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JAB1 NEGATIVELY REGULATES PTEN AND PROMOTES RESISTANCE TO TRASTUZUMAB IN HER2-POSITIVE BREAST CANCER

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by

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A

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DOCTOR OF PHILOSOPHY

by

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DEDICATION

This dissertation is dedicated to my parents and my brother for their love, constant support and encouragement. I love YOU!

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JAB1 NEGATIVELY REGULATES PTEN AND PROMOTES RESISTANCE TO TRASTUZUMAB IN HER2-POSITIVE BREAST CANCER

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HER2-positive breast cancer, which is characterized by the over-expression of the HER2 onco-protein, accounts for approximately 20% of all breast cancer cases. Trastuzumab (Herceptin), the first targeted therapy approved for HER2-positive disease, potently prevents the activation of signaling pathways downstream of HER2 and significantly improves patients' outcomes. However, resistance to trastuzumab is inevitable; such resistance can occur through reduced expression of PTEN protein. Jab1 is over-expressed in 50% of primary cancers and 90% of metastatic tumors. Our lab previously showed that depletion of Jab1 in combination with trastuzumab treatment up-regulated PTEN in mouse xenografts refractory to trastuzumab. PTEN was not detected in the control Jab1 knockdown. However, how Jab1 modulated trastuzumab responses and affected PTEN function was incompletely understood. The overall goal of my project was to identify the role of Jab1 in regulating PTEN and in contributing to trastuzumab resistance in HER2-positive breast cancer. I demonstrated that Jab1 mediated the post-translational regulation of PTEN by associating with PTEN and facilitating its degradation. I also found that the C-terminal end of Jab1 was required for Jab1 to induce degradation of PTEN. Furthermore, I showed that proteasome inhibitors failed to prevent PTEN degradation induced by Jab1 over-expression in breast cancer cells. Instead, the combination of lysosomal protease

inhibitors - E64D and pepstatin A - significantly impaired the ability of Jab1 to degrade PTEN. Further, I showed that silencing Jab1 increased trastuzumab's inhibitory effects on cell proliferation. In contrast, the introduction of Jab1 into breast cancer cells conferred resistance to trastuzumab. Taken together, my findings suggest that Jab1 negatively regulates PTEN and promotes trastuzumab resistance in HER2-positive breast cancer.

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LIST OF ABBREVIATIONS

AKT	Protein kinase B
ASCO	American Society of Clinical Oncology
ATG	Autophagy-related genes
ATP	Adenosine triphosphate
BCIRG 006	Breast Cancer International Research Group 006
BRCA	Breast Invasive Carcinoma
CAP	College of American Pathologists
CEP17	Chromosome enumeration probe 17
CMA	Chaperon-mediated autophagy
CP	Catalytic particle
CSN	Photomorphogenic-9 (COP9) signalosome
DCA	Drug-conjugated antibody
DCIS	Ductal carcinoma *in situ*
DUB	Deubiquitinating enzyme (deubiquitinase)
E1	Ubiquitin-activating enzyme
E2	Ubiquitin-conjugating enzyme
E3	Ubiquitin-protein ligase
EGFR	Epidermal growth factor receptor
FDA	Food and Drug Administration
FINHER	Finnish Herceptin
FISH	Fluoresecent *in situ* hybridization
Flag-PTEN	Flag-tagged PTEN

HER	Human epidermal growth factor receptor
HER2+	HER2 positive
HERA	Herceptin Adjuvant
His-Ub	His-tagged ubiquitin
Hsc70	Heat shock cognate 73 kDa protein
IHC	Immunohistochemistry;
ISH	*In situ* hybridization
Jab1	c-Jun activation domain-binding protein-1
JAMM	JAB1/MPN/Mov34 metalloenzyme
KO	Knockout
LAMP	Lysosomal-associated membrane proteins
LC3	Human microtubule-associated protein 1 light chain 3
Lys-Hsc70	Luminal form of Hsc70
MEF	Mouse embryonic fibroblast
MPN	Mpr1p, Pad1p-N-terminal
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
Myc-Jab1	Myc-tagged Jab1
NCCTG N9831	North Central Cancer Treatment Group 9831
NEDD4-1	Neural precursor cell-expressed developmentally down-regulated protein 4
NSABP B-31	National Surgical Adjuvant Breast and Bowel Project
PACS 04	Protocole Adjuvant dans le cancer du sein 04
PBD	PIP3 binding

PDK1	3-phosphoinositide-dependent protein kinase-1
PDZ	(PSD-95, Discs-large, ZO-1)-binding domain
PE	Phosphatidylethanolamine
PI3K	Phosphoinositide 3-kinase
PIP2	Phosphatidylinositol 4,5-bisphosphate
PIP3	Phosphatidylinositol 3,4,5-triphosphate
Poly-Ub	Poly-ubiquitination
RP	Regulatory particle
RTKs	Receptor tyrosine kinases
shRNA	Small hairpin RNA
siRNA	Small interfering RNA
TCGA	The Cancer Genome Atlas
TCPA	The Cancer Proteome Atlas
TKI	Tyrosine kinase inhibitor
Ub	Ubiquitin
Ub-K48	Lys48-linked poly-ubiquitin
Ub-K63	Lys48-linked poly-ubiquitin
UPS	Ubiquitin-proteasome system
VP-ATPase	Vacuolar proton pump ATPase
VPS	Vacuolar protein sorting
WWP2	WW domain-containing protein 2
XIAP	X-linked inhibitor of apoptosis protein

CHAPTER 1: INTRODUCTION

Summary list:

1. Breast cancer, a heterogeneous disease, is the most frequently diagnosed cancer in women and the second leading cause of cancer-related death for women worldwide.

2. HER2+ breast cancer is characterized by the amplification or over-expression of the HER2 oncogene/protein and accounts for about 15-20% of all breast cancers.

3. Trastuzumab (Herceptin) is the first targeted therapy approved for HER2+ breast cancer.

4. Though trastuzumab significantly improves patients' overall survival, resistance to the drug is inevitable.

5. Partial or complete loss of PTEN, which promotes p-Src-Y416 and p-AKT-S473, is one of the proposed key factors that leads to trastuzumab resistance.

6. Jab1, which is frequently amplified/over-expressed in breast cancer, potently promotes cell proliferation by binding to and causing rapid degradation of p27.

7. The ubiquitin-proteasome system accounts for the degradation of about 80% of intracellular proteins. In general, a protein is first attached to a K48-linked poly-ubiquitin chain, and then is transported to the proteasome for degradation.

8. The lysosomal proteolytic pathway does not require its target proteins to be poly-ubiquitinated and functions in three distinct ways: macro-autophagy, chaperone-mediated autophagy, and micro-autophagy. Proteins tagged with a K63-linked poly-ubiquitin chain have been found to be degraded by the lysosomal proteolytic pathway.

OVERVIEW OF BREAST CANCER

Breast cancer is the most frequently diagnosed cancer in women, which accounts for 23% of the total cancer cases worldwide and 29% of all new cancer cases in the United States (Figure 1) (1). Moreover, breast cancer is now the second leading cause of cancer-related death in women in both developed and developing countries, which accounts for 14% of the cancer deaths (1, 2).

Figure 1. Ten most popular cancer types based on estimated new cases in women in United States in 2014.

Breast cancer is the most frequently diagnosed cancer in women.

Adapted with permission from CA Cancer J Clin, Siegel, Ma, Zou, and Jemal, copyright 2014 (1)

Females

Breast	232,670	29%
Lung & bronchus	108,210	13%
Colorectum	65,000	8%
Uterine corpus	52,630	6%
Thyroid	47,790	6%
Non-Hodgkin lymphoma	32,530	4%
Melanoma of the skin	32,210	4%
Kidney & renal pelvis	24,780	3%
Pancreas	22,890	3%
Leukemia	22,280	3%
All Sites	**810,320**	**100%**

1. Development of breast cancer

Breast cancer or breast carcinoma is a complex and heterogeneous disease, comprising multiple tumor individual cells with distinctive histological patterns, different biological and clinical behaviors (3-5). Simply defined, breast cancer is initiated by the uncontrolled growth and proliferation of cells developing in an area of the breast (Figure 2) (5, 6). Specifically, most breast tumors arise at the junction between the terminal duct and lobule named terminal ductal lobular units (4).

Terminal ductal lobular units are comprised of luminal epithelial (or epithelial) and myoepithelial cells (5, 7). Mutations accumulated inside breast epithelial cells lead to the uncontrolled growth and proliferation of the cells and subsequently lead to breast tumor (Figure 2) (5, 6). As each mutation is accumulated, a non-invasive lesion can increase the risk of developing malignant or invasive breast cancer. Once cancer cells have invaded, the risk for developing metastasis significantly increases (Figure 2) (5). The lymph nodes are the primary site for breast cancer metastasis (5).

Figure 2. A simplified schematic of breast cancer progression.

Breast cancer is initiated by the genetic or epigenetic changes in breast (luminal) epithelial cells. Subsequent cell transformation and tumor progression are driven by the accumulation of additional genetic changes. The metastasis is formed when the tumor cells sustain proliferation and invade through the basement membrane, enter the vasculature, and establish a new tumor in a new microenvironment.

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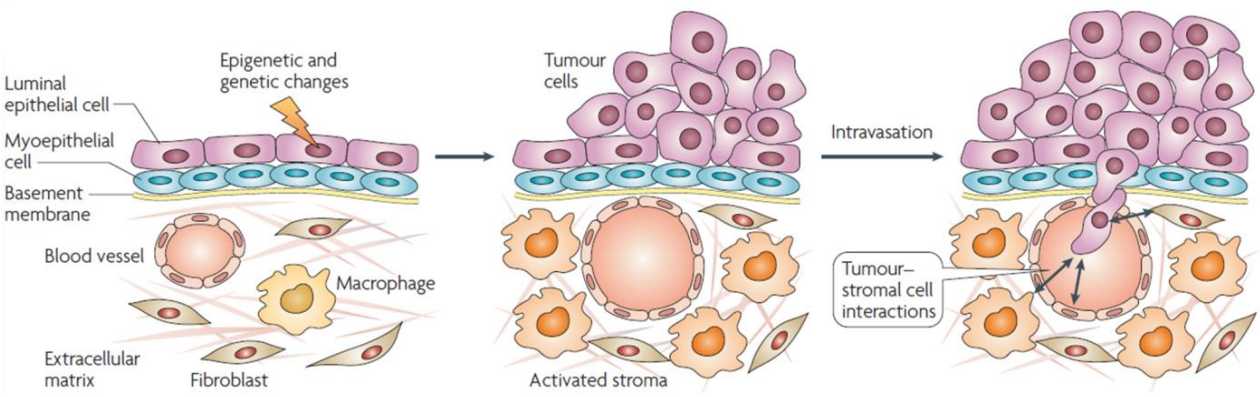

2. Classification of breast cancer

Traditionally, pathologists classified breast cancer into non-invasive and invasive types, based primarily on overall morphology, structural growth pattern, histological grade, and presence of lymph-node metastasis (4). Noninvasive breast carcinoma is detected when cancer cells fill the ducts but haven't spread into surrounding tissue, which is called ductal carcinoma in situ (DCIS). DCIS indicates a precursor to invasive breast cancer (3, 5).

Invasive breast cancer occur when the cancer cells have penetrated the lining of the ducts or lobules and are found in the surrounding tissues, such as fatty and connective tissues (5). The two most common histological subgroup of invasive breast cancer are infiltrating ductal and infiltrating lobular carcinomas (3). Compared with infiltrating ductal carcinoma, invasive lobular carcinoma is more likely to occur in older women, tends to be better differentiated, shows favorable outcomes, and metastasizes later (8-10). The other rare types of invasive breast tumor account for less than 10% of all cases include medullary, mucinous, tubular, papillary, metaplastic, and inflammatory types (3, 7). Patients with ductal, lobular, and medullary carcinomas have about 10-year survival rates, while mucinous and tubular carcinomas have relatively better overall outcomes (3, 7).

In the modern clinical practice, the emergence of fluorescence in situ hybridization (FISH) and advances in genomic profiling have provided new insights into breast cancer taxonomy (11). The initial study was done by Perou, Sorlie, and their colleagues who performed comprehensive analysis of genetics and molecular biology on different sets of cancer cell lines and tissues from breast cancer patients (12, 13).

Follow-up studies from these groups and from the Cancer Genome Atlas Network confirmed their findings that breast cancer can be defined into 4 major subtypes, luminal A, luminal B, basal-like and HER2-positive (HER2+) breast cancer which makes up about 80% of all breast cancer cases (table 1) (7, 11, 14, 15). The classification was primarily based on the presence or absence of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) (11). Luminal A and B tumors are generally associated with more favorable prognosis, compared to tumors lacking ER and PR expression such as HER2 and basal-like breast cancers (Figure 3) (11). The understanding of these heterogeneities has paved the way for the development of personalized targeted therapy in the treatment of breast cancer (table 1) (11). For comprehensive overviews of breast cancer, therapeutic implications, and updated reports on breast cancer incidence, readers are recommended to peruse excellent reviews by Sims et al (2007), Vargo-Gogola et al (2007), Weigelt et al (2009), and Bertos et al. (2011) (4, 5, 7, 11).

Table 1. Portraits of human breast tumour subtypes based on molecular pathology (11).

Abbreviations: minus signs indicate the negativity; plus signs indicate the positivity.

	Luminal A	Luminal B	HER2-positive	Basal - like
Cancer cell phenotype	Luminal	Basal luminal	Basal luminal	Basal
Molecular characteristics	ER+ PR-/+ HER2-	ER+ PR-/+ HER2+	ER- PR- HER2+	ER- PR- HER2-
Histological Grade	I (well differentiated)	III (poorly differentiated)	III (poorly differentiated)	III (poorly differentiated)
Incidence	40% Associated with increasing age	20%	18-20% More common at young age	15-20% Higher risk at young age
Predominant ethnicity			African American	African American
Approved therapy	Endocrine therapy (anti-estrogen or aromatase inhibitors)	Chemotherapy and/or endocrine therapy	Chemotherapy and/or HER2 targeted therapy (for ex., trastuzumab)	Heavy chemotherapy

Figure 3. Prognostic outcome for each breast cancer subtype by molecular profiling.

The HER2+ (ERBB2+) and basal subtypes demonstrate the worst prognoses, whereas the luminal subtype A and B show the more favorable outcome.

Abbreviation: HER2+, HER2 positive.

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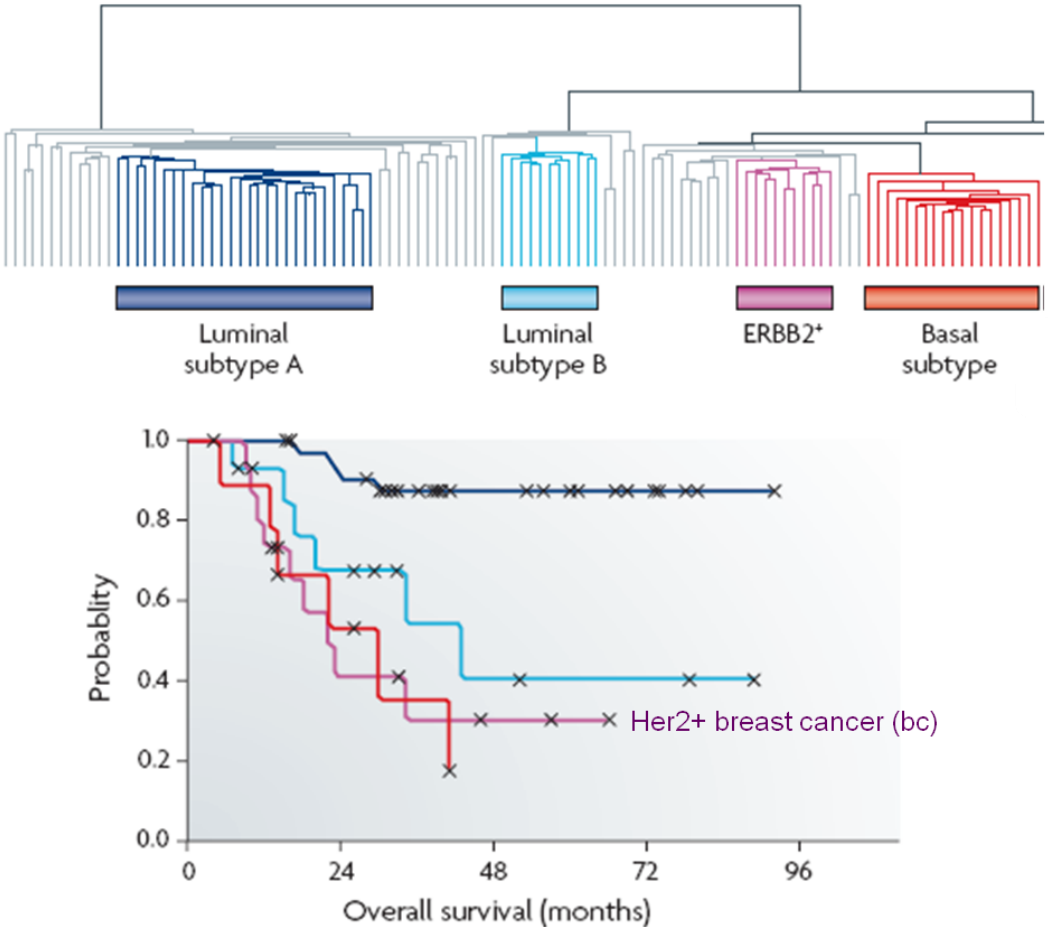

HER2-POSITIVE BREAST CANCER

In my studies, I focused on HER2+ breast cancer which is characterized by the amplification or over-expression of HER2 onco-gene or –protein

1. Biology of HER2 and the family of the epidermal growth factor receptors

Human epidermal growth factor receptor-2 (HER2) is a 185 kDa transmembrane receptor which is encoded by *HER2* gene located on the long arm of chromosome 17 (17q12–21.32) (16, 17). HER2 protein was first found to be homologous to neu, a rodent oncogenic growth factor receptor (18, 19). The neu oncogene was initially discovered in a carcinogen-induced rat-brain tumor model and was a homolog of the v-erbB (avian erythroblastosis virus) viral oncogene (16, 20). Later, HER2 was found to be amplified in human breast cancer cell lines and had tyrosine kinase activity similar to the other epidermal growth factor receptor (EGFR). The post-genome characterization of the human kinome completed the description of this HER family which comprise four members: ErbB1 (EGFR), ErbB2 (HER2), ErbB3 (HER3) and ErbB4 (HER4) (Figure 4) (16).

Their structures, in general, consist of 3 distinct domains, the extracellular ligand-binding domain, the transmembrane domain and the cytoplasmic kinase domain. The extracellular domain contains the ligand-binding cleft which mediates the dimerization of each receptor while the cytoplasmic domain contains the ATP binding pocket (Figure 4) (16). Once activated by a ligand, HER proteins undergo dimerization then recruiting ATP to their binding pocket and undergo trans-phosphorylation in the

tyrosine residues. These phosphorylated residues trigger various molecular pathways associated with tumor growth and progression (16). Of note, no known ligand has been found for HER2, which implies that HER2 activation preferred heterodimerization with the other members of the family (21). On the other hand, HER3 lacks ATP binding site and is catalytically inactive, which indicates that signaling functions of HER3 are mediated through the kinase activity of its heterodimeric partners (16, 22). Many studies have demonstrated that HER3 is not only the preferred partner for the activation of HER2 but HER2-HER3 dimer is also the most active signaling of the family (23-25).

One of the major and most well-studied pathways downstream of HER2 activation is the phosphatidylinositol 3-kinase (PI3K)/ protein kinase B (AKT) pathway (See section “Molecular mechanisms of actions of trastuzumab”) (23, 25-27). Preclinical and clinical studies showed the frequent activation of AKT in HER2 over-expressing human breast tumors, which promoted cell proliferation and survival (28, 29). For a comprehensive review on HER2 oncogen, its signaling and its tumorigenetic effects, please refer to review by Moasser (2007) (27).

Figure 4. Structures of the four members of the HER receptor family.

The HER receptor family is composed of four members, EGFR, HER2, HER3, and HER4. Each receptor has 3 distinct domains, the extracellular, transmembrane and cytoplasmic domains. The dimerization loop and the ligand-binding cleft on the extracellular domain were marked by dashed circles and black arrows, respectively. The ATP-binding pocket on the cytoplasmic domain was marked by white arrows. There is no known ligand reported for HER2.

Abbreviations: AR, amphiregulin; BTC, betacellulin; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; EPR, epiregulin; HBEGF, heparin-binding EGF; NRG, neuregulins; TGFα, transforming growth factor-α.

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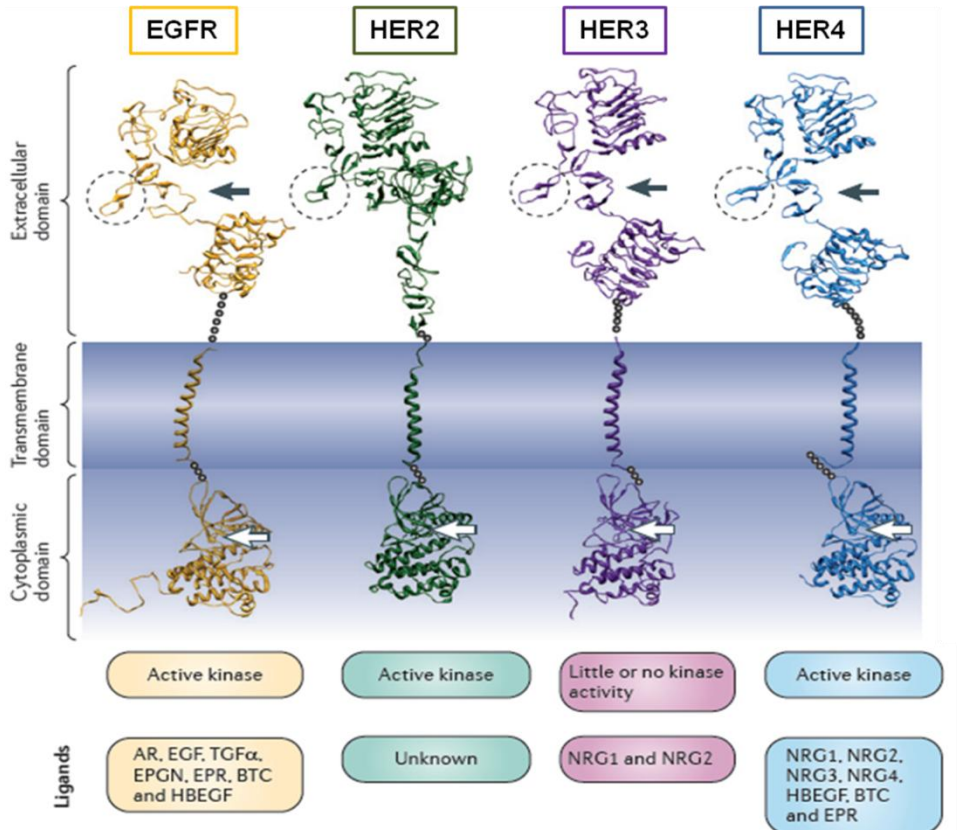

2. *Overview of HER2-positive breast cancer*

HER2+ breast cancer accounts for 15-20% of all breast cancer cases and is characterized by the amplification/over-expression of HER2 gene/protein (17, 30). In breast cancer, about 97% of cases with over-expression of HER2 protein were due to gene amplification (17, 31-33). Normally, HER2 is expressed at a low level on the surface of epithelial cells and is necessary for the normal development of many tissues, including those of the breast. In contrast, breast cancer cells can have up to 25-50 copies of the HER2 gene and up to 40-100 fold increase in HER2 protein expression which results in up to 2 million HER2 receptors per cell (27, 31, 32). This over-expression of HER2 drives continuous cell growth, proliferation and provides the cells survival advantages. These characteristics account for aggressive phenotype and poor prognosis of HER2+ breast cancer (Figure 3) (30, 34, 35). On the other hand, breast cancer with HER2 over-expression is unresponsive to endocrine therapy, and tends to metastasize to the brain (17, 27).

HER2 amplification is, however, not an indication of the late stage breast cancer (27). Several analyses from human breast tumors demonstrated that there was no significant difference in the levels of HER2 amplification/ over-expression among non-invasive disease, invasive disease, nodal metastasis and distant metastasis (33, 36-38).

3. *Methods of identifying HER2 positivity*

HER2 status is currently assessed by immunohistochemical (IHC) and/or *in situ* hybridization (ISH) analysis (17, 39-41). IHC evaluates HER2 protein expression, while ISH defines *HER2* gene copy numbers using a DNA probe which can be coupled to a fluorescent (FISH) (Figure 5) (40). However, approximately 20% of current HER2 testing may be inaccurate and there was significant variation among different laboratories (39). Therefore, the American Society of Clinical Oncology (ASCO) and the College of American Pathologists (CAP) have recommended guidelines in HER2 testing to ensure accuracy (42).

Briefly, breast cancer specimens initially undergo HER2 testing by a validated IHC assay (ie, HercepTest, Dako, Glostrup, Denmark) for HER2 protein expression (Figure 6) (39, 40). The scoring method for HER2 expression is based on the cell membrane staining pattern which gives the result of positive, equivocal or negative IHC (42).

If breast cancer specimens were scored with equivocal IHC (2+), the samples should then undergo validation using single-probe ISH assay (Figure 7) (42).

If the single-probe ISH raises an equivocal score, a dual-probe ISH assay will be performed. Dual-probe ISH use probes to detect both *HER2* copies and the centromere of chromosome 17 (chromosome enumeration probe 17, CEP17), which allows the determination of the HER2:CEP17 ratio (Figure 8) (41, 42).

In general, FISH testing gives more reliable results than IHC does, but it is more expensive (17). Other alternative methods for validating HER2 status, including reverse quantitative real-time polymerase chain reaction (qRT-PCR), have not yet been widely validated (40). For more complete views on the identification of HER2 positivity and

clinical challenges of each technique, readers are referred to recent reviews by Hanna et al (2014) and Perez et al (2014) (40, 41).

Figure 5. Detection of HER2 positivity in human specimens analyzed by different methods.

(**a**) IHC showing clusters of HER2-positive cells (brown staining).

(**b**) Double FISH/IHC staining showing the tumor cells displaying both HER2 protein over-expression (green membrane staining) and *HER2* gene amplification (red signals).

(**c**) Tumor analyzed by dual-probe ISH assay (HER2, black staining; CEP 17, red staining).

Abbreviations: CEP17, chromosome enumeration probe 17; FISH, fluoresecent *in situ* hybridization; IHC, immunohistochemistry; ISH, *in situ* hybridization.

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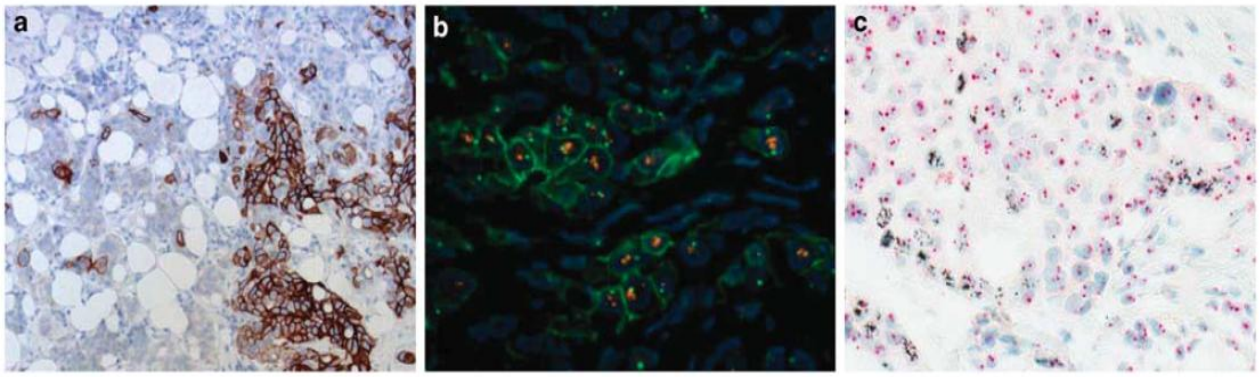

Figure 6. ASCO/CAP recommendations for evaluation of HER2 protein expression by IHC assay

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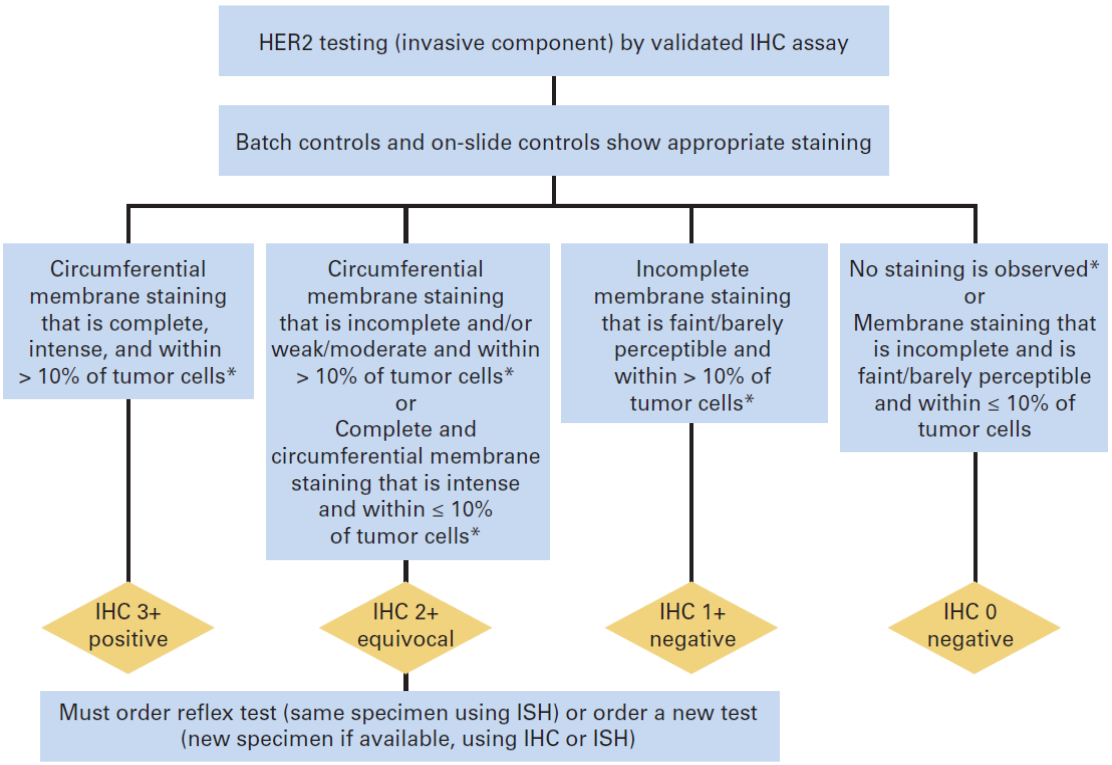

Figure 7. ASCO/CAP recommendations for evaluation of *HER2* gene amplification using single-probe ISH assay.

