nipple for the release of milk. Most of the epithelial cells are luminal and undergo functional differentiation during pregnancy to become secretory for milk production, which is largely stimulated by the hormone prolactin from the anterior pituitary (Tortora and Derrickson, 2006; Hennighausen and Robinson, 2006). The luminal cells are enclosed by basal, myoepithelial cells that are contractile (Figure 1B) and aid in the delivery of milk under the influence of the hormone oxytocin, which is released from the posterior pituitary upon suckling. The stroma, also called the mammary fat pad, refers to the connective tissue which is composed mostly of the adipocytes, fibroblasts, blood vessels, and neurons and embeds the extensive branches of epithelial cells. The mammary epithelial extracellular matrix containing the myoepithelial basement membrane and the stroma supports the mammary gland structure and acts as communicative bridge between the inner epithelium and the surrounding environment during the organ maturation and hormonal regulation (Wiseman and Werb, 2002). During pregnancy, the alveolar epithelium expands and produces milk during lactation, but after weaning where the level of prolactin wanes, it undergoes massive apoptosis and extensive remodeling process called involution (Hennighausen and Robinson, 2006).

Figure 1. The human mammary gland.

(A). Nomenclature of the ductal system. The terminal ductal-lobular unit (TDLU) is the functional unit of the mammary gland and consists of an ETD, extralobular terminal duct, and ductules. ITD stands for the intralobular terminal duct. Adopted from (Ohuchi et al., 1984). (B). Schematic diagram of human mammary gland lobule. Adapted (from Debnath et al., 2003).
1.1.2 Classification of breast cancer

Breast cancer is a clinically heterogeneous disease, and there are a number of variables that are taken into consideration to determine the prognosis and the likely response to therapies. Breast cancer can be classified by different criteria to serve different purposes, and examples of the breast cancer categorizations include histological type, grade, tumor size, involvement of lymph node, estrogen receptor (ER), progesterone receptor (PR) and HER2/Neu-receptor status, as well as protein and genetic markers. Tumor pathology refers to the histological appearance of the tumor tissue, and as the majority of breast cancers are derived from ductal and lobular epithelium, the most common forms of breast cancer under this category are carcinoma in situ (CIS) and invasive carcinoma. According to the MERCK Professional Manual, CIS refers to the proliferation and invasion of the cancer cells within the epithelial cells, while the invasive carcinoma denotes invasion of cancer cells to the surrounding tissues. The grading system is another form of the histological analysis that distinguishes between well differentiated (low grade), moderately differentiated (intermediate grade), and poorly differentiated (high grade) as cells progressively depart from the normal phenotype. Unsurprisingly, breast cancer of high grade has the worst prognosis. The use of stages for breast cancer follows the tumor, node, metastasis, or TNM classification, based on the size of tumor (T), presence of metastasis to the lymph nodes in the armpits, neck and chest (N) or the rest of the body (M). Staging is usually more accurately determined during surgery and is numerated from 0 to 4, with 0 being the pre-malignant stage and 4 being the ‘advanced’, or highly metastatic state.

The current most widely used molecular classification in clinical treatments is the receptor status, according to the presence (positive) or absence (negative) for hormonal receptors ER, PR, and HER2/Neu. ER status and probably PR contents, for example, are a good prognostic factor and a positive predictor of response to endocrine therapy (Andre and Pusztai, 2006). Overexpression of HER2, on the other hand, is generally associated with poor disease outcomes, and basal-like cells that express none of these receptors are considered more aggressive. The recent recommended guideline for the use of tumor markers in breast cancer by the American Society of Clinical Oncology is reviewed by (Harris et al, 2007). The molecular classification is constantly being updated with more large-scale genomic analyses of breast cancers and the discovery of subsets of molecular markers within the existing categories, which is hoped to improve selection criteria for patient treatment for maximum efficacy (reviewed in Andre and Pusztai, 2006).
1.1.3 Classical oncogenes in mammary gland tumorigenesis

In breast cancer, direct activation of proto-oncogenes to transform into oncogenes generally occurs by one of the three mechanisms: 1. point mutations or sequence changes in the coding or the promoter region of the gene, as in the case for the RAS family, 2. gene amplification, such as ERBB2 amplification in breast cancer and 3. gene arrangements, most frequently chromosome translocations which is a prominent form of oncogenic activation in chronic myelogenous leukaemia and Burkitts lymphoma (Oliveira and Fletcher, 2005). Indirect activation can also occur through suppression of normal regulatory mechanisms, by the inactivation of tumor suppressors such as p53, RB or BRCA1. Few examples of the classical oncogenes that are relevant in breast cancer have been outlined below.

1.1.3.1 HER2

HER2, also known as ErbB2, is a member of the epidermal growth factor receptor (EGFR) family of receptor tyrosine kinases (RTK) normally involved in signal transduction of cell growth and differentiation (Reese and Slamon, 1997). The HER family consists of HER1, 2, 3 and 4 and upon ligand binding, it can either homodimerize or heterodimerize with a different member of the family. However, HER2 is the preferential dimerisation partner of other members of the ErbB family, and its heterodimers are more stable and lead to more potent signaling (Cardoso et al, 2005). The proto-oncogene is located on chromosome 17q21-q22 and is amplified in 20 to 30% of human mammary carcinomas, which are associated with poor prognosis and increased disease recurrence (Cardoso et al, 2005). Because of its ability to predict response to treatment such as trastuzumab (Herceptin) or Lapatinib that target the HER2 signaling pathway, HER2 is a well used molecular marker routinely screened in breast cancer for prognosis. There is much in vivo evidence for HER2 oncogenic activity to date. MMTV-Neu transgenic mice overexpressing the activated rat homolog of ErbB2 develops multifocal adenocarcinoma and lung metastases in approximately 15 weeks after pregnancy (Muller et al, 1988). Similarly, transgenic mammary gland-specific expression of wild-type ErbB2 also leads to mammary tumor formation at an average age of 7 months and many of the mice developed secondary metastatic tumor in the lungs (Guy et al, 1992). More recently, Moody and colleagues demonstrated, using a doxycycline-inducible conditional mouse model, that tumor formation could be partially regressed upon withdrawing doxycycline. However, most mice exhibited recurrence, which was accompanied by upregulation of Snail and epithelial-to-mesenchymal transition (EMT) (Moody et al, 2005).
**1.1.3.2 c-Myc**

The family of *myc* proto-oncogenes code for proteins belonging to classical transcription factors with a characteristic basic helix-loop-helix leucine zipper (bHLHZ) domain and participate in various aspects of cell function, such as replication, cell growth, metabolism, differentiation and apoptosis (Liao and Dickson, 2000). The c-Myc protein heterodimerizes with Max, another transcription factor, and binds to an E-box element containing CAC(G/A)TG motif in the middle of the DNA recognition site (Haynes and Lane, 2001). The *c-myc* proto-oncogene, which is located on chromosome 8q24, is amplified in approximately 16% of breast cancer cases and is associated with decreased disease-free survival of the patient (Oliveira and Fletcher, 2005). *c-myc* expression is frequently upregulated in early breast lesions and the protein c-Myc is overexpressed in approximately 70% of breast tumors, which is correlated with poor clinical outcome (Liao and Dickson, 2000). *In vivo* models containing c-Myc transgene such as MMTV-Myc (Stewart *et al*, 1984), whey acidic protein (WAP) promoter-Myc (Schoenenberger *et al*, 1988), or tetracycline inducible system (D’Cruz *et al*, 2001) develop mammary tumors in 7 to 14 months in 50 to 80% of mice. c-Myc is now known to be a downstream effector of HER2 signaling (Haynes and Lane, 2001).

**1.1.3.3 Cyclin D1**

Cyclin D1 complexes with cyclin-dependent kinase (CDK) 4 and 6 during the G1 phase of the cell cycle to phosphorylate the retinoblastoma (Rb) family of tumor suppressors, to release the checkpoint on the entry into the DNA synthesis (S phase). Cyclin D1 protein is overexpressed in most breast cancer cases (Steeg and Zhou, 1998) and is associated with poor prognosis (Sutherland and Musgrove, 2004). The cyclin D1 gene, or *CCND1* is localized on chromosome 11q13, and the locus amplification has been found in up to 15% of breast cancer (Rennstam *et al*, 2001). As an activator of cell cycle progression, cyclin D1 overexpression leads to enhanced cell proliferation and has been shown to participate in the transition from the premalignant breast lesion to carcinoma (Weinstat-Saslow *et al*, 1995). Similarly, MMTV-cyclin D1 mouse models show mammary hyperplasia due to proliferative disturbances and eventually develop mammary adenocarcinoma (Wang *et al*, 1994).
1.2 Insulin-like Growth Factor type I Receptor (IGF-IR) pathway

1.2.1 Insulin-like growth factor type I receptor (IGF-IR) structure

Insulin-like growth factor type I receptor (IGF-IR) is a transmembrane tyrosine kinase cell surface receptor that undergoes dimerization and autophosphorylation of their C-terminal tyrosine residues upon binding of their ligands, IGF-I and IGF-II (Surmacz, 2000). The functional receptor is a heterotetramer, with two α and two β chains that are linked by disulfide bonds (Figure 2). The α chains participate in the ligand binding and provide the binding specificity, while the β subunits transmit the ligand-induced signal into the cell. There are three major domains that are recognized in the β subunit: a short juxtamembrane or transmembrane portion that provide docking sites to major signaling substrates such as insulin receptor substrates (IRS) 1-4 and src/collagen-homology (SHC) proteins, a tyrosine kinase domain containing the autophosphorylation tyrosine residue clusters, and the carboxy-terminal domain (C-terminus) that contains important residues necessary in the association of adaptor proteins required for IGF-I signaling (Dupont et al., 2003). Examples of proteins that associate with the C-terminal region of the IGF-IR include GRB10, 14-3-3 epsilon, and p85 subunit of phosphatidylinositol-3 kinase (PI3K) or SHPTP2 phosphatase.

![Figure 2. Structure of IGF-IR.](image)

The key residues and binding elements involved in signaling are depicted. Adapted from (Surmacz, 2000).
As its name suggests, IGF-IR is structurally and functionally homologous with the insulin receptor (IR) and they can heterodimerize to create an insulin/IGF-IR hybrid receptor. Structurally, the kinase domains of the two receptors are 80-90% identical. However, the C-terminal regions, which are important in the specificity of signaling pathway triggered through the recruitment of adaptor proteins, are only 40% equivalent. Tartare and colleagues demonstrated the C-terminal signaling specificity in signaling using chimeric receptors, which either contained the ligand-binding domain of IR and cytoplasmic segment of the IGF-IR or vice versa (Tartare et al, 1994). The functional analyses showed high correlation with the C-terminal region, more than the ligand-binding domain. Thus, the two receptors have distinct as well as similar physiological roles (Surmacz, 2000).

1.2.2 Downstream signaling

Upon the binding of its ligand, IGF-I, the IGF-IRs cluster and activate their tyrosine kinase to autophosphorylate and cross-phosphorylate the tyrosine residues in the beta chain, whereby the phosphorylated specific Tyrosine (Tyr) and Serine (Ser) residues create docking sites for the IGF-IR signaling molecules. Some of the most well known substrates are IRS-1 and SHC, both of which bind to Tyr 950 via phosphotyrosine binding (PTB) domain (Surmacz, 2000). The phosphorylated tyrosine residues of IGF-IR upon activation become the docking sites for adaptor proteins such as insulin receptor substrate (IRS)-1 and Grb-2, which lead to subsequent activation of downstream signaling (Figure 3). Although there are four different forms of IRS proteins in the mammalian system, only IRS-1 and 2 are the most prominent players in the IGF-IR or IR signaling (Surmacz, 2000). There are two major anti-apoptotic pathways induced by IGF-IR activation, which are covered in more depth in the following sections; 1. Lipid kinase PI3K/Akt and 2. GTPase Ras-Raf-ERK/MAPK pathway. PI3K/Akt is involved in mitogenesis, metabolism, cell adhesion and motility, while Ras/MAPK through the activation of Grb2 and Sos is implicated in cell growth and differentiation (Chitnis et al, 2008; Valentinis et al, 2001).
Figure 3. IGF-IR signaling pathway.

The IGF-IR has an extracellular α domain and a cytosolic β domain. Binding of IGF-I to the receptor leads to homodimerization of IGF-IR and activation of multiple downstream signaling pathways. Adapted from (Dupont et al 2003).

1.2.2.1 PI3K/p-Akt pathway

IRS-1 acts as a potent effector of IGF-IR by recruiting and phosphorylating various signaling molecules, especially PI3K, through the interaction of phosphotyrosine binding domains such as SH2 (Dupont et al, 2003). 3-phosphoinositide-dependent protein kinase 1 (PDK1), some isoforms of protein kinase C (PKC) and AKT kinases are activated upon increased level of lipid secondary messengers by PI3K and subsequent localization to the plasma membrane. The PI3K/p-Akt pathway is a major downstream effector of IRS-1 and is important in cell growth, survival and metabolism. Akt function covers several biological processes such as cell-cycle progression, growth and apoptosis through the regulation of key molecules, including mTOR, IKK, and BAD (Tao et al, 2007). The enhanced cell proliferation induced by Akt occurs in both anti-apoptotic and pro-cell division manner, as it also impedes the expression and function of cell cycle inhibitors such as, p21, p27, Chk1, and GSK3 (Chitnis et al, 2008). p70S6 kinase is another effector of Akt, which elevates the expression of cyclin D1 to initiate the cell cycle progression (Hashemolhosseinin et al, 1998).
1.2.2.2 Ras/MAPK pathway

The Ras/MAP cascade of kinases of the Ras/MAPK pathway are activated downstream of the IGF-IR signaling when IRS-1 associate with the GRB2/SOS complex. The Ras/MAPK pathway is also involved in a wide variety of biological processes, such as cell growth and differentiation. The downstream components of the Ras GTPase include RAF, MEK and extracellular signal-regulated kinase (ERK) kinase/MAPK proteins. The main role of Ras signaling in G1 to S phase of cell cycle progression often mediated by the IGF-I or other growth factor signaling is to inactivate RB, as indicated by studies where cells with RB knockout no longer require RAS activity (Mittnacht et al, 1997; Peeper et al, 1997). Ras increases cyclin D1 activity, through transcriptional elevation and protein stabilization and also participates in the assembly of cyclin D1 with CDK4 or 6 (Aktas et al, 1997; Lavoie et al, 1996; Winston et al, 1996). The indispensable role of cyclin D1 in the Ras-mediated cell proliferation is demonstrated by the resistance to H-Ras induced mammary tumor development in mice that lack cyclin D1 (Yu et al, 2001).

1.2.3 Involvement in breast cancer

The involvement of insulin-like growth factor-I receptor (IGF-IR) in breast cancer is very well established. Elevated expression of IGF-IR in breast carcinoma cells lines and the potent mitogenic effect of IGF-I (more than IGF-II) was initially noted by Cullen et al in 1990 (Cullen et al, 1990) and the area has received considerable attention with numerous subsequent studies in last two decades. The critical role of IGF-IR in breast cancer was consistently confirmed by knockdown studies such as with antibodies or siRNA, where inhibition of IGF-IR effectively abrogated cell proliferation in different breast cancer cell lines. IGF-IR was also required for anchorage-independent growth, and ectopic expression of dominant negative IGF-IR lacking the C-terminus showed that the C-terminal region was vital for transformation and tumorigenesis (Surmacz et al, 1998). IGF-IR has been also shown to be elevated in estrogen receptor (ER)-positive breast cancer cells and that its level is correlative with the ER and PR status in primary tumors (Peyrat et al, 1988).

The importance of IGF-IR in the breast cancer mortality was further heightened when Kim et al demonstrated that overexpression of IGF-IR in immortalized normal human mammary epithelial cells MCF10A was sufficient to cause epithelial-to-mesenchymal transition (EMT) (Kim et al, 2007). EMT of primary cancer cells is implicated in the tissue invasion and metastasis, which is the lethal factor in most cancers. Stable overexpression of constitutively active CD8-IGF-IR fusion protein in MCF10A not only led to full transformation, but also conferred migratory ability to the cells,
drastically downregulating epithelial adherens junctions and increasing the transcription of Snail (Kim et al., 2007). The study done by Kim et al., therefore, made a hallmark that IGF-IR is involved in virtually all stages of breast cancer progression.