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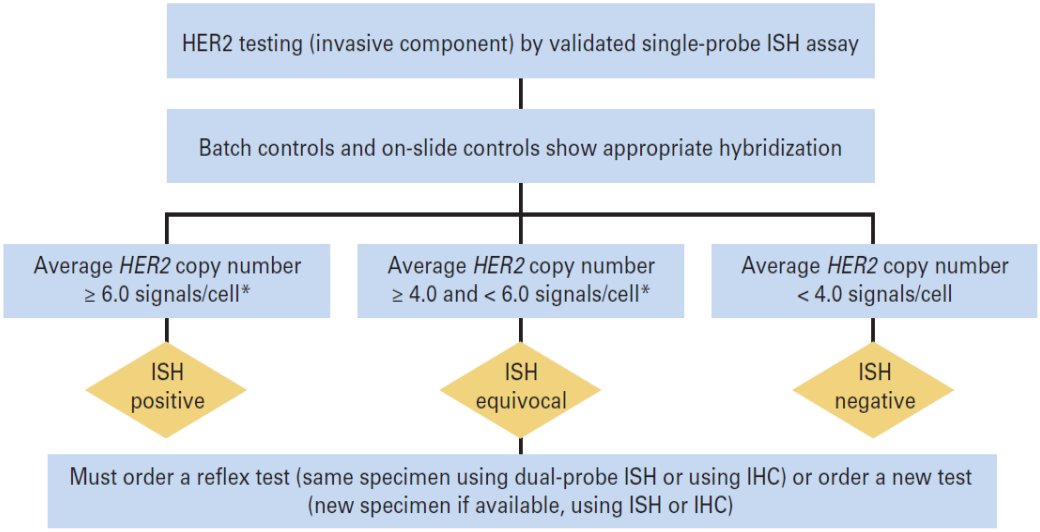

Figure 8. ASCO/CAP recommendations for evaluation of *HER2* gene amplification using dual-probe ISH assay.

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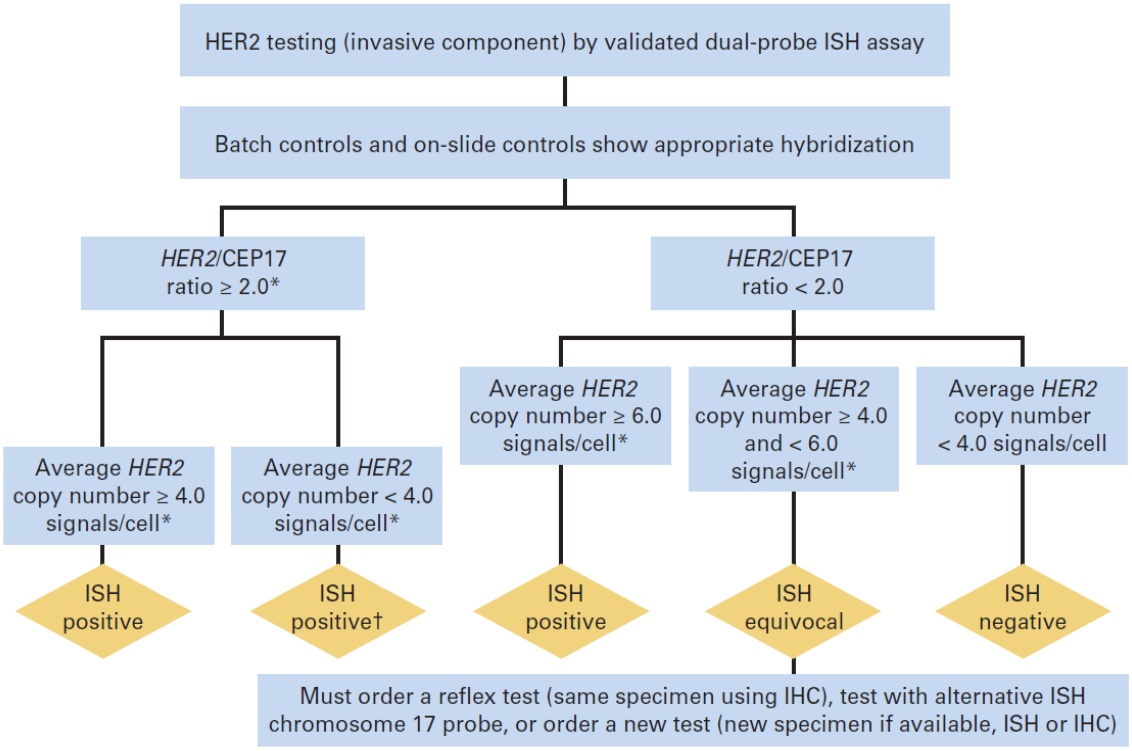

4. HER2 targeted treatment for HER2-positive breast cancer

The discovery of HER2 over-expression and its role in driving breast cancer progression led to numerous efforts to develop agents targeting HER2 and the other members of HER family. Currently in clinical use, approaches that target HER2 can be divided into 3 major types: monoclonal antibodies, drug-conjugated antibodies (DCAs), and small molecular tyrosine kinase inhibitors (TKIs) (Figure 9) (43).

Monoclonal antibodies bind to and prevent HER2 dimerization with itself or with other receptor tyrosine kinases (RTKs). The first targeted therapy which was approved by the Food and Drug Administration (FDA) was the humanized monoclonal antibody called trastuzumab (43). Clinical studies have shown that combinations of trastuzumab with standard chemotherapy produce far better response rates than does chemotherapy alone. The triumph of trastuzumab gave rises to another FDA-approved monoclonal antibody called pertuzumab and a FDA-approved DCA called T-DM1 (43).

T-DM1 consists of 1 molecule of trastuzumab which is covalently linked to 3 or 4 molecules of emtansine (DM) (Figure 9). DM alone is too toxic to be used in patients. However, T-DM1 targets this toxic compound specifically to HER2-over-expressing cells and thus spares the cells with low HER2 over-expression (43). In clinical setting, T-DM1 has been shown to increase progression-free survival and overall survival in patients with HER2+ advanced breast cancer. Moreover, T-DM1 showed a more favorable toxicity profile compared with trastuzumab plus docetaxel (43). For detailed information on T-DM1, including its activity, safety, and clinical trials, please see reviews of LoRusso's and Boyraz's (2011 and 2013, respectively) (44, 45).

TKIs are small molecules that can cross the cellular membrane and bind to the ATP-binding pocket of kinase receptors to inhibit their catalytic activities (Figure 9) (46). However, TKIs are far less specific than therapeutic antibodies and may be associated with a higher risk of toxicity. The first TKIs approved by the FDA for HER2+ metastatic breast cancer was lapatinib (46). Lapatinib potently inhibits the kinase activity of both HER1 and HER2. Besides lapatinib, other TKIs that are in active clinical trials are afatinib (BIBW-2992) and neratinib (HKI-272) (Figure 9). For more information on TKIs and related clinical data, please refer to Vu et al (2014) (46).

In my studies, I focused on the effects of trastuzumab in HER2+ breast cancer.

Figure 9. HER2 targeted therapy currently used in clinical practice for HER2-positive breast cancer.

There are currently 3 main approaches to inhibiting HER2 and its family members: 1) Monoclonal antibodies, including trastuzumab and pertuzumab to inhibit HER2 dimerization; 2) Small molecule tyrosine kinase inhibitors (lapatinib, neratinib, and afatinib) to inhibit HER2 kinase activity; and 3) drug-conjugated antibodies to selectively target toxic compounds to breast cancer cells over-expressing HER2.

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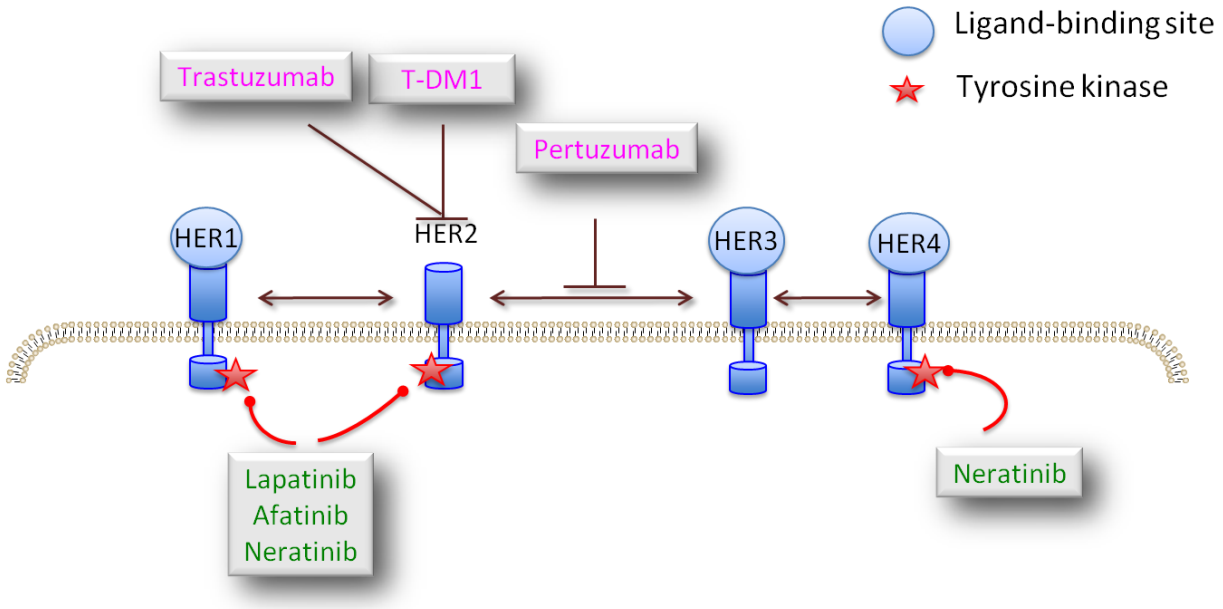

5. Impacts of trastuzumab in patients with HER2-positive breast cancer

5.1. The clinical impacts of trastuzumab in women with early HER2-positive breast cancer

Trastuzumab has proven synergism with a variety of chemotherapeutics on women with early HER2+ breast disease. In adjuvant (postoperative) setting, results from 5 large trials (HERA, FinHer, NSABP B-31, BCIRG006, N9831) have demonstrated that the addition of trastuzumab consistently reduced the risk of recurrence up to 50% and improve overall survival up to 30% (43, 46-50).

5.2. The clinical impacts of trastuzumab on women with HER2-positive metastatic breast cancer

In the metastatic setting, trastuzumab monotherapy was well-tolerated and resulted in objective response rate up to 34% for a median duration of 9 months (51, 52).

Later, a pivotal phase III trial compared standard chemotherapy alone (doxorubicin/epirubicin and cyclophosphamide or paclitaxel) with chemotherapy plus trastuzumab in patients with HER2+ metastatic disease. The findings demonstrated that compared with chemotherapy alone, trastuzumab plus chemotherapy was associated with significant improvements in time to disease progression (7.4 months vs 4.6 months), objective response rate (50% vs 32%), survival (median survival, 25.1 vs. 20.3 months), and a 20% reduction in the risk of death (53).

Furthermore, trastuzumab in combination with other HER2-targeted therapies such as lapatinib and pertuzumab have been shown to critically prolonged disease-free survival time and improve overall survival in patients with HER2+ metatstatic breast cancer (54-58).

5.3. Side effects of trastuzumab

Trastuzumab is relatively non-toxic, especially when compared with chemotherapy (59). Whereas diarrhea and rash are the common side effects, the most serious effect of trastuzumab is cardiac dysfunction. Yet this symptom can be improved by using the standard medical management or by adjusting the dose and duration of trastuzumab (17, 49, 53, 59, 60).

In early clinical studies with trastuzumab, cardio-toxicity was observed in a small percentage of patients, though this toxicity was more notable in patients concurrently given anthracyclines (53). Therefore, it is recommended that trastuzumab should not be combined with anthracyclines for the treatment of patients with HER2+ breast tumors.

MOLECULAR MECHANISMS OF ACTIONS OF TRASTUZUMAB

Trastuzumab prevents HER2 activation, thus inhibits HER2-driven cell growth and proliferation through 2 well-studied mechanisms: (1) facilitating HER2 degradation (61) and (2) down-regulating PI3K/ AKT/ PTEN and Src pathways (Figure 10) (62). My studies focus on the effects of trastuzumab on these pathways.

Figure 10. Effects of trastuzumab on the downstream cascades of HER2 activation.

Upon HER2 dimerization and activation, PI3K is triggered leading to an increase in the cellular levels of PIP3. PIP3 binds to and activates other kinases, such as PDK1 and AKT. AKT, in turn, causes rapid degradation of p27, resulting in cell survival and drug resistance. PTEN, antagonizes HER2 signaling by de-phosphorylating PIP3 and p-Src. Trastuzumab, by inhibiting HER2 dimerization, turns off PI3K/AKT and Src signalings, promotes PTEN function, and subsequently inhibits cell proliferation and tumor progression. Smooth lines represent direct effects; dash lines represent indirect effects. Abbreviations: AKT, protein kinase B; PDK1, 3-phosphoinositide-dependent protein kinase-1; PI3K, class IA phosphatidylinositol 3-kinase; PIP2, phosphatidylinositol 4,5 bi-phosphate; PIP3, phosphatidylinositol 3,4,5 tri-phosphate; PTEN, phosphatase and tensin homolog deleted on chromosome 10.

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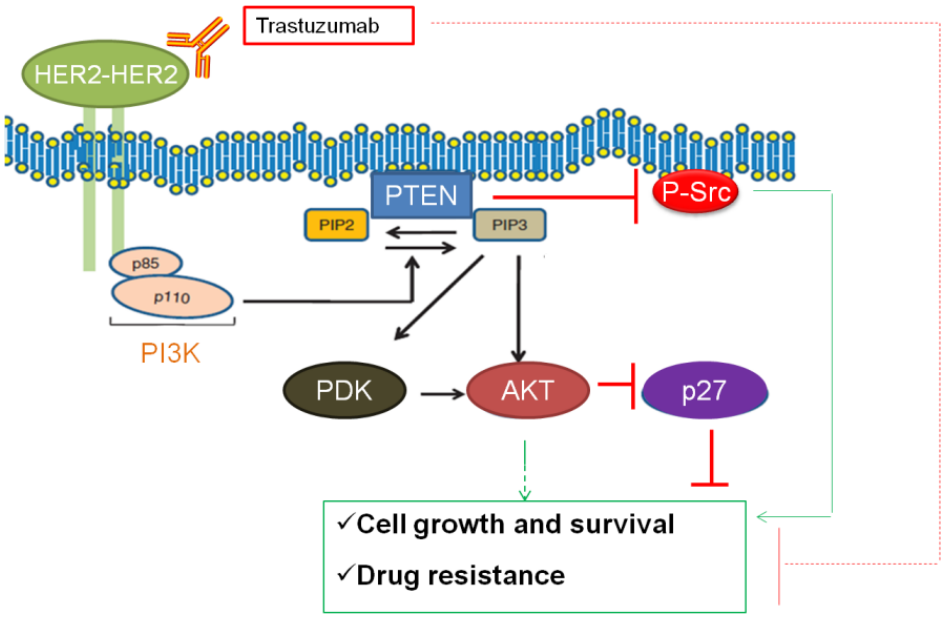


1. PI3K/AKT/PTEN and Src signaling – The major downstream cascades of HER2 activation

1.1. PI3K/AKT pathway

- Class I phosphatidylinositol 3-kinases (PI3Ks)

PI3Ks are heterodimers composed of p110 catalytic and p85 regulatory subunits (Figure 10) (64).

In response to HER2 dimerization and activation, PI3K is recruited to the membrane by direct interaction of its p85 subunit with activated HER2s. The interaction activated p110 catalytic which in turn phosphorylates phosphatidylinositol 4,5 bi-phosphate (PIP2) at the 3-position of the inositol ring, converting it to phosphatidylinositol 3,4,5 tri-phosphate (PIP3). PIP3 acts as a docking site for proteins that contain the pleckstrin homology domain, such as the protein kinase B (AKT) and 3-phosphoinositide-dependent protein kinase-1 (PDK1) (Figure 10) (64).

The PI3K pathway is frequently activated in human cancer which provides the cancer cells survival and proliferation advantages (65, 66). For a comprehensive review about PI3K regulation and function, readers are referred to Liu et al (2009) (64).

- Protein kinase B (AKT)

AKT, a serine–threonine kinase, is the key effecter of PI3K signaling in cancer (63). Once PIP3 is generated by PI3K, AKT is translocated to the membrane and dock its pleckstrin homology domain to PIP3 (Figure 10) (64, 67). The resulting conformational change in AKT at the membrane allows AKT to be phosphorylated at

serine 473 (S473) and get fully activated (68, 69). Once AKT has been activated, it can phosphorylate other target proteins, including p27, which results in rapid p27 degradation (Figure 10) (64, 70). p27 is a critical component of the cell-cycle machinery which controls cells' exit from G1 phase and entry into S phase during development and tumorigenesis (71). Therefore, activated AKT potently promotes cell cycle progression and subsequently cell proliferation (70, 72).

Increase in AKT activation which is reflected through the increase in p-AKT-S473 has been found to be correlated with HER2 over-expression in human breast carcinoma. Moreover, AKT activation is the direct consequence of activating mutation in PI3K or through the loss of function of the tumor suppressor gene, PTEN (64).

1.2. Phosphatase and tensin homolog deleted on chromosome 10 (PTEN)

- PTEN structure

The PTEN tumor suppressor gene, also referred to as *MMAC* (mutated in multiple advanced cancers), was identified by homozygous deletion mapping of the human chromosome 10q23 in cancer (73, 74). PTEN protein contains 403-amino acid which has dual-specific tyrosine and lipid phosphatase functions (74, 75).

PTEN structure is composed of 4 domains: the PIP3 binding domain (PBD), the phosphatase domain, the C2 domain and the C-terminal tail (Figure 11) (75). The extreme N terminus of PTEN resides in the PBD which is essential for membrane localization and PTEN catalytic activity. Next to the PBD is the phosphatase domain which has the catalytic pocket (75). The C2 domain is required for PTEN to bind to the phospholipid membranes (76). The phosphatase and C2 domains together make up

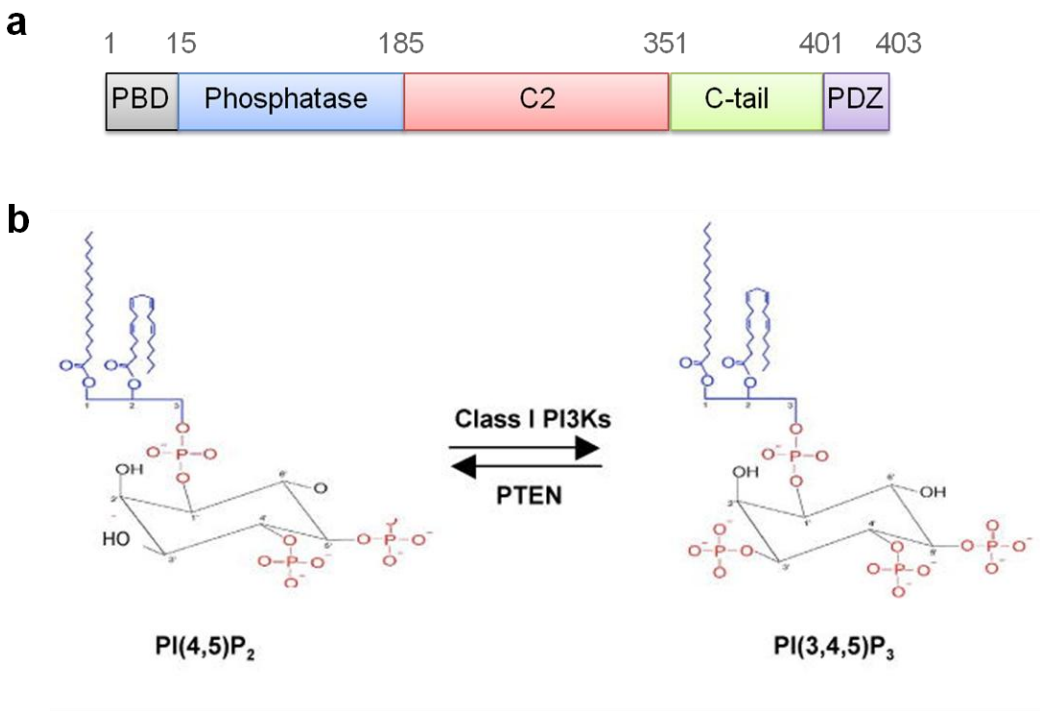
the minimal catalytic region of PTEN (75, 77). The C-terminal tail, which contains a PDZ (PSD-95, Discs-large, ZO-1)-binding domain, is important for the regulation of PTEN stability and its interaction with other proteins (75, 76, 78, 79).

Figure 11. Schematic representation of PTEN structure and function.

(**a**) PTEN contains 4 distinct domains: the PBD, the phosphatase domain, the C2 domain and the C-terminal tail with the PDZ motif.

(**b**) PI3Ks phosphorylate PI(4,5)P2 to generate PI(3,4,5)P3. PTEN antagonizes PI3Ks effect by removing phosphate group on PI(3,4,5)P3 and converting it back to PI(4,5)P2. Modified with permission from Nat Rev Drug Discov, Liu, Cheng, Roberts, and Zhao, copyright 2009 (64)

Abbreviations: PBD, PIP3 binding domain; PDZ (PSD-95, Discs-large, ZO-1)-binding domain.



❖ PTEN functions as a lipid phosphatase

The lipid phosphatase function of PTEN is regulated through its binding to the plasma membrane (75, 79, 80). The association step results in a conformational change of PTEN and activation of its phosphatase domain to convert PIP3 to PIP2 (Figure 11) (77, 80, 81). The trigger for PTEN dissociation is unknown.

The PIP3 generated by PI3Ks, is therefore, tightly regulated by PTEN (77, 81). The biological link between the PI3K pathway and PTEN was demonstrated by the finding that deletion of PTEN elevated intracellular PIP3 levels and thus activated AKT in numerous cell types including breast cancer cells (77, 81-83). Therefore, the loss of PTEN results in unrestrained signaling by the PI3K/AKT pathway (81). As such, PTEN with its lipid phosphatase function negatively regulates many cellular processes, including cell proliferation and survival (Figure 10) (63, 81).

❖ PTEN functions as a tyrosine phosphatase

PTEN is well-studied for its lipid phosphatase function, yet there are few research investigating the regulation of PTEN tyrosine phosphatase. It was recently found that in breast cancer cell lines, PTEN loss was associated with an increase in the phosphorylation of the oncogenic Src at tyrosine 416 (Y416). The study also demonstrated that PTEN and Src proteins interacted and that PTEN de-phosphorylated p-Src-Y416 peptide *in vitro* (84).

❖ Post-translational regulation of PTEN stability

The activity of PTEN is regulated at many levels, including DNA methylation, transcriptional regulation, micro-RNA-directed mRNA degradation, posttranslational

modifications (phosphorylation and ubiquitination) or through protein-protein interaction (Figure 12) (63, 85, 86). These mechanisms are modulated to maintain appropriate PTEN activity, thereby regulating the cellular content of PIP3, p-AKT and p-Src (86, 87).

In my studies, I focused on the post-translation regulation of PTEN, specifically through phosphorylation and ubiquitination.

Figure 12. Regulation of PTEN stability and function by different mechanisms.

PTEN can be regulated through epigenetic modification such as DNA methylation of PTEN promoter. At the transcriptional level, PTEN can be regulated by multiple transcription factors. PTEN mRNA can also be mediated by micro-RNA. The post-translational regulation of PTEN includes protein-protein interaction, ubiquitination, oxidation, and phosphorylation which affect the activity and function of PTEN.

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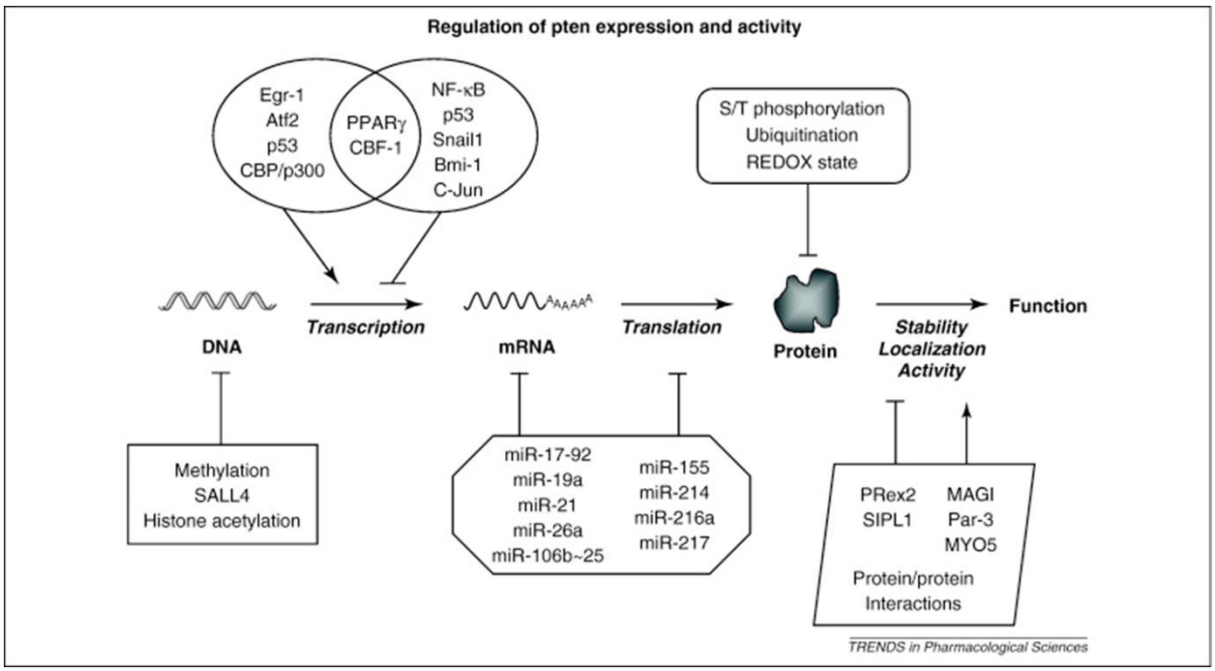

- Regulation of PTEN through phosphorylation

Under normal condition, PTEN exists in the “closed” conformation in the cytoplasm, in which, its C-terminal tail “masks” the C2 domain (Figure 13) (78, 88). This C-tail contains a cluster of serine and threonine residues, which are phosphorylated by casein kinase 2 (89). Specifically, phosphorylation of serine 380 (S380), threonine 3802/383 (T382/383) in the C-tail of PTEN appears to keep PTEN in the “closed” and less active state (78, 90). De-phosphorylation or alanine mutation of these sites (S380A, T382A, and T383A) “opens” PTEN and leads to its translocation to the membrane (80, 90). The “open” conformation results in an elevation in PTEN lipid phosphatase activity yet provokes a decrease in PTEN's stability, in comparison with “closed” PTEN (Figure 13) (78, 80, 90).

- Regulation of PTEN by poly-ubiquitination

Many studies have currently supported a dominant role of the ubiquitin proteasome pathway in controlling PTEN's stability (Figure 13). They found that treatment of cancer cells with proteasome inhibitors enhanced the levels of PTEN protein. Also, several E3 ligases for PTEN have been proposed (86, 87). Three such candidates are NEDD4-1 (neural precursor cell-expressed developmentally down-regulated protein 4), WWP2 (WW domain-containing protein 2), and XIAP (X-linked inhibitor of apoptosis protein). NEDD4-1 is the first proposed E3 ligase for PTEN and promotes PTEN degradation through ubiquitin-proteasome system in non-small cell lung cancer (Figure 13) (91, 92). Moreover, NEDD4-1 was found to facilitate PTEN mono-ubiquitination which mediates PTEN import to the nucleus (91, 93). However, the exact mechanism and the nucleus function of PTEN regulated by NEDD4-1 are unclear. WWP2, a NEDD4-like E3 ubiquitin ligase, was found in a pull-down analysis

for interacting partners of PTEN. Endogenous WWP2 interacts with and poly-ubiquitylates PTEN, then targets PTEN for degradation (94). XIAP is a member of the inhibitor of apoptosis family of proteins, was discovered to regulate PTEN poly-ubiquitination and degradation in breast cancer (95). Interestingly, the group who discovered WWP2 could not confirmed the previous conclusion that NEDD4 was an E3 ligase for PTEN (94). Therefore, it seems that much remains to be discovered regarding how PTEN ubiquitination is controlled and if it is cell context dependent.

Figure 13. Regulation of PTEN function and activity by phosphorylation and ubiquitination.

Cytosolic phosphorylated PTEN is stable but display minimal activity. Once PTEN is de-phosphorylated and recruited to the membrane, PTEN is more active but less stable compared to the cytosolic PTEN. On the other hand, PTEN turnover can be regulated by ubiquitination, specifically through interacting with an E3 ligase, NEDD4-1. Poly-ubiquitylated PTEN remains in the cytoplasm and is targeted for degradation by the proteasome, whereas mono-ubiquitination of PTEN translocates PTEN into the nucleus, where its function remains unclear.

Abbreviation: NEDD4-1, neural precursor cell-expressed developmentally down-regulated protein 4; P, phosphor group; Ub, ubiquitin.