In other recent studies, transgenic overexpression of IGF-IR resulted in perturbed mammary ductal morphogenesis such as marked hyperplasia and impaired ductal elongation, and accelerated tumor formation, with mice developing palpable tumors in approximately 8-11 weeks of age (Carboni et al., 2005; Jones et al., 2006). Interestingly, histological and cytokeratin immunohistochemical analyses indicated that some of IGF-IR-induced mouse mammary tumors exhibited features of ErbB2/Ras tumors, while more advanced tumors shared characteristics of Wnt-oncogene tumors (Jones et al., 2006). In general, ErbB2/Ras pathway leads to solid, nodular tumors with little stroma and metaplastic myoepithelial cells, while Wnt oncogene forms ductular tumor with abundant stroma and lymphocytic infiltration (Rosner et al., 2002). However, IGF-IR did not specifically fit under those categories, and this may support that IGF-IR can cross-talk with multiple cellular signaling cascades to cause rapid transformation and hyper-proliferation. As well as in breast cancer, IGF-IR is involved in multiple types of cancers (Tao et al., 2007) due to its broad spectrum of signaling pathway activation and gene regulation (Dupont et al., 2003). For example, Creighton and colleagues identified more than 800 genes whose expression was influenced by IGF-I (Creighton et al., 2008).

Importantly, IGF-IR has also been implicated in clinical resistance to trastuzumab (Herceptin) in breast cancer. Overexpression of IGF-IR has been shown to antagonize downstream effects of trastuzumab on G1 cyclins and Cdk2 inhibitor p27Kip1 (Lu et al., 2001) and unique heterodimerization and crosstalk of IGF-IR with HER-2 has been suggested to contribute to trastuzumab-resistance (Nahta et al., 2005; Knowlden et al., 2005). This called for a closer examination of IGF-IR signaling as a potential therapeutic target (reviewed in Tao et al., 2007 and Chitnis et al., 2008).

### 1.3 Pleckstrin-homology domain Interacting Protein (PHIP)

#### 1.3.1 Discovery and characterization

PHIP, or pleckstrin-homology domain interacting protein, was first cloned and characterized as a 902-amino acid (aa)-long variant 9 (Farhang-Fallah et al., 2000), which was involved in mitogenesis and insulin receptor-mediated GLUT4 translocation in myoblasts (Farhang-Fallah et al., 2002). It was
discovered as a novel strong binding partner of IRS-1 through yeast two hybrid screening of murine 10.5-day embryonic cDNA library using the PH domain from rat IRS-1 as bait, followed by screening of mouse thymus and human fetal brain cDNA libraries. The 902-aa protein, though not sharing significant sequence homology with any previously known proteins, was found to harbor two characteristic bromodomain (BD) structural motifs that were most similar (44% identity, 61% homology) to that of mouse CREB-binding protein (CBP) (Farhang-Fallah et al, 2000). This suggested that the BD motif in the PHIP family might confer them the function of transcriptional co-activators.

NCBI mouse database (NCBI Build 36; mm8) identifies at least four variants of PHIP, from alternative splicing and multiple translational initiation start sites (Figure 4) (Podcheko et al, 2007). My study focuses on the larger 1,821-aa, 206kDa isoform of PHIP (denoted as PHIP1), which contains two additional WD repeat domains to the smaller variant 9. The human version of PHIP1 shares 96% primary amino acid sequence identity with the mouse counterpart. Based on the fact that the smaller variant of PHIP was involved in the insulin signaling pathway, PHIP1 was identified and characterized in the context of pancreatic β-cell function, as it was the most prominently expressed isoform in the whole-cell lysates of the mouse islets and insuloma cell lines MIN6 and INS-1 cells through immunoblot analysis (Podcheko et al, 2007).

Immunohistochemical and immunoblot analyses in pancreatic β-cells showed that PHIP1 was confined to the nucleus. (Podcheko et al, 2007), though interestingly the 902-aa isoform of PHIP was shown to selectively interact with IRS1 and IRS2 PH domains as a downstream target (Farhang-Fallah et al, 2000). During the functional analyses, adenoviral PHIP1 overexpression stimulated insulin-like growth factor 1 (IGF-I) -dependent and -independent proliferation of INS-1 cells through PKB phosphorylation and transcriptional regulation of cyclin D2 expression, while PHIP1 knock-down inhibited IRS-2-dependent mitogenesis (Podcheko et al, 2007). The fact that PHIP1 alone could drive the cyclin D2 transcription was demonstrated by promoter-reporter gene analysis in INS-1 cells transiently transfected with firefly luciferase gene downstream of the promoter fragment of mouse cyclin D2. These results consolidated PHIP1 as a novel downstream effector of IGF-IR signaling pathway with crucial functions in the regulation of cellular growth and apoptosis.
1.3.2 PHIP1 structure and functional domains

As revealed by sequence analysis for conserved protein motifs by PFAM database, the 1821-aa PHIP1 was found to contain eight WD40 repeats (residues 171 to 211, 214 to 253, 256 to 299, 310 to 349, 354 to 393, 408 to 452, 455 to 495, and 498 to 542) on its N-terminus and two bromodomains (BD) (residues 1158 to 1261 and 1318 to 1423) on the C-terminus, representing a new member of the WD40-repeat (WDR) protein family. PHIP1 additionally contains two putative nuclear localization signals (NLSs), as identified by the PredictNLS program, and the human PHIP is 96% identical in sequence with the predicted mouse PHIP isoform (Podcheko et al, 2007). The full-length hPHIP1 was first cloned in our laboratory by reverse-transcriptase PCR from MCF-7 cells to produce 5.53 kb cDNA that correspond to the complete open reading frame (ORF) of the predicted genome sequence.

1.3.2.1 WD40-repeat domain

WD40-repeat domains consist of 40 to 60 amino acids, characteristically starting with glycine-histidine (GH) dipeptide 11 to 24 residues from the N-terminus and ending with a tryptophan-aspartic acid (WD) at the C-terminus. The WD40 domains are usually referred to as ‘WD-repeats’, as most proteins characterized by this motif contain a cluster of 4 or more tandem repeats. The motif was first identified in 1986 in the transducin $\beta$ subunit by Fong et al (Fong et al, 1986) and the protein family has been expanding with research and discovery. The WD-repeat proteins are diversified by sequence differences in the two variable regions within the WD-repeat as well as by

Figure 4. Different variants of PHIP.

The originally identified isoform was the 902-aa variant 9, and the one concerned in this project is the human PHIP1, which is the WD40-containing 206 kDa protein on the very top. Adapted from (Podcheko et al, 2007).
the number of structural repeats that are present. The common functionality of the family is to serve as a scaffold to coordinate multi-protein complex formations and facilitate protein-protein interaction (Li & Roberts, 2001; Neer et al, 1994). Examples of the WD-40 repeat proteins include β subunit of heterotrimeric G proteins, Apaf-1, most of α and β-COP proteins, chromatin assembly factor-1 (CAF-1), and the substrate specific adaptor in the CUL4-DDB1 E3 ubiquitin ligase complex (which is further discussed in the next section). The β subunit of the G protein has been the classically studied member of the protein group, and the only protein for which the crystal structure has been elucidated (Sondek et al, 1996). Given the conservation of the domain, WD-repeat proteins are thought to probably take a shape of a circularized β-propeller, with each repeat comprising a four-stranded antiparallel β sheet deduced from the β subunit of the G protein. As represented by the diverse members of the group, WD40-repeat containing proteins serve a wide range of biological regulatory functions, such as signal transduction, cell cycle regulation, RNA processing, chromatin and cytoskeleton assembly (Li & Roberts, 2001). However, there are many areas that need investigation to better understand this protein family. For example, there are a number of WD-repeat proteins, with PHIP1 also named as WDR11, that currently have no known function registered in the Human Gene Nomenclature Database.

1.3.2.2 Bromodomains

The bromodomain was initially discovered by Tamkun et al in Drosophila melanogaster Brahma protein, which is closely related to the SWI2/SNF2 family of transcriptional co-activators (Tamkun et al, 1992). Bromodomain was also identified in a number of eukaryotic transcriptional factors and co-activators, including GCN5 and p300/CREB histone acetyltransferases (HAT) (Haynes et al, 1992).

The fold of the bromodomain contains four α helices to form a four-helix bundle arranged in anti-parallel twist. The double bromodomain, such as in TAFII250 and in PHIP1, the two domains are held by the electrostatic interface by the interaction between the glutamate and lysine residues in the interface of the two domains, and the two loops on the opposite side to the N-terminal and C-terminal ends form a hydrophobic pocket. Interestingly, the presence of both conserved amino acids as well as the variability from residue insertions and deletions occur in the loop region, hinting that the region is likely involved in substrate specificity (Marmorstein and Berger, 2001).

The structural analysis of the human P300/CREB-associated factor (PCAF) by Dhalluin and co-workers (Dhalluin et al, 1999) was the first study that gave insight into the function of the bromodomain. Because GCN5/PCAF was a member of the histone acetylase (HAT) protein family, the authors tested whether the bromodomain targeted the acetyl-lysine residues on histone H4 in
NMR titration experiments and showed that the interaction was specific (Dhalluin et al, 1999). Further studies by Jacobson et al, which analyzed functional and structural characteristics of TAFII250, indicated that fully acetylated histone H4 peptide interacted with the double bromodomain with a stoichiometric ratio of 1:1, as determined by isothermal titration calorimetry, while no binding was detected with the non-acetylated peptide (Jacobson et al, 2000). As bromodomains can bind to acetylated histone lysine residues, they may promote interaction of the HATs with chromatin to begin transcriptional activation (reviewed in Marmorstein and Berger, 2001; Loyola and Almouzni, 2004). In a model of bromodomain function, Marmorstein and Berger proposed that factor acetyltransferase (FAT)-mediated acetylation of a transcriptional activator and facilitation of its DNA binding recruits the SWI/SNF remodeling and subsequently the HAT domain complex through sequential interactions between the bromodomains. This, in turn, leads to the acetylation of histones within the promoter region and ultimately the recruitment of transcription-initiation complex, such as TATA box binding protein (TBP), TAF250 and RNA polymerase II, thus increasing the gene transcription and activation (Marmorstein and Berger, 2001). Therefore, the presence of these highly functional domains supports that PHIP is a novel transcriptional co-activator with important roles in many aspects of cell life-cycle.

1.3.3 PHIP1 as DCAF14

WDR proteins are becoming an emerging topic of interest within the scientific community. There are still a number of WDR proteins less well understood and some have been newly classified as DCAFs, or DDB1 and CUL4-associated factors, suggested to be putative substrate receptors for CUL4-DDB1 ubiquitin ligase complex (Lee and Zhou, 2007). PHIP1 has been recently discovered as a subunit of the CUL4-DDB1 E3 ubiquitin ligase, known in alias as DCAF14, which suggests of more diverse function for PHIP1 in the control of regulatory and signaling proteins.

1.3.3.1 Ubiquitin-Proteasome Pathway (UPP) and CUL4-DDB1 Ubiquitin Ligase

The ubiquitin-proteasome pathway (UPP) is a protein-degradative mechanism whereby 80-90% of the intracellular proteins are proteolyzed after being targeted for ubiquitination. Ubiquitin (Ub) is a 76-amino acid polypeptide, which post-translationally modifies the substrate by covalent transfer, which involves conjugation of the Ub to the ε-amino group of the lysine residue via the C-terminus to form the isopeptide bond. The conjugation takes place in three consecutive steps, involving three different enzymes, E1, E2 and E3. E1 is the ubiquitin-activating enzyme, which adds the Ub onto itself by a ATP-requiring process. E2, the ubiquitin-conjugating enzyme, transfers this ubiquitin to a
cysteine residue of the enzyme from E1. Ubiquitin ligase, E3, finally transfers the bound Ub molecule on E2 to the amino −NH₂ side chain of the lysine residue in the target protein, forming a peptide bond (Lafarga et al, 2002). These three steps are repeated numerous times and the polyubiquitin chain is recognized and degraded by the proteasome. The specificity of protein turnover is provided in part by the E3 enzymes. This is exemplified by the fact that there is only one E1, several E2s, but hundreds of E3 enzymes (O’Connell and Harper, 2005). Non-degradative function of the UPP include monoubiquitination, which plays a role in protein-protein interaction, protein trafficking and localization, facilitation of endocytic internalization, regulation of plasma membrane protein activity, and in histone coding, such as in the methylation of H3 and gene silencing in yeast (Wang et al, 2006).

The cullin-RING finger ligases (CRL) are members of the RING-finger domain-containing class of ubiquitin ligases. The E3 ubiquitin ligase that utilizes the cullin subunit as a scaffold in a multiprotein complex was first reported in 1997 (Skowyra et al, 1997; Feldman et al, 1997). There are seven cullins in vertebrates (CUL1, 2, 3, 4A, 4B, 5, and 7), and while the different cullins form variations of E3 ubiquitin ligase (Petroski and Deshaies, 2005; O’Connell and Harper, 2007; Figure 5), the insight into the spatial configuration of the largest family of E3 ubiquitin ligases was derived from crystal structure of the archetypical Cul1-Rbx1-Skp1-F boxSkp2 SCF ubiquitin ligase complex by Zheng and co-workers (2002). In this CRL, Skp1 acts as the adaptor and F-box proteins as the substrate receptors. Cullins have an extended and rigid structure that contains a long stalk and a globular domain. The N-terminal region of cullin binds with the substrate receptors through specific adaptor molecules and the C-terminal, globular domain interacts with the RING finger protein Rbx1/ROC1/Hrt1, which forms a two-subunit catalytic core and provides the site of E2 ubiquitin-conjugating enzyme recruitment (Zheng et al, 2002).

There is evidence that PHIP1 is part of the CUL4 E3 ligase complex, which is composed of DDB1 (damaged DNA binding protein), as the adaptor and DCAF as the substrate receptor (Figure 5). DDB1 contains 21 WD40-like repeats which folds into three β-propeller domains, where the double-propeller pocket participates in the substrate presentation and the third domain binds to the cullin (Li et al, 2006). In 2006, Angers and co-workers reported on structural and proteomic analyses of DDB1-CUL4A-ROC1 complex bound to the simian virus 5 V protein (SV5V), which ubiquitinates and degrades STAT1 to inhibit the host antiviral response, in order to elucidate the molecular architecture of the enzyme complex as well as to identify its protein interaction networks. Interestingly, PHIP1 was identified as one of the substrate receptors of CUL4-DDB1 complex during the tandem-affinity analyses. As CUL4-DDB1 E3 ligases have been reported to be involved in
histone ubiquitination, methylation and responses to DNA damage (Wang et al, 2006; Higa et al, 2006), this enthralling new discovery potentiates the role of PHIP in cell survival and function.

**Figure 5. E3 ubiquitin ligases of different Cullin proteins.**

Substrates become poly-ubiquitinated by the E3 ubiquitin ligases and targeted for protein degradation by 26S proteasome. The E3 ubiquitin ligases have conserved molecular assembly composed of the core Cullin-Rbx1 and a determinant of specificity (Skp1-F-box, BTB, EloBC/BC/SOCS-box, DCAF or FBW8 depending on the Cullin type). Adapted from (O’Connell and Harper, 2007).

### 1.3.3.2 Involvement of Cul4 in breast cancer

In support of role of cullin-RING ubiquitin ligase in breast cancer, cul-4, the human homologue for the *Caenorhabditis elegans* cul-4 gene, was found to be amplified and overexpressed in primary breast cancer by Chen and colleagues in 1998. In the study, cul-4 was amplified in 16% of untreated primary breast cancers and overexpressed in 47% of the cases, as determined by RNA *in situ* hybridization. Likewise, in 14 breast cancer cells analyzed, the authors showed that cul-4 gene was both amplified and overexpressed in only 3 cell lines, but overexpressed without amplification in the majority, which indicated that cul-4 could accumulate via other mechanisms and the upregulation
may play a role in tumorigenesis (Chen et al, 1998). In a recent study by Schindl et al, overexpression of cul-4 gene has been related to poor outcome in lymph node-negative invasive breast cancer through immunohistochemistry analyses of 167 human breast cancer specimens, where moderate to high levels of cul-4 were observed in over 50% of the cases. The data provided further evidence that CUL-4 may participate in tumor development and progression (Schindl et al, 2007).