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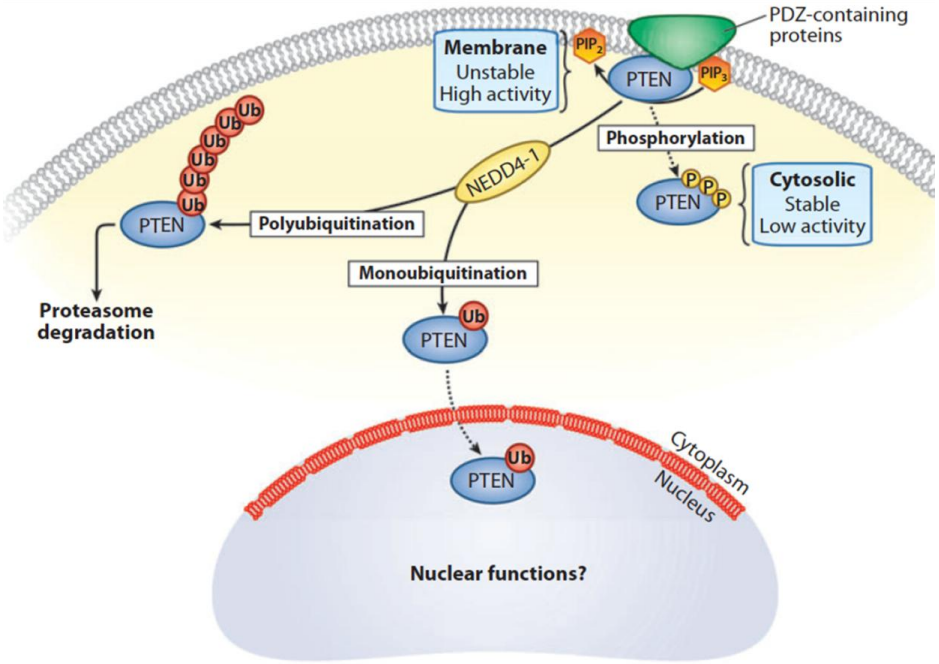

❖ The loss of PTEN in breast cancer

The importance of PTEN in cellular function is underscored by the frequency of its aberrant expression in various cancer types (63, 87).

PTEN mutation is most frequent in endometrial cancers, approximately 50% (~50%). However, only ~5% of PTEN is mutated in breast cancer (73, 87). On the other hand, PTEN protein is lost or reduced in about 48% of human invasive breast carcinoma which is correlated with poor prognosis (96, 97). In HER2+ breast cancer, low expression of PTEN is seen in ~25% of all cases (98). The other group found that the loss of PTEN was observed in 36% of HER2+ breast cancer patients with stage IV disease (99).

It is interesting to note that, no germline or somatic mutations have been detected specifically in the phosphorylation cluster (S380/T382/383) at the C-tail of PTEN (100). There are two proposed explanations for these observations. One hypothesis is that the presence of dominant negative mutations within PTEN tail is embryonic lethal. The other hypothesis is that these mutations are not germane to carcinogenesis (100).

In general, given the important role of PTEN in tumor progression, subtle variation in PTEN expression has been experimentally demonstrated to have significantly impacts on tumor susceptibility and progression (Figure 14) (101, 102).

Figure 14. Correlation of PTEN expression levels and tumorigenesis.

Complete or partial loss of PTEN differently affects the outcomes of breast cancer.

Abbreviations: PD, poorly differentiated; WD, well-differentiated.

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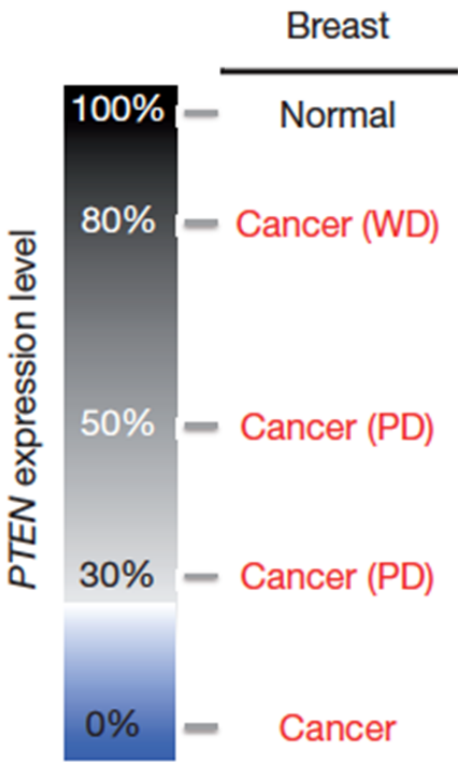


1.3. Src

Src is a protein product of *c-Src* (Src) gene belongs to the Src family of kinases (SFKs), a group of non-RTKs. The *Src* gene, discovered in the 1970s, is the oldest 'proto-oncogene' in mammalian cells (103).

The most well-studied function of Src is its interaction with activated transmembrane RTKs, including activated HER2 (103-105). Through its interactions, Src directly transduces survival signals to downstream effectors such as PI3Ks and AKT (105). Therefore, Src is a potent transforming and tumorigenic factor and its activation is seen in many human tumor types, including HER2-over-expressing breast cancer (103).

For comprehensive reviews about Src regulation and function, readers are referred to Yeatman (2004) and Zhang et al (2012) (103, 105).

2. Molecular effects of trastuzumab on PI3K/AKT/PTEN and Src signaling

A well-known proposed mechanism of trastuzumab action is the elevation in PTEN function accompanied by the reduction in PI3K/AKT and Src signaling.

Several groups have demonstrated that trastuzumab specifically disrupts HER2-Src interactions, leading to inactivation of Src. This, in turn, causes increased PTEN phosphatase activity which results in de-phosphorylating AKT at S473 and Src at Y416. Moreover, by inhibiting HER2 activation, trastuzumab abolished HER2-activated PI3K activity, which also reduces p-AKT-S473 level (table 2).

Table 2. Molecular effects of trastuzumab on the expression levels of PI3K/AKT/PTEN and Src.

	HER2 activation	Trastuzumab treatment
PI3K	↑	↓
p-AKT-S473	↑	↓
p-Src-Y416	↑	↓
PTEN	↓	↑

MECHANISM OF TRASTUZUMAB RESISTANCE

1. Overview of trastuzumab resistance

Though trastuzumab with or without chemotherapy has significantly prolonged patients' survival, data from large randomized trials showed that about 50% of patients did not respond to trastuzumab with anthracycline and cyclophosphamide (53). Also, about 74% of patients did not experience a response to first-line trastuzumab monotherapy (52). This can be referred to as primary resistance (43).

Moreover, secondary resistance to trastuzumab eventually developed in a large number of patients who initially responded (43). In an adjuvant setting, about 70% of patients with early-stage HER2+ breast cancer relapse after one year of initial treatment (47).

Despite numerous studies trying to identify the resistant mechanism and find ways to overcome it, the molecular mechanisms underlying the resistance are not well defined. Some of the compensatory pathways and downstream signaling aberrations such as loss of PTEN function or increase in Src activation have been implicated.

2. The loss of PTEN and the constitutive active AKT in conferring trastuzumab resistance

Nagata and colleagues (2004) was the first to show that patients with PTEN-deficient tumors had markedly lower overall response rates to trastuzumab plus taxane than did patients with wild-type PTEN (35.7% vs. 66.7%) (99). The group also find that

the loss of PTEN which led to constitutive active AKT significantly contributed to both inherent and acquired resistance to trastuzumab (99). Later, other group demonstrated that in tumor specimens from metastatic breast cancer patients who were treated with trastuzumab, the loss of PTEN was significantly correlated with the poor response rate to this therapy (106). In contrast, restoration of PTEN expression suppressed tumor formation and progression and enhanced the anti-proliferative function of trastuzumab (83, 98, 107).

3. The role of Src in promoting trastuzumab resistance

Zhang and colleagues (2011) were the first to identify that Src signaling was significantly up-regulated in both primary and acquired trastuzumab resistance. Furthermore, they found that PTEN deficiency also led to Src hyperactivation which in turn promoted trastuzumab resistance (84). However, the correlation between Src activation and trastuzumab resistance has not been supported in clinic, and the trials using Src inhibitor in combination with trastuzumab have not yielded any significant results.

JAB1

1. Overview of Jab1 and its structure

Jab1 (c-Jun activation domain-binding protein-1) protein, encoded by a *COPS5* gene, was initially identified as a coactivator of c-Jun, a transcription factor involving in

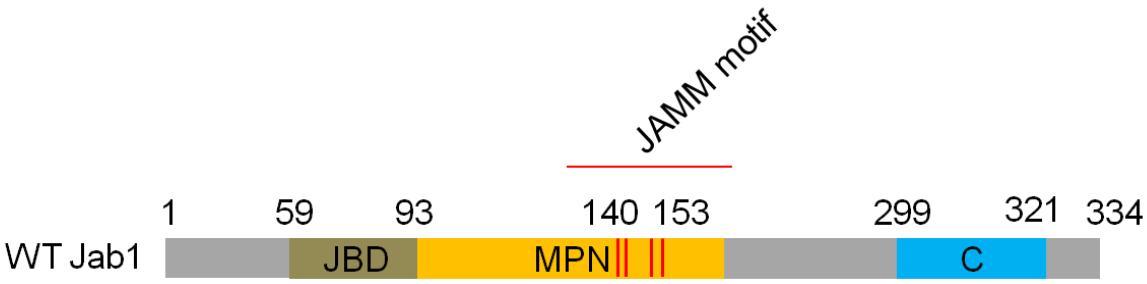
numerous cellular processes, such as cell proliferation and tumorigenesis (108, 109). Later, Jab1 was discovered to be the fifth component of the photomorphogenic-9 (COP9) signalosome (CSN) complex which was called CSN5. CSN is a large protein complex which regulates multiple cellular processes in both mammalian and plant cells (110). To date, more than 20 proteins have been identified to interact with Jab1, including other CSN subunits, which suggest the diverse roles of Jab1 in regulating cellular processes (109, 111).

Jab1 protein contains 3 distinct domains, namely c-Jun binding domain (JBD), Mpr1-Pad1-N-terminal (MPN) domain and the C-terminal domain (Figure 15) (109, 112). The MPN domain has the metalloenzyme motif (JAMM) which is essential for mediating Jab1 interaction with other proteins and for its isopeptidase activity (110-112).

Figure 15. Jab1 structure.

Jab1 protein is of 334-amino-acid long with 3 identified domains: JBD domain, MPN domain and unidentified C-terminal domain. Within the MPN domain, positions of the residues forming the JAMM motif are indicated by red lines.

Abbreviations: JAMM, JAB1/MPN/Mov34 metalloenzyme; JBD, c-Jun binding domain; MPN, Mpr1p, Pad1p-N-terminal.



2. Jab1 as a coactivator of c-Jun

Jab1 potently enhances the transcriptional function of c-Jun by physically interacting with c-Jun protein (Figure 16) (108). c-Jun is a well-studied oncogene whose aberrant expression and activity have been detected in numerous cancer, including breast tumors. Over-expression of Jab1, therefore, aggressively drives c-Jun activity to promote tumorigenesis (Figure 16) (108).

3. Jab1 as a negative regulator of p27

Jab1 directly binds to p27 and induces its nuclear export and subsequent degradation (Figure 16) (113, 114). Our group previously examined the relationship between Jab1 and p27 and their association with prognosis in clinical specimens of invasive breast carcinoma (115, 116). We found that in the group of tumors that had elevated Jab1 protein levels, p27 protein expression was significantly down-regulated ($p = 0.02$). Moreover, we noticed that breast tumors with high p27 and low Jab1 protein expressions showed better prognosis compared with tumors with low p27 and high Jab1 protein levels. The correlations between reduced expression of p27 and over-expression of Jab1 were also observed in other human tumors and were comprehensively reviewed by Shackleford and Claret (2010) (109). These findings confirm that Jab1 is a potent negative regulator of p27 in different cancer types.

4. Jab1 as a member of the CSN with isopeptidase activity

The CSN is a multisubunit complex located mainly in the nucleus and a regulator of various cellular and developmental processes, including cell-cycle control, transcription, and DNA-damage response (110, 117). The CSN consists of 8 subunits, namely CSN-1, -2, -3, 4, -5, -6, -7 and -8 in mammals (109, 117).

Jab1 is one of two members of the CSN that contains the MPN domain (the other one is CSN6) (110-112). Jab1 MPN domain contains a metalloprotease motif referred to as JAMM (Jab1/MPN/Mov34 metalloisopeptidase) or MPN+ motif (Figure 15) (118). The MPN domain of CSN6 does not contain a JAMM motif (110, 111, 117).

The JAMM motif is involved in zinc ion coordination and provides the active site for isopeptidase activity. JAMM motif can catalyze ubiquitin from ubiquitinated proteins that are tagged for degradation (110, 112, 117, 118). Thus, Jab1 with JAMM motif was proposed to have deubiquitinating activity; yet it has not been experimentally validated (111). Mutations in the conserved histidine (His) or aspartic acid (Asp) residues of the Jab1/JAMM motif abolish this activity (110, 118, 119).

5. The role of Jab1 in cancer progression

Besides p27, Jab1 can mediate the nuclear export and cytoplasmic degradation of the tumor suppressor p53 (Figure 16) (120, 121). Through these mechanisms, Jab1 over-expression has been demonstrated to drive cell proliferation, inhibit apoptosis, and impair cellular DNA damage repair (109, 121, 122).

Transgenic mouse model with over-expression of Jab1 showed enhanced proliferation (123). In contrast, inhibition of Jab1 resulted in delayed tumor growth in murine xenografts (124).

Moreover, analysis from specimens of patients with invasive breast carcinoma showed that Jab1 expression was elevated in 50% of primary breast tumors and 90% of metastatic lesions. However, its expression is low or absent in normal adult breast tissue (116).

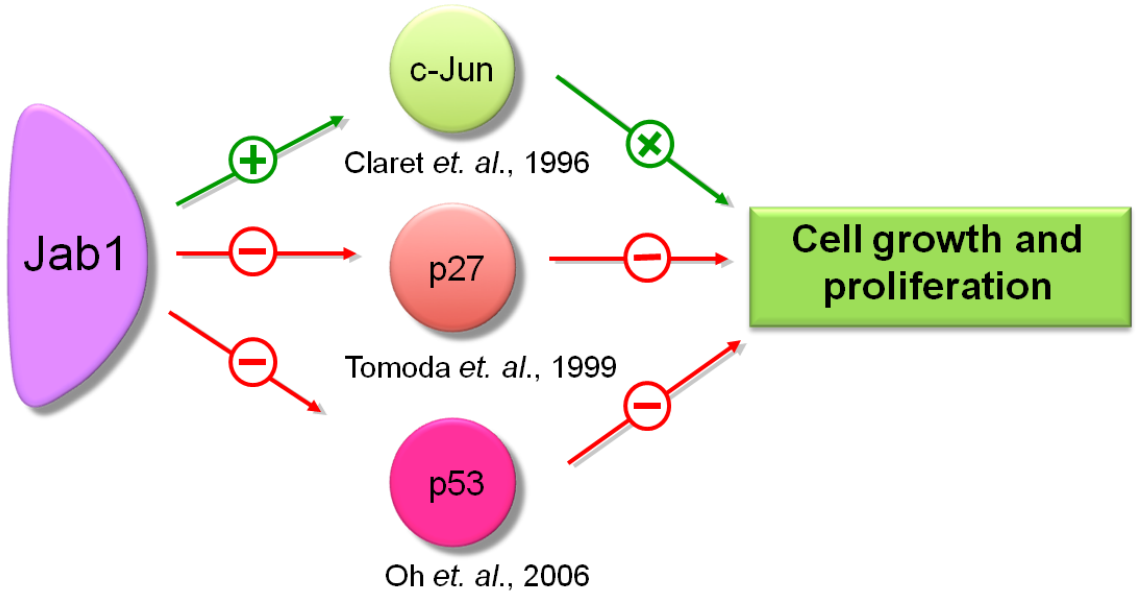
In conclusion, Jab1, through working as part of the CSN holocomplex and independently of the CSN, promotes cell growth and survival (Figure 16). Furthermore, high expression of Jab1 was associated with poor outcome in breast cancer patients. For comprehensive summary of Jab1 activity, its target proteins and its role as a part of CSN complex, please refer to Wei et al (2008) and Terry et al (2010) (109, 110).

Figure 16. Simplified model of direct targets and functions of Jab1.

Jab1 was first discovered to bind to and enhance c-Jun transcription function. Later, Jab1 was found to associate with and promote rapid degradation of p27 and p53 through proteasomal pathway. With these functions, Jab1 potently facilitates tumor cell growth and proliferation.

Green arrows and plus (+) signs indicate Jab1-positive interaction; red arrows and minus (-) signs indicate Jab1-negative interaction.

Modified from Cell Div, Shackleford and Claret, 2010 (109)



TWO MAJOR MECHANISMS TO DEGRADE INTRACELLUALR PROTEINS

Protein degradation, as well as synthesis, is essential to normal activity of the cell. To degrade intracellular proteins, all eukaryotic cells have two major mechanisms, namely the ubiquitin–proteasome system (UPS) and the lysosomal proteolytic pathway (125). The proteasome mainly degrades ubiquitinated proteins. The lysosomal proteolytic pathway, in contrast, does not require protein to be ubiquitinated for degradation and can degrade other macromolecules and cell organelles.

THE LYSOSOMAL PROTEOLYTIC PATHWAY AND PROTEIN DEGRADATION

1. Overview of the lysosomal proteolytic pathway

Lysosomes are acidic (pH 4.5–5) catabolic organelles found in all mammalian cells except for mature erythrocytes. The lysosome is often described as a ‘‘cellular garbage can’’ that is responsible for the disposal and recycling of old-age or damaged cellular macromolecules and organelles (Figure 17). After the targets are digested by lysosomal enzymes, the products are recycled back to the cytosol via diffusion and specific transport channels or released to the extracellular space by exocytosis (Figure 17) (126).

Lysosomes contain more than 50 hydrolases including proteinases, phosphatases, nucleases, and lipases, which function only in the highly acidic pH inside the lysosomal lumen (126).

Lysosomal membrane components are not well characterized (126). In general, they are composed of integral membrane protein 2 (also known as SCARB2), vacuolar proton pump ATPase (VP-ATPase), transport proteins, and membrane-associated proteins such as lysosomal-associated membrane proteins 1 and 2 (LAMP-1 and LAMP-2). VP-ATPase is ubiquitously expressed and drives protons against their electrochemical gradient into the lysosome in an ATP-dependent manner. VP-ATPase is critical for the maintenance of acidic pH inside lysosome (126). LAMP-1 and LAMP-2, single span transmembrane proteins, make up approximately 50% of the lysosomal transmembrane proteins and play pivotal role in maintaining the structural integrity of the lysosomal compartment. In addition, LAMPs also regulate lysosomal trafficking and exocytosis (126).

Figure 17. The role of the lysosome in clearing cytosolic components.

Accumulated macromolecules or damaged organelles are delivered to lysosome for degradation. Lysosome can break down proteins, lipid, and glycogen for the synthesis of new proteins and production of energy. Levels of amino acids, free fatty acids, and sugars circulating in blood or in the extracellular media have a direct impact on the function of lysosome.

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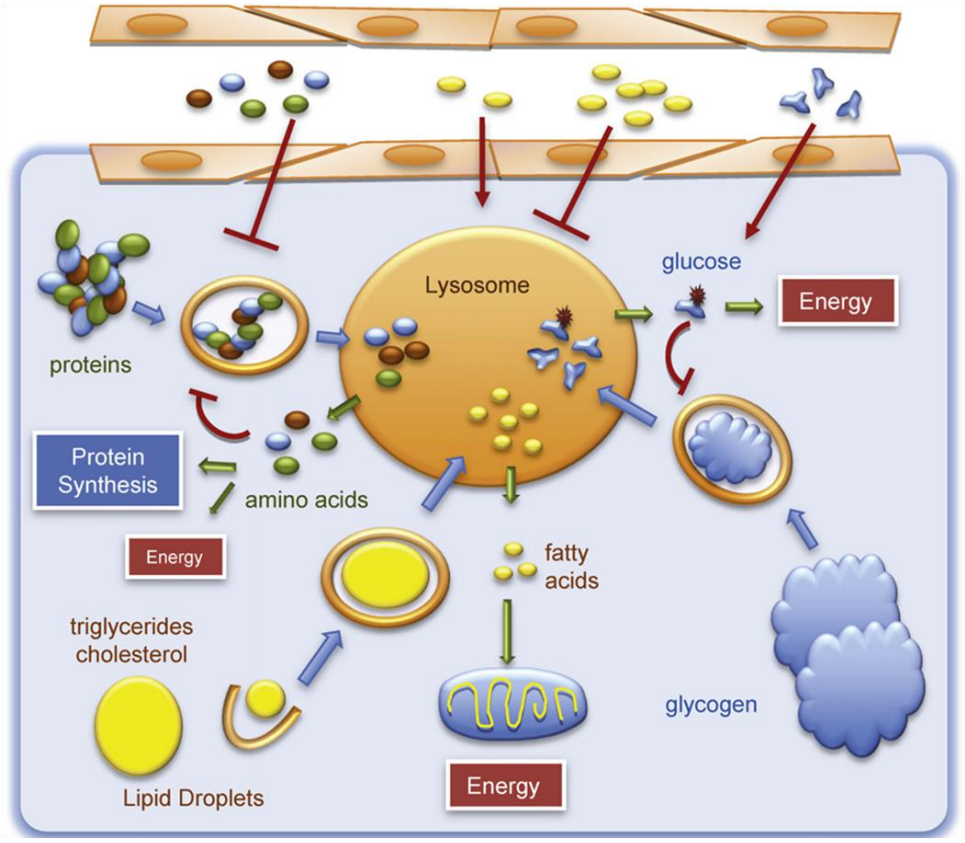


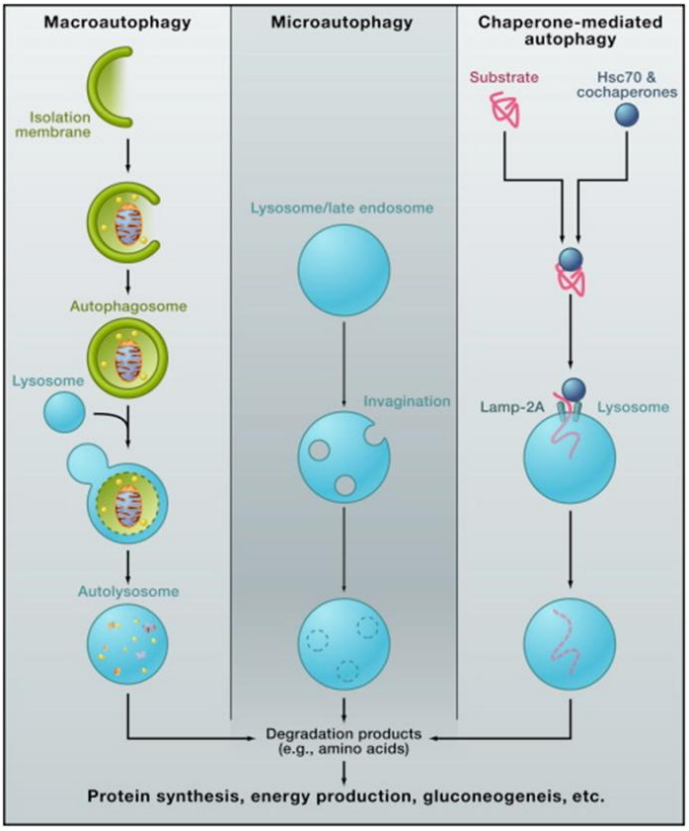
Currently, there are three basic ways to deliver substrates to lysosome for degradation (128). They are macro-autophagy (or autophagy), micro-autophagy, and chaperone‑mediated autophagy (CMA) (Figure 18). Two of them, autophagy and micro-autophagy, are high-capacity processes that can directly sequestered multiple cytosolic components (soluble proteins and organelles) and degrade them in the lysosomal lumen. In contrast, CMA only modulates the lysosomal degradation of specific cytosolic proteins on a one-by-one basis (128). The three pathways and their comprehensive physiological and pathological relevance have been extensively reviewed in Sridhar et al (2012), Mizushima et al (2011) (128, 129).

Figure 18. Three major types of the lysosomal proteolytic pathway.

Macro-autophagy (autophagy) is characterized by the formation of autophagosome which then fuses with the lysosome to form autolysosome where all internal materials are degraded. In micro-autophagy, small pieces of the cytoplasm are directly engulfed by inward invagination of the lysosomal membrane. Chaperone-mediated autophagy, the most selective form, only degrades proteins and is mediated by the cytosolic Hsc70 and the lysosomal membrane LAMP-2A. After all, resultant degradation products can be used for new protein synthesis, energy production, and gluconeogenesis.

Abbreviations: Hsc70, heat shock cognate protein of 73 kDa; LAMP-2A, lysosomal-associated membrane proteins.

Adapted with permission from Cell, Mizushima and Komatsu, 2011 (129)



2. Macro-autophagy (autophagy)

2.1. Overview of autophagy

Autophagy, the most well-studied form of lysosomal proteolytic pathway, is an essential physiological process for cell survival during starvation (Figure 18) (129).

Autophagy was first described as mechanisms for ‘in bulk’ degradation. However, emerging evidence suggests that there is selective form of autophagy, specifically in degrading intracellular proteins, which will be described below. In the selective autophagy, ubiquitinated protein are recognized by a specific subset of proteins known as cargo-recognition proteins such as p62 (also known as sequestosome 1) (128, 130, 131).

Genetic screenings of autophagy in yeast have identified that more than 30 different genes (known as ATGs or autophagy-related genes) participate in the execution and regulation of macroautophagy. Among them, ATG1–10, 12–14, 16, and 18 are considered the ‘‘core ATG proteins’’ and are required for autophagosome formation (129, 132-135).

2.2. The selective autophagy process

Autophagy is a multi-step process to degrade a substrate protein which can be divided in three main steps: (1) initiation and phagophore formation, (2) autophagosome formation and substrate sequestration, and (3) fusion of autophagosome and lysosome and the degradation of the substrates (127, 129, 134).

❖ Initiation and phagophore formation

Cellular stresses, including reactive oxygen or reactive nitrogen species (ROS, RNS), or nutrition starvation activate AMP-activated protein kinase (AMPK) and inhibit mammalian target of rapamycin (mTOR) to signal autophagy through ATG1-containing complex (127). Once initiated, a kinase complex formed by Beclin-1 (autophagy-related Bcl2-interacting ATG6 homolog) and VPS34 (vacuolar protein sorting 34) triggers downstream events leading to activation and formation of ATG12/ATG5 complex (127, 133, 134). The resulting ATG12/ATG5 conjugate forms a complex with a homodimer of ATG16, which assembles on a membrane structure termed the phagophore or isolation membrane (Figure 19) (127, 132-134).

❖ Autophagosome formation and substrate sequestration

After the phagophore is formed, ATG8 (a yeast homolog of mammalian microtubule-associated protein light chain 3, LC3-I) is activated by cascade of other ATG proteins. ATG8/LC3-I is then conjugated onto phosphatidylethanolamine (PE) in the lipid bilayer of the membrane. This reaction results in the formation of lipidated LC3-I called LC3-II or ATG8–PE, in yeast (Figure 19) (134, 136).

The growing ends of the phagophore eventually meet and fuse to form the double-membrane structure of the autophagosome. LC3-II is present on both surfaces of the isolation membrane (134, 136). ATG12/ATG5/ATG16 preferentially localizes on the outer surface of the membrane and dissociates from the membrane upon completion of the autophagosome (Figure 19) (134).

In selective autophagy, the phagophore engulf ubiquitinated proteins through p62 (131, 137). p62 sequesters target proteins to the autophagosome through directly association with LC3 (137, 138). Impairment of autophagy from this step is therefore, partially reflected by the accumulation of p62 (129, 139, 140).

- Fusion of autophagosome and lysosome and the degradation of target substrate

Autophagosome finally fuses with a lysosome to form autolysosome. Inside autolysosome, the infusion of hydrolases and the acidification results in the complete degradation of cargo into their constituent components, including LC3-II and p62 as well as p62-conjugated proteins (135).

The acidification is mediated by the VP-ATPase. Inhibition of the activity of VP-ATPase by bafilomycin A or concanamycin A blocks the lysosomal pumping of H+ resulting in less acidic pH environment and inhibiting functions of lysosomal enzymes (Figure 19) (140). Importantly, it was also proposed that bafilomycin A also blocks the fusion of autophagosomes with lysosomes though the mechanism is not fully understood. In general, the overall consequences of bafilomycin A is to prevent the degradation of sequestered materials, which is reflected by the accumulation of LC3-II and p62 (table 3) (140, 141). The summary of autophagy process is described in Figure 19.

Figure 19. Overview of selective autophagy and its inhibition.

Autophagy is triggered by increased ROS level leading to the formation of various ATG complexes and subsequently autophagosome. The fussion of autophagosome and lysosme forms autolysosome which degrades all the internal contents, including LC3, p62 and target ubiquitinated substrates.

Abbreviations: 3-MA, 3-Methyladenine; AMPK, AMP-activated protein kinase; ATG, autophagy-related gene; LC3, microtubule-associated protein light chain; RNS, reactive nitrogen specifies; ROS, reactive oxigen species; Ub, ubiquitin; VP-ATPase, vacuolar proton pump ATPase; VPS, vacuolar protein sorting.