1.4 Hypothesis and Rationale

The study of PHIP1 in islet β-cell function and survival demonstrated that PHIP1 is a positive regulator of cellular growth and that it potentiates the mitogenic effect of IGF-I. Additionally, adenoviral overexpression of PHIP1 in NIH3T3 cells promoted proliferation, indicating a growth-promoting ability of PHIP1. Consistent with this finding, preliminary analysis suggested that PHIP1 protein levels were markedly elevated in a number of human breast cancer cell lines compared to normal human mammary epithelial cell line (HMEC). Additionally, one of the MMTV-PHIP1 transgenic mouse lines established in the laboratory developed mammary hyperplasia within 8 months of age upon multiple pregnancy. Given this supportive preliminary information and the well-established implication of IGF-IR in breast cancer, we hypothesized that PHIP1 will play an important role in mammary gland tumorigenesis and oncogenic transformation. We further aimed to study PHIP1 action in molecular signaling that drives breast cancer tumorigenesis.
Chapter 2. Materials and Methods

2.1 Reagents and antibodies

Polyclonal rabbit antibody against the bromodomain of human PHIP was generated in our laboratory using glutathione S-transferase (GST)-PHIP fusion protein corresponding to residues 1548-1821 of mouse PHIP1 injected into rabbits and purifying the rabbit sera through a GST affinity column, as previously described (Podcheko et al., 2007). Mouse monoclonal antibody against the hemmaglutinin (HA) epitope (Cat. # sc-7392) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The rabbit polyclonal antibody against tubulin (Cat. # 2148) was purchased from Cell Signaling Technology (Danvers, MA). The mouse monoclonal antibody specific for Cyclin D2 (Cat. # MS-213-PO) was purchased from Thermo Fisher Scientific (Rockford, IL) and mouse antibody against β-actin (Cat.# A3853) from Sigma Aldrich (St. Louis, MO). The secondary antibodies, both the goat anti-mouse HRP-conjugate (Cat. # 170-5047) and goat anti-rabbit HRP-conjugate (Cat. # 170-5046) were purchased from Biorad Life Science (Hercules, CA). The rabbit ExactaCruz was purchased from Santa Cruz (Cat. # sc-45043). The GIPZ and TRIPZ shRNA lentiviral constructs expressing a scrambled control or shRNA against human PHIP1 (V2THS_174590, V2THS_174591, V2THS_174592) were obtained from Open Biosystems, Thermo Fisher Scientific (Huntsville, AL). The HA-PHIP1-MSCV retroviral vector was previously generated in our laboratory by a post-doctoral fellow, where the full-length human PHIP1 cDNA with a triple HA tag on the N-terminus was cloned into the Murine Stem Cell Virus (MSCV) backbone from Clontech. For transfection reagents, PEI was purchased from Sigma (Cat. # 408727) and Lipofectamine 2000 was purchased from Invitrogen (Cat. # 11668-027). MG132 proteasomal inhibitor was purchased from Calbiochem (Cat. # 474790) as part of the Proteasome Inhibitor Set 1 (Calbiochem, Cat. # 539164).

2.2 Cell culture

MCF10A cells stably overexpressing constitutively active CD8-IGF-IR (‘MCF10A-IGFR’) or IRS-1 or IRS-2 (MCF10A-IRS1 or 2) were kindly provided by Dr. Adrian V. Lee at Baylor College of Medicine. Parental MCF10A or MCF10A-derived stables were all maintained in DMEM-F12 (Invitrogen, Cat. # 11330-032) containing 5% horse serum (Gibco, Cat. # 16050-122), 1% penicillin/streptomycin (BioWhittaker, Cat. # 17-602E), 20 ng/mL EGF (Peprotech, Cat. # AF-100-15), 10 μg/mL insulin (Sigma, I-1882), 0.5 μg/mL hydrocortisone (Sigma, Cat. # H-0888) and 100 ng/mL cholera toxin (Sigma, Cat. # C-8052). For MCF10A-IGFR cell line, the medium additionally
contained 5 μg/mL puromycin (Sigma, Cat. # P-8833) to maintain the selective pressure for the stable expression of the CD8-IGF-IR transgene. For subculturing MCF10A, cells were washed once with 1x PBS and trypsinized with 1 mL of 0.05% trypsin in 10 cm dish and incubated for 20 minutes in 37 °C. MCF10A, MCF-7, and NIH/3T3 were obtained from American Type Culture Collection (ATCC) and cells within passage number 10 were used in experiments. 293T, MDA-MB-231 and NIH/3T3 cells were cultured in DMEM (Wisent, Cat. # 319-005-CL) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Wisent, Cat. # 090-150) and 1% penicillin/streptomycin. MCF7 cells were grown in DMEM/F12, 10% FBS and 1% antibiotics.

For cell cryopreservation, freezing media containing 90% FBS and 10% DMSO (Sigma, Cat. # D-2650) was generally used, except for MCF10A, which was prepared by 70% MCF10A growth medium, 20% horse serum, and 10% DMSO. Approximately 2 million to 5 million cells in 1 mL of freezing media were stored in cryotubes.

### 2.3 Virus production and transduction

The 293T cell line was used for both lentivirus and retrovirus production. The cells were plated at 70% confluency the day before transfection on 100 mm culture dishes. For lentivirus production, 9 μg of GIPZ or TRIPZ plasmid (Open Biosystems), 9 μg psPAX.2 (packaging) and 4.5 μg of MD2.G (envelope) plasmids were added to 5 mLs of serum-free, antibiotic-free DMEM (Wisent) and incubated at room temperature (RT) for 5 minutes, after which 20 μL of 2 mg/mL polyethylenimine (PEI; Sigma) was added and allowed to complex with the plasmids for further 20 minutes. For doxycycline-induced protein knockdown in MCF10A-IGFR or MDA-MB-231 TRIPZ stables, lentivirally infected and drug selected cells were incubated in 10% FBS DMEM containing 1 μg/mL doxycycline (Sigma, Cat. # D-9891). The medium was refreshed every 24 hours for 3 days before being lysed for Western blot analyses.

Similarly, for the retrovirus production, 10 μg MSCV and 10 μg of pCl-Ampho (RetroMax) plasmids were transfected into 293T cells. 6 hours after transfection, 5 mLs of DMEM containing 20% FBS (no antibiotics) were added per culture dish and incubated at 37 °C and 5% CO₂ for 48 hours. The spent medium containing lenti- or retrovirus was filtered through 0.45 μm pore (made of mixed cellulose esters; Millipore, Cat. # SLHA033SS), aliquoted, and stored in -80°C. Before being used, the virus was rapidly thawed in a 37°C waterbath.

For transduction, the cells to be infected were seeded the day before at 40-60% confluency in 60 mm dishes before for lentiviral infection or at 100,000 cells per well on 6-well plates for retroviral infection. The transduction mixture comprised a 1:1 ratio of the viral supernatant and the growth
medium, and 4-8 μg/mL polybrene (= hexadimethrine bromide; Sigma, Cat. # H-9268). The final volumes of the mixture were 3 mLs for 60 mm dishes and 1 mL for 6-well plates. For retroviral infection, cell 6-well plates were spun at 1,800 rpm for 45 minutes at RT. The media was changed the next morning to fresh growth medium and drug selection was started 48 hours after. The concentrations of antibiotics used were 2 μg/mL for puromycin (Sigma, Cat. # P-8833) and 800 μg/mL for G418 (Bioshop, Cat. # GEN418.5). For puromycin, the period of selection was 5-7 days, while for G418, it was minimum 10 days, or until all of the non-infected control cells died.

2.4 siRNA transfection for transient knockdown experiments

For MDA-MB-231, 100,000 to 200,000 cells were plated per well on a 6-well plate 24 hours before the transfection. SMARTpool PHIP-siRNA (Dharmacon, Cat. # M-019291-00) and Scrambled-siRNA (Dharmacon, Cat. # D-001210-02) were resuspended in the siRNA buffer (Dharmacon Cat. # B-002000-UB-015) at the final concentration of 20 μM. For each well, the cells were washed twice with serum-free media (SFM) before transfection. 100 nM of siRNA and 0.8% of Lipofectamine 2000 (Invitrogen, Cat. # 11668-027) were added to 1 mL SFM and incubated at RT for 20 minutes to allow complex formation before being added to the cells. The transfection medium was aspirated 6 hours later and changed to growth medium. The cells were analyzed after being incubated for minimum 48 hours. For transfection of larger number of cells, the volumes were adjusted by appropriate proportions.

For transfection of MCF10A-IGFR cells for the proliferation assay, 50,000 cells were seeded per well on 12-well plates the day before the transfection. 100 nM of siRNA and 0.4% Lipofectamine2000 were mixed in serum-free DMEM/F12 for 25 minutes and added onto the cells. During the 25 minutes, the attached cells were washed twice with plain DMEM/F12 with no other supplements. The transfection mixture was incubated with cells overnight (16 hours) and aspirated and replaced with MCF10A starvation medium containing 0.5% horse serum, 1% penicillin/streptomycin, 20 ng/mL EGF, 10 μg/mL insulin, 0.5 μg/mL hydrocortisone and 100 ng/mL cholera toxin the next morning. The day of the transfection was designated as Day 0 and the serum-reduced MCF10A media was replaced every 2 days until Day 8. The cells were counted on Days 2, 4, 6 and 8 with Beckman Coulter as explained further in Section 2.5.

For MCF-7 cells, reverse transfection method was used to transiently knockdown PHIP. For transfections in 6-well plates, 200 nM of siRNA was mixed with 0.5% Lipofectamin 2000 by first separately incubating each of them in 250 μL of serum-free DMEM/F12 and further incubating for 20 minutes at RT after combining the two components to make a final volume of 0.5 mL (per well;
final siRNA concentration 100 nM). This was mixed with 1 mL of freshly trypsinized and resuspended MCF7 cells at concentration of 200,000 cells/mL in the growth medium (10% FBS in DMEM/F12 but without antibiotics) and incubated overnight at 37 °C 5% CO₂. The transfection media was aspirated and changed to growth medium the next day. For the treatment with IGF-I (Cell Signaling, Cat. # 8917), 10,000 cells were reverse transfected in growth medium overnight in 96-well plate and washed once with plain DMEM/F12 the next morning. The cells were starved in SFM for 24 hours, after which cells were treated with 100 ng/mL IGF-I in DMEM/F12 for 48 hours and measured for growth using the MTT assay as mentioned in the next Section.

2.5 Cell proliferation assays

For cell counting on the hemocytometer, cells were trypsinized with 0.05% trypsin (with EDTA) and blocked with 20% FBS. The volumes were adjusted to maximize the cell concentration per mL. 10 μL of cell suspension was mixed with 10 μL of trypan blue and loaded onto the hemocytometer. The four 16-squares at each corner were counted and averaged. The total cell number was calculated by: [averaged cell number x 2 x 10⁴ x Volume of cell resuspension]. When counting cells on the Beckman Coulter Counter Z2 (Beckman), the cells were prepared similarly and the lower threshold used was 6.0 μm. Usually, 50 – 100 μL of cell suspension was diluted in 10 mL of isotone solution before subjecting the measurement. The proliferation assay using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) reagent (Sigma, Cat. # M-2128) was performed by adding 20 μL of 5 mg/mL filter-sterilized MTT dissolved in PBS per 100 μL cell culture media (overall MTT concentration 1 mg/mL) on a 96-well plate and incubating at 37 °C 5% CO₂ for 3 hours and 30 minutes. The media was carefully removed and the purple formazan precipitates were dissolved in 150 μL MTT solvent (4 mM HCl, 0.1% NP40 in isopropanol) per well. The plate was then covered with aluminium foil and agitated on an orbital shaker for 15 minutes, after which the absorbance was read at 595 nm on the spectrophotometer.

2.6 Soft agar anchorage-independent colony formation assay

For the soft agar assay, which looks at anchorage-independent colony formation, 10,000 or 20,000 MCF10A cells infected with AdPHIP virus the day before were suspended in MCF10A growth media containing 0.4% agarose, low-melting point (Sigma, Cat. # A-9414) plated on top of a basal layer with 1% agarose in complete MCF10A media. For the specific procedure, 3% agarose in PBS was autoclaved and re-dissolved in a microwave prior to use. For the basal layer, the agarose was
mixed 1:3 with MCF10A complete medium and 2 mL of 1% agarose was put per well of a 6-well plate. For the top layer, one set of triplicates was prepared by mixing 1.08 mL of 3% agarose, 2.92 mL MCF10A growth media and 4 mL of cell suspension (40,000 or 80,000 cells total) and then plated on top of the basal agar at 2 mL per well. The wells were maintained by adding 0.3-0.5 mL of media every 3-4 days. Colonies were stained with MTT (2 mg/ml in PBS) by incubation for 4 h at 37 °C. Cells were photographed with Nikon ECLIPSE TE2000-U microscope and SimplePCI6 software. Regarding the number of MOI used during the adenovirus infection, viral titre was measured by immunohistochemistry for AdGFP using AdEasy Viral Titre Kit (Agilent, Cat. # 972500), which detects only live viral particles, but for AdPHIP, values of titre derived from measuring the absorbance was used, which does not differentiate between live and dead particles (where OD_{260nm} = 1 x 10^{12} viral particle/mL). The actual degree of GFP fluorescence was used to determine the appropriate volume of virus used in the experiment, which in the study was MOI of 10 for AdGFP and MOI of 650 for AdPHIP.

For the soft agar assay for the MCF10A-PHIP retrovirally infected stable cell line, very similar procedure was used with minor modification. For the bottom layer, SeaPlaque low-melting agarose (Lonza, Cat. # 50101) was dissolved in 10 mL 1x PBS to concentrations of 2.1% by heating in the microwave for approximately 2 minutes. The agarose solutions were quickly mixed with pre-warmed MCF10A growth medium (in 40 °C waterbath) in 1:2 and plated on to 6-well plates in minimum of triplicates with 1.5 mL mixture per well. The gel was allowed to solidify in the room temperature for 30 minutes, during which time the top layer was prepared. Similar to the bottom layer, 1.7% of agarose was heated in 1x PBS and cooled to 40 °C before being mixed with MCF10A medium containing 20,000 cells (for triplicates, or (n+1) x 5,000 for n replicates) and plated on top of the bottom layer in volumes of 1.5 mL per well. 0.5 mL of MCF10A growth medium was added drop-wise every 3 to 4 days for 14 days. The colonies were stained with MTT as described above.

2.7 SDS-PAGE Electrophoresis and Western Blotting

Cells were lysed in hot 6x SDS reducing buffer (150 mM Tris-HCl pH 6.8, 30% glycerol, 10% SDS, 3% β-mercaptoethanol, and 0.002% bromophenol blue) and scraped off the culture dishes or plates with a scraper. The lysates were boiled for minimum five minutes at 100 °C and measured for total protein concentration by the Bradford assay (Sigma, Cat. # B-6916). 30 to 50 μg of cell lysates were loaded and run on 10 to 12% polyacrylamide gels in 1x SDS running buffer (25 mM Tris-HCl, 190 mM glycine, 0.1% SDS pH 8.6) at 150V for 60 minutes to separate the proteins by size alongside a pre-stained protein marker (Fermentas, Cat. # SM0671). After the size fractionation by the SDS-PAGE electrophoresis, the proteins were transferred on to polyvinylidene fluoride (PVDF)
membranes (Millipore, Cat. # IPVH00010) at 110V for 90 minutes in transfer buffer (25mM Tris-HCl, 192mM glycine, 20% (v/v) methanol, pH 8.3).