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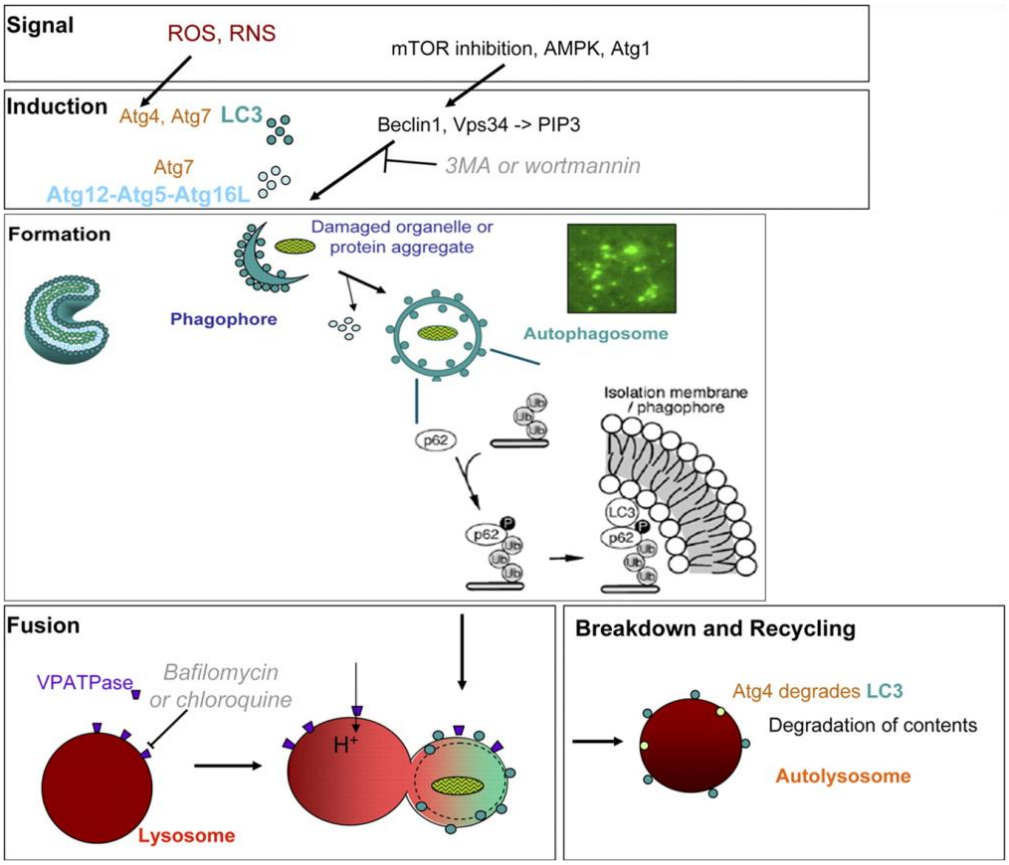


Table 3. Molecular effects of autophagy inhibition (140).

	Inhibition of late autophagy (Bafilomycin A)	Inhibition of autophagy initiation such as ATG5 depletion
ATG5	No change	Not detected
p62	↑	↑
LC3I/II	↓	Not formed

3. Micro-autophagy

Micro-autophagy is descrised as the sequestration process of a cytosolic region directly by the lysosomal membrance. The steps involve: (1) invagination of vacuole, (2) internalization of portions of cytsolic components, and (3) degradation of all the components (Figure 18) (129). Micro-autophagy also involves the direct engulfment of organelles, such as the peroxisome and the nucleus (127).

There have been some discoveries on yeast micro-autophagy. However, mammalian micro-autophagy remains a “mysterious” process due to the absence of mammalian homologs for the micro-autophagy genes discovered in yeast (127, 129, 143).

4. Chaperon-mediated autophagy (CMA)

4.1. Overview of the CMA

CMA is a highly selective form of autophagy that has only been described in mammalian cells (128). CMA plays a pivotal role in removing misfolded proteins and providing amino acids during prolonged periods of starvation. CMA is active in almost all cells, although basal and inducible levels of CMA activity vary depending on the cell types and cellular conditions.

Alterations of CMA have been linked to different human pathologies such as Parkinson’s disease, and several lysosomal storage disorders (144). However, little is known about the contribution of CMA to cancer biology. Recently, activation of CMA

has been found in various cancer cell lines and human tumor biopsies, including breast tumors. Moreover, suppression of CMA by knocking down LAMP-2A in lung cancer cells significantly reduced cancer cell proliferation and tumor growth (145).

4.2. The CMA process

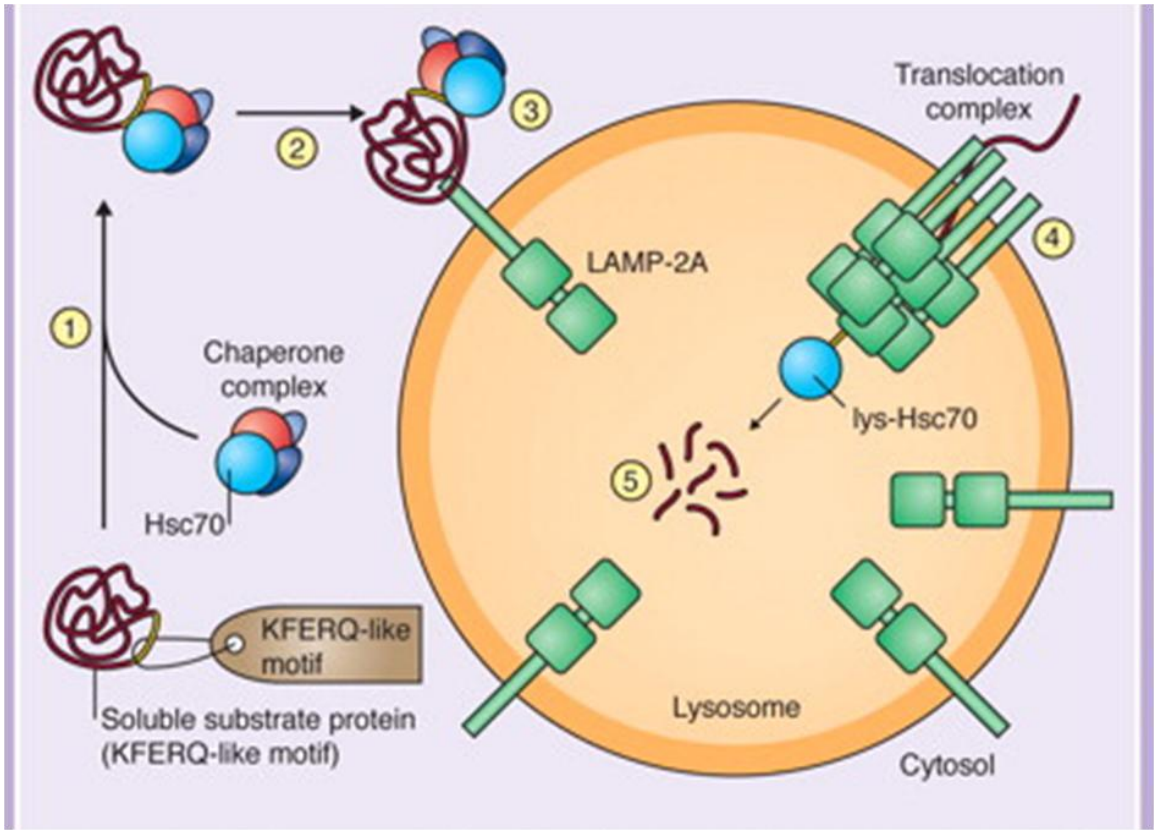
A protein gets degraded by CMA through five steps: (1) substrate recognition and lysosomal targeting; (2) binding of the substrate to LAMP-2A; (3) unfolding of substrate protein; (4) substrate translocation across the lysosomal membrane; and (5) substrate degradation in the lysosomal lumen (Figure 20) (146). All the first four steps take place in the cytosol.

Figure 20. Overview of the CMA process.

CMA is a five-step process in which a protein containing the KFERQ-like motif is first recognized by Hsc70. Hsc70 transports the protein to the LAMP-2A complex on the lysosomal membrane. The protein is translocated across the lysosomal membrane through LAMP-2A and lys-Hsc70; and finally gets degraded in the lysosomal lumen.

Abbreviation: lys-Hsc70, lysosomal-Hsc70.

Modified with permission from J Cell Sci, Kaushik, Bandyopadhyay, Sridhar, Kiffin, Martinez-Vicente, Kon, Orenstein, Wong, and Cuervo, 2011 (146)



❖ Substrate recognition and lysosomal targeting

A protein considered to be a CMA substrate has to contain a pentapeptide motif in its amino acid sequence which is biochemically related to the KFERQ-like motif (147, 148). KFERQ-like motifs have been found in 30% of cytosolic proteins (144, 147). By definition, KFERQ-like motif contains 5 types of amino acids: (1) a glutamine (Q) residue, at the beginning or end of the sequence; (2) one of the two positively charged amino acids, lysine (K) or arginine (R); (3) one of the four hydrophobic amino acids, phenylalanine (F), valine (V), leucine (L) or isoleucine (I); (4) one of the two negatively charged amino acids, glutamic acid (E) or aspartic acid (D); and (5) either a positive or hydrophobic amino acid as the fifth one (148).

The motif is then recognized by a heat shock cognate protein of 73 kDa (Hsc70) in the cytosol (149). Moreover, the motif must be presented on the exterior of the folded target protein for Hsc70 to bind to it. Having multiple KFERQ-like motifs does not increase the affinity of a substrate for the chaperone, or its rate of degradation by the CMA.

❖ Binding of substrate to LAMP-2A

The substrate-chaperone complex is targeted to the lysosomal surface, where it interacts with the cytosolic tail of LAMP-2A (Figure 20) (150).

LAMP-2A is normally present at the lysosomal membrane as monomers. To transport the substrate across the membrane, LAMP-2A forms a multi-protein complex (Figure 20) (151).

Binding of the substrate to LAMP-2A is a limiting step for the CMA. Levels of LAMP-2A at the lysosomal membrane are tightly controlled and constitute a regulatory

mechanism for the CMA (144, 152).

- Unfolding of substrate protein

Once the substrate binds to LAMP-2A, it undergoes the unfolding step which is thought to be mediated by Hsc70 and some of its cochaperones. It is important to note that only soluble cytosolic proteins that can be unfolded by Hsc70 and thus are considered CMA substrates. This process is expected to be completed before the multimer LAMP-2A complex is fully assembled (144).

- Substrate translocation across the lysosomal membrane

After unfolding, the substrate is translocated into the lysosomal lumen through LAMP-2A which is assisted by a luminal form of Hsc70 (lys-Hsc70) (Figure 20) (144, 153). As soon as the substrate is translocated into the lumen, LAMP-2A disassembles from its multimer complex into monomer where other substrates can bind again. The dissociation of LAMP-2A was thought to occur in an Hsc70-dependent manner (144).

Lys-Hsc70 is not the result of internalization of (cytosolic) Hsc70 through CMA, thought Hsc70 itself may be a CMA substrate (144, 153). This was supported by the experiment in which blockage of CMA did not affect the content of lys-hsc70 in lysosomes (154). Instead, Lys-Hsc70 was proposed to be internalized to the lysosomal compartment through endosomal-lysosomal fusion (144).

- Substrate degradation in the lysosomal lumen

Once in the lysosomal lumen, the substrate is rapidly degraded (in 5–10 min) by lysosomal proteases (Figure 20) (144).

In conclusion, CMA is a very specific and highly regulated process of protein degradation. From the experimental view, a substrate must be recognized and associated with both Hsc70 and LAMP-2A to be degraded by the CMA. The requirements for a CMA substrate are summarized in table 4 (144).

Readers interested in CMA, its regulation and its physiological as well as pathological roles are referred to comprehensive reviews by Kaushik et al (2011) and Cuervo et al (2014) (144, 146).

Table 4. Requirements and methods to identify a CMA substrate.

Characteristics of CMA substrate	Assay(s)
Presence of KFERQ-like motif in the sequence	Sequence and structural analyses
Increased protein level of the target due to CMA inhibition (depletion of Hsc70 or LAMP-2A)	siRNA and western blot analyses
Association with Hsc70 and LAMP-2A	Co-immunoprecipitation
Association with LAMP-2A at the lysosomal membrane	Co-immunoprecipitation with LAMP-2A from isolated lysosomes

UBIQUITIN-PROTEASOME SYTEM - A COMMON WAY TO DEGRADE CELLULAR PROTEINS

The ubiquitin-proteasome system (UPS) is considered the major pathway in regulating protein turnover. The UPS, which accounts for the degradation of about 80% of intracellular proteins, degrades ubiquitin-conjugated proteins by 26S proteasomes. Substrates of the UPS include soluble proteins of the cytosol and nucleus, and proteins of the endoplasmic reticulum that have been ejected into the cytoplasm. The process of protein degradation by the UPS is described in details below (155).

1. Ubiquitin-conjugating machinery

1.1. Ubiquitin

Ubiquitin (Ub) is a 76-amino-acid long protein. Ubiquitination (also referred to as ubiquitylation) is a process in which Gly76 residue of Ub is coupled to a Lys residue of another protein through an isopeptide bond (155). A Ub itself has 7 lysine (Lys) residues (Lys6, Lys11, Lys27, Lys29, Lys33, Lys48 and Lys63), which means seven possible homotypic linkage types and multiple possible heterotypic chains can be formed (155, 156). As a result, a ubiquitinated protein can gain or lose its function and/or be degraded, depending on the types of Ub attached to it (Figure 21) (155).

1.2. Ubiquitin conjugating cascade

The process of ubiquitin conjugation involves 3 steps (Figure 21) (155). First, Ub

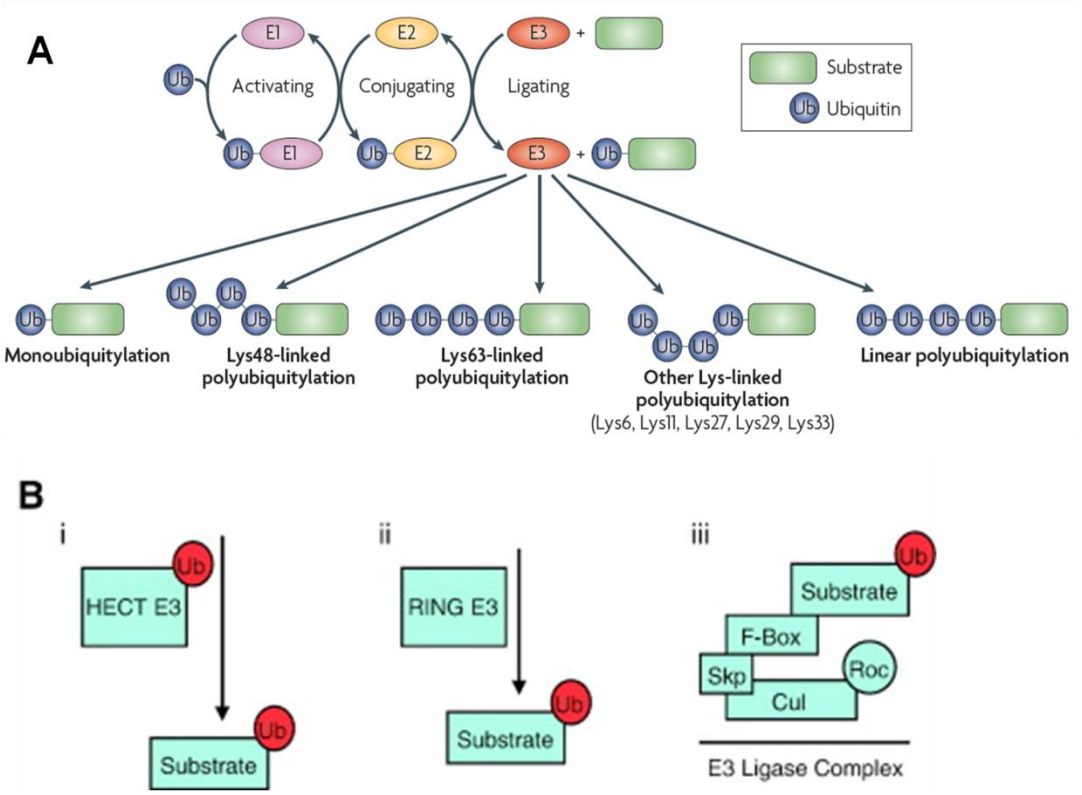
is activated through forming a covalent bond with the ubiquitin-activating enzyme (E1) in an ATP-dependent manner. Second, E1 transfer the Ub to the ubiquitin-conjugating enzyme (E2). Third, the ubiquitin ligase (E3) recognizes its specific substrate and transfers Ub from the E2 to a substrate lysine to make ubiquitinated protein. The completion of one ubiquitination cycle results in a mono-ubiquitinated substrate. The ubiquitin chain can then be lengthened by the E2 and E3 (Figure 21). Also, the fates of ubiquitinated proteins depend on the amount of ubiquitin added and the configuration of the linkages (155, 157).

Figure 21. The ubiquitin conjugating cascade.

(**A**) The process of protein ubiquitination involves 3 steps: (1) E1 activates Ub in an ATP-dependent manner; (2) an E2 enzyme transfers Ub from E1; and (3) an E3 enzyme facilitates the transfer of Ub to a target protein. The substrate can be mono-, multi- or poly-ubiquitinated through different linkages. Adapted with permission from Nat Rev Mol Cell Biol, Dikic, Wakatsuki, and Walters, copyright 2009 (155)

(**B**) Three classes of E3 ligases have been identified: (i) HECT, (ii) RING, and (iii) multi-subunit complexes such as the SCF complex. Adapted with permission from J Mol Cell Cardiol, Willis and Patterson, copyright 2006 (157)

Abbreviations: HECT, homologous to E6-AP C terminus; Lys, lysine; RING, really interesting new gene; SCF, Skp1-Cullin-F-box; Ub, ubiquitin.



❖ The fates of poly-ubiquitinated proteins

Among different poly-Ub linkages, ubiquitin chains linked via Lys48 (K48) or Lys63 (K63) are the best characterized so far (155). Whereas K48 chains are more globular, K63 chains are more extended and elongated (Figure 21) (157). Additionally, it is well-supported that proteins tagged with K48-linked poly-Ub represent a signal for proteasomal degradation (156, 158, 159). In contrast, growing evidence suggest that substrates tagged with K63-linked chain are not targeted for proteasomal degradation (156, 160, 161). Instead, K63-linked chain affects protein localization, cellular signaling and is currently shown to direct substrates for lysosomal degradation (143, 161-163). However, the later mechanism is not fully understood.

1.3. Deubiquitination

The regulation and maintenance of ubiquitinated proteins is the fine tune between the ubiquitination and the de-ubiquitination (155). The later is catalyzed by deubiquitinating enzymes (DUBs) which cleavage Ub from ubiquitinated substrates, especially prior to proteolysis of the proteins. Therefore, DUBs play a pivotal role in recycling Ubs and in maintaining the sufficient pool of free Ubs. Moreover, DUBs can also reverse the effects of ubiquitination, such as preventing protein degradation (Figure 22) (164).

Figure 22. Protein ubiquitination in comparison with protein phosphorylation.

Protein phosphorylation involves the covalent attachment of a phosphate (P) to proteins, mainly through serine, threonine, or tyrosine residues. Phosphorylation is facilitated by protein kinases and reversed by protein phosphatases.

Protein ubiquitination involves the covalent attachment of ubiquitin (Ub) to proteins through lysine residues. This reaction is mediated by a cascade of E1, E2 and E3 enzymes. Ubiquitination process is reversed by deubiquitinase enzymes.

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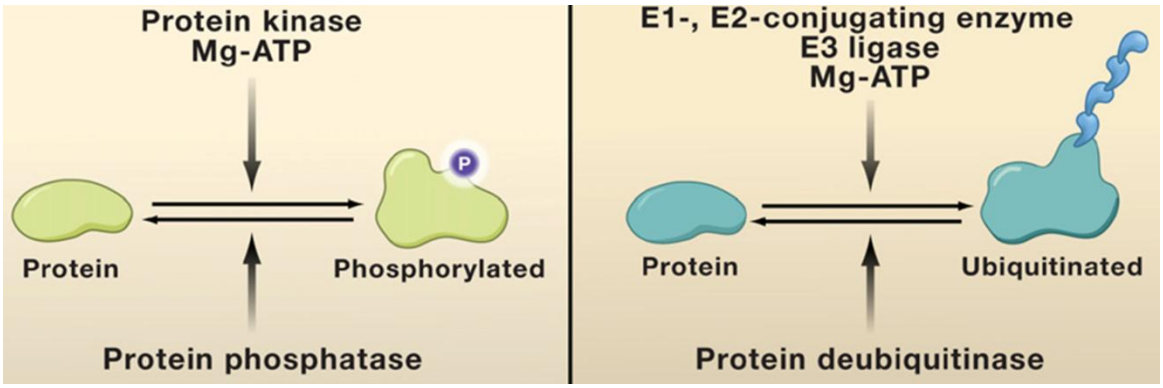


2. 26S proteasome and protein degradation

The final step of a poly-ubiquitinated protein is its degradation by the 26S proteasome, forming a complete cycle of the UPS process.

The 26S proteasome is a large 2.1 MDa complex, containing two separated subcomplexes - the 20S core catalytic particle (CP), and the 19S regulatory particle (RP) (165). The 26S proteasome is activated by the binding of a 19S complex to each end of the 20S cylinder (Figure 23). While 19S RP governs access of substrates to the core, 20S CP contains proteolytic active sites accounting for substrate degradation (165). The active sites of the CP are very sensitive to the 26S proteasome inhibitors, such as MG115, MG132 and lactacystin (165, 166).

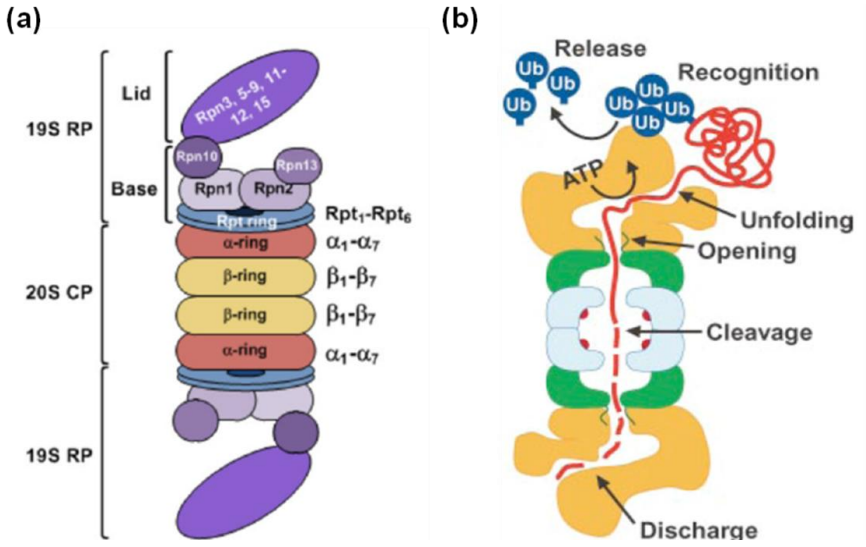
To get degraded by a 26S proteasome, first, a poly-ubiquitinated protein is recognized by and bound to specific subunits in the 19S RP through its poly-Ub chain(s). Next, the poly-Ub chains are removed by DUB(s) residing on the 19S RP. After this, the substrate gets unfolded by ATPases also residing in the base of the 19S RP. Then the unfolded polypeptide is inserted into the proteolytic chamber via an open gate in the 20S CP in an ATP-dependent manner. In the lumen of the CP, the unfolded polypeptides are broken down by the proteases (Figure 23) (166).

Figure 23. Structure of the 26S proteasome and the degradation process of a poly-ubiquitinated protein.

(**a**) Structure of the 26S proteasome. The 26S proteasome is comprised of two subcomplexes - the 20S CP, and the 19S RP. The 19S RP is composed of 17 core subunits that are divided into the lid and base subcomplexes. The 20S is a cylindrical stack of four heptameric rings, the two peripheral rings (α subunits) and the two central rings (β subunits). Adapted with permission from Nat Rev Mol Cell Biol, Weissman, Shabek, and Ciechanover, copyright 2011 (165)

(**b**) Diagram of the 26S proteasome combined with the predicted activities during the degradation of a poly-ubiquitinated protein. Briefly, the 19S RP assists in recognizing and unfolding the target, removing the Ubs, opening the gate of 20S CP, and then directing the unfolded polypeptide into the lumen of the 20S CP for breakdown. Adapted with permission from Trends Plant Sci, Vierstra, copyright 2003 (166)

Abbreviations: ATP, adenosine triphosphate; CP, catalytic particle; RP, regulatory particle.



PROTEOLYSIS: FROM THE LYSOSOME TO THE UBIQUITIN- PROTEASOME DEGRADATION

1. The brief history of the lysosome and the UPS

Even though the UPS is the most studied and common pathway in protein degradation, lysosome was the first mechanism implicated in controlling protein turnover (167).

Lysosome was discovered in early 1953 followed by an important discovery of micro-autophagy, the basic functional mechanism of the lysosome. For a long time, the lysosomal proteolytic pathway was thought to be “bulk” or non-specific degradation. Long after, several findings indicated that lysosomal degradation could be specific and was mediated by the recognition of a conserved and well-defined motif in target proteins (KFERQ motif). The process of recognizing the specific substrates was later known as the CMA (167).

The UPS was not discovered until late 1970s when some studies showed that specific and general inhibitors of lysosomal proteases have different effects on different populations of proteins (such as long-lived and short-lived proteins) (167). From this finding, it was suggested that there were different proteolytic machineries function in the cell. Through various studies on cell-free proteolytic system, the three enzymes that are involved in the UPS were finally purified by Ciechanover and colleagues, which were E1, E2 and E3, as described above (167-170). After that, another important advance in the discovery of the UPS was done by Hough and colleagues, who partially purified and characterized an alkaline protease that degraded ubiquitin conjugates of lysozyme, but not untagged lysozyme, in an ATP-dependent manner (167, 171). This

protease finding, which later became known as the 26S proteasome, is the stepping stone in the completion of the known UPS nowadays (167).

2. The intergration of the UPS and the (selective) lysosomal pathway

The UPS and the lysosomal proteolytic pathways are completely distinct in the ways they functions. However, they both go through 4 common steps, including: (1) target selection and recognition, (2) delivery of the target to the proteolytic machinery, (3) target degradation in the proteolytic core and (4) recycling of constituent amino acids (143).

There are several factors contributing to how a protein is recognized, such as the half-life of a protein or the types of Ub linkages. However, the precise mechanism is not completely understood (143). As mentioned above, one of the most well-studied factors for determining the "fate" of a protein is whether the protein is attached via a chain of Ub linked at K48 or K63 (K48 or K63-linked polyUb, respectively). While K48-linked polyUb directs proteins to 26S proteasomes for destruction, proteins tagged with K63-linked chains are targeted for lysosomal degradation (Figure 21) (143, 158, 162).

The relative contribution of each degradation pathway varies between cell types. In the cells cultured under stress-free conditions, proteasomal degradation predominates (accounts for about 80% of intracellular protein degradation) (172). However, in muscle cells, lysosomal pathways (specifically macro-autophagy) can account for up to 40% of degradation of proteins (143, 172). Moreover, the proteasome is itself degraded by starvation-induced macro-autophagy (173).

Several studies showed that some cancer cells up-regulated either CMA or macro-autophagy in response to the chemical blockage of the proteasome (144, 174, 175). It was reasoned that the induction in macro-autophagy or CMA may serve as an alternative mechanism to protect the cells against toxicity of accumulated aggregate proteins (144). Yet in what conditions CMA or macro-autophagy is activated is not clear.

Interestingly, compromised CMA impairs the function of the UPS (176). The mechanism is unknown, though it was suggested that CMA affected the turnover of some of specific proteasome subunits leading to altering proteasome assembly (144, 173). Similarly, macro-autophagy deficiency also suppresses proteasome function indicated by the increase in proteasomal substrates (Figure 24) (177).

In addition, CMA was also found to cross-talk with macro-autophagy during starvation (176). When CMA is stimulated, macro-autophagy is first induced and then inhibited (154, 176). Conversely, CMA is constitutively active in cells deficient in macro-autophagy, yet little is known about the molecular modulators of all these crosstalks (Figure 24) (178).

In conclusion, the UPS, the macro-autophagy and the CMA are strictly connected to each other. Understanding the molecular determinants that efficiently reroute a protein substrate from one proteolytic pathway to another would allow us to better manipulate the various proteolytic systems and apply it to anti-cancer therapy. For comprehensive reviews and experts' opinions on the integration of the UPS and lysosomal proteolytic pathway, please refer to articles by Wong and Cuervo (143, 144).

Figure 24. Crosstalk between different proteolytic systems.

Different proteolytic systems are wired through multi-levels of interactions to maintain cellular homeostasis. The inhibition or activation in one system could leads to the elevation in the others to prevent toxicity to the cells.

Abbreviations: PD, Parkinson's disease; HD, Huntington's disease; SMA, spinal muscular atrophy.