Membranes were then blocked to reduce non-specific signals for 30 to 60 minutes on a rocker with 5% skim milk powder dissolved in 1x TBST (50mM Tris-Cl pH 7.5, 150 mM NaCl, 0.05% Tween-20). After several rinses with 1x TBST, the membranes were cut at appropriate marker lanes and incubated with primary antibodies at 4°C for 20 hours (overnight). The primary antibodies were diluted in 5% bovine serum albumin (BSA; Bioshop, Cat. # ALB001.500) in 1x TBST containing preservative 0.5% NaN₃.

The membranes were washed three times with 1x TBST for 10 minutes per wash and incubated with anti-mouse or anti-rabbit HRP-linked secondary antibody for 1 hour at RT on a rocker. The secondary antibodies were diluted in 5% skim milk in 1x TBST. For anti-rabbit HRP secondary antibody (Biorad, Cat. # 170-5046), dilution of 1:10,000 was used in 10 mL of 5% skim milk in 1x TBST and anti-mouse HRP-conjugate (Biorad, Cat. # 170-5047) was diluted to 1:2,000 in 5% skim milk in 1x TBST for all the experiments. Following three more washes for 10 minutes each with 1x TBST, the membranes were subjected to ECL solution (Thermo Scientific, Cat. # PI34080/DDEL) for 60 seconds before being developed on autoradiography films (Denville Scientific, Cat. # E3018).

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Table 1. Table of antibodies used in the study with their dilutions

2.8 Immunoprecipitations

HA-PHIP1 immunoprecipitations were performed on 293T cells transfected with MSCV-HA-PHIP or MIEV (MSCV-IRES-EGFP vector) control plasmid on 10 cm culture dishes at least 48 hours prior
to the experiment. The HA-PHIP protein was first enriched through nuclear fractionation, thereby reducing the cytoplasmic content and decreasing the potential non-specific signals. The cells were washed twice with ice cold 1x PBS and the buffer was aspirated as much as possible before adding 500 µL of Buffer A (10 mM HEPES pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 0.5 M DTT) containing protease inhibitors (1x Protease Inhibitor Cocktail with EDTA from Bioshop Cat. # PIC001.1, 1 mM NaF, 1 mM Na₃VO₄ and 2 mM PMSF) per 10 cm dish and scraping the cells off the plate on a pan of ice. The cell lysates were incubated on ice for 15 minutes and added 10% stock solution of NP-40 to a final concentration of 0.5%, after which they were vortexed for 10 second each. The nuclei were pelleted by centrifugation at 6500 rpm for 20 seconds on a microcentrifuge. The supernatant, which contains the cytoplasmic proteins, were usually transferred to fresh eppendorf tubes in case of a future analysis.

The nuclear pellet was lysed in 1x RIPA buffer (50 mM Tris-HCl pH 7.4, 1% NP-40 (v/v), 0.25% Na-deoxycholate (w/v), 150 mM NaCl, 2 mM EDTA and 0.1% SDS) containing protease inhibitors (1x Protease inhibitor cocktail, 1 mM NaF, 1mM Na₃VO₄ and 2mM PMSF) added immediately before use. To fully release the nucleic content, the nuclear lysates were sonicated twice for 10 seconds at Power Level 3 and centrifuged at 10,000 rpm for 10 minutes at 4 °C. The supernatant was transferred to fresh eppendorf tubes while the cellular debris was discarded. The nuclear protein lysate in the 1x RIPA buffer was subjected to preclearing with 50 µL of protein-A 50% slurry (Millipore, Cat. # 16-156) per 1 mL for 30 minutes at 4 °C with gentle rocking. Meanwhile, the IP-antibody-IP-matrix complex was prepared by adding 50 µL of suspended (25% v/v) IP matrix and 2 µg of IP antibody (10 µL of p53, Santa Cruz Cat. # sc-6243) into 500 µL of 1xPBS and incubating at 4 °C on a rotator for 1.5 hours. The IP-antibody-IP-matrix complex was subsequently washed twice with 1x PBS, and centrifuged briefly at 13,000 rpm for 1 min at 4 °C. The precleared lysates were then immunoprecipitated with the complexed HA-antibody and protein A-matrix for 2 hours at 4 °C on a rotator in a total volume of 1 mL of 1x RIPA. After the incubation, the eppendorf tubes were microcentrifuged at 13,000 rpm for 30 seconds to pellet the IP matrix and washed with 1x RIPA (with protease inhibitors) for 3 or 4 times. To release the immunoprecipitated protein 35 µL of 6xSDS lysis buffer was added to the pellet and the samples were boiled at 100 °C for 5 minutes, vortexed briefly, and centrifuged at 13,000 rpm for 5 mins and loaded on the SDS-acrylamide gels for Western blot analysis.
2.9 Propidium iodide (PI) staining and FACS analysis

For the FACS analysis, cells to be analyzed were fixed in 70% ethanol and stained with propidium iodide (PI). For analyzing the effect of PHIP1 knockdown on cell-cycle progression, 200,000 MDA-MB-231 cells were seeded per well onto 6-well plates in 10% FBS DMEM in six replicates and transfected with 100 nM siRNA (siScrambled or siPHIP) the next day as per described in Section 2.4. The next day, the medium was changed to either 0.5% FBS or 5% FBS and incubated in 37 °C and 5% CO₂ for 48 hours. The cells were prepared for FACS by washing once with sterile 1x PBS and trypsinizing with 0.05% trypsin for 5 minutes. The cells were centrifuged for 5 minutes at 1,500 rpm in 10% FBS DMEM growth medium and the supernatant was carefully aspirated. The cell pellet was thoroughly resuspended with 0.5 mL PBS and 10 mLs of 70% ethanol was added and fixed overnight at -20°C, or until staining. PI staining solution consisted of 1 mg/mL PI (Molecular Probes, Cat. # V-13242), 0.2% TritonX-100 and 0.2 mg/mL of RNAseA (DNAse-free; Sigma, Cat. # R-6513) all in 1x PBS. For staining, fixed cells were centrifuged at 3,000 rpm for 3 minutes, after which the 70% ethanol was removed as much as possible. The cell pellet was washed once with 1x PBS by suspending and centrifugating briefly at 5 minutes, 1,500 rpm. 1 mL of the PI solution was added to each 15 mL falcon tube and the thoroughly resuspended cell pellet was incubated at room temperature for 30 minutes in the dark. For the FACS analysis, the cells in the PI staining solution were transferred to glass tubes and the data were acquired on the FACS (Yoda) with settings optimized manually. 16,000 cells were counted per flow measurement. The raw data analyses were performed using the WinMDI 2.9 software.

2.10 RNA Isolation and real-time quantitative PCR (RT-qPCR)

Total RNA was isolated from cells using the Qiagen RNeasy Mini Kit (Qiagen, Cat. # 74104) according to the manufacturer’s instructions. Briefly, 200,000 to 500,000 cells seeded per well on 6-well plates were lysed in 600 µL RLT buffer and centrifuged in QIAshredder spin column for 2 min at 13000 rpm 4 °C. Then same volume of 70% EtOH (made with RNase-free water) was added to the flow through and mixed 5-10 times by pipeting. The mixture was re-centrifuged in fresh RNeasy spin columns at 13000 rpm for 15s at 4 °C twice in 600 µL and the flow-through was discarded. Then 700µl of Buffer RW1 was added and centrifuge for 15s at 13000 rpm at 4 °C, where the flow-through was again discarded. Afterwards, 500µl Buffer RPE was added to the RNeasy spin column and spun at 13000 rpm for 15s at 4 °C. The flow-through was discarded and the last step was repeated once more. For eluting the RNA, the RNeasy spin column was placed in a new 1.5mL
eppendorf tube and 30 µl RNAse-free water was added directly to the spin-column membrane and centrifuged at 13000 rpm, 1 min, 4 °C. The flow through was measured for RNA concentration on a spectrophotometer. Following extraction, total RNA was treated with rDNase I (Ambion, Cat. # AM1906) to remove any DNA contamination. For reverse transcription, High Capacity cDNA Reverse Transcriptase Kit was used (Applied Biosystems Cat. # 4368813). Per reaction, 2 µL of 10x RT Buffer, 0.8 µL of 25x dNTP mix (100 mM), 2 µL 10x RT Random Primers, 1 µL Multiscribe reverse transcriptase, 4.2 µL RNAse-free H2O and 10 µL of RNA (1 µg total) were mixed on ice and put into a thermocycler at 25 °C for 10 min, 37 °C for 120 min, 85 °C for 5s and 4 °C for indefinite. The resulting cDNA (10 ng per reaction or 2.5ng/µl) was used for amplification in quantitative real-time PCR (qPCR). cDNA (4 µL/well) was added to a qPCR mixture (6 µL/well) containing 3.475 µL RNAse-free H2O, 1 µL of 10x PCR buffer, 0.6µL of 50 mM MgCl2, 0.2 µL of 50 µM Primer Mix, 0.2 µL of 10 mM dNTP mixture, 0.2 µL ROX reference dye, 0.3 µL SYBR Green I (stock diluted 1:1000 in RNAse-free water) and 0.025 µl (or 125 U) Platinum Taq Polymerase (Invitrogen, Cat. # 10966-083)). For PCR amplification, the qPCR was set to 95 °C for 3min, 40 cycles of PCR, 95 °C for 10 s, 65 °C for 15 s, 72 °C for 20 s, 95 °C for 15 s, 60 °C for 15 s, and 95 °C for 15 s and performed in the ABI Prism 7900 HT Sequence Detection System (Applied Biosystems).

<table>
<thead>
<tr>
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Table 2. Sequence of qPCR primers used in the study
Gene specific oligonucleotide primers were designed using the Primer Quest SciTool from Integrated DNA Technologies (IDT). The expression level of the various transcripts was calculated using the comparative \( C_T \) method as described in the ABI Manual in detail. The values were normalized to that of the TATA-binding protein (TBP), which is a ubiquitous transcription factor, whose gene is frequently used for comparative qPCR analyses.
Chapter 3. Results

3.1 PHIP1 is overexpressed in primary breast tumor samples of MMTV transgenic mouse models and in MCF10A stable cell lines overexpressing members of the IGF-IR pathway.

One of the common mechanisms of a proto-oncogene-mediated oncogenic transformation is overexpression of the protein product, and the differential expression profile of PHIP observed in early screening of human breast cancer cell lines led us to question its potential role in the mammary gland tumorigenesis. The initial Western blot analysis of human breast cancer cell lines indicated that PHIP1 was notably upregulated in cancerous cell lines compared to human mammary epithelial cell line (HMEC) (Janet Farhang-Fallah, unpublished data), which are primary normal cells usually harvested from tissues removed during breast reduction surgeries (Stampfer et al., 1993). As PHIP1 was previously found to be a downstream nuclear co-activator of the IGF-IR signaling (Podcheko et al., 2007), this prompted us to look at whether PHIP1 could be also differentially expressed in the MCF10A stable cell lines overexpressing the constitutively active, truncated IGF-IR (CD8-IGF-IR) as well as those with IRS-1 or -2 overexpression. Whole cell lysates subjected to the immunoblot analyses indicated that PHIP1 was indeed distinctively present in the MCF10A cell lines with ample IGF-IR signaling molecules.

To extend and confirm the finding that PHIP1 was more abundantly present in tumor-derived cells compared to that of the normal, we performed Western blot analysis on the crude lysates of the breast tumors derived from transgenic mice of FVB strain overexpressing classical breast oncogenes Wnt, Neu, and IGF-IR (Figure 6). The Western blot revealed more robust level of PHIP1 in breast tumor samples of the oncogenic MMTV mice compared to that of the FVB wild-type female mouse. The results suggested that PHIP protein levels could be elevated in different in vivo breast cancer models.

3.2 MCF10A-PHIP1 stable cell line spontaneously silences ectopic PHIP1 expression at the protein level.

In order to study the oncogenic property of human PHIP1, we attempted to generate PHIP1-overexpressing MCF10A stable cell line, which would enable us to do long-term experiments, such as in vivo orthotopic injections, soft agar anchorage-independence colony formation assay and 3D
Figure 6. PHIP is manifested in the primary breast tumor samples of MMTV transgenic mouse models of classical oncogenes, and in MCF10A stable cell lines with constitutively active IGF-IR pathway.

(A). PHIP expression profile in mouse mammary gland tissue samples of different breast tumor models. MMTV-Neu (N) represents a mouse model with overexpressed growth receptor ErbB2 and MMTV-Wnt (W) represents overexpression of the differentiation marker Wnt, which often results in EMT and metastasis. N85 and N135 refer to two independent mice of MMTV-Neu strain. Likewise, W204, W225 and W248 are three independent MMTV-Wnt mice. MMTV-IGFR (RJ) similarly overexpress human IGF-IR in the mammary gland. N and W breast tumor samples were provided by Dr. Liu from Dr. Zacksenhaus’ lab in University of Toronto, while RJ samples were obtained from Dr. Moorehead at the University of Guelph. The Western blot was performed only once. (B). Densitometry analysis of the Western blot for PHIP expression in mouse breast tumors normalized against the values from actin. The error bars indicate standard deviation of 6 scannings of the one Western blot from (A). (C). Expression of PHIP in MCF10A stable cell lines overexpressing components of the IGF-IR pathway. MCF10A-IRS1 (lane 2) and MCF10A-IRS2 (lane 3) cells are MCF10A cells that were infected with retrovirus coding for
wild-type human IRS-1 and IRS-2 genes containing HA tags (as shown in the blot). The Western blot was performed only once.

growth in Matrigel™. The technical problem with the production of viable retrovirus was troubleshooting at multiple levels. Firstly, three retroviral packaging cell lines, Phoenix, 293T and PT-67, were tested for transfection efficiency and retroviral titre, with a GFP-positive retroviral vector (MIEV, or MSCV-IRES-EGFP-vector) for better visibility of the procedures. As recommended by the Nolan laboratory at Stanford University, the NIH/3T3 cell line was used to test the viral titre as it is easily infected with retrovirus compared to other mammalian cell lines. PT-67, a NIH/3T3-based packaging cell line, was relatively difficult to transfect with Lipofectamine2000, and required a long-term selection with a high concentration of G418 (at 500 μg/mL). Generally, stable PT-67 transfectants as separate colonies are screened and tested for viral titre. Also, upon testing for the presence of functional virus by using the supernatant from PT-67 cells transfected with MIEV to infect NIH/3T3, there were very few GFP positive cells and the titre proved to be too low, requiring the laborious selection of high-titre PT-67 clones. Thus the utilization of PT-67 was discontinued. Phoenix cells, being 293T-based, had the advantage of easy transfectivity. However, transfecting MSCV alone did not produce functioning retrovirus. It was later realized that the Phoenix cell line had lost its stable expression of Gag-Env-Pol, as it required pCl-Eco, a helper plasmid that contained the retrovirus structural genes, in order to generate retrovirus capable of infecting NIH/3T3 cells. Unfortunately, pCl-Eco was ecotropic and could not produce retrovirus compatible with human cells. Therefore, pCl-Ampho was obtained from University of Montreal as a generous gift and amphotrophic retrovirus was generated by co-transfecting pCl-Ampho and pMSCV-HA-PHIP or pMIEV into 293T cells and collecting the culture media 48 hours post-transfection. Transduction protocol was also optimized by performing spin-infection, minimizing the volume of infection mixture to reduce viral Brownian motion, testing different virus versus medium ratios, and adding appropriate amount of polybrene to neutralize surface charge on the cell membrane and enhance viral adsorption (Davis et al, 2002). MIEV was used in every transfection and infection as a visual marker and a positive control for the presence of active retrovirus. Upon optimizing the retrovirus production and transduction protocols, I proceeded to generate both NIH/3T3 and MCF10A stable cell lines by spin-infecting cells on six-well plates. Cells were infected every second day three times, and drug selection was started 48-72 hours after the last infection.