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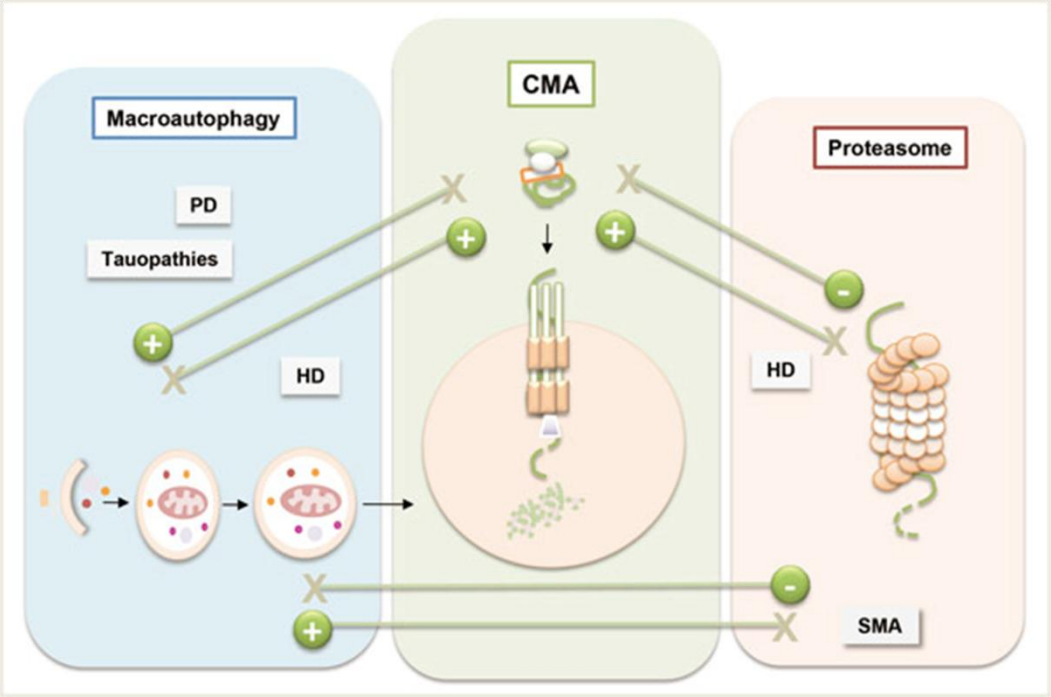


HYPOTHESIS AND SPECIFIC AIMS

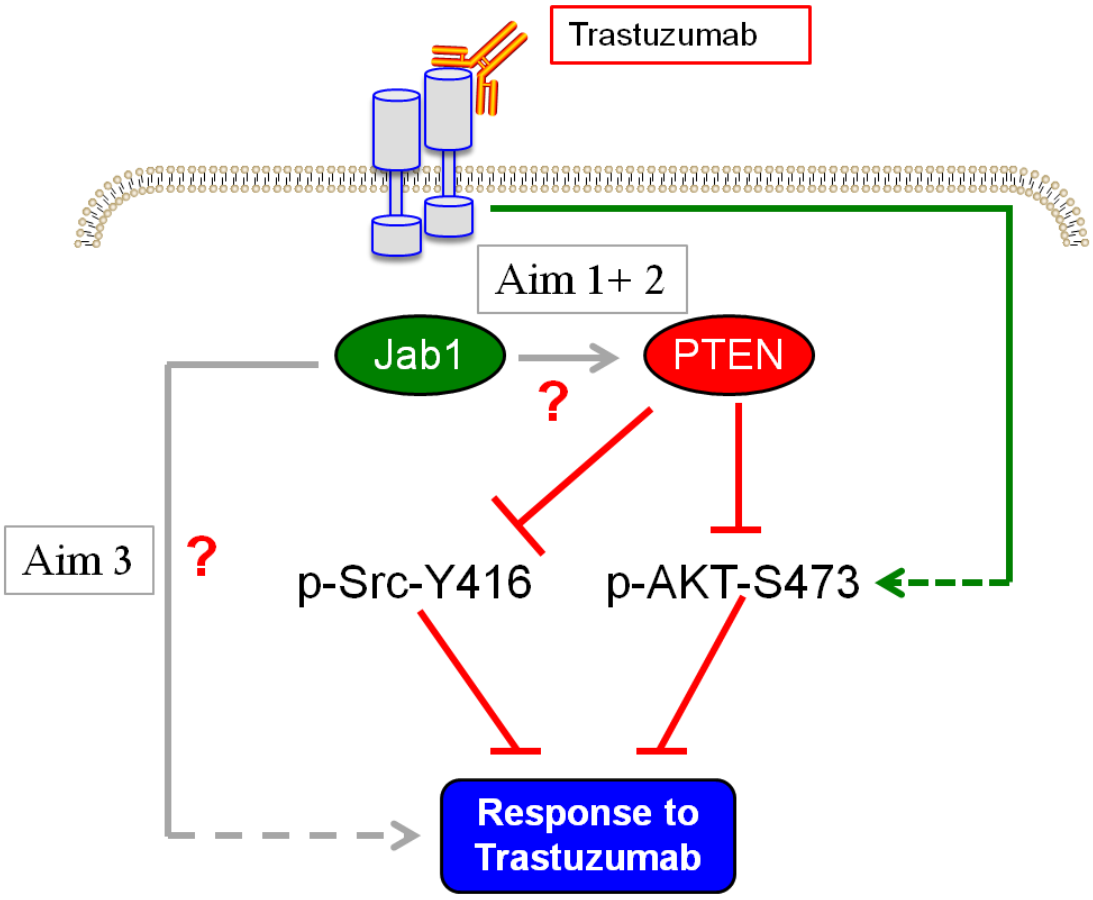
Over-expression or an increase in the copy number of Jab1 is frequently found in various cancers, contributing to tumor progression and drug resistance. In breast cancer, Jab1 is over-expressed in 50% of primary cancers and 90% of metastatic tumors, while its expression is low or absent in normal adult breast tissues. Mechanistically, Jab1 promotes cell cycle progression and cell survival by facilitating the rapid degradation of p27. Moreover, our group has shown that, in xenograft mouse models refractory to trastuzumab, silencing Jab1 up-regulates both p27 and PTEN protein levels in response to trastuzumab compared to control Jab1 knockdown. Down-regulation of p27 or PTEN has been implicated in promoting trastuzumab resistance in HER2+ breast cancer. However, the significance of Jab1 in mediating PTEN expression and the cells' responses to trastuzumab is unknown. The goal of my dissertation is to understand the role of Jab1 in regulating PTEN expression and trastuzumab resistance in HER2+ breast cancer. My central hypothesis is that Jab1 negatively regulates PTEN expression and promotes resistance to trastuzumab in HER2+ breast cancer.

The specific aims of my dissertation project illustrated in Figure 25 are:

1. To identify the role of Jab1 in regulating PTEN expression and function (Chapter 3).
2. To elucidate the mechanism by which Jab1 suppresses PTEN expression (Chapter 4).
3. To characterize the role of Jab1 in conferring trastuzumab resistance (Chapter 5).

Figure 25. Proposed hypothesis model of Jab1 role in regulating PTEN expression and trastuzumab resistance in HER2-positive breast cancer.

Red lines indicate the inhibition; green lines indicate the activation; and black lines represent unknown effects.



CHAPER 2: MATERIALS AND METHODS

Cell culture

The human embryonic kidney 293T cells, the HER2+ human breast cancer cell lines BT474 and SKBR3 were obtained from ATCC. BT474 cells were cultured in Dulbecco's modified Eagle medium (DMEM), and SKBR3 was maintained in Roswell Park Memorial Institute 1640 medium (RPMI 1640). Trastuzumab-resistant C5 and C6 cells, which were derived from BT474 cells and kindly provided by Dr. Timothy Kute (Wake Forest University, Winston-Salem, NC), were maintained in DMEM with 2 μg/ml of trastuzumab (179). Paired MEF WT and MEF-Atg5 KO cells were kindly provided by Dr. Jiyong Liang (MD Anderson Cancer Center, Houston, TX). Paired MEF-p62 WT and MEF-p62 KO were generously given by Dr. Maria Diaz-Meco (Sanford-Burnham Medical Research Institute, La Jolla, CA) (180). All the MEF cells were cultured in DMEM supplemented with 5% fetal bovine serum (FBS). The other media used in this study were supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100 μg/ml). All the cells were incubated at 37°C in a humidified chamber supplemented with 5% CO2.

Cell proliferation MTS assay

Cell viability was assessed using a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay (Promega). Cells were seeded for 24 hours before the indicated treatments. After 5 days, the cell proliferation was assessed by adding the MTS solution into each well. Wells with cell-free media were used as negative controls.

The plates were incubated for the indicated times at 37°C in a humidified 5% CO_2 atmosphere. The color developed was read with an enzyme-linked immunosorbent assay reader at 490 nm. The percentage of cell growth inhibition was calculated by comparing the growth inhibition of the treated cells with that of the untreated cells. The results are the mean values ± standard deviations.

Reagents and antibodies

Trastuzumab was kindly provided by Genentech/Roche. The proteasome inhibitors MG-132 and lactacystin were purchased from Calbiochem. The calpain inhibitor, pepstatin A, and bafilomycin A were from Sigma-Aldrich. E64D was obtained from Santa Cruz. The anti-Jab1, anti-p27, and anti-Myc antibodies were from Santa Cruz Biotechnology, BD Biosciences, and Roche, respectively. Antibodies against p-PTEN (S380/T382/T383), p-Src (Y416), p-AKT (S473), total Src, total AKT and ATG5 were from Cell Signaling Technology. The anti-p62 antibody was from PROGEN Biotechnik. Antibodies against LC3 and Hsc70 were purchased from Novus Biologicals. Anti-β-actin and anti-Flag antibodies were from Sigma-Aldrich.

Western blot

Total cell lysates was prepared using lysis buffer supplemented with phosphatase inhibitors and complete protease inhibitor tablet (Roche). The lysates were then separated by SDS-PAGE, transferred to PVDF membrane (GE Healthcare) and blocked with 5% nonfat milk in PBS with 0.1% Tween-20 (PBST). Membranes were incubated with primary antibodies

overnight at 4˚C then washed with PBST. Secondary antibody was incubated with membrane for 1-2 hour(s) at room temperature. Immunoreactive bands were detected with horseradish peroxidase–conjugated secondary antibodies using the Western Lightning Chemiluminescence Reagent (Thermo Scientific). β-Actin was used as the loading control for all blots.

Small interfering RNA transfection

Small interfering (si) RNA against Jab1 (siJab1) was purchased from Ambion/Life Technologies while siPTEN and siHsc70 were from Dharmacon. The Pepmute siRNA transfection reagent (SignaGen) was used according to the manufacturer's instructions to deliver the siRNA into cells. 48 or 72 hours after transfection, the cells were collected for Western blotting or real-time polymerase chain reaction (RT-PCR).

Generating shRNA stable cells from C5 and C6

JAB1 shRNA and luciferase (Luc) shRNA oligonucleotides as a control were cloned into a retrovirus pSIREN-RetroQ system (Clontech) according to the manufacturer's instructions. The packaging cell line 293T was co-transfected with the helper vectors pCGP, pVSVG vectors together with either Jab1-shRNA vector DNA or Luc-shRNA vector DNA using the Lipofectamine PLUS reagent (Invitrogen). At 48 hours after transfection, the supernatant was collected and filtered through 0.45-μm syringe filters, supplemented with Polybrene to a final concentration of 8 μg/ml, and used to transduce the C5 and C6 cells for 16 hours. After incubation, the medium was replaced with DMEM with 10% FBS. Stable clones were

selected following treatment with puromycin at 1.0 µg/ml for 2 weeks. Positive clones were further confirmed by western blot analysis, and clones were maintained in 0.4 µg/mL puromycin.

PTEN lipid phosphatase assay

PTEN lipid phosphatase activity was assessed by its ability to de-phosphorylate PIP3 to release free phosphate groups using Echelon's Malachite Green Phosphatase Assay kits (Echelon Bioscience). Briefly, cells were lysed in phosphatase inhibitor–free lysis buffer according to the manufacturer's protocol. PTEN proteins were immunoprecipitated from lysates with anti-PTEN antibodies overnight following several thorough washes in reaction buffer. The substrate, PIP3, was then added into the complexes, which were incubated at 37°C. The reaction without immunoprecipitated complexes was performed in parallel as the negative control. After 1 hour, the reaction supernatants were transferred to a 96-well plate and incubated with Malachite Green Solution at room temperature. The color developed was measured at 650 nm using an enzyme-linked immunosorbent assay microplate reader. The amount (in pmol) of free phosphate group released during each reaction was determined by linear regression analysis against a standard phosphate curve.

Immunohistochemistry

The p-SRC-Y416 expression was assessed by immunohistochemical analysis on formalin-fixed and paraffin-embedded tumors from mice injected with C5shLuc or C5shJab1 lines. The monoclonal antibody used was specific for p-SRC-Y416 (diluted 1:50; Cell Signaling).

Briefly, heat-induced retrieval of p-SRC-Y416 antigen was conducted, and the slides were incubated with the primary antibody overnight. An immunoreaction was detected with the LSAB+kit from DakoCytomation. We used 3,3′-diaminobenzidine as the chromogen and hematoxylin as the counterstain. Tumor cells were considered positive for p-SRC-Y416 when cytoplasmic staining was present, regardless of the intensity. The immunohistochemistry results were evaluated by a pathologist in our lab. This study protocol was approved by the institutional review board of MD Anderson Cancer Center

qRT-PCR

Total RNA was extracted using the Trizol total RNA isolation reagent (Invitrogen) according to the manufacturer's instructions. The RT-PCR assay was performed with 2 μg of total RNA using the TaqMan mRNA assay kit (Life Sciences/Life Technologies), and the expression levels of the mature mRNA were measured using the same kit following the manufacturer's protocol. A reaction without reverse transcriptase was performed in parallel to ensure the absence of genomic DNA contamination. PTEN Primers used were designed and purchased from Life Technologies (Assay ID: Hs02621230_s1).

Plasmid constructs and transfection

Jab1 (334 amino acid) was amplified by PCR, and the cDNA coding for full-length (1-334) was cloned with an N-terminal Myc tag in the pcDNA3.1 vector (Invitrogen) to generate pcDNA3.1-WT-Myc-Jab1. The N- and C- terminal deletion mutants ending at codons 48, 93, and 299 (Jab1ΔN, Jab1ΔJBD, and Jab1ΔC, respectively) were

produced by inserting stop codons at the mentioned positions, which generated the, pcDNA3.1-Myc-Jab1ΔN, pcDNA3.1-Myc-Jab1ΔJBD, and the pcDNA3.1-Myc-Jab1ΔC. pcDNA3.1-Myc-Jab1ΔMPN was generated by deleting amino acids from 138-151 of WT Jab1. Site directed mutagenesis was performed using the QuikChange Lightning Site- Directed mutagenesis kit (Stratagene). The entire ORF of all constructs and mutations were checked by DNA sequencing (Beckman Coulter Genomics).

The plasmids pCMV-Flag-PTEN and pCMV-Flag-PTEN-3A were generously provided by Dr. Maria-Magdalena Georgescu (The University of Texas Southwestern Medical Center, Dallas, TX) (88).

Lipofectamine 2000 (Invitrogen) was used according to the manufacturer's instructions for the transfection of plasmid DNA into the cells. 48 or 72 hours after transfection, the cells were subjected for different treatment or collected for further analysis by Western blotting.

Immunoprecipitation

Cell lysates was prepared using lysis buffer supplemented with phosphatase inhibitors and complete protease inhibitor tablet (Roche). Five hundred micrograms of cell lysate were incubated with appropriate antibodies overnight at 4°C. Prewashed protein A/G agarose beads (Upstate) were then added into Ab-lysate mixers and rotated for 2-4 h at 4°C. The beads were washed three times with lysis buffer and centrifuged for 10 min at 5,000 × *g*. Proteins were eluted from the beads with 20 µl of loading buffer and subjected to Western blotting as described above.

Nuclear and cytoplasmic fractionation

Fractionations of the cells were performed using an NE-PER nuclear and cytoplasmic extraction reagent kit (Thermo Scientific/Pierce) according the manufacturer's specifications.

Cycloheximide (CHX) study

293T cells stably expressing Flag-tagged wild-type PTEN (WT PTEN) or Flag-tagged mutant PTEN-3A (PTEN-3A) were incubated with cycloheximide (CHX) (50 μg/ml) and harvested at the indicated times. Whole protein lysates were extracted and subjected to Western blotting with an anti-FLAG antibody for transfected PTEN; anti–β-actin antibody was used as the loading control.

***In vivo* ubiquitination assay**

293T cells were co-transfected with control vectors and/or plasmids encoding Flag-PTEN, Myc-Jab1, and His-tagged WT ubiquitin (His-Ub-WT) or His-tag mutated Ub from lysine to arginine at site 48 or 63 (K48R or K63R). His-Ub-WT and mutated isoforms were generously given by Dr. Shiaw-Yih Lin (MD Anderson Cancer Center, Houston, TX). Part of the lysates were used for western blot analsys. The rest of the lysates were subjected to immunoprecipitation in which the expressed His-tag Ub proteins were immunoprecipitated using Nickel beads against His-tag. Subsequently, Western blott against the Flag tag was performed to detect ubiquitinated PTEN.

Bioinformatic and statistical analysis

Gene and protein expression data from the TCGA Breast Invasive Carcinoma (BRCA) (n=744) were downloaded from cbioPortal (181, 182) (http://www.cbioportal.org/public-portal/). The samples were grouped by mRNA expression levels of *COPS5* (*Jab1* gene), *WWP2*, or *NEDD4*. Expression levels of the given genes were defined as High (≥ upper quartile) and Low (≤ lower quartile).

Protein expression data were downloaded from the TCPA BRCA (n=750) (183) (http://app1.bioinformatics.mdanderson.org/tcpa/_design/basic/index.html). The samples were grouped by protein expression levels of Jab1 measured by reverse phase protein array (RPPA), in which protein levels were defined as High (≥ upper quartile) and Low (≤ lower quartile). For the two datasets, PTEN protein levels, measured by RPPA, were analyzed for each group and represented by box-and-whisker plots. The p-value was calculated using the Mann–Whitney test. *P* values <0.05 were considered statistically significant.

For the *in vitro* and *in vivo* analysis, experiments were repeated at least three times. The data were presented as means ± standard deviations. Statistical calculations were performed with Microsoft Excel analysis tools. Differences between individual groups were analyzed using a *t* test. *P* values <0.05 were considered statistically significant. Quantification of protein levels was done using ImageJ software from the National Institutes of Health (http://rsb.info.nih.gov/ij).

CHAPTER 3: THE ROLE OF JAB1 IN REGULATING PTEN EXPRESSION AND FUNCTION IN HER2-POSITIVE BREAST CANCER

RATIONALE

One of the mechanisms contributing to trastuzumab resistance in HER2+ breast cancer is the reduced expression of PTEN protein levels, which results in constitutive active AKT. However, it is not fully understood how PTEN protein is down-regulated in breast cancer despite the lack of PTEN gene mutation or promoter methylation (98, 99). Therefore, it is critical to understand the mechanism driving PTEN protein low expression and to effectively restore PTEN level in HER2+ breast cancer. Our group previously showed that in xenograft mouse models refractory to trastuzumab, silencing of Jab1 together with trastuzumab treatment up-regulated PTEN protein level, which was not observed in control Jab1 knockdown mice (unpublished data). However, the functional correlation between Jab1 and PTEN was not understood. In this chapter, *my goal is to examine whether the inhibition of Jab1 alone is sufficient to restore PTEN protein expression and function in HER2+ breast cancer.*

RESULTS

1. Jab1 silencing but not trastuzumab treatment restores PTEN protein expression in HER2-positive breast cancer cell lines resistant to trastuzumab

1.1. Trastuzumab treatment does not affect the proliferation and downstream signaling of HER2 in HER2-positive breast cancer cells that were made resistant to this drug

I first evaluated trastuzumab inhibitory effects on the growth of a HER2+ breast cancer cell line, BT474 and two BT474-derived trastuzumab-resistant lines, C5/BT474 (i.e., C5) and C6/BT474 (i.e., C6). As presented in Figure 26A (left graph), trastuzumab inhibited the proliferation of BT474 (blue line), but had no effects on C5 and C6 cells (red and green lines). IgG does not affect the growth of any of these cell lines (Figure 26A, right graph). Also, I demonstrated that HER2 protein levels were relatively similar among the three lines, BT474, C5 and C6 (Figure 26B). The unchanged in HER2 protein indicates that the responses of the cells to trastuzumab treatment are dependent on HER2 downstream alterations rather than the levels of HER2 *per se*.

Next, I identified the effects of trastuzumab on downstream signaling targets of HER2 in BT474, C5 and C6 cells which are PTEN and AKT phosphorylation at serine 473 (p-AKT-S473). I treated theses cells with either IgG or increasing concentrations of trastuzumab and examined the changes in protein levels by western blot. As mentioned in chapter 1, the key mechanism by which trastuzumab inhibited cancer cell proliferation was to increase PTEN protein expression and decrease p-AKT-S473 (table 2). Consistently, I showed that in trastuzumab-responsive BT474 cells, increased treatment with this drug enhanced PTEN level accompanied by the decrease in p-AKT-S473 level (Figure 27A). In contrast, trastuzumab did not alter PTEN or p-AKT-S473 levels in C5 and C6 cells (Figure 27B).

These data, together, confirm that trastuzumab shows no effect on either cell proliferation or downstream signaling of HER2 in trastuzumab resistant cells.

Figure 26. Effects of trastuzumab on HER2-positive breast cancer cells that are sensitive or resistant to trastuzumab.

(A) Growth rates of BT474, C5 and C6 lines treated with trastuzumab (left graph) or with control IgG (right graph) were measured by MTS assay. Experiments were repeated three times. The data were presented as means ± standard deviations. Differences between individual groups were analyzed using a t test. P values <0.05 were considered statistically significant, which were represented by the asterisk (*).

(B) Western blot showing protein expressions of HER2 protein in BT474, C5 and C6 lines.

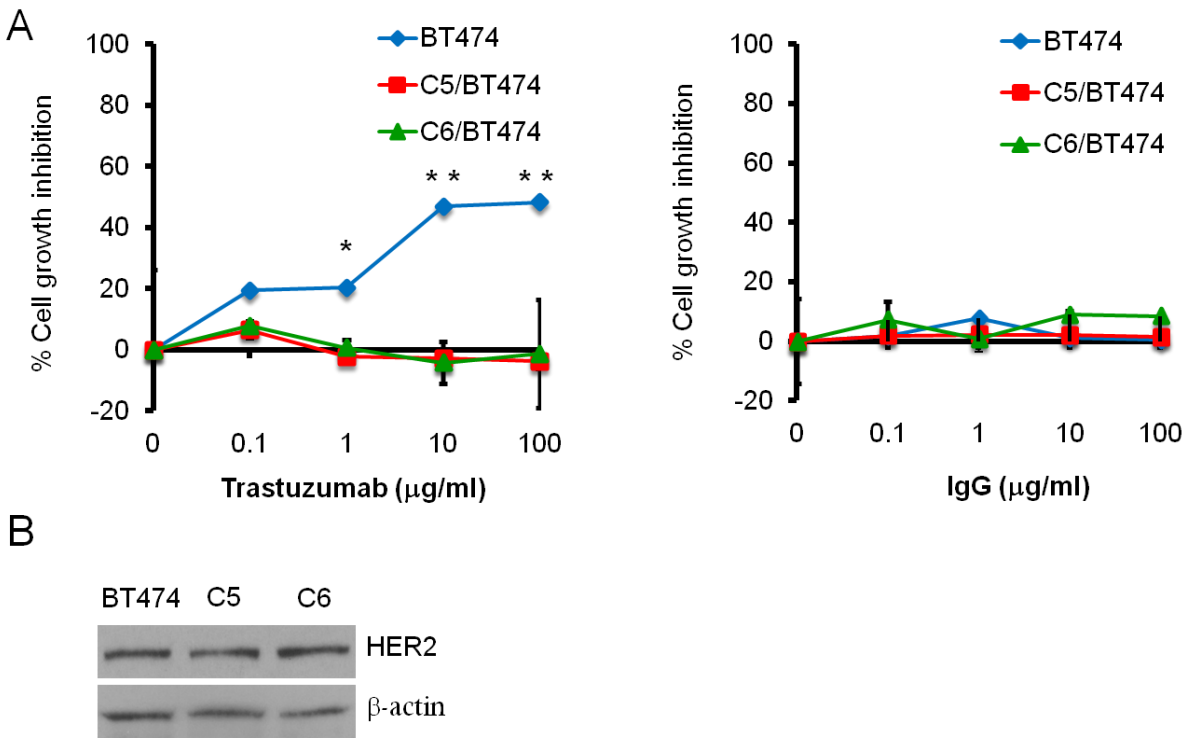
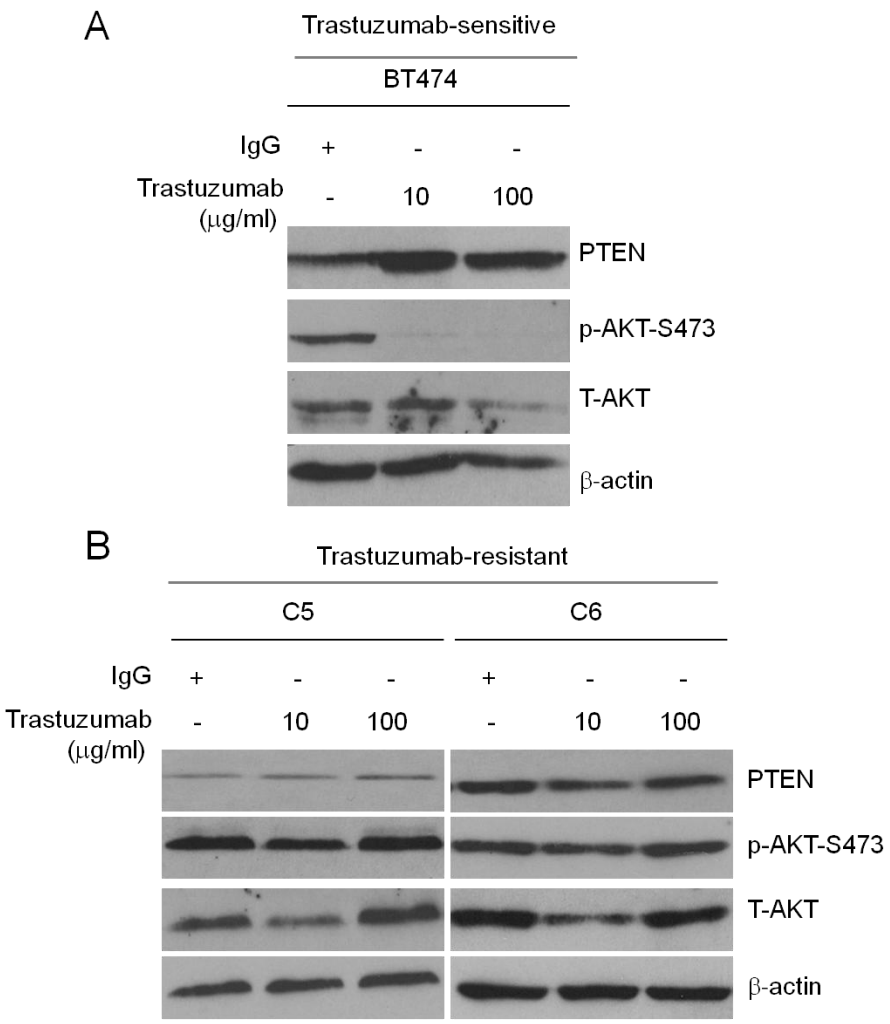


Figure 27. Effects of trastuzumab on the downstream signaling of HER2 in HER2-positive breast cancer cells that are sensitive or resistant to trastuzumab.

Western blot showing changes in PTEN and p-AKT-S473 levels in **(A)** BT474 and **(B)** C5 and C6 lines treated with control IgG or increasing concentrations of trastuzumab.



1.2. Inhibition of Jab1 up-regulates PTEN protein expression in HER2-positive breast cancer cells resistant to trastuzumab

To evaluate the effect of Jab1 on PTEN protein levels, I first transiently depleted Jab1 using small interfering (si) RNA in BT474, C5 and C6 cells. I found that in trastuzumab sensitive BT474 cells, transient silencing of Jab1 slightly increased PTEN protein while decreased p-AKT-S473 without affecting total AKT (T-AKT) levels (Figure 28). In trastuzumab resistant C5 and C6 cells, Jab1 knockdown showed stronger effect on up-regulating PTEN, compared to this effect observed on BT474 cells (Figure 28).

To confirm the finding that depletion of Jab1 increased PTEN protein level in trastuzumab resistant cells, I generated stable *Jab1* knockdown cells in C5 and C6 cells called C5shJab1 and C6shJab1. C5shLuc and C6shLuc, in which the *luciferase* gene was depleted, were used as the control knockdown cells. As presented in Figure 29A, the levels of PTEN protein were increased by more than 2-fold as a result of stable inhibition of Jab1 in the C5shJab1 and C6shJab1 cells, compared to the shLuc cells. Consistently, p-AKT-S473 levels were markedly decreased in C5shJab1 and C6shJab1 cells, compared to C5shLuc and C6shLuc cells (Figure 29A). Quantitative data from repeated experiments confirmed my finding that suppression of Jab1 leads to the increase in PTEN protein expression in trastuzumab resistant cells (Figure 29B).

Figure 28. Transient depletion of Jab1 increases PTEN and decreases p-AKT-S473 levels in trastuzumab-sensitive and trastuzumab-resistant cells.

Western blot representing the changes in protein expressions of Jab1, PTEN, p-AKT-S473 and T-AKT in BT474, C5 and C6 cells transfected with mock oligonucleotide (siCtrl) or siRNA against Jab1 (siJab1) for 72 h.