Excitingly, Western blot of cells shortly after the antibiotic selection with G418 showed robust expression of HA in MCF10A (Figure 7). This, however, was unexpectedly diminished with sub-culturing (Figure 8), rendering the cell line unusable for long-term analysis, such as orthotopical
Figure 7. Retroviral infection of MCF10A leads to robust expression of HA.

(A). MSCV-HA-PHIP retroviral vector expression in 293T cells. 293T cells were transfected with MSCV-HA-PHIP (lane 1, MSCV-PHIP), MSCV-empty or MIEV-control vector as controls. Cell lysates were probed with anti-PHIP or anti-tubulin antibodies. The Western blot is a representative of at least 3 independent experiments (N=3). (B). MCF10A cells were infected with MSCV-PHIP retrovirus three consecutive times (lane 2, MCF10A-PHIP III) or with MIEV retrovirus as control (lane 1, MCF10A-MIEV) and cells were selected with G418 for 10 days. Lane 3 represents MCF10A cells infected with MSCV-PHIP retrovirus 6 times but without drug selection. Cells were collected on day 12 after the last infection and lysates were probed for expression of recombinant HA-PHIP using anti-HA antibodies. Lane 4 represents Phoenix cells transfected with MSCV-HA-PHIP, which was used as a Western blot control for HA-PHIP expression levels. The figure shown is a representative of 2 Western blots (N=2). (C). Fluorescent pictures of MCF10A-MIEV and NIH/3T3-MIEV control stable cells 48 hours after the third retroviral infection, plated on 6-well plates. Cells were lysed for Western blot analysis on day 12 after the last infection, and same degree of fluorescence was observed. The GFP photographs are representative figures from at least 3 independent experiments (N=3). (D). MCF10A cells infected with either MSCV-PHIP or MIEV-control virus and were selected for 10 days in G418. Selected
cells were expanded and 5,000 cells were then seeded on soft agar for 14 days. Cells were stained with 2 mg/mL MTT and imaged using Nikon digital camera. The experiment was successfully performed only once.

Figure 8. Loss of HA-PHIP expression in the MCF10A stables.

(A). A Western blot showing the significant decrease of HA-PHIP signal in MCF10A-PHIP III that previously had robust HA (lane 3). Lane 1 and 2 respectively represent non-infected MCF10A-parental and MCF10A-MIEV as HA negative controls. MCF10A-PHIP VI (lane 4) is MCF10A cells infected with PHIP retrovirus for six times but not selected with G418. NIH/3T3-PHIP III (lane 6) refers to NIH/3T3 cells infected with PHIP retrovirus three times under the same conditions as MCF10A cell line, and NIH/3T3-MIEV (lane 5) acts as its GFP control. MCF10A-CD8-IGFR (lane 7) and MDA-MB-231 (lane 8) were included as PHIP positive control. Phoenix/MSCV-PHIP (lane 9) indicates Phoenix cells that were transfected with MSCV-PHIP vector and acts as both PHIP and HA positive control. The figure is a representative of 2 Western blots (N=2). (B). The earliest passage of MCF10A-PHIP III (lane 2) and MCF10A-vector control (lane 1) that had been cryopreserved after first subculture following G418 selection for 10 days were thawed and analyzed for HA signal, which was present. The Western blot was run only once (N=1).

inoculation into SCID or nude mice to see tumor formation in vivo. In another round of infection, single colonies were isolated, but none gave the HA signal, despite the continued presence of GFP in the cells infected with GFP-only control virus. At first, the disappearance of HA signal in MCF10A-PHIP was surmised to arise from the environmental factors that may modulate PHIP expression in order to maintain the cellular homeostasis, such as the concentration of growth components in the MCF10A medium, cell density, and the presence of G418. Upon consulting Dr. Senthil Muthuswamy,
Figure 9. PHIP is targeted for protein degradation in MCF10A-PHIP stable cell line.

(A). HA-PHIP mRNA levels in MCF10A-PHIP stable cell line were quantified using RT-qPCR analysis. Recombinant PHIP cDNA was amplified using primers designed to the C-terminal region of the human PHIP mRNA and was normalized to an ubiquitous control gene, in this case TATA-binding protein (TBP). The statistical analysis was done by Student’s t-test. The qPCR was performed once with replicates of 3 independent wells (n=3). The error bars indicate standard deviation. (B). MCF10A-PHIP cells (lanes 4, 5 and 8, 9) that had previously lost the expression of HA-PHIP were plated in 200,000 cells per well on 6-well plates. On the next day, the cells were treated with 5 µM MG132 proteasomal inhibitor or its solvent DMSO in MCF10A growth medium for either 4 or 16 hours and lysed at the indicated time points in 6x SDS. The Western blot membranes were blotted for HA and p53, which was used as a positive control to MG132 as it is a well-known target of ubiquitination. The first lane indicates MCF10A-vector cells that had neither DMSO nor MG132 in the medium during the 4 hours. The Western blot is a representative of 2 independent experiments (N=2).

one of the co-developers of the MCF10A culture system, we were advised that the cells may be compensating as a feedback response to the probable excessive activation of the insulin pathway triggered by the ectopic overexpression of the wild-type PHIP. Therefore, it was highly recommended to reduce insulin levels in the MCF10A media (personal communications). Unfortunately, changes in those conditions did not seem to influence the PHIP levels. The specificity of the current antibody was also suspected, despite the unfailing presence of a thick band for the positive control 293T-MSCV-PHIP lysate, and new WDR-targeting antibody from the crude rabbit sera was extracted. The antibody gave a clean band, but to our disappointment, the size did not correspond to PHIP, possibly indicating the presence of another WDR-containing isoform.

Surprisingly, RT-qPCR analysis using human PHIP primers targeting the C-terminal region...
revealed that PHIP was in fact being overexpressed in the stable MCF10A cell lines at the transcript level (Figure 9A). This led us to question whether PHIP was specifically being targeted for protein degradation. To test this hypothesis, the MCF10A stable cells were treated with MG132, a proteasomal inhibitor, and lysed at either 4 or 16 hours after treatment. Intriguingly, HA signal was at least partially restored on the Western blot (Figure 9B), suggesting that PHIP was being proteolytically degraded, rather than being silenced at the transcriptional level, which sometimes can occur in retrovirally generated stable cell lines (Swindle et al., 2004). One possible explanation behind this occurrence may be that PHIP auto-regulates its own protein levels, as a putative binding partner to the DDB1-Cul4 ubiquitin ligase complex (Angers et al., 2006), which will be discussed further in the Discussion, Chapter 4. Unfortunately, this aspect could not be investigated further due to time and reagent limitations.

3.3 AdPHIP1 is not the ideal system for the soft agar colony-forming assay

In favor of our hypothesis that PHIP1 is involved in oncogenic transformation of breast epithelial cells, initial analysis with the MCF10A-PHIP1 stable cell line resulted in positive colony formation on soft agar in 2 weeks post-seeding (Figure 7), indicating that the cells could acquire the ability to grow in the absence of anchorage with PHIP1 expressed approximately twice the physiological level. However, due to the spontaneous silencing of the ectopic PHIP1 after a number of rounds of sub-culturing, we attempted to replicate the soft agar colony formation assay using an alternative means of protein overexpression. It was previously shown that adenovirus coding for human PHIP1 (AdPHIP) with HA epitope could successfully overexpress the protein in INS-1 cells (Podcheko et al., 2007). Thus to circumvent the silencing in the stable cell lines, we attempted using AdPHIP as an alternative means to examine the effect of full-length PHIP1 overexpression. Although soft agar assays typically take 3-4 weeks, there have been reported cases of adenovirus used in the long-term experiment, such as in Lee et al. (2003), where transduction of lung cancer cells with adenovirus expressing truncated (defective) IGF-IR led to a significant suppression of colony formation in a soft agar clonogenic assay. Encouragingly, the authors had also previously reported successful colony formation using adenovirus after 3 weeks (Lee et al., 1996). Upon testing the virus on MCF10A cells for the degree of overexpression, the virus was able to reproducibly produce HA signal in a dose-dependent manner (Figure 10). Unfortunately, however, despite multiple attempts, the HA-PHIP1 expression could not be maintained over one week and thus no colony
Figure 10. Soft agar results with MCF10A cells infected with PHIP1 adenovirus.

(A). MCF10A-cells infected with AdGFP (second panel) or AdPHIP (third panel) were plated on low-melting agarose (soft agarose) and stained with MTT 16 days after seeding. MCF10A-ERBB2 (first panel panel), which stably overexpress constitutively active ERBB2 receptor, was kindly donated by Dr. Senthil Muthuswamy and was used as the positive control in the assay. The figures are a representative of 5 independent experiments (N=5). (B). MCF10A parental cells were infected with different doses of either AdGFP or AdPHIP and were analyzed for dose-dependent overexpression of HA-PHIP 48 hours post-infection. The figure is representative of 2 independent Western blots (N=2). (C). MCF10A cells that had been concurrently infected with those seeded on soft agar were maintained on 6-cm plates and lysed on the designated dates (Days 4, 9 and 14) for Western blot analysis of HA-PHIP signal. Day 0 was taken as the day of the adenoviral infection. The Western blot is a representative of 5 independent assays (N=5).
was observed even after 20 days. MCF10A-ERBB2, which has stable amplified gene expression of constitutively active, truncated ERBB2, was used as the positive control and it produced visible colonies in every soft agar assay attempted. Likewise, the MCF10A cells infected with GFP adenovirus, which was used as the negative control, did not show any colony formation, which validated the soft agar assay technique.

3.4 TRIPZ shRNA-PHIP constructs fail to maintain PHIP knockdown.

One of the typical approaches used in cancer research to study the effect of a protein is the generation of shRNA stables in the cancerous cell line of interest. Stable knockdown of the protein under study allows one to investigate tumor cell growth in vivo through xenograft implantation or orthotopic injections, as well as numerous in vitro assays such as anchorage-independent growth in soft agar, 3D acini formation in Matrigel, and migration assays.

In an attempt to create an optimal model system that will enable us to study the role of PHIP in multiple aspects of breast cancer, we pursued to obtain the tet-inducible shRNA-TRIPZ plasmids. One major benefit of employing an inducible system in MCF10A-based cell line would be the ability to investigate the effect of the knockdown of a protein under study at different time points of the acinar morphogenesis, as well as being able to show reversibility and the specificity of an observation associated with the protein that is being knocked down.

For the generation of the stable cell lines to be used in our experiments, MCF10A-IGFR were infected three times every second day with appropriate TRIPZ-shRNA lentivirus, and were exposed to 1 µg/mL doxycycline for minimum 72 hours before being FACS-sorted by TurboRed (Figure 11C) versus 7-AAD, a cell viability marker. Initial analysis indicated that there was some degree of PHIP knockdown (Figure 11A), which was supported by the proliferation assay (Figure 11B). However, subsequent Western blot indicated no change in the protein level, and neither the amount of doxycycline nor the length of the exposure time influenced the outcome (Figure 11D). To validate that the TRIPZ shRNA-PHIP constructs from the Openbiosystems were indeed able to knockdown PHIP, and to exclude the possibility that such obliteration of knockdown could be a MCF10A-specific effect, the TRIPZ lentiviral stable cell lines were re-generated in MDA-MB-231 (Figure 12). Removal of cells that do not express the shRNA transgene by puromycin selection was considered important, as transfection and expression of the TRIPZ constructs in 293Ts gave dubious results multiple times and this observation was suspected to be due to masking of the visible knockdown by high PHIP levels from the non-transfected cells. Also, in an attempt to mimic the constitution of the SMARTpool siRNA from Dharmacon where there are multiple siRNA sequences that collectively
lead to efficient PHIP knockdown, a plate of MDA-MB-231 was infected with ‘pooled’ TRIPZ lentivirus generated by transfecting three different shRNA-PHIP constructs at equal ratios to 293T.

Fortuitously, Western blot analysis of cells straight after puromycin selection indicated a partial reduction in PHIP levels in shRNA_174591 and shRNA_174592 after 72 hours exposure to 1 µg/mL doxycycline (Figure 12C). To test whether this observation could be associated with a biological effect, a proliferation assay was performed using two different methods; one by enzymatic MTT growth assay and the other by directly counting cell number with Beckman Coulter Z2 (Figure 12D). To our astonishment and inconsistent to the previous results where PHIP knockdown led to inhibition of cell growth, the shRNA stables in fact grew better than the Scrambled control cells. Western blot of the cells revealed that there was no observable PHIP knockdown at the protein level (Figure 12E, F). However, though the lentivirally generated shRNA stable cell lines were deemed unfit to be used in the study, this phenomenon might be a further important and intriguing suggestion that PHIP is regulated at multiple stages and such potentially intricate regulation might be subjected to another exciting investigation.
**Figure 11 Analyses of MCF10A-IGF-IR TRIPZ stable cell lines.**

(A). MCF10A-IGFR cells infected with lentivirus generated from TRIPZ-shRNA-Scrambled (lane 1), -GAPDH (lane 2) or -174592 (lane 3) were treated with 1 µg/mL doxycycline for 72 hours following puromycin selection and were blotted for PHIP, E-cadherin, IGF-IR and tubulin on the Western blot. The Western blot represents n=1. (B). MTT assay was performed to assess the growth pattern of newly generated shRNA stables corresponding to (A). 5,000 cells of MCF10A-IGFR-TRIPZ-shRNA-Scrambled or shRNA-174592 were plated onto 96-well plates in four replicates (n=4) in either starved medium, which consisted of plain DMEM/F12 (serum free), or regular MCF10A growth medium containing 5% horse serum and other components. The day of seeding was designated as Day 0. The media were changed every two days and replenished with fresh doxycycline. The cell proliferation was measured on every second day using the MTT assay, and wells only containing the media without cells were used as MTT negative control. The proliferation assay was performed once. The statistical analyses were done by two-way ANOVA. The astrix refers to statistical significance between ‘Scrambled Growth’ and ‘174592 Growth’ groups. ** = P <0.01 *** = P <0.001 (C). 72 hours post-treatment with doxycycline, MCF10A-IGFR stables of TRIPZ-shRNA-Scrambled or TRIPZ-shRNA-174592 were photographed under the Nikon fluorescence microscope, which show the
robust presence of doxycycline-induced red fluorescence (RFP). The photographs are a representative of at least 3 independent experiments. (D). PHIP1 knockdown in MCF10A-IGFR TRIPZ stables was assessed under different conditions of amount of doxycycline used (0, 0.5 or 1.0 µg/mL) or the length of treatment (for 3 days or 6 days after seeding). The cells lysed were blotted with PHIP, IGF-IR or tubulin. Medium was changed every day with fresh doxycycline, and MCF10A-vector (last lane on the right) was used as the negative control for IGF-IR. The Western blot was performed only once.

Figure 12. Validation of TRIPZ constructs in 293T and MDA-MB-231 cells.
(A). 293T cells were transfected with different shRNA constructs with the TRIPZ backbone plasmid, including 174590 (lane 6), 174591 (lane 7) and 174592 (lane 8). In order to mimic the synergistic effect of having multiple shRNA as in SMARTpool siRNA, the three TRIPZ-shRNA plasmids were co-transfected in 293T in 1:1:1 ratio (lane 5). These were compared to 293T transfected with TRIPZ-shRNA-Scrambled (lane 4) and 293T treated with only PEI without DNA (lane 3). 293T transfected with MSCV-PHIP (lane 2) was used as a PHIP positive control as well as for the test of PEI functionality, which was compared to 293T transfected with MSCV empty vector (lane 1). The Western blot is a representative of at least 3 independent experiments (N=3).  

(B). MDA-MB-231 cells were infected with lentiviruses generated from TRIPZ-shRNA-174590, 174591, 174592 or from pooled transfection, and selected with puromycin for 5 days. The treatment of doxycycline was performed following the drug selection, and pictures show MDA-MB-231 TRIPZ stables after 72 hours of 1 µg/mL doxycycline treatment. The photographs are a representative of at least 2 independent assays (N=2).  