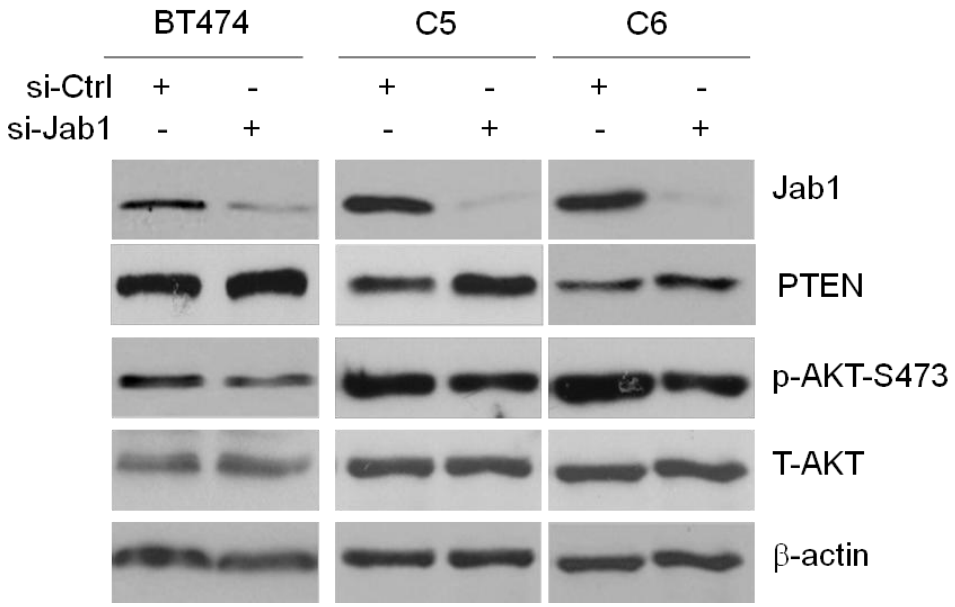
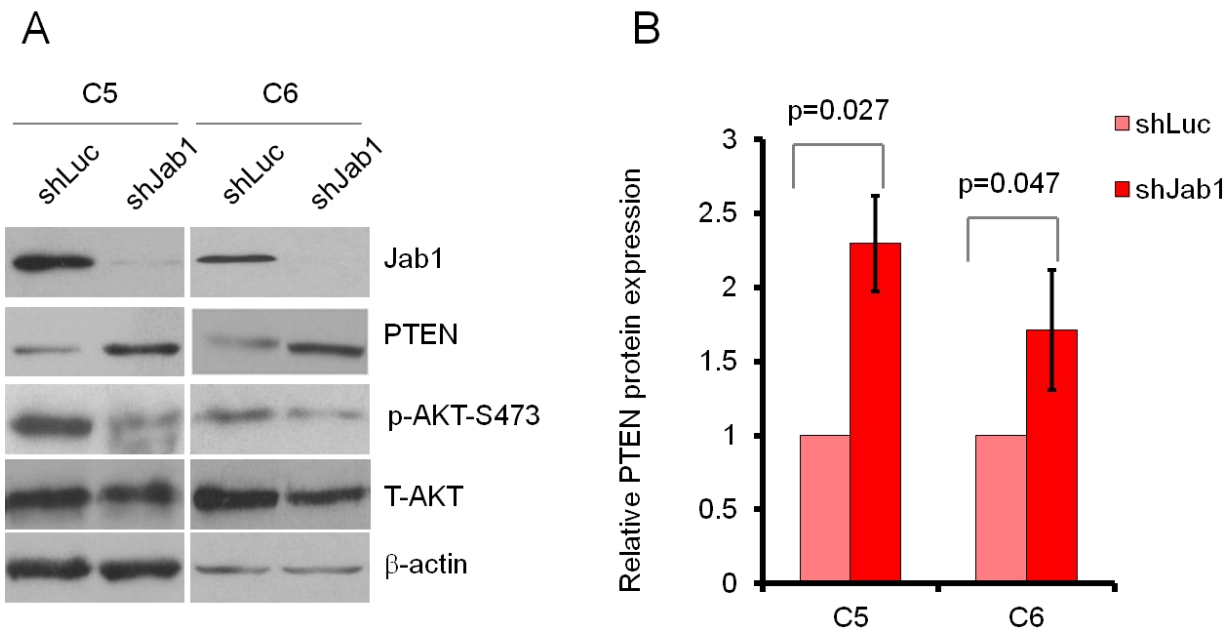


Figure 29. Stable knockdown of Jab1 increases PTEN and decreases p-AKT-S473 levels in trastuzumab-resistant cells.

(A) Western Blot analysis of indicated proteins in C5shLuc and C5shJab1, or C6shLuc and C6shJab1.

(B) Bar graph shows the relative fold changes in PTEN proteins between C5shLuc and C5shJab1, or C6shLuc and C6shJab1. The relative fold change represents the protein expression value of PTEN in cells bearing shJab1 normalized to the expression value of PTEN in cells bearing control shLuc.

Experiments were repeated three times. The data were presented as means ± standard deviations. Differences between individual groups were analyzed using a t test. P values <0.05 were considered statistically significant.



2. Jab1 is inversely correlated with PTEN protein expression in patients with invasive breast cancer

Next, to identify if Jab1 was also clinically associated with PTEN, I analyzed the correlation between Jab1 mRNA and PTEN protein expressions using The Cancer Genome Atlas (TCGA) Breast Invasive Carcinoma (BRCA) dataset. The correlation between Jab1 protein and PTEN protein expressions was also analyzed using The Cancer Protein Atlas (TCPA) BRCA dataset. I found that in both the TCGA and TCPA BRCA datasets, higher PTEN protein levels were associated with lower Jab1 mRNA or protein levels. Similarly, PTEN protein expressions were lower in the group with high expressions of Jab1 mRNA or protein (Figure 30). I also noticed that Jab1 protein (Figure 30B) showed much stronger association with PTEN protein expression than Jab1 mRNA did (Figure 30A). In general, this negative correlation between Jab1 and PTEN are consistent with my experimental findings that silencing Jab1 resulted in elevation in PTEN protein expressions in breast cancer.

As discussed in chapter 1, previous work of other groups showed that PTEN protein expressions were negatively regulated by E3 ligases which are NEDD4-1 and WWP2 (91, 92, 94). Therefore, I compared the degree of clinical correlation between Jab1 and PTEN with these two proteins and PTEN using the TCGA BRCA data, since protein expressions for WWP2 and NEDD4 were not available for TCPA BRCA. In contrast to prior experimental data, WWP2 mRNA and PTEN protein are not clinically correlated while NEDD4-1 mRNA shows positive correlation with PTEN protein expression in human breast tumors (Figure 31A and B, respectively).

In general, my findings strongly argue that high expression of Jab1 is causally linked to low expression of PTEN in human breast cancers.

Figure 30. Jab1 amplification is correlated with low PTEN protein expression in clinical specimens of invasive breast carcinoma.

Differences in protein levels of PTEN between High and Low groups of **(A)** Jab1 mRNA or **(B)** Jab1 protein expressions in TCGA BRCA (n=744) and TCPA BRCA (n=750) datasets, respectively. In box-and-whisker plots, horizontal bold bars indicate the medians, boxes indicate 25th to 75th percentiles, and whiskers indicate minimum and maximum values. P value was calculated by Mann–Whitney U test. P values <0.05 were considered statistically significant.

Data analysis was performed by Tyler Moss, MD Anderson Cancer Center, Houston, TX.

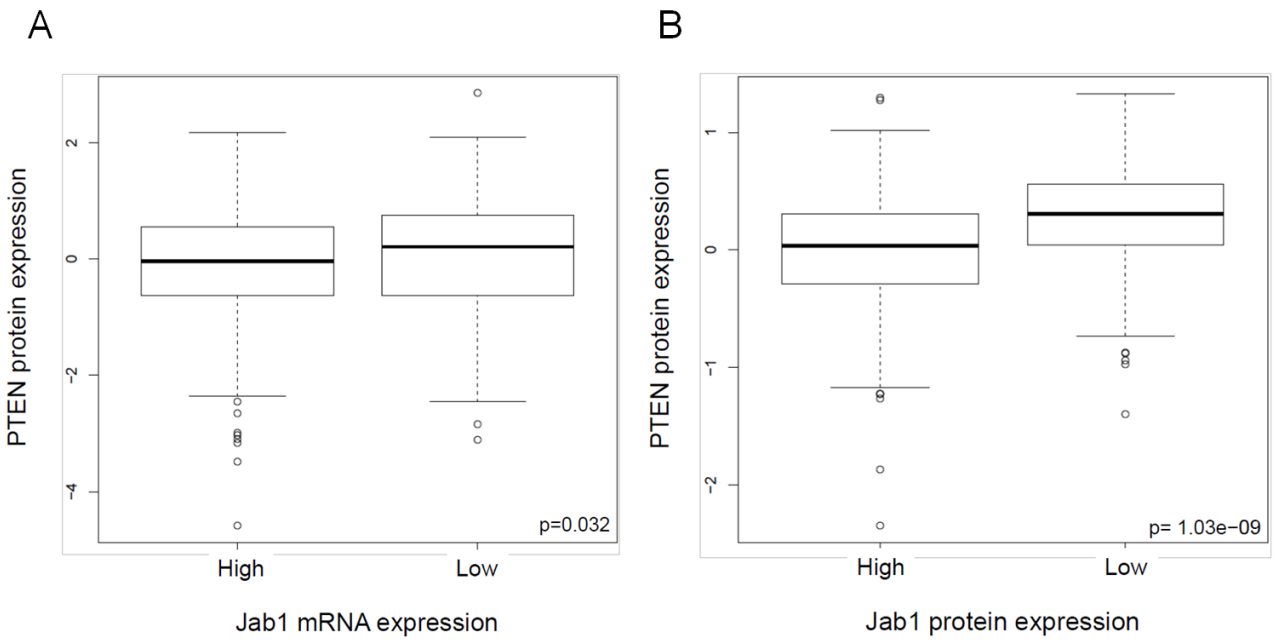
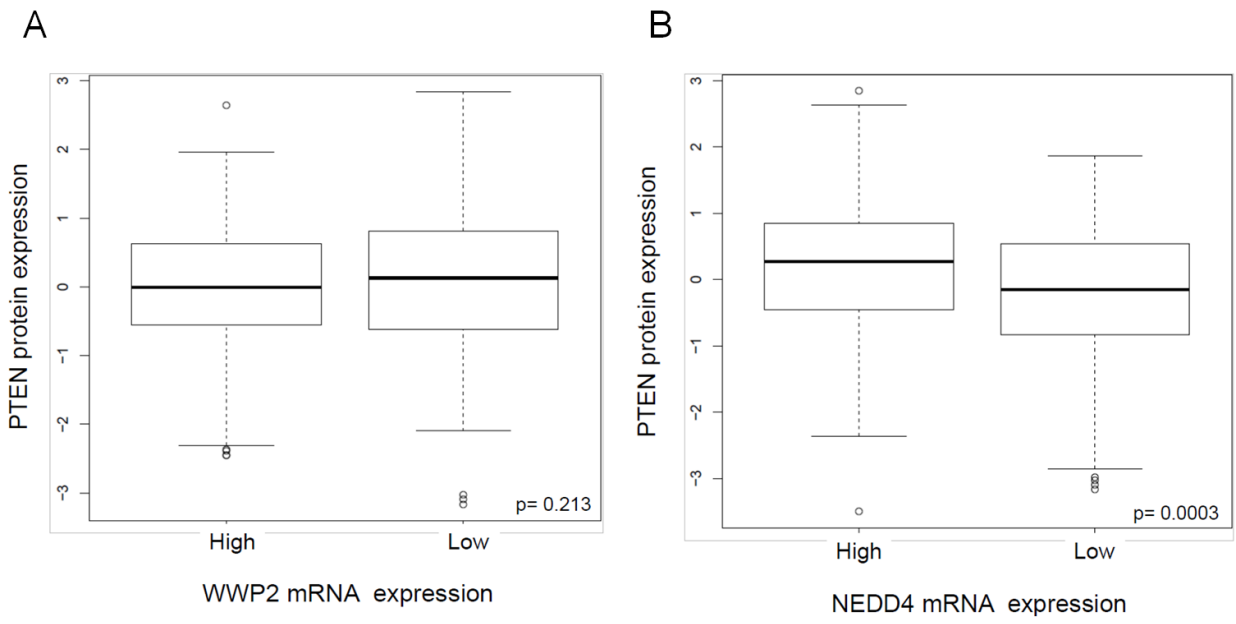


Figure 31. The correlation of PTEN protein with mRNA levels of other regulators of PTEN.

Box-and-whisker plots representing differences in protein levels of PTEN between **(A)** WWP2-High and WWP2-Low and **(B)** NEDD4-High and NEDD4-Low cases in TCGA BRCA dataset (n=744). P value was calculated by Mann–Whitney U test. P values <0.05 were considered statistically significant.

Data analysis was performed by Tyler Moss, MD Anderson Cancer Center, Houston, TX.



3. Inhibition of Jab1 stabilizes PTEN which results in increases in lipid and tyrosine phosphatase functions

PTEN is well-known for its phosphatase function (86). Therefore, I next examined whether the increases in PTEN protein levels due to depletion of Jab1 also led to the increase in the lipid and tyrosine phosphatase functions of PTEN. As the effects of Jab1 on altering PTEN protein expression were more significant in the two trastuzumab-resistant C5 and C6 cells compared to trastuzumab-sensitive BT474 cells (Figure 26), I used the C5 and C6 cells to examine how Jab1 regulated PTEN activities.

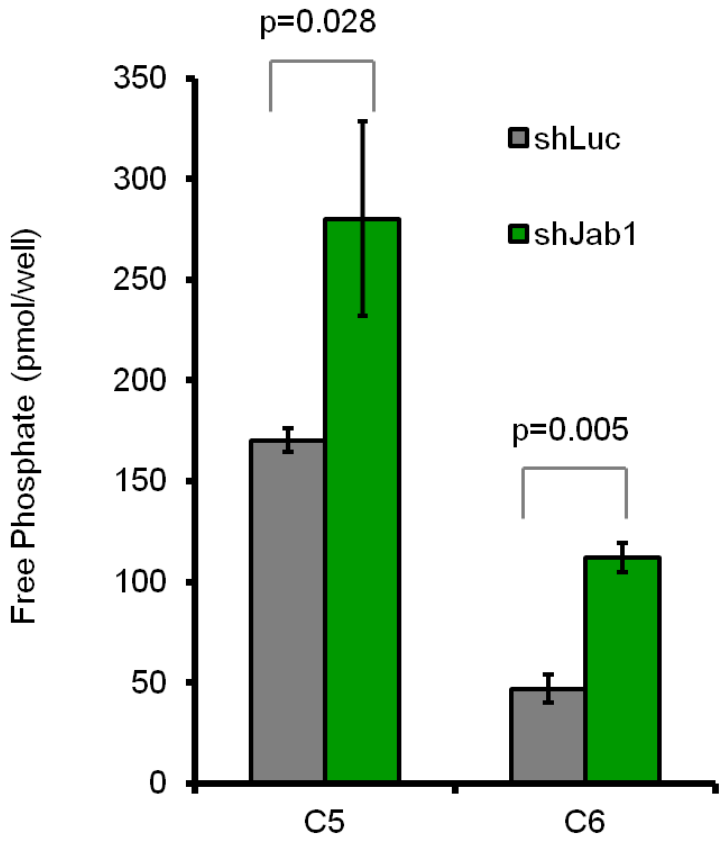
3.1. Jab1 negatively regulates PTEN lipid phosphatase activity

I first tested the impact of silencing Jab1 on PTEN lipid phosphatase activity by measuring the ability of PTEN to de-phosphorylate PIP3 using Malachite Green Phosphatase Assay. As shown in Figure 32, the lipid phosphatase activity was two-fold higher in shJab1-bearing C5 and C6 cells than in control shLuc-bearing C5 and C6 cells. The data indicate that inhibition of Jab1 enhances PTEN lipid phosphatase activity.

Figure 32. Depletion of Jab1 enhances PTEN ability to de-phosphorylate PIP3.

Bar graph indicating differences in free phosphate group released during PTEN phosphatase reactions in C5 and C6 cells stably transfected with shLuc or shJab1. The changes in free phosphate relatively reflect the changes in PTEN lipid phosphatase activity between these cells.

Experiments were repeated three times. The data were presented as means ± standard deviations. Differences between individual groups were analyzed using a t test. P values <0.05 were considered statistically significant.



3.2. Jab1 negatively regulates PTEN tyrosine phosphatase activity

In addition to its lipid phosphatase activity, PTEN was reported to specifically de-phosphorylate p-Src at Y416 (84). Therefore, I tested whether the depletion of Jab1 also altered p-Src-Y-416 levels in C5 and C6 cells. As shown in Figure 33A, stable silencing of Jab1 decreased p-Src-Y416 levels in these cells, which was confirmed by quantitative data from repeated experiments (Figure 33B).

Next, I examined whether depletion of Jab1 also affected p-Src-Y416 levels in paraffin-embedded tumors from mice injected with C5shLuc or C5shJab1 lines. The immunohistochemical analysis demonstrated that knockdown of Jab1 in C5 tumor xenografts significantly decreased p-Src-Y416 compared to control Luc knockdown (Figure 34). This result implied the increase in PTEN tyrosine phosphatase activity due to inhibition of Jab1 in xenograft mice.

Together, these data indicate that silencing Jab1 results in increased PTEN tyrosine phosphatase function in breast cancer.

Figure 33. Knockdown of Jab1 enhances PTEN ability to de-phosphorylate p-Src-Y416 in HER2-positive breast cancer cells.

(A) Western Blot analysis of indicated proteins in C5shLuc and C5shJab1, or C6shLuc and C6shJab1.

(B) Bar graph shows the relative fold changes in p-Src-Y416 proteins between C5shLuc and C5shJab1, or C6shLuc and C6shJab1. The relative fold change represents the protein expression value of p-Src-Y416 in cells bearing shJab1 normalized to the expression value of p-Src-Y416 in cells bearing shLuc. Experiments were repeated three times. The data were presented as means ± standard deviations. Differences between individual groups were analyzed using a t test. P values <0.05 were considered statistically significant.

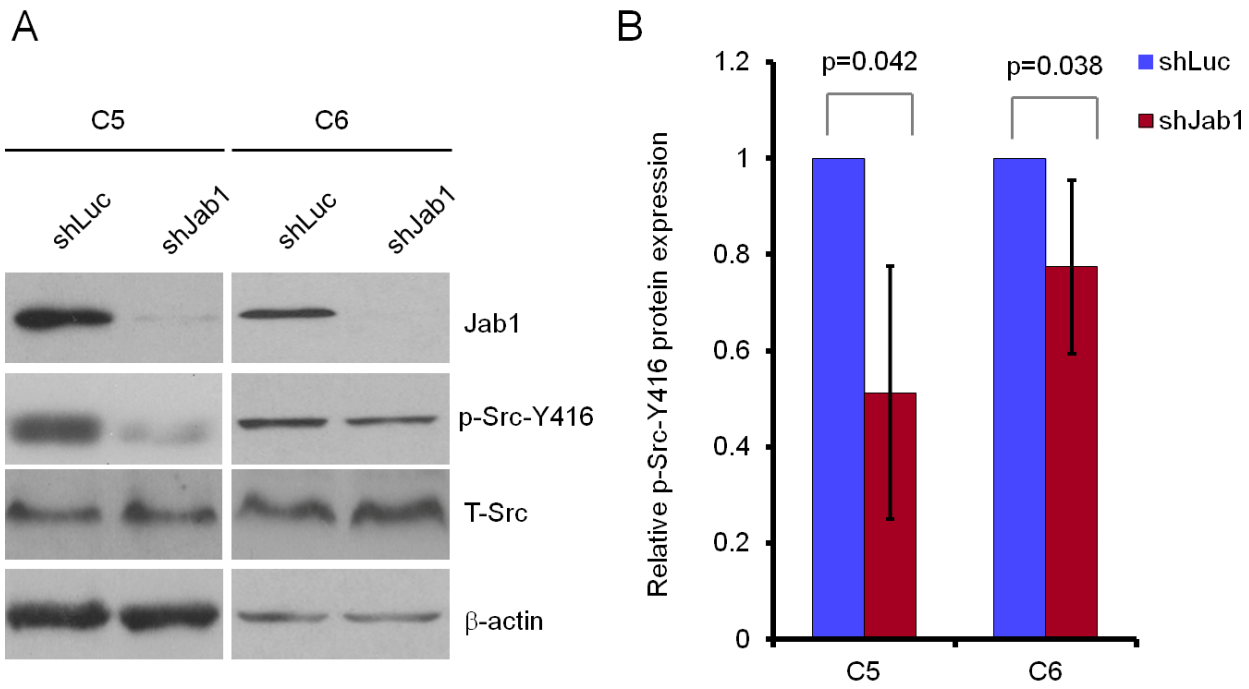
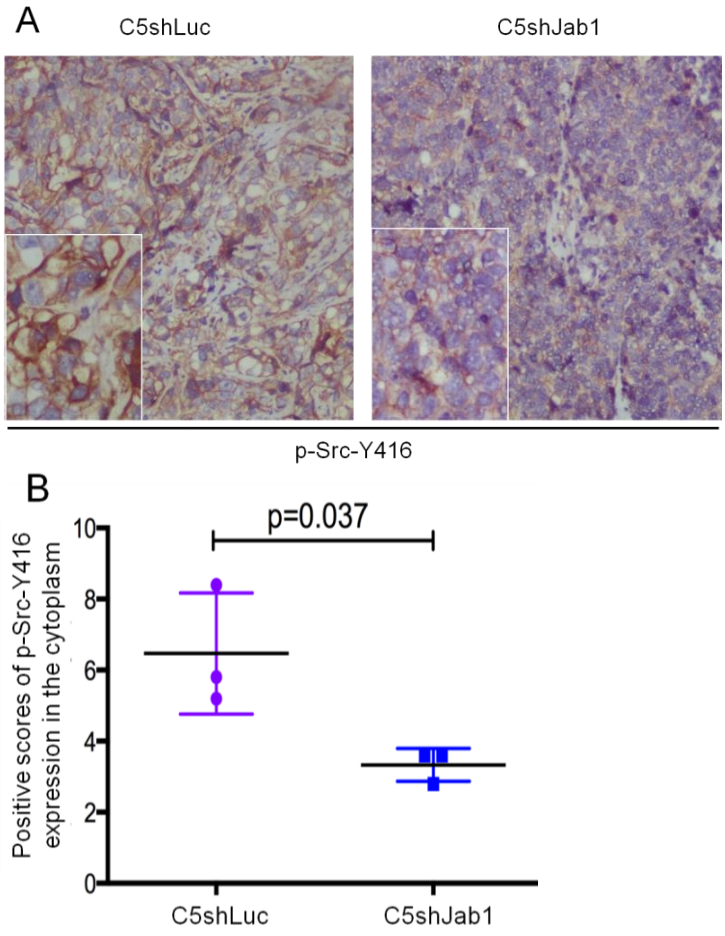


Figure 34. Knockdown of Jab1 decreases p-Src-Y416 in mouse xenografts.

(A) and **(B)** p-Src-Y416 expressions in paraffin-embedded tumors of mice injected with C5shLuc or C5shJab1 lines.

(A) Immunohistochemical staining of p-Src-Y416. Tumor cells were considered positive for p-Src-Y416 when cytoplasmic staining was present (brown cells). Scale bar, 100μm. Immunohistochemical staining was performed by Ronghua Zhang, MD Anderson Cancer Center, Houston, TX.

(B) Graph representing the positive scores of p-Src-Y416 expression in the cytoplasm. The scoring was performed by Sumei Wang, MD Anderson Cancer Center, Houston, TX (n=3 tumor slides per group).



3.3. Knockdown of Jab1 decreases p-Src-Y416 in a PTEN-dependent manner

Next, I investigated if Jab1 *per se* was able to mediate the level of p-Src-Y416 without PTEN expression. To address this question, I depleted Jab1 in MDA-MB-468, a PTEN-deficient breast cancer cells to examine how this affected p-Src-Y416 level. As presented in Figure 35, silencing of Jab1 did not alter p-Src-Y416 expression in MDA-MB-468 cells.

Next, I silenced PTEN using siRNA in stable Jab1 knockdown cells, C5shJab1 and C6shJab1. C5shLuc and C6shLuc were used as paired controls for Jab1 knockdown. I noticed that in C5 cells with normal PTEN expression (siCtrl), silencing of Jab1 consistently decreased the expression of p-Src-Y16 about 50% (Figure 36A). However, once PTEN was inhibited by siPTEN, inhibition of Jab1 did not show any effect on p-Src-Y416 level (Figure 36A). Similar results were observed for the pair of C6shLuc and C6shJab1 (Figure 36B).

In conclusion, these data suggest that Jab1 alters p-Src-Y416 expression in a PTEN-dependent manner.

Figure 35. Depletion of Jab1 in PTEN-deficient breast cancer cells shows no effect on p-Src-Y416 level.

Western blot showing protein expressions of Jab1, PTEN, p-Src-Y416 and T-Src in MDA-MB-468 cells transfected with siCtrl or siJab1 for 72 h.

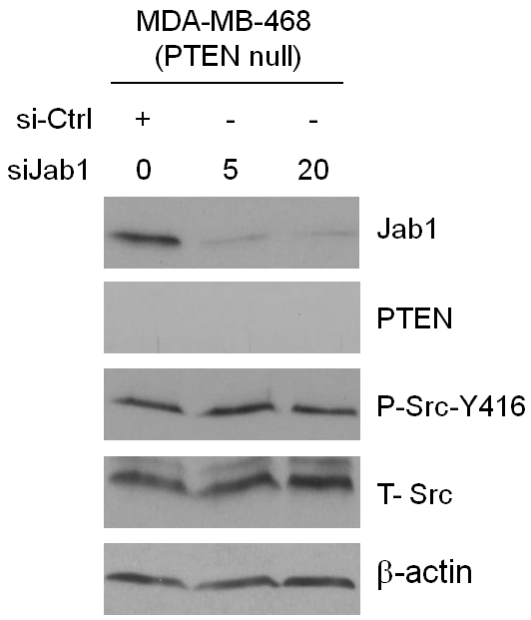
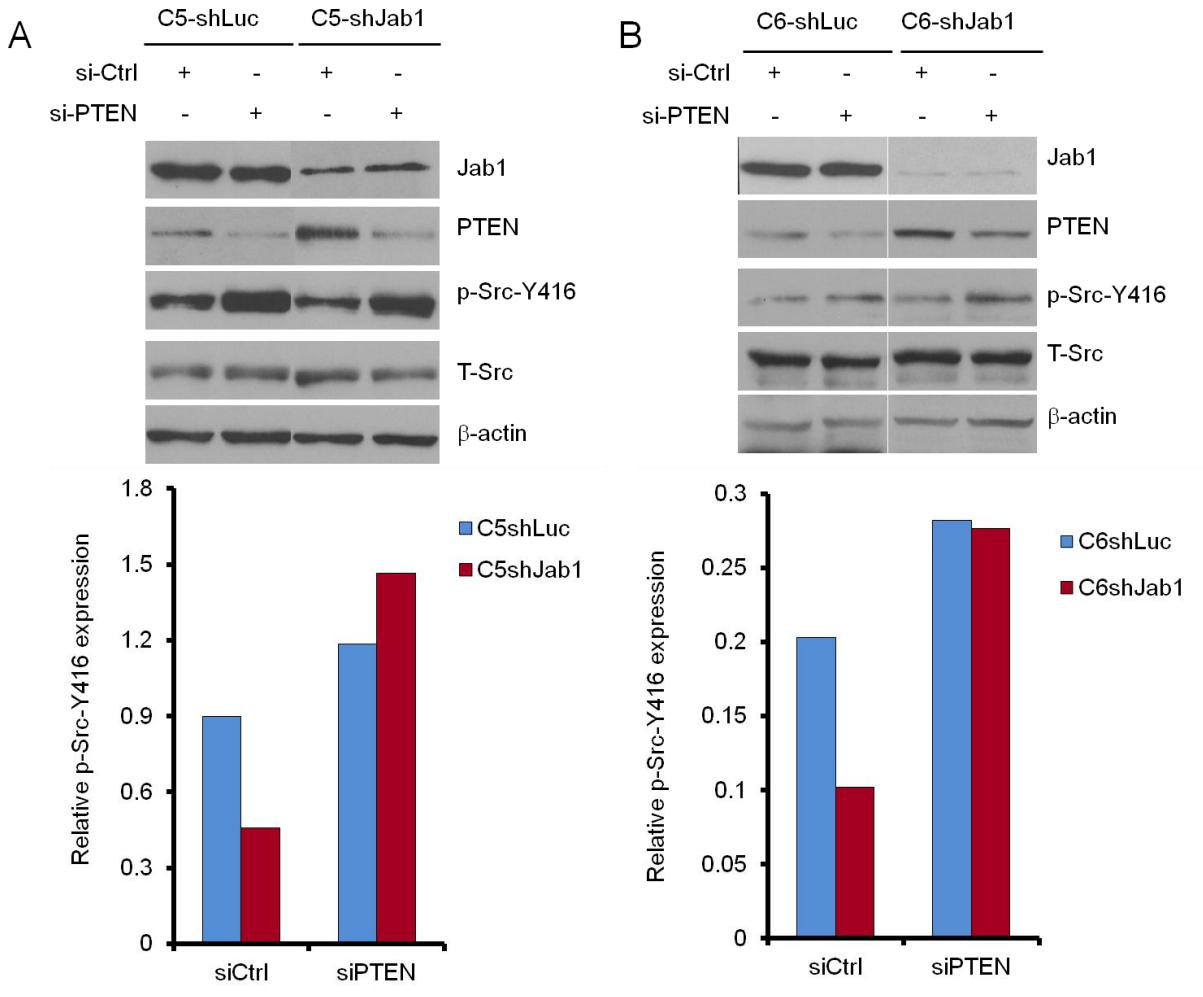


Figure 36. The loss of PTEN impairs the effect of Jab1 on altering p-Src-Y416 expression level.

(Top) Western Blot analysis of Jab1, PTEN, p-Src-Y416 and T-Src in **(A)** C5shLuc/C5shJab1 and **(B)** C6shLuc/C6shJab1 cells that were transiently transfected with siCtrl or siPTEN.

(Bottom) Densitometry of relative fold changes in protein levels of p-Src-Y416 in **(A)** C5shLuc/C5shJab1 and **(B)** C6shLuc/C6shJab1 cells that were transiently transfected with siCtrl or siPTEN.



CONCLUSION

My studies in this chapter demonstrate that depletion of Jab1 is sufficient to stabilize PTEN protein in HER2+ breast cancer cells.

Specifically, I found that silencing Jab1 affected PTEN more significantly in trastuzumab resistant cells (C5 and C6) compared to trastuzumab-sensitive cells (BT474). Based on the protein expression of Jab1 in BT474, C5 and C6 cells (Figure 28), I hypothesized that Jab1 level was higher in C5 and C6 cells which implied that depletion of Jab1 would create more significant effect on these cells than on BT474 cells. This hypothesis was further examined in chapter 5.

In addition, my studies showed that Jab1 expression was negatively correlated with PTEN protein expression in human breast invasive carcinoma specimens (Figure 30). Breast tumors with high Jab1 tend to have low PTEN while tumors with low Jab1 tend to have high expression of PTEN. I also observed that there was a stronger correlation between Jab1 protein and PTEN protein than between Jab1 mRNA and PTEN protein. There are two possible reasons for this difference: (1) TCGA BRCA may contain the mutated Jab1 *gene* which may not be correlated with PTEN expression and (2) the amplification of Jab1 gene does not always reflect the over-expression of Jab1 protein.