(C). The MDA-MB-231 TRIPZ stables that had undergone puromycin selection and 72 hour-treatment with doxycycline was run on the Western blot to analyze degree of PHIP knockdown. The stable cell lines were generated by infecting MDA-MB-231 cells with lentivirus mixed with growth medium in 1:1 ratio and 8 µg/mL of polybrene. Three independent TRIPZ shRNA constructs 174590 (lane 4), 174591 (lane 5), 174592 (lane 6) as well as from pooled infection of all three lentiviral types (lane 3) were compared to stables of TRIPZ-shRNA-Scrambled (lane 1) and shRNA-GAPDH (lane 2). The Western blot was performed successfully only once (N=1).  

(D). 100,000 cells of MDA-MB-231 TRIPZ-shRNA-173491, 174592 and Scrambled were seeded onto 6-well plate in 5% or 0.5% FBS media and counted with Beckman Coulter every two days for 6 days. Each datapoint represent six replicates (n=6), and the media containing fresh doxycycline was replaced every 2 days throughout the experiment. The experiment is a representative of two independent experiments (N=2).  

(E+F). The MDA-MB-231 TRIPZ stables that were used in the proliferation assay as in (D) and grown under either 0.5% or 5% FBS were also maintained in 6-well plates and lysed on Day 2, 4 and 6 for Western blot analyses. The Western blot was performed only once (N=1).

3.5 PHIP1 is implicated in cancer cell proliferation

As PHIP1 is a known downstream regulator of the IGF-IR pathway, we assessed whether PHIP was implicated in IGF-IR-mediated mitogenesis, and thus the effect of siRNA-mediated knockdown of PHIP on the growth of MCF10A-CD8-IGF-IR cell line was examined. siRNA-PHIP–treated MCF10A-IGF-IR cells reproducibly displayed significant growth inhibition compared to the siRNA-Scrambled-treated counterparts, as shown in Figure 13. More convincingly, as the protein level of PHIP was partially restored on Day 8 with the time-dependent degradation of the siRNA from the cells, the cell growth accordingly peaked, suggesting that PHIP is intimately involved in the cell
Figure 13. PHIP knockdown inhibits the growth of MCF10A-IGFR cells.

(A). Proliferation assay of MCF10A-IGFR with PHIP knockdown. 50,000 MCF10A-IGFR cells were seeded on 12-well plate and transfected with either siScrambled or siPHIP on Day 0 with Lipofectamine 2000. The medium was changed the next day to reduced serum medium containing 0.5% horse serum and other constituents. Cells were counted using Beckman Coulter counter (n=6 per group) for Days 2, 4, 6, and 8 and Statistical analysis was done by two-way ANOVA test. *** P<0.0001. The figure shown is a representative of at least 2 independent experiments (N>2).  

(B). The cells that had been transfected concurrently with those for the proliferation assay were lysed on each time points of the experiment and analyzed on Western blot for the presence of siRNA-mediated PHIP knock-down. The Western blot is a representative of 3 independent analyses (N=3).  

(C). MCF10A-IGFR were plated on 6-well plate and transfected with either siScrambled or siPHIP overnight. The next day, the medium was changed to either 0.5% or 5% horse serum DMEM/F12 and the cells were incubated for 48 hours before lysis with 6x SDS lysis buffer. The lysates were blotted for cyclin D1 and cyclin D2 as well as p-Akt to see the effect of PHIP knockdown under complete (lanes 3, 4) and reduced (lanes 1, 2) serum conditions. The Western blot was performed only once.  

(D). MCF10A-IGFR were similarly treated as (C) but grown in 5% horse serum for 48 hours before lysis in 6x SDS for Western blot or RLT Buffer for qPCR. Total RNA was extracted from MCF10A-IGFR with no treatment (lane
1), with siScrambled (lane 2) or siPHIP (lane 3) transfection and were reacted with primers against human p21. Statistical analysis was done by one-way ANOVA where * refers to P < 0.05 and *** represents P < 0.0001. The qPCR was performed once (N=1) with n=6 per datapoint.

**Figure 14. PHIP1 knockdown inhibits the growth of both ER positive and ER negative cell lines.**

(A). MCF7 cells were reversely transfected with either siScrambled or siPHIP in 96-well plate (10,000 cells/well) in five replicates and starved overnight, after which (+) cells were treated with 100 ng/mL IGF-I for 48 hours in the presence of 1% serum. Parental cells (first two lanes) were included as positive control for mitogenic response to IGF-I. The degree of cell proliferation was measured by the MTT assay, and media-only wells were included as negative controls for the MTT reagent. Statistical analyses were done by performing one-way ANOVA. ns = not significant. ***=P <0.0001. The experiment was successfully performed only once (N=1). (B). Using the same ratio, 200,000 MCF7 cells were reversely transfected on to 6-well plates, starved overnight and were either further starved for 48 hours in 1% serum (-; lanes 1, 3, 5) or treated with 100 ng/mL IGF-I under the same condition (+; lanes 2, 4, 6). Cells were collected on the same time point as when the MTT assay was performed, and lysates were blotted for PHIP, p-Akt, p-MAPK and actin. The Western blot is a representative of 3 independent experiments (N=3). (C+D). In the preliminary analysis, 200,000 MDA-MB-231 cells were plated on 6-well plate and transfected with either

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### A.

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Statistical analysis was done by one-way ANOVA where * refers to P < 0.05 and *** represents P < 0.0001. The experiment was successfully performed only once (N=1).

### B.

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### C.

**Figure 14. PHIP1 knockdown inhibits the growth of both ER positive and ER negative cell lines.**

(A). MCF7 cells were reversely transfected with either siScrambled or siPHIP in 96-well plate (10,000 cells/well) in five replicates and starved overnight, after which (+) cells were treated with 100 ng/mL IGF-I for 48 hours in the presence of 1% serum. Parental cells (first two lanes) were included as positive control for mitogenic response to IGF-I. The degree of cell proliferation was measured by the MTT assay, and media-only wells were included as negative controls for the MTT reagent. Statistical analyses were done by performing one-way ANOVA. ns = not significant. ***=P <0.0001. The experiment was successfully performed only once (N=1). (B). Using the same ratio, 200,000 MCF7 cells were reversely transfected on to 6-well plates, starved overnight and were either further starved for 48 hours in 1% serum (-; lanes 1, 3, 5) or treated with 100 ng/mL IGF-I under the same condition (+; lanes 2, 4, 6). Cells were collected on the same time point as when the MTT assay was performed, and lysates were blotted for PHIP, p-Akt, p-MAPK and actin. The Western blot is a representative of 3 independent experiments (N=3). (C+D). In the preliminary analysis, 200,000 MDA-MB-231 cells were plated on 6-well plate and transfected with either
siScrambled or siPHIP with Lipofectamine 2000. The transfection mixture was replaced with 10% FBS DMEM 6 hours after transfection. The cells were counted 48 hours after on the hemocytometer and lysed for Western blot, which was incubated with anti-PHIP and anti-actin antibodies to see the siRNA-mediated knockdown of PHIP. The figures are a representative of at least two independent experiments (N>2).

Figure 15. PHIP knockdown in MDA-MB-231 arrests G1 to S phase transition of cell cycle.

(A) MDA-MB-231 cells were treated with siRNA-Scrambled (=siScram) or siRNA-PHIP (=siPHIP) in six replicates and starved overnight the next day in 0.5% FBS DMEM. The cells were then incubated in either 0.5% or 5% FBS media for further 24 hours, after which they were stained with propidium-iodide (PI) and sorted by FACS. 16,000 cells were measured during the flow-through and the values were averaged across the replicates (n=6). The experiment was performed only once (N=1). (B) Table showing the results of statistical analyses on the processed FACS data. Two-way ANOVA was performed between the pairs indicated. ns = not significant; *** P<0.0001. (C). Average values (in %) of proportions of cells in different phases of the cell cycle for the bar graph in (A). (D). MDA-MB-231 cells were plated on 6-well plates and were transfected with siRNA Scrambled or PHIP as described
previously. The cells were lysed in RLT buffer for qPCR analysis 48 hours post-transfection in the growth medium and the cDNA reverse transcribed from the extracted RNA were reacted with primers against C-terminal region of PHIP transcript (PHIP C-TER), 3’UTR of PHIP (PHIP 3’UTR), p21, cyclin D1 or cyclin D2. Each datapoint represents two independent samples of triplicate values each (n=6 total). The comparative Ct method was used to calculate the fold difference, using the TBP values as the baseline, and the Student’s t-test was used for the statistical analyses. The error bars indicate the standard deviation.

proliferation. The level of cyclin D2, but not cyclin D1, was also reduced on the Western blot, and this effect could not be overridden by the presence of high serum (5% equine serum) (Figure 13C), confirming that cyclin D2 expression is regulated by PHIP consistent to the previous finding by (Podcheko et al, 2007). In order to support that downregulation of PHIP inhibits the enhanced cellular proliferation induced by IGF-I, MCF7 was used as another cell model. MCF7 is an estrogen receptor (ER)-positive luminal epithelial cell line that responds mitogenically to IGF-I (Dufourny et al, 1997; Dupont et al, 2001). These cells were reversely transfected with siRNA and were starved in serum-free medium overnight before being treated with the physiological concentration of IGF-I (100 ng/mL) for 48 hours. As expected, measurement of the relative live cell number by the MTT assay demonstrated that the mitogenic effect of IGF-I was curtailed in cells transfected with siRNA-PHIP (Figure 14A). Changes in the level of phosphor-Akt was checked on the Western blot to signify the cellular response to the IGF-I treatment (Figure 14B). Interestingly, the first knockdown experiment using MCF7 seemed to exhibit dampening of the phosphorylated Akt levels with PHIP downregulation. As overexpression of PHIP was previously shown to promote the phosphorylation of Akt in INS-1 cells (Podcheko et al, 2007), the experiment was repeated twice more in order to validate whether PHIP knockdown prohibited Akt phosphorylation in MCF7 cells. Unfortunately, subsequent Western blots failed to reproduce the initial observation.

Preliminary cell counting of MDA-MB-231 48 hours after transfection with siRNA indicated that there was a significant difference in the cell number between siScrambled and siPHIP (Figure 14C). When there was reasonable knockdown, lower cell number was constantly observed in siPHIP wells compared to siScrambled. As a noticeable number of floating cells were observed during the siRNA transfection, we also attempted to address whether the reduced cell number with siPHIP transfection was due to increased apoptosis or cell cycle arrest. FACS analysis with Propidium Iodide (PI) staining on MDA-MB-231 with PHIP knockdown grown under two different serum conditions (0.5% and 5% FBS) indicated that PHIP knockdown induces G1 arrest without significantly affecting the degree of apoptosis (Figure 15A, B). This was extended further by performing qPCR on cell cycle regulators to see how the knockdown of PHIP modulated them to prohibit the cell cycle
progression. Upon approximately 90% reduction of PHIP in siRNA-PHIP treated MDA-MB-231 cells compared to siRNA-Scrambled controls, cyclin D2 was again visibly diminished, while p21 was slightly increased (Figure 15). Intriguingly, qPCR analysis on MCF10A-IGFR cells showed a dramatic increase in p21 that was inversely dose-dependent to the amount of PHIP present in the cell (Figure 13D).

3.6 PHIP1 may complex with p53

p21 belongs to the Cip/Kip family of cyclin-dependent kinase inhibitor (CKI) that mainly inhibits the activity of cyclin E/cdk2 (Gartel et al, 2005). p21 is also a well-known transcriptional target of p53 (El-Deiry et al, 1993) and is required for the p53-dependent induction of G1 arrest in human cancer cells (Waldman et al, 1995). Because knockdown of PHIP1 increased the level of p21, and the magnitude of change was larger in MCF10A-based cells that contained wild-type p53 compared to MDA-MB-231, which has mutated p53 (Lacroix et al, 2006), it prompted us to question whether PHIP1 was directly involved in the transcriptional regulation of p21 by inducing proteasomal degradation of the protein (for example, by activating MDM-2 through phosphorylation of Akt (Ogawara et al, 2002; Zhou et al, 2001) or by potentially complexing with p53 and acting as a co-repressor. Another lab member had also previously shown preliminary data that PHIP interacts with p53 in MCF7 (Alexey Podcheko, unpublished data). The 293T cell line, which contains abundant levels of wild-type p53, was chosen as the model system for the co-immunoprecipitation (co-IP) experiment, in order to avoid co-transfecting the cells with two plasmids to introduce both HA-PHIP and a differently tagged-p53. Additionally, antibody manufacturing companies commonly use 293T as a source of the p53 protein to test their antibodies.

Unlike what was expected, transient overexpression of HA-PHIP by transfecting MSCV-HA-PHIP into 293T did not decrease the level of p53. HA-IP protocol was optimized by performing nuclear fractionation on the transfected cells to enrich HA-PHIP in the lysate similar to the methods used in (Waldman et al, 1997), and by strictly limiting the incubation time with the antibody-agarose bead complex to two hours in 4 oC to minimize protein degradation. After ensuring a successful HA-IP, the membrane was stripped and re-probed with p53 antibody, and ExactaCruz secondary antibody was used to eliminate unwanted signals from the IgG fragments. Remarkably, a thicker band on the HA-IP lane compared to the control was observed (Figure 16B). The reverse co-IP, where p53 was pulled down first, likewise gave a much stronger HA band in another set of trial (Figure 16C). However, as later mentioned in the Discussion, this phenomenon was not consistently observed in the other attempts.
Figure 16. PHIP complexes with p53.

(A). Overexpression of PHIP in 293T does not change p53 levels. 293T cells were transfected with MSCV-empty vector or MSCV-PHIP with PEI. 48 hours post-transfection, the cells were subjected to nuclear fractionation into cytosolic (lanes 1, 2) or nuclear compartments (lanes 3, 4). The lysates were blotted for PHIP or p53 on the Western blot. The Western blot is a representative of three independent experiments (N=3). (B). Inputs (lanes 1-4) equal to (A), where HA-PHIP from cytosolic and nuclear compartments of 293T cells transfected with either MSCV-control or MSCV-PHIP were detected with HA antibody. As PHIP was only found to be in the nucleus, the nuclear extract was subjected to immunoprecipitation with HA antibody, and immunoblotted for both HA and p53 antibodies (lane 5 and 6). The immunoprecipitation experiment was successfully performed only once (N=1). (C). Reciprocal immunoprecipitation was performed likewise, where nuclear fractionation was performed on 293T cells transfected with MSCV-vector or MSCV-PHIP to enrich the concentration of PHIP and p53. The inputs are the nuclear lysates before undergoing immunoprecipitation (lanes 1, 2). The nuclear lysates that were subjected to immunoprecipitation with p53 antibody and immunoblotted with both p53 and HA antibody are represented in lanes 3 and 4. ExactaCruz rabbit secondary antibody was used for the detection of immunoprecipitated p53, in order to eliminate the detection...
of heavy and light chains of the IgG that could conceal the signal for p53 at around 50 kDa. The immunoprecipitation assay was successfully performed only once (N=1).
Chapter 4. Discussion and Future Directions

4.1 PHIP1 is differentially expressed in cancer cells.

Western blot on the mammary tumor lysates of MMTV mouse models indicated that PHIP1 was more prominently present in samples containing oncogenes compared to the control wild-type mouse, which is a compelling evidence that PHIP1 is implicated in breast cancer. However, there needs to be more data in order to make a more definitive conclusion:

First of all, there should have been more than one age-mate wild-type (WT) samples to take into account the possible existence of variation among individual animals and between mouse strains. Unfortunately, the initial analysis which included at least two control samples used refined preps of mammary epithelial cells isolated using the Mammary Stem Cell Enrichment Kit (Stem Cell) and lysed in 1x RIPA buffer, which yielded absolutely no PHIP1 band on the Western blot. It was later realized that due to the nuclear confinement of PHIP, sonication for minimum of 10 seconds was necessary to release PHIP1 from the cellular nuclei into the solution to enable its detection with the antibody. Second caveat of this crude approach was that tumors that arise from different oncogenes have characteristic phenotypes and that the loading control informs us neither of the proportion of the proteins derived from the stromal versus epithelial cells nor of the degree of contribution from different cell types within the tumor tissues. Although there are examples in the literature where crude lysates of the mammary tissues processed with RIPA or PBS/NP-40-based buffers are used (Romieu-Mourez et al, 2003; Wagner et al, 2003), it would still have been preferable to do epithelial cell extraction and lyse the cells in 6xSDS lysis buffer or in 1x RIPA and sonicated before running the Western blot. Along the same line, one should note that while the tumor tissues are mostly comprised of epithelial mass, the tissues obtained from the WT mouse was the whole mammary gland, which contains both epithelial and stromal components. Therefore, as well as having more WT tissue samples, there needs to be a better extraction of mammary epithelial cells for it to be a more appropriate control.

In addition to what was found in this study, there should be additional analyses of clinical human breast tissues. Correlation or association studies, such as RT-qPCR analyses for PHIP1 mRNA levels on human breast tissues from various clinical stages (from premalignant to advanced) may provide better insight into at what stage PHIP1 is more intensely present during the tumor development, if a trend is present. Also, looking into gene amplification of human PHIP gene using Southern blot or fluorescent in situ hybridization (FISH) as part of an epidemiological study or
prognostic evaluations may increase its clinical value and potential widespread use as a diagnostic tool. Combining multiple approaches will better consolidate or confirm the finding that PHIP1 is specifically upregulated in human breast cancer. For example, an alternative approach to delineate the expression of PHIP among different breast cancer types includes performing Tumor microarray (TMA) of breast tumors at various clinical stages. However, there may be a few limitations to this approach due to the inherent nature of detecting protein, which is the very final product of a gene expression. For example, even if the intensity of the PHIP staining is more robust and disorganized in human breast tumor samples per unit area, they still do not inform us of whether the strong staining was the result of another oncogenic event that led to excessive proliferation and disorganization of the epithelial cells that already expressed PHIP at high levels before any transformation occurred or whether the overexpression of PHIP had a role in the tumor initiation. Additionally, the PHIP antibody has been shown multiple times on Western blot to detect both the 206 kDa isoform and another around 100 kDa. Therefore, it is possible that the results from the staining do not correspond to the endogenous, full-length wild-type PHIP1 under investigation. The signal might originate from mutated, truncated or stabilized PHIP with intact BD domain, which is the target of the antibody.

Screening the established mammary epithelial cell lines that cover characteristic stages of the disease progression is another option to support the data from the clinical samples. However, it should be noted that a major limitation in breast cancer research is the lack of multiple control cell lines. One of the most prominently used non-tumorigenic control cell is the MCF10A, which is a spontaneously immortalized human mammary epithelial cell line originated from the breast tissue of a 36-year-old patient with fibrocytic changes (Soule et al, 1990). It displays many physiological characteristics of the non-diseased breast, such as the requirement of growth factors for survival, anchorage-dependent growth in culture, and lack of tumorigenicity in nude mice (Soule et al, 1990). A more significant attribute of the MCF10A line in the study of mammary development and dysfunction is its ability to form 3D acinar structures in reconstituted basement membrane, or Matrigel, that resemble the glandular epithelium seen in in vivo (Debnath et al, 2002; Muthuswamy et al, 2001). Thus, despite a number of cytogenetic abnormalities such as deletion of p16 and p14ARF-containing locus and amplification of c-myc, and the basal phenotype of MCF10A (being ER negative) (Debnath et al, 2003), MCF10A is widely used as the “normal” cell line for the functional analyses of numerous breast cancer-related oncogenes. One recent example would be the generation and study of stable MCF10A cell lines that stably overexpress constitutively active IGF-IR (Kim et al, 2007) as mentioned in the Introduction. While it may be feasible to make sound conclusions on the effect of an oncogene if the expression levels significantly differed between vector and
transgenic cell lines, the expressive pattern of a protein from comparing multiple breast cancer cell lines to only one or two controls might not be as informative if the levels were not visibly distinct between normal and diseased samples. For example, we were initially excited to find that PHIP1 levels were significantly higher in the stable cell lines that overexpressed components of the IGF-IR signaling pathway, when compared to the vector control (Figure 6C). As PHIP1 was previously found to be a downstream positive effector of the IGF-I signaling pathway, it well supported our hypothesis that PHIP may be involved in the IGF-IR-induced transformation. However, this observation could not be replicated, and the PHIP levels between MCF10A parental cells and MCF7 or MDA-231 were also almost indistinguishable on a number of Western blots. Therefore, there needs to be a larger sample size of currently used breast cancer cell lines as well as data from clinical human breast cancer samples in order to confidently make the claim that PHIP1 is differentially expressed in cancer compared to the normal state.

Additionally, it should be noted that cyclin D2, a transcriptional target of PHIP, is downregulated in breast cancer, evidenced both by Evron et al (2001) and ONCOMINE, which is a widely used bioinformatic resource among many cancer researchers (Rhodes et al, 2004). In at least four different studies preceding the analyses by Evron et al, cyclin D2 was found to be abundantly present in HMECs which had limited life span, whereas it was noted to be absent in almost all breast cancer cell lines. Comprehensive series of reverse transcription (RT)-PCR confirmed that cyclin D2 mRNA level was lost in breast cancer cell lines as well as primary breast carcinomas when compared to HMEC, and Western blot demonstrated that cyclin D2 is also lost at the protein level in primary breast carcinomas (Evron et al, 2001). Interestingly, similar to PHIP, cyclin D2 was detected in both luminal and myoepithelial cells obtained from normal mammoplasty specimens. The authors, more importantly, showed that random methylation of the CpG-rich region of the cyclin D2 promoter in breast cancer cells was associated with the loss of cyclin D2 expression in breast cancer, in contrast to the expectation that cyclin D2 will likely act as a positive growth regulator by accelerating the G1 to S phase transition, which is the case for gastric or germline tumors (Takano et al, 2000; Schmidt et al, 2001). Indeed, hypermethylation of the CpG-rich regions on the cyclin D2 promoter occurred in almost half of the primary breast carcinomas, and the treatment of MCF7 and MDA-MB-231 with methyltransferase inhibitor 5-aza-dC or histone deacetylase inhibitor trichostatin-A (TSA) restored the cyclin D2 expression. As cyclin D2 promoter hypermethylation was also observed in ductal carcinoma in situ (DCIS), the authors suggested that loss of cyclin D2 expression may be an early event in the transformation event that precede the malignant stage of an invasive carcinoma (Evron et al, 2001).
Therefore, in order to clarify this apparent discrepancy and to test whether wild-type PHIP was directly involved in cellular transformation, we attempted to stably overexpress human PHIP in the non-tumorigenic MCF10A cell line and aimed to study the changes in cell phenotype, which is discussed further in the next section.

4.2 PHIP1 as a component of the nuclear proteasome and transcriptional machinery

In this study, we attempted to address the potential oncogenic property of PHIP1 by generating cell lines that stably overexpressed wild-type (WT), full-length protein. Thus MCF10A cells were infected with retrovirus, which stably incorporates the reversely transcribed DNA from its viral RNA genome into the host chromatin, and achieved approximately twice the level of protein present in the GFP control as semi-quantitatively determined by the immunoblot analysis. Despite the initial promising results where colony formation was observed on the soft agar assay, the protein levels soon diminished within 2 weeks of G418 selection, in contrast to the durable 3-4 fold expression in the mRNA transcription indicated by the Real-Time quantitative PCR (RT-qPCR) and the non-fading presence of GFP in the MIEV-infected control cell line. Upon treatment of the cells with MG132, a 26S-proteasome inhibitor, HA signal was at least partially recovered, suggesting that HA-PHIP1 may be targeted for ubiquitination and proteasomal degradation. Infection with PHIP adenovirus, which we used as an alternative, also failed to sustain sufficient levels of the protein for the soft agar colony formation assay and the initial robust expression of HA-PHIP rapidly declined within a week. There are a number of areas of consideration that may explain this phenomenon, including possible existence of PHIP autoregulation and protein stabilization of mutated or defective PHIP1.

An unexplored area of PHIP1 function in important cellular processes is in the interconnection between the ubiquitin-proteasome pathway (UPP) and transcriptional complexes. PHIP1 is also known as the DDB1 and CUL4-associated factor 14 (DCAF14), where DCAF denotes a general group of WD40-containing proteins that act as substrate receptors within various DDB1-CUL4 ubiquitin ligases (Lee and Zhou, 2007). The role of PHIP1 as a component of the E3 RING-finger ubiquitin ligase is currently unknown. However, a number of speculations may be made based on previous findings and proposed models that support the existence of nuclear proteasomes and the involvement of proteasomal complexes in transcription activation and regulation.

Several studies involving immunolocalization and biochemical fractionation indicated that proteasomes and their subunits were not only localized in the cytoplasm but also in the nucleus (Adori et al, 2006; Lafarga et al, 2002; Brooks et al, 2000). Although the exact nuclear proteasomal
content varied according to cell type, cell density and growth conditions, the nuclear ubiquitin-proteasome system (nUPS) has been reported to degrade cell-cycle regulators, transcriptional factors and tumor suppressors, such as p53 (Mikecz, 2006). The nUPS also plays important roles in nuclear gene expression and quality-control. In 2003, Muratani and Tansey proposed a model for how the Ub-proteasome system may control the transcriptional machinery. In their unified model, the regulation of transcription by Ub-proteasome system occurs in a step-wise manner in a very short period of time to inhibit any excess gene expression by an activator or the RNA polymerase II complex (pol II). The authors proposed that the Ub-proteasome is also recruited to the promoter site, probably mediated by direct binding to an activator or those of the basal transcriptional machinery. The Ub-proteasome acts to ubiquitinate the transcriptional composite, such as the activator, pol II, histones and other members, which subsequently attracts the components of the proteasome. Thus, the interaction between pol II and proteasome only occurs when the activator and the transcriptional machinery are bound to the promoter, and the degradation of the activator by the recruited proteasome prohibits transcription re-initiation. It is possible that PHIP1 is involved in the process, as summarized in Figure 17. The BD domain of PHIP may aid correct positioning of the co-activator by binding to specific acetylated histone residues, while WD40 repeats act as protein scaffold. One study validating this model has demonstrated that the steroid receptor coactivator (SRC) directly interacts with LMP2 (Low Molecular mass Polypeptide 2), which is a component of catalytic β subunit of the 20S proteasome, and that LMP2 is required throughout the estrogen receptor-mediated gene transcription. Through chromatin immunoprecipitation (ChIP) assays using primers spanning the whole genomic region of the ER promoter and the coding region of an ER target, the authors of the study showed that LMP2 was recruited on the entire sequence of the target gene when transcriptionally activated by the estradiol (E2) treatment. More intriguingly, sequential knockdown of the transcription initiation complex and the time-course analyses showed the cyclic nature of component recruitment and supported the requirement of SRC-mediated LMP2 recruitment in ER-mediated gene transcription (Zhang et al, 2006).

Similar to the failure of stable protein expression of ectopic PHIP1 observed in retrovirally infected MCF10A cells, lentivirally-infected GIPZ or TRIPZ-shRNA-PHIP1 knockdown in
Figure 17. A model for role of PHIP1 as part of the ubiquitin-proteasome complex and in the regulation of transcription initiation.

(A). The CUL4-DDB1 E3 Ubiquitin ligase ubiquitinates the activator, RNA pol II, histones and other TFs to recruit the proteasomal complex. PHIP, or DCAF14, would act as a substrate receptor to aid correct positioning of the complex subunits and specificity of the substrate. (B). The proteasomal complex degrades activator, so that re-initiation of transcription does not occur. (C). The proteasomal complex moves along with pol to modify chromatin structure and also disenages RNA pol II at the end of transcription or at the DNA damage sites. Pictures modified from (Muratani and Tansey, 2003), and (Jackson and Xiong, 2009).
MCF10A-CD8-IGF-IR cell line could not be sustained. Because we have no knowledge of how PHIP is regulated, it is difficult to interpret why the overexpression or silencing could not be maintained, especially when we had been able to successfully knockdown other proteins of interest studied in the laboratory using the same GIPZ backbone vectors. Currently, there is no information on any existing compensatory mechanism that responds to long-term reduction in PHIP1 levels. However, it could be possible that PHIP1 is very tightly regulated similar to the autoregulative network of p53. The autoregulation of PHIP1 might include a feedback loop where a protein under the control of PHIP1 acts as a sensor and leads to PHIP1 activation or degradation. Also, PHIP1 may directly or indirectly transcriptionally activate the ubiquitin ligase complexes to its own destruction. Because PHIP1 does not harbor a DNA-binding region, it probably would co-operate with an activator or repressor transcription factors to regulate its target genes.

In this study, PHIP1 was found to complex with p53 in 293T cell model. The co-IP experiment was repeated five times, but because the results were not consistent in all of the attempts, despite the fact that the pull down of p53 never failed, identification and optimization of conditions that are conducive to PHIP binding with p53 are needed to better understand the biological function of PHIP within the context of cell cycle regulation and tumor progression. It could be that when the co-IP did work, the cells were under an unknown stress (for example, excessive confluency and serum-starvation). Also, the fact that HA-IP was not as easy to reproduce as p53 may be attributed to: the relative abundance of the available epitope; the protein’s different sensitivity to proteases (as changing the manufacturer for Protease Inhibitors Cocktail seemed to decrease the HA-IP efficiency); or the different specificity and the affinity of the HA antibody compared to that of p53. Other approaches to demonstrate that PHIP participates in transcriptional downregulation of p21, would include luciferase assay with co-transfection of pGL3 luciferase reporter vector with p21-responsive promoter or chromatin-immunoprecipitation (ChIP) using primers that span the p21 promoter site along with PHIP overexpression via adenovirus or transfection. These are subjected to more in-depth analyses, which could not be performed in this study due to time limitations. However, from the fact that PHIP1 can bind to p53, the PHIP1 regulatory network might include overlapping components involved in the p53 autoregulation (reviewed in Lu, 2009), which could be another area for investigation.

Moreover, data from large-scale screening such as mass spectrometry and cDNA microarray analyses in the future would greatly aid in identifying gene targets of PHIP1 as well as suggesting its binding partners, which could include proteasomal subunits that may play a role in the transcriptional initiation and control of those target genes. The findings should be confirmed by
immunoprecipitation assay and by the use of specific inhibitors such as lactacystin that blocks 20S activity or silencing reagent such as siRNA for a particular protein component for the functional analyses.

Interestingly, PHIP1 mutations in non-BRCA1/2 breast cancer have been reported in a Ph.D. thesis from Karolinska Institutet, Stockholm, Sweden (Maguire, 2005, unpublished data). BRCA1/2 is the major genetic predisposition to breast cancer so far known. In the study, data from genome wide linkage scan and cDNA microarray were screened and a 2.8Mb region of shared haplotype on chromosome 6q14.1 was identified in a total of 31 families analyzed. The 2.8 Mb region contained six known genes, one of which was PHIP. Of the 53 sequence alterations discovered in the six genes, there were no frameshift or nonsense mutations, and PHIP gene was specifically found to contain a missense mutation, Leu1093Pro. In support of PHIP1 mutation in breast cancer, Arason and colleagues very recently performed genome-wide searches for linkage on 9 Icelandic and 13 additional Nordic multiple-case non-BRCA1/2 families to map highly penetrant loci. The three highest signals were reported to be located at chromosomes 6q, 2p and 14q at respective heterogeneity multipoint logarithm of odds (HLOD) scores in all families at 3.27, 1.66 and 1.24 (Arason et al, 2010). Thus, the authors concluded that Chromosomes 2p, 6q and 14q were candidate sites for genes indicative of high risk for breast cancer and suggested a polygenic model of additive probability with joint effect of the risk-factor genes (Arason et al, 2010).