Moreover, I demonstrated that elevation in PTEN protein level due to depletion of Jab1 was accompanied by the increases in PTEN tyrosine and lipid phosphatase functions (Figures 32-35). As mentioned in chapter 1, PTEN converts PIP3 to PIP2 which results in decreased p-AKT-S473 level and increased free phosphor group (82, 83, 184). Consistently, in this chapter, I demonstrated that the increase in PTEN level

due to Jab1 knockdown was corroborated with the increase in PTEN ability to de-phosphorylate PIP3 and p-AKT-S473 (Figures 28, 29, and 32). In addition, I demonstrated that Jab1 knockdown, by enhancing PTEN protein expression, resulted in decreased p-Src-416 level (Figures 33-35) which indicated the increase in PTEN tyrosine phosphatase activity (84). In general, I identified that the negative effects of Jab1 on phosphatase function of PTEN were consistent with the major effect of Jab1 in negatively decreasing PTEN protein levels.

In conclusion, all these results together suggest that inhibition of Jab1 stabilizes PTEN protein which subsequently leads to increased PTEN lipid and tyrosine phosphatase functions. The molecular mechanisms of Jab1 that governs PTEN expression will be the focus of Chapter 4.

CHAPTER 4: THE MOLECULAR MECHANISMS BY WHICH JAB1 SUPPRESSES PTEN EXPRESSION

RATIONALE

The activity of PTEN can be regulated at many levels, including transcriptional regulation, posttranslational modifications (phosphorylation and ubiquitination) or through protein-protein interaction (86). Studies in Chapter 3 demonstrated that Jab1 negatively regulated PTEN in HER2+ breast cancer cell lines. *The goal of the studies in this chapter is to elucidate the mechanism of Jab1 that mediates PTEN expression.*

RESULTS

1. Jab1 mediates the posttranslational regulation of PTEN expression

1.1. Jab1 physically associates with PTEN protein

First, I investigated whether Jab1 regulated PTEN at a transcriptional level. I performed quantitative real-time PCR (qRT-PCR) to analyze the effect of Jab1 depletion on PTEN mRNA levels in BT474 and C5 cells. I found that neither transient nor stable knockdown of Jab1 significantly affected PTEN mRNA levels in BT474 or C5 cells (Figure 37A and B). This suggests that Jab1 may mediate post-transcriptional regulation of PTEN.

I next investigated whether PTEN associated with Jab1 by immunoprecipitation (IP). For endogenous interaction analysis, lysates of a HER2+ breast cancer cell line SKBR3 were immunoprecipitated with an anti-Jab1 or anti-PTEN antibody and then subjected to Western blotting with anti-PTEN and anti-Jab1 antibodies. SKBR3 cells immunoprecipitated with IgG antibody were used as controls. As presented in Figure 38A, endogenous PTEN was pulled down by Jab1 antibody and endogenous Jab1 was immunoprecipitated with PTEN antibody in SKBR3 line. For exogenous interaction analysis, I transfected 293T cells with Flag-tagged PTEN (Flag-PTEN) and Myc-tagged Jab1 (Myc-Jab1) then performed the IP. I showed that exogenous Flag-PTEN and Myc-Jab1 cross co-immunoprecipitated in 293T cells (Figure 38B).

These results together strongly suggest that Jab1 and PTEN proteins physically interact.

Figure 37. Silencing of Jab1 does not affect PTEN mRNA expression.

(A) *(top)* Western blot and *(bottom)* qRT-PCR analyses of PTEN expressions in BT474 cells transiently transfected with siCtrl or increasing concentrations of siJab1.

(B) *(top)* Western blot and *(bottom)* qRT-PCR analyses of PTEN expressions in C5shLuc and C5shJab1 cells. Experiments were repeated three times. The data were presented as means ± standard deviations.

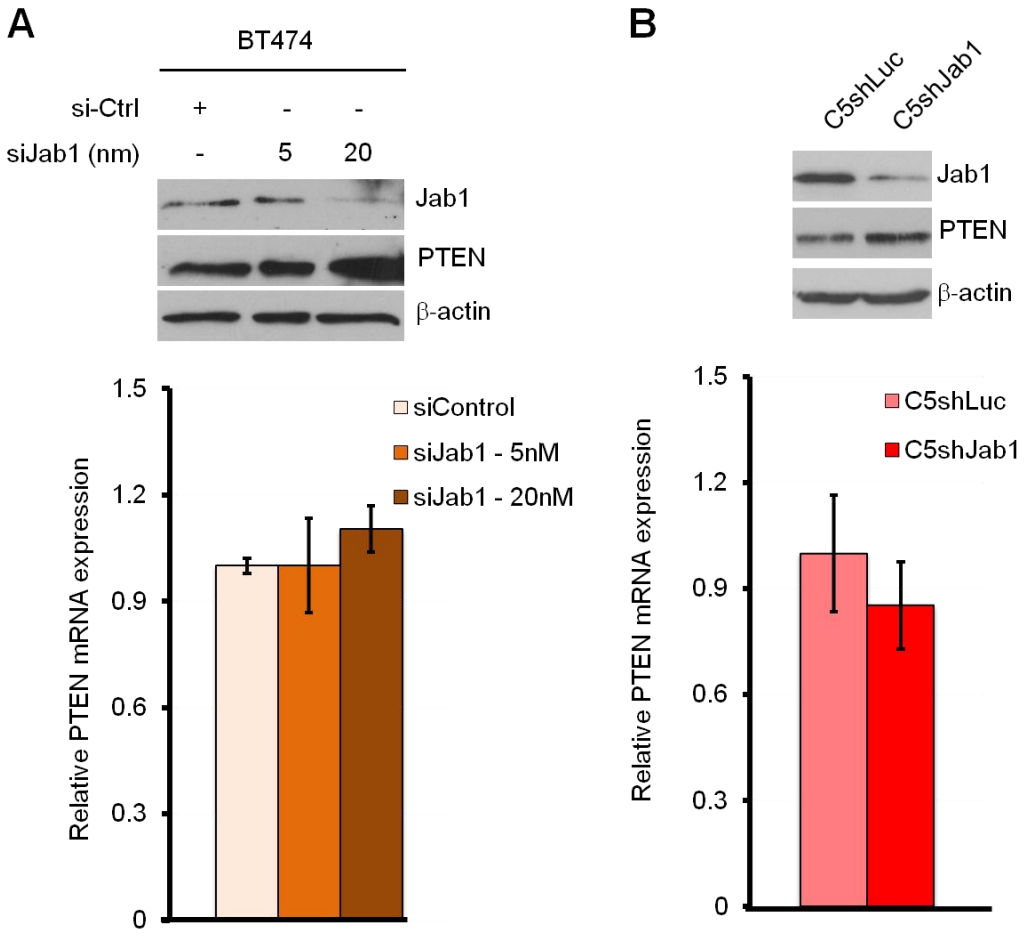
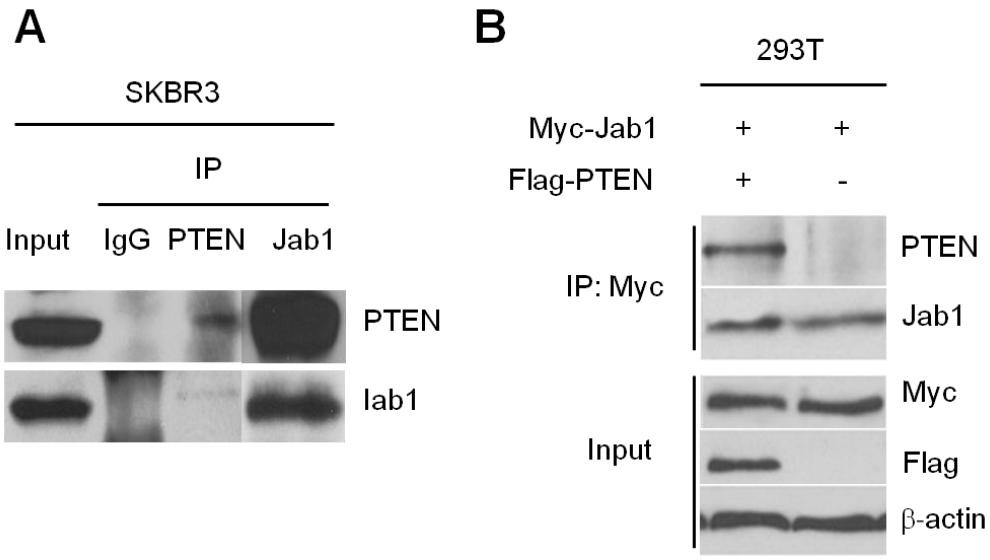


Figure 38. Jab1 and PTEN proteins cross co-immunoprecipitate.

Co-immunoprecipitation analysis of **(A)** endogenous and **(B)** exogenous Jab1 and PTEN in SKBR3 and 293T cells, respectively.

Minus signs (-) indicates empty vector transfection.



1.2. C-terminal end of Jab1 is required for Jab1 to regulate PTEN expression

Next, I identified the domain of Jab1 that was required for its association with PTEN. I used plasmids bearing wild-type (WT) Jab1 or a series of Jab1 deleting mutants that were previously generated in my lab (109). These mutants were deleted at the N-terminal (Jab1ΔN), the C-terminal (Jab1ΔC), the c-Jun binding domain (Jab1ΔJBD), or the MPN domain (Jab1ΔMPN) of Jab1 (Figure 39A).

Each of these mutants was co-transfected with Flag-PTEN followed by IP in 293T cells. The 293T cells transfected with empty vector or WT Jab1 alone were used as controls. Consistent with my previously presented data, Jab1 and PTEN associated (Figure 39B, lane 4). I further demonstrated that, compared to WT Jab1, the mutants Jab1ΔMPN, Jab1ΔN, and Jab1ΔJBD, had more than 3-fold increase in their abilities to immunoprecipitate PTEN (Figure 39B, lanes 4, 6, 7, and 8). In contrast, Jab1ΔC had a decreased ability to immunoprepitate PTEN of approximately 2-fold compared to WT Jab1 (Figure 39B, lanes 4 and 5).

Next, to verify that the C-terminal end of Jab1 is required for Jab1 to associate with PTEN, I over-expressed increasing amounts of WT Myc-Jab1 (WT Jab1) or Myc-Jab1ΔC (Jab1ΔC) into BT474 and SKBR3 cells and compared their effects on endogenous PTEN levels. Consistent with the IP data, while over-expression of WT Jab1 decreased endogenous PTEN protein expressions in a dose-dependent manner, Jab1ΔC showed no effect on PTEN level in BT474 and SKBR3 cells (Figure 40A and B, respectively). Similar findings were observed when I co-transfected Flag-PTEN with increasing amounts of either WT Jab1 or Jab1ΔC in 293T cells. Jab1 WT gradually

degraded exogenous Flag-PTEN while Jab1ΔC lost the ability to degrade exogenous Flag-PTEN (Figure 41).

To rule out the possibility that the differences in the association of these isoforms with PTEN was affected by their protein expression or subcellular localization, I transfected each of these constructs alone into 293T cells and examined their protein levels and their localizations. I found that all Jab1 WT and mutant constructs expressed similar protein levels and exhibited a cytoplasmic localization in 293T cells (Figure 42A and B, respectively).

Taken all together, my findings indicate that Jab1 regulates PTEN through protein-protein interaction and that this function requires the intact C-terminal end of Jab1.

Figure 39. Wild-type and mutants of Jab1 show different abilities to immunoprecipitate PTEN.

(A) Schematic representation of WT Jab1 and its deleting mutants.

(B) Immunoprecipitation analysis of exogenous Flag-PTEN with exogenous WT Myc-Jab1 (WT Jab1) or different mutants of Myc-Jab1.

Minus signs (-) indicates empty vector transfection.

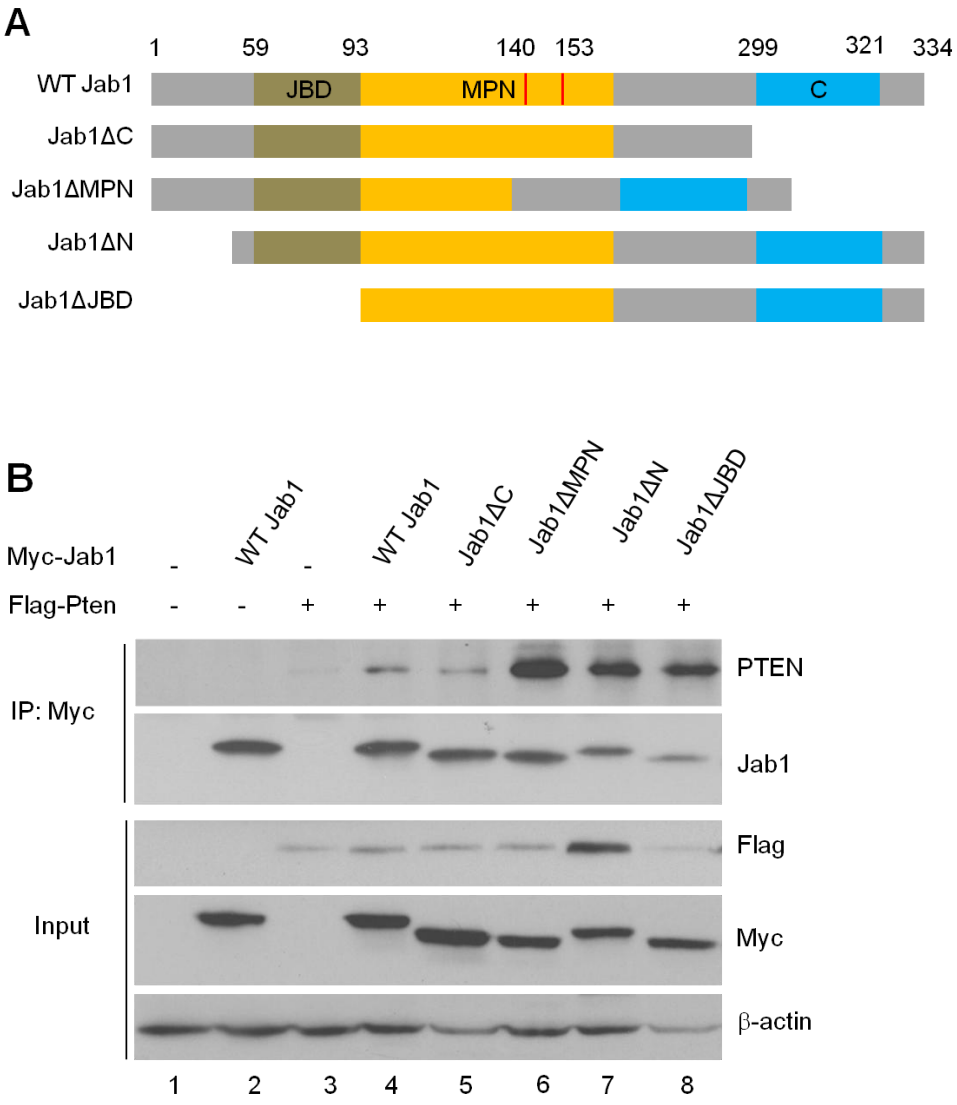


Figure 40. Deletion of the C-terminal end of Jab1 impairs its ability to degrade endogenous PTEN in HER2-positive breast cancer cells.

Western Blots showing the changes in expression levels of endogenous PTEN in **(A)** BT474 and **(B)** SKBR3 transfected with increasing amounts of WT Jab1 (*left panel*) or Jab1ΔC (*right panel*).

Minus signs (-) indicates empty vector transfection.

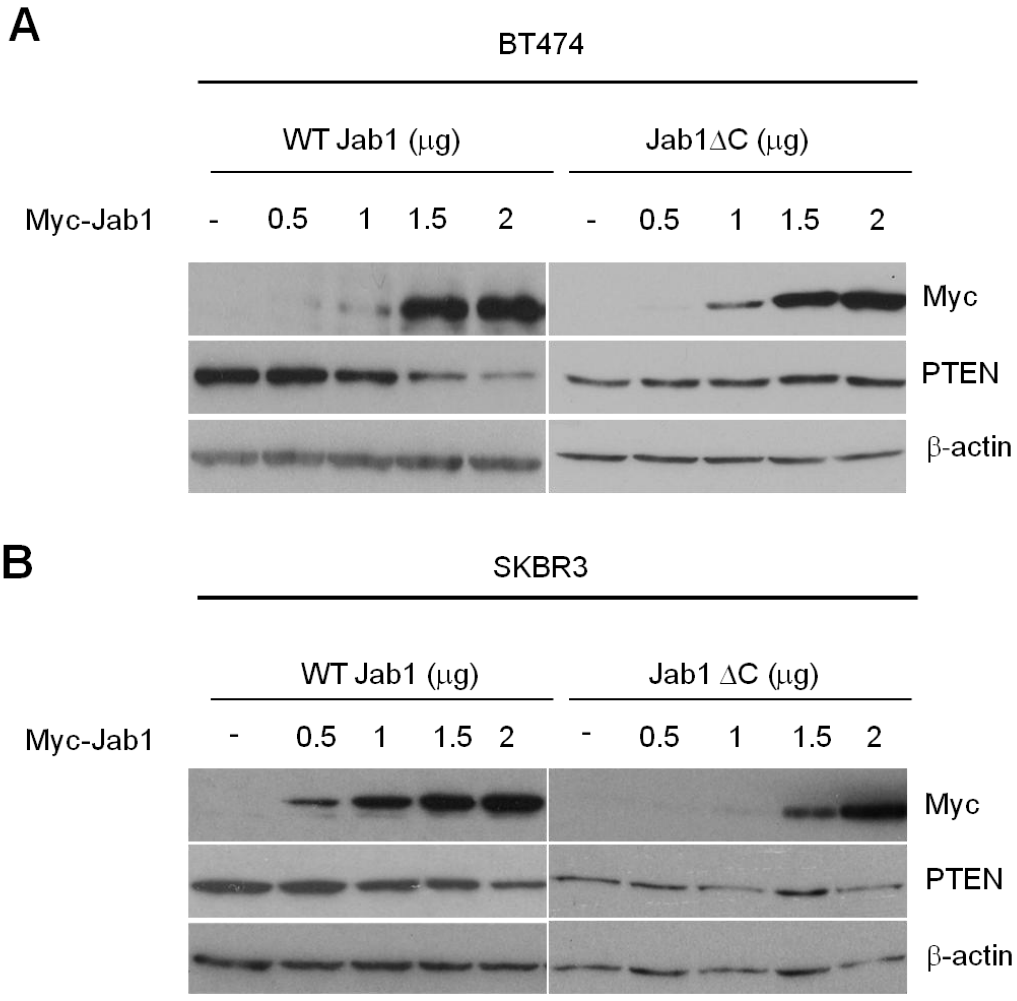


Figure 41. Deletion of the C-terminal end of Jab1 abolishes its ability to degrade exogenous PTEN.

Western Blots showing the changes in expression levels of exogenous Flag-PTEN in 293T cells co-transfected with increasing amounts of WT Jab1 (*left panel*) or Jab1ΔC (*right panel*).

Minus signs (-) indicates empty vector transfection.

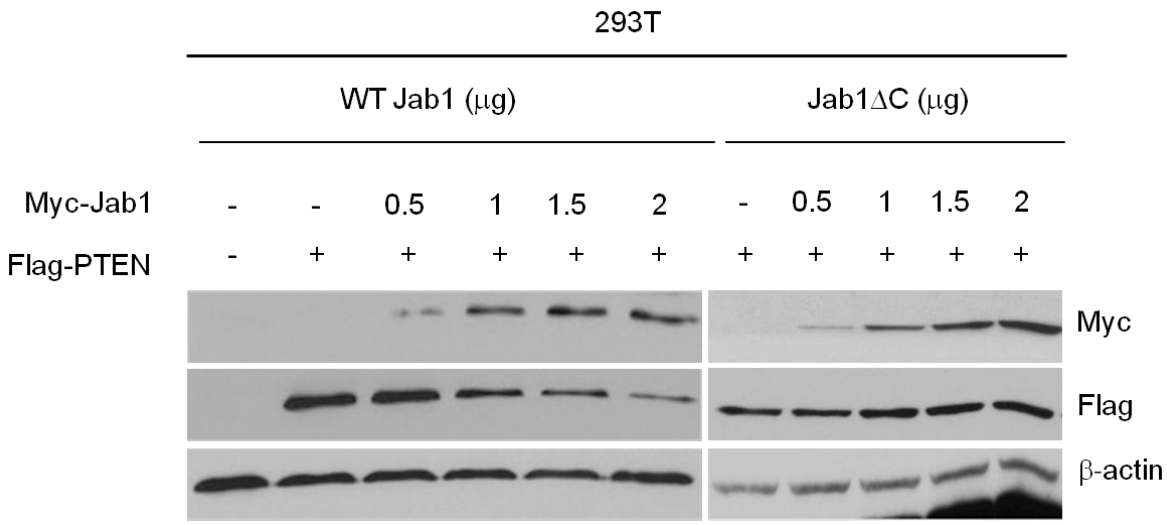


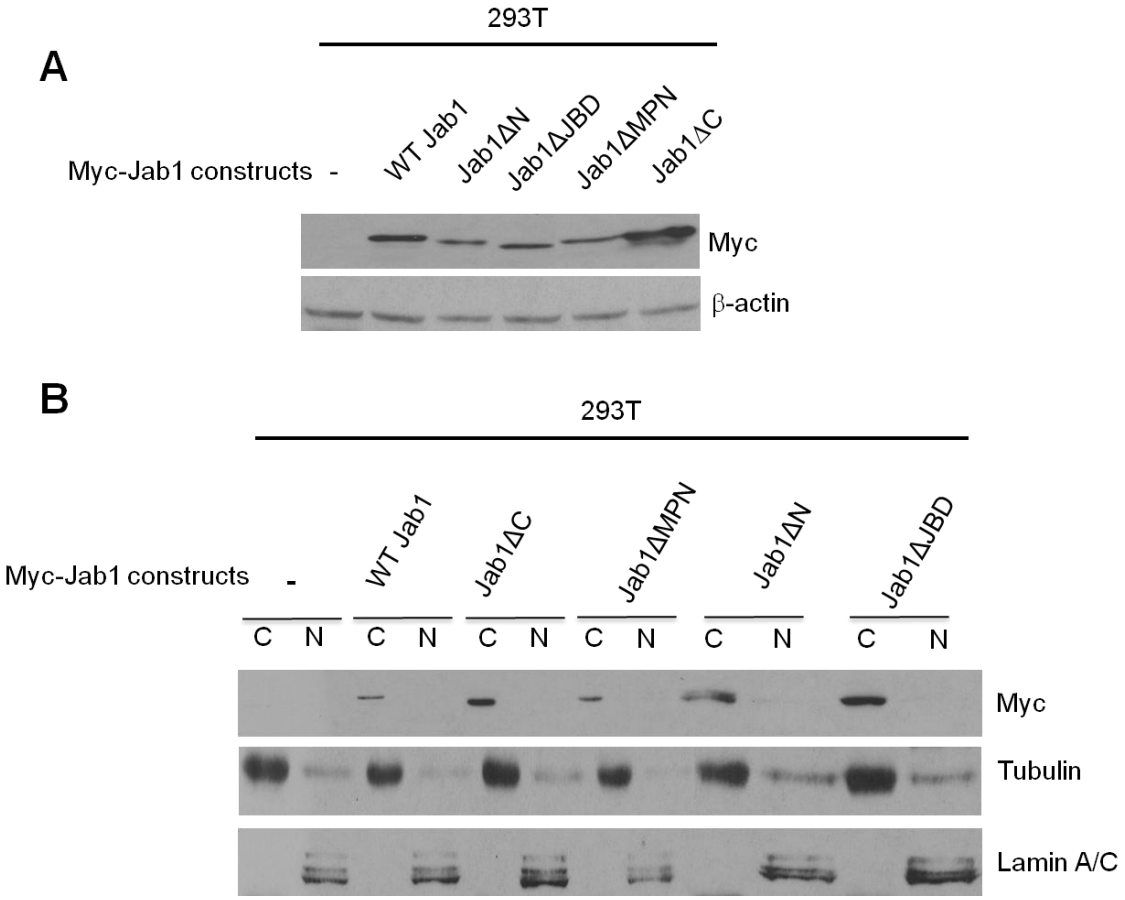
Figure 42. Exogenous wild-type Jab1 and different mutants of Jab1 show similar level of protein expression and exhibit cytoplasmic localization.

(A) Western Blot representing total protein expressions of WT Jab1 and its mutants in 293T cells.

(B) Nuclear-cytoplasmic fractionations of transfected WT Jab1 or different mutants of Jab1 in 293T cells.

Minus signs (-) indicates empty vector transfection.

Abbreviations: C: cytoplasmic fraction; N: nuclear fraction.



2. Jab1 interacts with phosphorylated and de-phosphorylated PTEN

PTEN is frequently down-regulated in breast cancer, and PTEN mutations contribute to its low expression in tumors (86, 87). Herein, I examined if PTEN mutations affected the association with Jab1. Structurally, PTEN consists of a PIP3-binding domain, a phosphatase domain, a C2 domain, and a C-terminal tail with a PDZ binding motif (Figure 43A) (86). As mentioned in chapter 1, the C-terminal tail of PTEN, specifically the phosphorylation clusters of S380/T382/T383, mediated PTEN stability and its interactions with other proteins (87, 90). Therefore, I focused my study on this cluster region of PTEN (Figure 43A, red lines). First, I compared the interaction of Jab1 with WT PTEN and of Jab1 with the phosphorylation-defective PTEN-3A. The PTEN-3A is a mutant in which the three phosphorylation sites, S380, T382 and T383 of PTEN were mutated to un-phosphorylatable alanine residues (Figure 43B). I co-transfected the 293T cells with Myc-Jab1 and either Flag- WT PTEN (WT PTEN) or Flag-PTEN-3A (PTEN-3A). The 293T cells transfected with empty vector or Flag-PTEN alone were used as controls. Using the co-IP, I demonstrated that PTEN-3A bound Jab1 more efficiently than WT PTEN did (Figure 43C, lanes 5 and 4).

To examine whether the increased binding of PTEN-3A to Jab1 led to its faster degradation by Jab1, I transfected 293T cells with either WT PTEN or PTEN-3A in the presence or absence of Jab1. As compared to WT PTEN, the introduction of Jab1 completely degraded PTEN-3A (Figure 44A, lanes 2 and 4). I then compared the degradation kinetics of WT PTEN with PTEN-3A. I transfected WT PTEN or PTEN-3A with or without Jab1 into 293T cells followed by short-term blockade of translation with cycloheximide (CHX). The protein expressions of WT PTEN or PTEN-3A in the

presence or absence of Jab1 at different time points were measured using Image J and were blotted in Figure 44B. I showed that compared to WT PTEN alone (blue line), the turnover of WT PTEN was much faster in presence of Jab1 (red line), which is consistent with my previous data. In addition, I demonstrated that the half-life of PTEN-3A (Figure 44, green line) was shorter compared to the half-life of WT PTEN (Figure 44, blue line). I further showed that in the presence of Jab1, PTEN-3A (Figure 44, pink line) was degraded about 4 times faster compared to PTEN-3A alone (Figure 44, green line).

Taken together, these findings suggest that Jab1 facilitates the degradation of both WT PTEN and PTEN-3A.

Figure 43. De-phosphorylation of PTEN at the clusters of Serine380 and Threonine382/383 increases PTEN binding to Jab1.

(A) Schematic representation of WT PTEN and its phosphorylation-defective PTEN-3A mutant (the three un-phosphorylatable alanine residues S380A, T382A and T383A were marked by red lines).

(B) Western blot showing the expressions of exogenous p-PTEN (S380/T382/T383) and (total) PTEN in 293T cells transfected with Flag-WT PTEN or Flag-PTEN-3A.

(C) Immunoprecipitation analysis of Myc-Jab1 with Flag-WT PTEN or Flag-PTEN-3A. Minus signs (-) indicates empty vector transfection.

All experiments were performed in 293T cells.

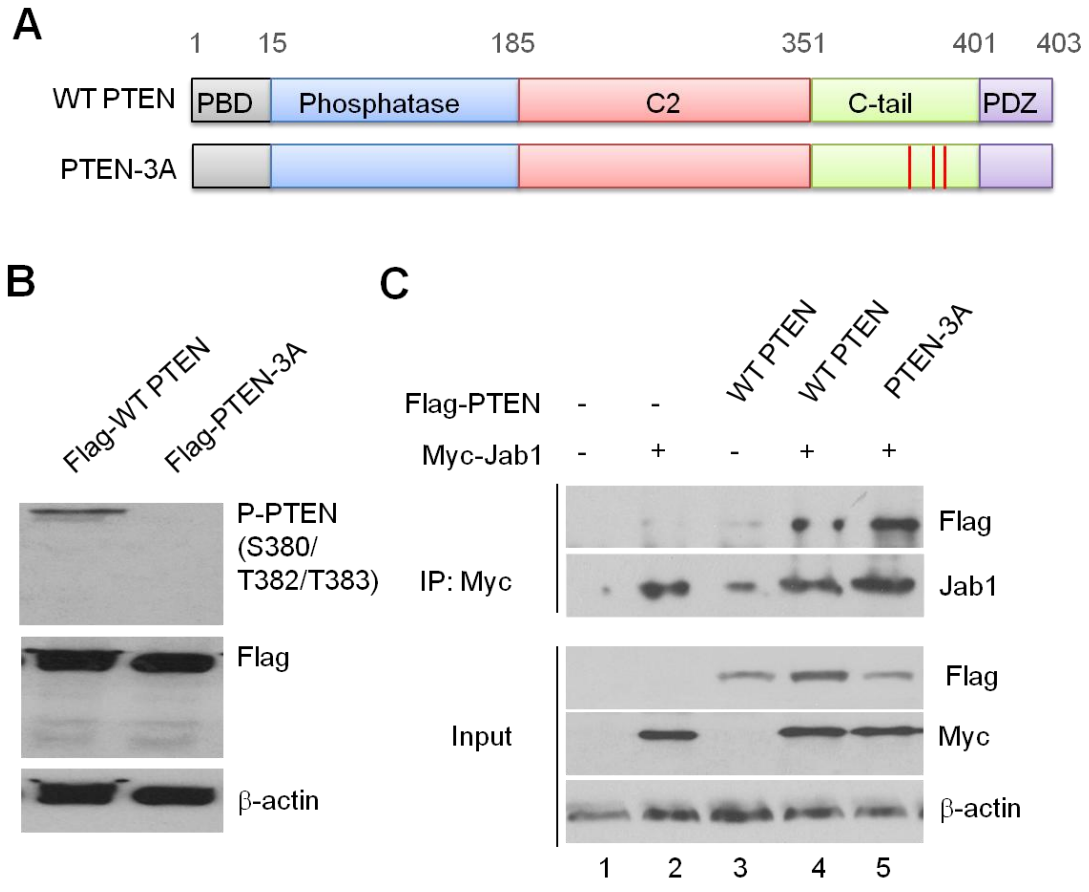


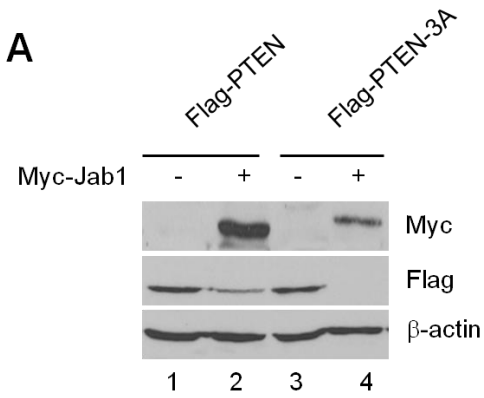
Figure 44. In the presence of Jab1, mutant PTEN-3A is degraded faster by Jab1 compared to wild-type PTEN.