Based on these results, it is possible that in a normal cell, such as MCF10A, the ectopically expressed PHIP1 is also subjected to the autoregulatory mechanism as for the endogenous protein, thus leading to its downregulation. Analogously, in cancerous state, this regulatory function may be disrupted or evaded through PHIP1 mutation, leading to accumulation of potentially oncogenic product that leads to uncontrolled cell proliferation. This may occur at the coding region or at the post-translational level that results in the stabilization of protein at an active state. This prospect of the existence of PHIP1 mutation in cancer is akin to p53, which was first discovered in SV40-transformed cells in the late 1970s and was originally identified as an oncogene because it could transform NIH/3T3 mouse fibroblasts similar to ras or myc (Weisz et al, 2007). However, p53 was later re-classified as a tumor suppressor when only the mutated form was found to be transformative (Finlay et al, 1989; Weisz et al, 2007). There is currently limited knowledge on PHIP mutational status in cancer and it is another research area needing more thorough investigations.
4.3 Role of PHIP1 in cell-cycle progression

The G1 to S phase transition in cell cycle progression is regulated by the D- and E-type cyclins with their cyclin-dependent kinases CDK2, 3, and 4/6. The cyclin D-CDK4/6 complex leads to the phosphorylation and inactivation of tumor suppressor Retinoblastoma (RB), which releases the E2F transcription factors for the subsequent transcription of cyclin E and other genes that promote G1 progression and drive the DNA replication in the S phase. There are three types of the D-type cyclins, cyclin D1, D2 and D3. Unlike the first two D-type cyclins, Cyclin D3 is strongly expressed in benign tumors of steroid hormone secreting endocrine cells and is also implicated in pathogenesis of malignant lymphoma (Doglioni et al, 1998; Bartkova et al, 1998). Additionally, cyclin D3 may have a dual role in cell proliferation and terminal differentiation (Bartkova et al, 1994).

The current knowledge on the role of PHIP1 in cell cycle regulation is its ability to directly and specifically induce cyclin D2 expression in INS-1 cells, an effect which was enhanced with IGF-I stimulation (Podcheko et al, 2007). However, adenoviral overexpression of PHIP1 had no effect on cellular cyclin D1 levels (Podcheko et al, 2007) and neither did the knockdown of PHIP1 in MCF10A-CD8-IGF-IR or MDA-MB-231 as presented in this study. The prominent form of cyclin D involved in breast cancer is cyclin D1, which has been long established to be a putative proto-oncogene (Bartcova et al, 1994; Wang et al, 1994; Weinstat-Saslow et al, 1995; Steeg and Zhou, 1998). It has been previously shown that ablation of cyclin D1 in vivo leads to specific protection against cancers induced by neu and ras oncogenes while having no influence on the tumorigenesis driven by c-myc or Wnt-1 (Yu et al, 2001). In the same study, cyclin D2 was upregulated in the mammary tumors of MMTV-Myc and MMTV-Wnt females (Yu et al, 2001). The fact that PHIP1 downregulation had no effect on cyclin D1 levels unlike cyclin D2 in MCF10A-IGFR cell model might suggest that PHIP1 works more distinctively with pathways that activate cyclin D2, such as the Myc- or Wnt-1-mediated signaling.

Cyclin D2 is transcriptionally regulated by the Myc/Max/Mad network (Bouchard et al, 2001), a family of basic helix-loop-helix-leucine zipper (bHLH-LZ) proteins that activates cyclin E-Cdk2 complex by sequestering p27Kip1 into cyclin D2-Cdk4 complexes (Bouchard et al, 1999). As demonstrated by the oligonucleotide microarray analysis of Myc-induced genes in primary human fibroblasts (Coller et al, 2000), as well as lack of proliferative response to Myc in cyclin D2 knockout cells, cyclin D2 is a crucial downstream effector of Myc (Bouchard et al, 1999). Myc promotes cell cycle progression by activating the expression of cdc25A, Cdk4 and cyclins D2, -E, and -A, while downregulating the expression of growth arrest genes such as p21, p27, p15 and gas1
(Gartel and Shchors, 2003). Induction of cyclin D2 expression by Myc involves MycBoxII (within the transactivation domain, or TAD)-mediated recruitment of TRRAP, a component of the PCAF/GCN5 and TIP50 HAT complexes, and preferential histone H4 acetylation at a single nucleosome (Bouchard et al., 2001). It could be that PHIP1, being also a positive transcriptional regulator of cyclin D2, directly or indirectly contributes to this Myc-dependent control of cyclin D2 expression. PHIP1 might facilitate the recruitment or correct positioning of transcriptional machinery after the acetylation of histone residues by the HAT complexes via the interaction with bromodomains. Or, PHIP might be involved in the transcriptional activation by ubiquitinating the Myc nuclear factor to enable the transcription of its target genes by Pol II as per model proposed by Muratani and Tansey. Also, it has been previously shown that activation of PI3K/Akt and MAPK pathways regulate the Myc-mediated transcription through the phosphorylation and degradation of its alternative heterodimer binding partner Mad1 (Zhu et al., 2008). Mad1 competes with Myc for binding with Max for heterodimerization, thus suppressing the Myc transcriptional activity, which necessitates the obligatory binding partner Max (Zhu et al., 2008).

In MDA-MB-231, FACS analysis with PI staining indicated that the growth inhibition observed was mainly due to growth arrest rather than increased apoptosis. In this study, PHIP1 knockdown was shown to significantly increase p21 mRNA level in two different cell lines, in MDA-MB-231 and MCF10A-CD8-IGF-IR. This could indicate that PHIP1 acts to repress p21 expression in normal state through unknown mechanism. Although the main activation of p21 expression is p53, p21 can be transcriptionally regulated in either a p53-dependent or p53-independent manner, when stimulated by different agents, such as phorbol ester (PMA), transforming growth factor-β (TGF-β) or nerve growth factor (NGF) (Abbas and Dutta, 2009). Interestingly, p21 is also a direct repressive target of c-Myc, as demonstrated by the oligonucleotide microarray analysis in primary human fibroblasts by Coller et al (Coller et al., 2000). Additionally, Gartel and colleagues showed that c-Myc repressed p21 at the promoter region 119 bp upstream of the transcription start site, in common with the Sp1 binding sites (Gartel et al., 2001). Because Myc was shown to interact with the C-terminal zinc finger domain of Sp1, which is required in the transcription of p21, Myc likely downregulates p21 expression in part by sequestering Sp1 (Gartel et al., 2001). Intriguingly, c-Myc and p21 has also been shown to reciprocally regulate each other through protein-protein interaction (Kitaura et al., 2000). p21 can block c-Myc-Max complex formation on the E-box by binding to the Myc Box II region, while binding of c-Myc to the C-terminal region of p21 partially rescued the DNA polymerase δ-PCNA activity that is inhibited by p21 (Kitaura et al., 2000). Therefore, in the context of breast cancer, it may be that upregulation of
p21 from knockdown of PHIP1 leads to inhibition of c-Myc activity and thus the downregulation of cyclin D2. Given the overlapping gene targets of c-Myc with that of PHIP1, it will be interesting to see whether c-Myc requires the presence of PHIP for its normal function.

The intricate regulation of Myc by the proteasomal degradation is another area that may involve PHIP1. It has long been recognized that the two highly conserved regions on the N-terminal region of Myc protein family, called the Myc Boxes I and II, provide the signals for protein degradation via the proteasome (Flinn et al, 1998). It has been previously shown that when treated with proteasome inhibitors, c-Myc stably associates and accumulates in the nucleoli, which might be the site for sequestrating and degrading excess c-Myc (Arabi et al, 2003). Similarly, there are a number of ubiquitin ligases known to regulate c-Myc activity such as SCF\textsubscript{Skp2} (von der Lehr et al, 2003; Kim et al, 2003), SCF\textsubscript{Fbw7} (Moberg et al, 2004; Yada et al, 2004; Welczer et al, 2004), and HectH9 (Adhikary et al, 2005). CUL4-DDB1 E3 ubiquitin ligase has also emerged as a novel regulatory mechanism of Myc activity in 2010 by Choi et al. The authors identified a new substrate receptor in a mass spectrometry of Flag-tagged Myc in HeLa cells called TRPC4AP (transient receptor potential cation channel, subfamily C, member 4-associated protein)/TRUSS (tumor necrosis factor receptor-associated ubiquituous scaffolding and signaling protein), which complexed with CUL4-DDB1 E3 ligase to selectively degrade Myc through the proteasome (Choi et al, 2010). However, though TRPC4AP/TRUSS negated Myc function in this case, one should not rush to conclude that the same would apply for PHIP1. The mechanisms of Myc regulation are complex and vary depending on the cellular context. There are cases of dual roles of the same protein, such as p300/CBP, which usually acts as a co-activator of Myc transcriptional targets but under certain conditions, such as inhibition of cellular HDAC, leads to Myc degradation via acetylation of several lysine residues between TAD and DNA-binding domain (Faiola et al, 2005).

Along with the idea that PHIP1 may cooperate with Myc for its function, another area that needs further investigation is the role of PHIP1 in three dimensional (3D) culture system, which more closely mimics the physiological microenvironment of the mammary gland than the plastic dish (Vargo-Gogola and Rosen, 2007). As mentioned previously, single MCF10A cells undergo acinar morphogenesis into a 3D structure when embedded in Matrigel. The cells initially proliferate (Day 1-8) and then organize into apico-basally polarized epithelial cells that encircle a central hollow lumen formed by apoptosis due to the differential action of serine threonine kinase Akt in the two populations (Day 6-9). This can well be performed using the MCF10A cells cultured on Matrigel (Debnath et al, 2002; Debnath et al, 2003). There have been examples where the activity of a protein differs drastically when cells are embedded in extracellular matrix compared to a monolayer.
example, while long-term activation of Myc in MCF10A did not induce apoptosis when grown in a monolayer, more than 60% cell death was observed within acini (Zhan et al., 2008). Also, a subset of genes was found to be alternatively spliced in cells growth under 3D culture conditions compared to 2D on oligonucleotide microarray, suggesting that the cell geometry can influence the posttranscriptional gene regulation (Li et al., 2006). Therefore, it would be interesting to see the function of PHIP1 in 3D contexts which more closely resemble the physiological environment. Additionally, because the repressive function of Myc may be more dominant in some cellular context (Zhan et al., 2008; Evan et al., 1992, Nilsson et al., 2003), a concurrent activation of anti-apoptosis response may be required for the Myc-driven transformation (Zhan et al., 2008). It could be this cell survival signaling PHIP1 is responsible for during the tumor initiation.

4.4 Putting PHIP1 in the context of breast cancer and IGF-IR signaling

As mentioned in the Introduction, insulin-like growth factor 1 receptor (IGF-IR) is highly implicated in breast cancer, and is involved in virtually all stages of tumor development by providing the proliferative, anti-apoptotic and pro-metastatic signals. For example, early studies indicated that in malignant breast cancer, IGF-IRs are overexpressed approximately 14-fold, with 2-4 fold higher levels of autophosphorylation compared to the normal tissue controls. When combining the increased receptor function with increased protein expression, the overall IGF-IR activity could mount up to 40-fold elevation in cancer (Resnik et al., 1998). However, increasing evidence has suggested that IGF-IR can have different cellular effects at various cancer stages. Schnarr et al (2000), for example, observed that IGF-IR and IRS-1 were downregulated in cells that were poorly differentiated, which represent cells of advanced stages of human breast cancer. In fact, the authors found that IGF-IR and IRS-1 expressions correlated better with tumor progression than loss of the ER expression (Schnarr et al., 2000). Similarly, as demonstrated by Bartucci and co-workers (2001), IGF-IR plays different roles between ER positive and ER negative cell lines. While IGF-I could act as a chemoattractant for both cell types and induce cell motility, it was only found to be mitogenic and anti-apoptotic in ER positive cell lines (Bartucci et al., 2001). Currently, the consensus is that IGF-IR can have different cellular effects depending on its signaling through IRS-1 or IRS-2, where IRS-1 has a proliferative function and IRS-2 has a more metastatic role (Denley et al., 2007; Deepali, 2008). This is supported by a number of studies which demonstrated that overexpression of IRS-1 or IGF-IR reduced estrogen growth requirements and enhanced survival (Surmacz, 1995; Guvakova et al., 1997) and that in ER negative cell line, IRS-1 overexpression is not sufficient to enhance IGF-I mediated cell proliferation (Jackson et al., 1999). One of reports supporting the role of IRS-2 in cell motility is by Zhang et al
which revealed greater phosphorylation of IRS-2 than IRS-1 during IGF-I-mediated cell motility, and co-localization of IRS-2 with focal adhesion kinase (FAK) in the leading edge of filopodia of motile cells.

The key finding in this study was that PHIP1 knockdown could significantly inhibit the growth of MCF10A-CD8-IGF-IR. CD8-IGF-IR is constitutively active regardless of the presence of the IGF-I ligand, and is composed of human T-cell CD8α extracellular domain fused with the IGF-IR β cytoplasmic subunit (Carboni et al, 2005; Kim et al, 2007). It has been previously demonstrated that CD8-IGF-IR is highly oncogenic, where MMTV transgenic mice overexpressing CD8-IGF-IR develop salivary and mammary tumors as early as 8 weeks of age (Carboni et al, 2005). Similarly, stable CD8-IGF-IR overexpression in MCF10A was sufficient to cause growth factor-independent growth, colony formation on the soft agar, and tumor formation in immunocompromised mice at Day 15 when injected orthotopically to the mouse mammary gland (Kim et al, 2007). More importantly, overexpression of CD8-IGF-IR disrupted the formation of normal MCF10A cell mammary acini when grown in 3D Matrigel, resulting in the filling of the luminal space due to cell hyperproliferation and inhibited apoptosis. Additionally, the authors provided the evidence that upregulation of constitutively active CD8-IGF-IR could lead to direct downregulation of epithelial cell markers such as E-cadherin and α-catenin and upregulation mesenchymal markers such as vimentin, fibronectin and α-SMA, thus inducing epithelial-to-mesenchymal transition (EMT) (Kim et al, 2007), which supported the fact that IGF-IR could be involved in every stage of breast tumor development, from the initial oncogenic transformation to tumor dissemination. The fact that PHIP knockdown inhibited the growth of highly aggressive cell line is a very exciting finding, as it indicates that IGF-IR necessitates the presence of PHIP1 for its cell proliferative function. This notion would have been strengthened if the growth characteristic of MCF10A-vector cell transfected with siScrambled had been also included in the same growth curve, as this will validate the CD8-IGF-IR-mediated enhanced cell proliferation under reduced serum conditions as previously reported by Kim and colleagues, as well as showing whether PHIP1 knockdown alone could completely rescue the hyperproliferative phenotype of CD8-IGF-IR overexpression into vector-like growth pattern. As a downstream regulator of IGF-IR pathway, it is possible that the role of PHIP1 also differs depending on the growth factor receptor status and the stage of tumor development metastatic stage. Additionally, performing the proliferation assay with MCF10A stable cell lines which overexpress IRS-1 or IRS-2 and comparing the data between the two cell lines may provide an insight into whether PHIP works downstream of any particular IRS and if so, what specific role it plays when activated by the IGF-IR.
Lastly, from the fact that PHIP1 is involved in CD8-IGF-IR-mediated cell proliferation and transcriptional regulation of cyclin D2, PHIP1 may also function to potentiate the synergistic effect of different mitogens, such as estrogen, which enhances the IGF-I-mediated upregulation of type D cyclins and cyclin E (Dupont et al, 2000). Moreover, there have been a number of studies that illustrate how different mitotic signaling pathways can cooperate, despite recruiting and activating different docking proteins. Fleming and colleagues, for instance, showed that although the degrees of overlap vary in different cell lines, the distinct upstream signaling molecules recruited by IGF-IR and EGFR are recruited to both enhance Akt activity (Fleming et al, 2006). Because PHIP1 was previously shown to induce Akt phosphorylation via unknown mechanism (Podcheko et al, 2007), an indirect modulation of Akt activity by PHIP1 might be another point of cross-talk between two growth factor pathways. It has been well established that the estrogen pathway synergistically elicits mitogenic responses by interacting with the insulin/IGF-I pathway in breast epithelial cells at various crossing points such as induction of IRS1 expression of estrogen and binding of the ER with p85 subunit of the PI3K and tyrosine kinase Src (Zhang et al, 2005; Bernard et al, 2006) or the more aggressive tumor formation through the synergist effect of cyclin D1 and c-Myc (Yong et al, 2007).
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