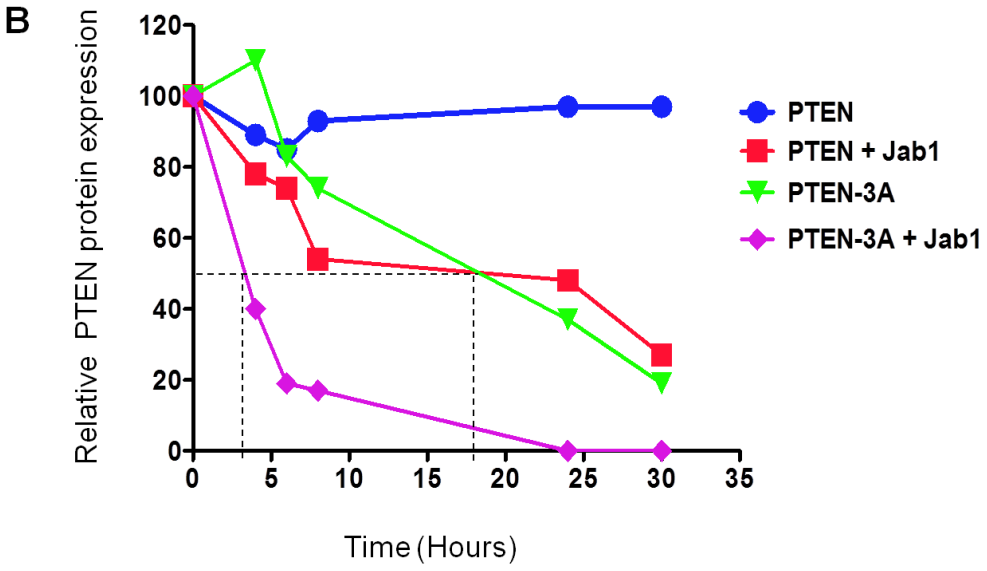
(A) Western Blot showing changes in protein expressions of exogenous WT PTEN or PTEN-3A, in the presence or absent of Jab1.

(B) Line graph representing the degradation kinetics of WT PTEN or PTEN-3A with or without Jab1.

Minus signs (-) indicates empty vector transfection.

All experiments were performed in 293T cells.





3. Jab1 does not regulate PTEN protein expression through the UPS in HER2-positive breast cancer

3.1. Proteasome inhibitors increase PTEN poly-ubiquitination but do not prevent PTEN degradation mediated by Jab1

I have shown that Jab1 mediated post-translational regulation of PTEN, which decreased PTEN protein stability and subsequently PTEN phosphatase activity. I next elucidated the mechanism by which Jab1 controlled PTEN protein expression. As mentioned in chapter 1, the majority (~80%) of intracellular proteins were reported to go through the UPS for degradation, including PTEN (86, 87). To address whether Jab1 also facilitated PTEN degradation through the UPS, I first tested if PTEN stability is mediated through the UPS pathway. I transfected the 293T cells with Flag-PTEN and treated the cells with either DMSO as a control or MG-132, a potent inhibitor of 26S proteasome. The changes in ubiquitination pattern of exogenous PTEN between the two treatment groups were then examined. Consistent with previous work (92, 94, 95), I observed a robust increase in PTEN poly-ubiquitin (poly-Ub) pattern with the addition of MG-132 compared with DMSO treatment (Figure 45).

Next, to determine if blocking proteasome activity restored PTEN expression in the presence of Jab1, the BT474 cells were transfected with increasing amount of Myc-Jab1 and then were treated with either DMSO or MG-132. I found that similar to DMSO treatment, MG-132 failed to prevent PTEN degradation due to over-expression of Jab1 in BT474 cells (Figure 46). To verify the results, I transfected BT474 cells with the same amount of Myc-Jab1 and treated the cells with either DMSO or different

proteasome inhibitors (MG-132, LLnL, and Lactacystin). Figure 47A showed that compared to DMSO treatment, these inhibitors prevented p27 destruction when Jab1 was over-expressed, which is consistent with prior work of others. This restoration in p27 protein expression also indicates that the proteasomal activity was blocked by these inhibitors. In contrast, treatment with these inhibitors did not prevent PTEN degradation induced by over-expression of Jab1 (Figure 47A). Quantitative data from repeated experiments confirmed my findings that these proteasome inhibitors failed to prevent PTEN degradation in the presence of Jab1 (Figure 47B). These results together imply that Jab1 does not facilitate PTEN degradation through the 26S proteasome pathway.

Figure 45. MG-132 treatment results in the accumulation of PTEN poly-ubiquitination.

In vivo ubiquitination analysis of exogenous PTEN poly-ubiquitination patterns in 293T cells treated with DMSO or MG132.

Abbreviations: His-Ub, His-tagged ubiquitin; IB, immunoblot (western blot); IP, immunoprecipitation. Minus signs (-) indicates DMSO treatment or empty vector transfection.

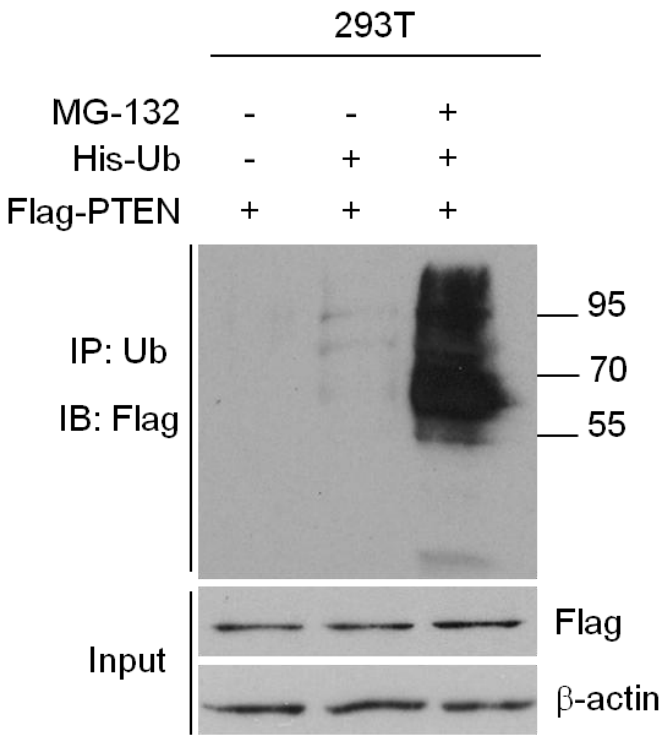


Figure 46. Over-expression of Jab1 facilitates PTEN degradation in BT474 cells treated with either DMSO or MG-132.

Western blot showing protein expressions of endogenous PTEN in BT474 cells transfected with increasing amounts of Myc-Jab1 and then treated with DMSO or MG-132.

Minus signs (-) indicates empty vector transfection.

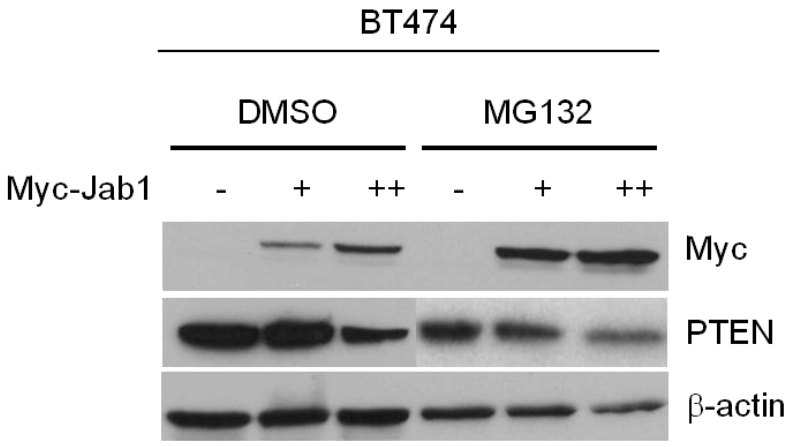
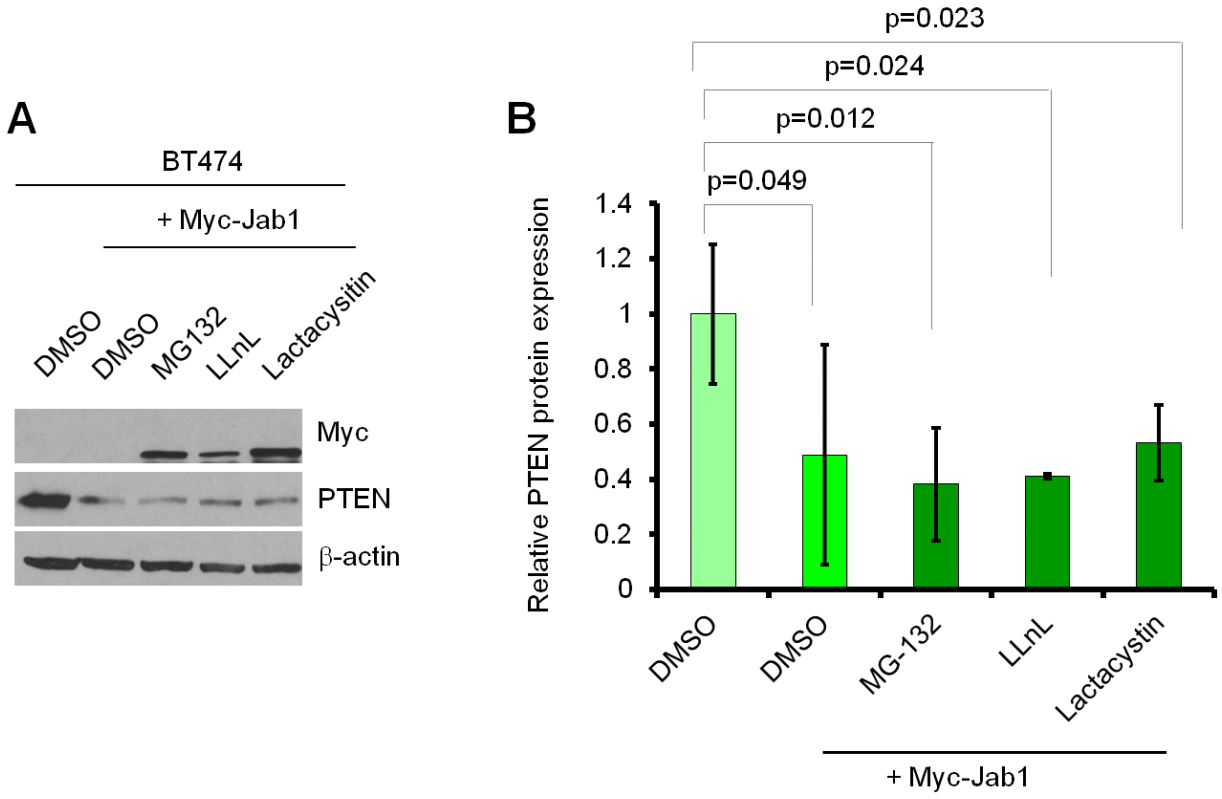


Figure 47. Proteasome inhibitors do not prevent PTEN degradation induced by over-expression of Jab1.

(A) Western blot showing protein expressions of endogenous PTEN, and p27 in BT474 cells transfected with empty vector or the same amount of Myc-Jab1 and then treated with DMSO or different proteasome inhibitors.

(B) Graph representing the means of PTEN protein expression levels from repeated experiments as described in **(A).** Experiments were repeated three times. The data were presented as means ± standard deviations. Differences between individual groups were analyzed using a t test. P values <0.05 were considered statistically significant.



3.2. In the presence of Jab1, proteasome inhibitors fail to accumulate PTEN poly-ubiquitination

If a protein is degraded through the UPS, the addition of a proteasome inhibitor will lead to the increase in protein expression and the accumulation in protein poly-Ub (166). I identified that though proteasome inhibitors resulted in the accumulation of PTEN poly-Ub, the treatment failed to prevent PTEN degradation induced by Jab1. Therefore, I next evaluated the effects of proteasome inhibitors on PTEN poly-Ub in the presence of Jab1. I co-transfected the 293T cells with the same amounts of Flag-PTEN, His-tagged ubiquitin (His-Ub) and increasing amount of Myc-Jab1. 293T cells transfected with either Flag-PTEN or His-Ub alone were used as controls. After treating the cells with MG-132 to block the 26S proteasome activity, I performed the *in vivo* ubiquitination assay to examine the changes in the ubiquitination pattern of PTEN. As presented in Figure 48, lanes 1, 2 and 3, PTEN was ubiquitinated in the presence of His-Ub. However, I found that even with the addition of MG-132, over-expression of Jab1 markedly decreased PTEN ubiquitination (Figure 48, lanes 3, 4, 5, and 6).

As discussed in chapter 1, Jab1 contains the MPN domain, which was thought to de-ubiquitinase ubiquitinated proteins (Figure 39A) (110, 112, 118). Therefore, to rule out the possibility that Jab1 facilitated the de-ubiquitination of PTEN, I over-expressed the Jab1 mutant lacking the MPN domain (Jab1ΔMPN) into 293T cells and examined the changes in PTEN ubiquitination. Similarly, PTEN ubiquitination was decreased by the over-expression of Jab1ΔMPN, even in the presence of MG-132 (Figure 48, lanes 7, 8, and 9). In addition, I noticed that PTEN was ubiquitinated less effectively in the presence of Jab1ΔMPN than in the presence of WT Jab1 (Figure 48,

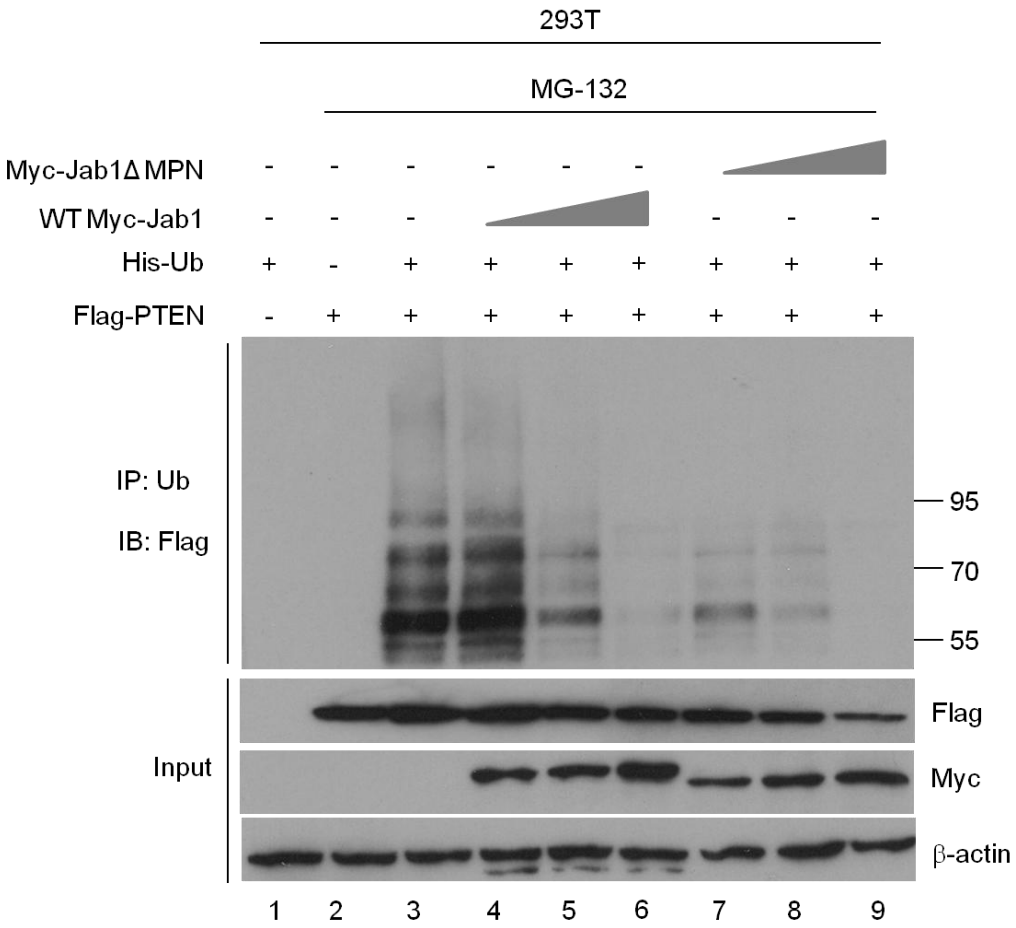
lanes 4 and 7). These data suggests that the decreases in PTEN ubiquitination are not mediated by the potential de-ubiquitinase activity of Jab1.

Together, these findings strongly argue that the UPS is not the major mechanism underlying the effect of Jab1 on PTEN stability.

Figure 48. Over-expression of either wild-type Jab1 or mutant Jab1ΔMPN reduces PTEN poly-ubiquitination.

In vivo ubiquitination analysis of Flag-PTEN poly-ubiquitination in 293T cells transfected with increasing amounts of WT Myc Jab1 or Jab1ΔMPN and treated with MG132.

Minus signs (-) indicates empty vector transfection.



4. Jab1 mediates PTEN degradation through the lysosomal pathway in HER2-positive breast cancer

4.1. Inhibition of lysosomal enzymes impairs Jab1 ability to degrade PTEN

As mentioned in chapter 1, the UPS pathway usually mediated the degradation of proteins attached to K48-Ub chains; the lysosomal proteolytic pathway regulates substrates modified by K63-linked polyUb (143). To determine whether the polyUb chain of PTEN was linked at K48 or at K63, I co-transfected Flag-PTEN with either Ub-WT, K48R-mutant ubiquitin (Ub-K48R), or K63R-mutant ubiquitin (Ub-K63R) in 293T cells and performed *in vivo* ubiquitination assays. The controls were 293T cells transfected with either Ub-WT or Flag-PTEN alone. The K48R- or K63R-mutant in which the K (lysine) was mutated to the R (arginine) lost the ability to form the poly-ubiquitin chains. I found that mutation at K48 of Ub did not significantly affect the ubiquitination pattern of PTEN compared to WT Ub (Figure 49, lanes 5 and 3). Instead, I noticed that mutation at K63 of Ub markedly reduced PTEN poly-ubiquitination compared with Ub-WT or Ub-K48R (Figure 49, lanes 4, 3 and 5). These results implied that PTEN poly-Ub chain was predominantly linked at K63. Therefore, I hypothesized that Jab1 mediated PTEN degradation mainly through the lysosomal system.

To address this hypothesis, I first examined the effect of the lysosomal enzyme inhibitors- E64D and pepstatin A- on PTEN protein expression in BT474 cells transfected with increasing amounts of myc-Jab1. I found that this combined treatment led to an increase in the conversion of LC3-I to LC3-II accompanied by an accumulation of p62 compared with DMSO-treated cells (Figure 50A). As discussed in

chapter 1 (table 3), the increase in LC3 and p62 indicated impaired lysosomal function (185). Similar to my previous findings, in the control DMSO treatment, increases in Jab1 led to gradual decreases in PTEN (Figure 50A, left panel). However, in the E64D and pepstatin A treatment, PTEN was not degraded by over-expression of Jab1 (Figure 50A, right panel). Quantitative data from repeated experiments confirmed that lysosomal inhibitors completely restored PTEN expression, even when Jab1 was over-expressed (Figure 50B).

Figure 49. Mutation of ubiquitin at lysine 63 reduces PTEN poly-ubiquitination compared to wild-type ubiquitin or mutation of ubiquitin at lysine 48.

In vivo ubiquitination analysis of PTEN poly-ubiquitination in 293T cells co-transfected with Flag-PTEN and either Ub-WT, Ub-K48R, or Ub-K6R, then treated with MG132. Minus signs (-) indicates empty vector transfection.

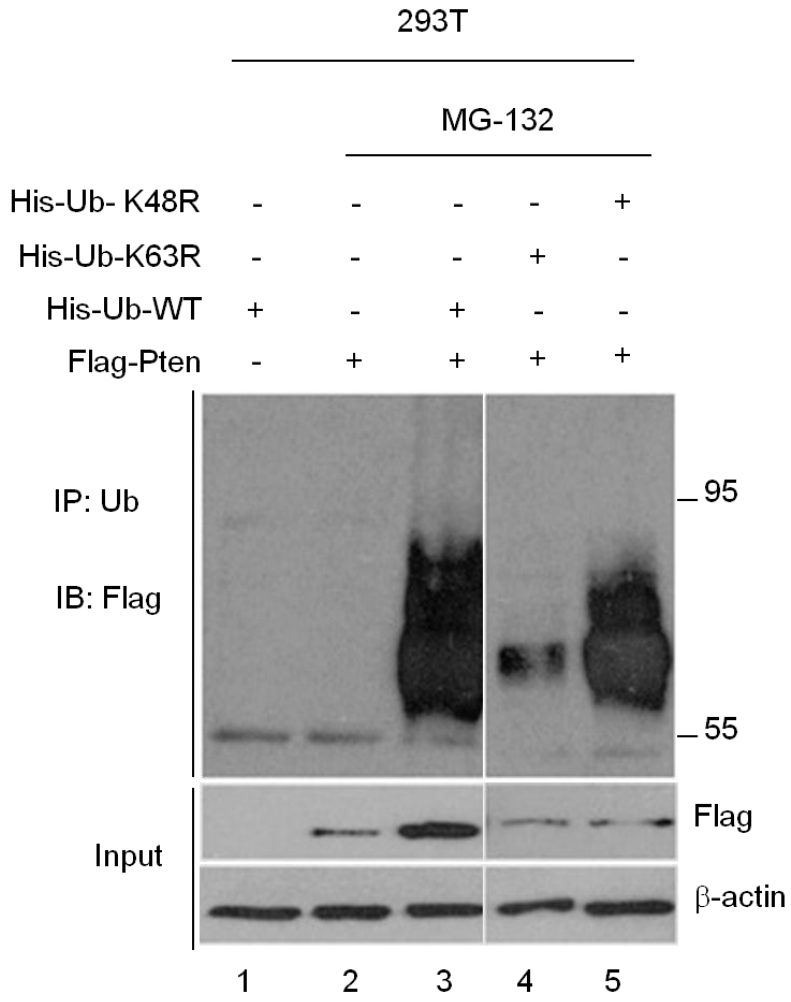
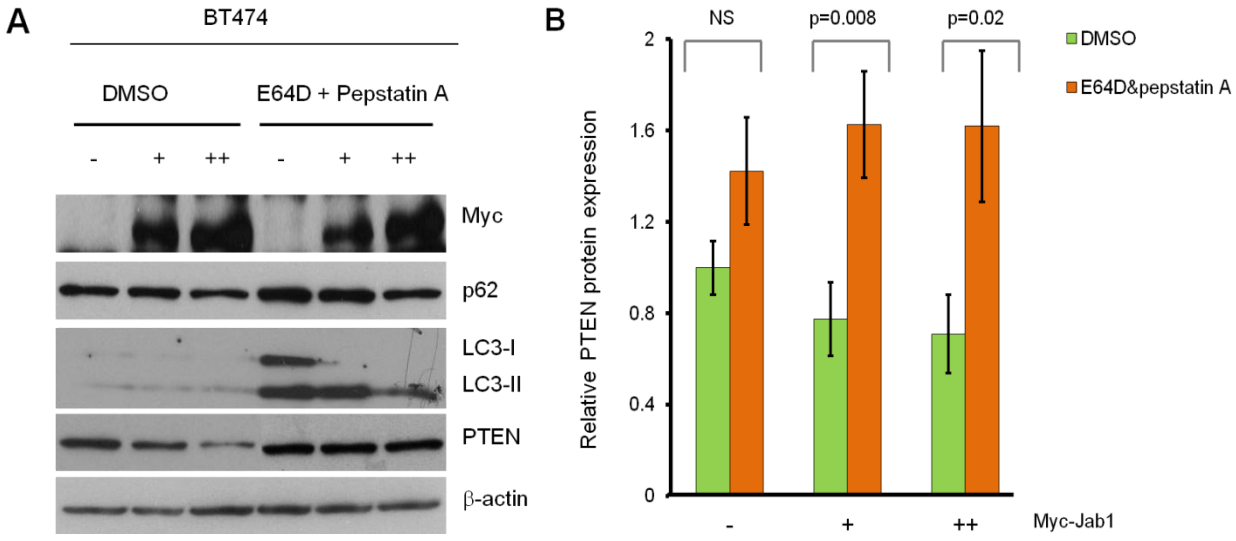


Figure 50. Inhibitors of lysosomal enzymes prevent PTEN degradation induced by over-expression of Jab1.

(A) Western blot showing protein expressions of autophagy markers (p62 and LC3) and PTEN in BT474 cells transfected with increasing amounts of Myc-Jab1 and then treated with DMSO or lysosomal enzyme inhibitors E64D and pepstatin A.

(B) Bar graph representing the means of PTEN protein expression levels from repeated experiments as described in **(A).** Experiments were repeated three times. The data were presented as means ± standard deviations. Differences between individual groups were analyzed using a t test. P values <0.05 were considered statistically significant.

Minus signs (-) indicates empty vector transfection.



4.2. PTEN degradation-mediated by Jab1 does not occur through the macro-autophagy pathway

As discussed in chapter 1, in mammalian cells, there are three distinct pathways that deliver proteins for lysosomal degradation: macro-autophagy, chaperone-mediated autophagy (CMA), and micro-autophagy (143). Among these mechanisms, macro-autophagy is the best-characterized pathway and has been found to directly mediate the degradation of poly-ubiquitinated-proteins through their association with p62. I first investigated whether macro-autophagy delivered PTEN for lysosomal degradation. I treated the BT474 cells with a macro-autophagy inhibitor bafilomycin A (142). I observed that p62 protein was accumulated while LC3-II was not formed in bafilomycin A–treated cells compared with DMSO-treated cells (Figure 51), which indicates that macro-autophagy flux is defective (table 3). However, bafilomycin A had minimal effect on PTEN levels (Figure 51).

Next, I compared the effect of Jab1 on PTEN in paired MEF WT cells and the MEF cells lacking an essential macro-autophagy-related gene ATG5 (MEF-ATG5 KO). I found that p62 protein was accumulated while LC3-II was not formed in MEF-ATG5 KO cells compared with MEF WT cells (Figure 52A), which is consistent with the previous work of others (table 3). My western blot and quantitative analysis shown in Figure 52A and B indicated that PTEN was gradually degraded due to the over-expression of Jab1 in both MEF WT and MEF-ATG5 KO cells.

In conclusion, these data demonstrate that neither pharmacologic inhibition of macro-autophagy nor genetic removal of a critical macro-autophagy gene prevents degradation of PTEN induced by over-expressing Jab1.

Since direct inhibition of macro-autophagy did not rescue PTEN expression, I investigated whether p62 was required for Jab1-mediated PTEN degradation. I compared the effects of over-expressing Jab1 on PTEN in MEF-p62 WT and MEF-p62 KO cells. I found that depletion of p62 also failed to prevent degradation of PTEN mediated by Jab1 over-expression (Figure 53A and B). Taken all data together, I conclude that although Jab1 requires functional lysosomes to degrade PTEN, this degradation does not occur through the macro-autophagy pathway.

Figure 51. Bafilomycin A has minimal effects on PTEN protein expression levels.

Western blot showing the changes in protein expression levels of ATG5, autophagy markers (p62 and LC3-II), and PTEN in BT474 treated with DMSO (0) or increasing concentrations of bafilomycin A.

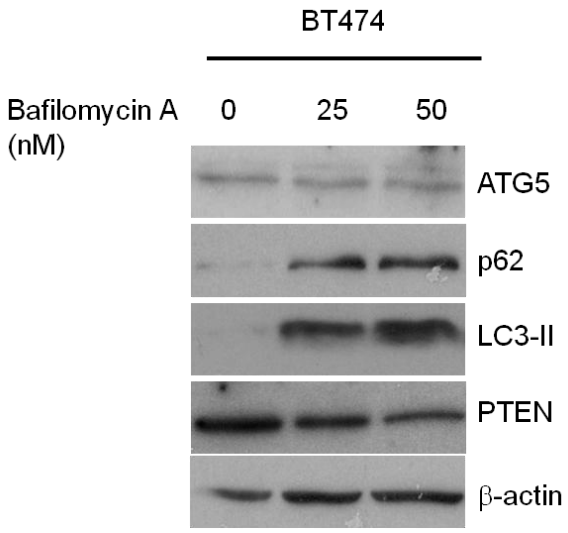


Figure 52. Jab1 facilitates PTEN degradation in MEF WT and MEF-ATG5 KO.

(A) Western blot showing protein expressions of ATG5, autophagy markers (p62 and LC3) and PTEN in MEF WT and MEF-ATG5 KO transfected with increasing amounts of Myc-Jab1.

(B) Bar graph representing the means of PTEN protein expression levels from repeated experiments as described in **(A).** Experiments were repeated three times. The data were presented as means ± standard deviations. Differences between individual groups were analyzed using a t test. P values <0.05 were considered statistically significant.

Minus signs (-) indicate empty vector transfection.

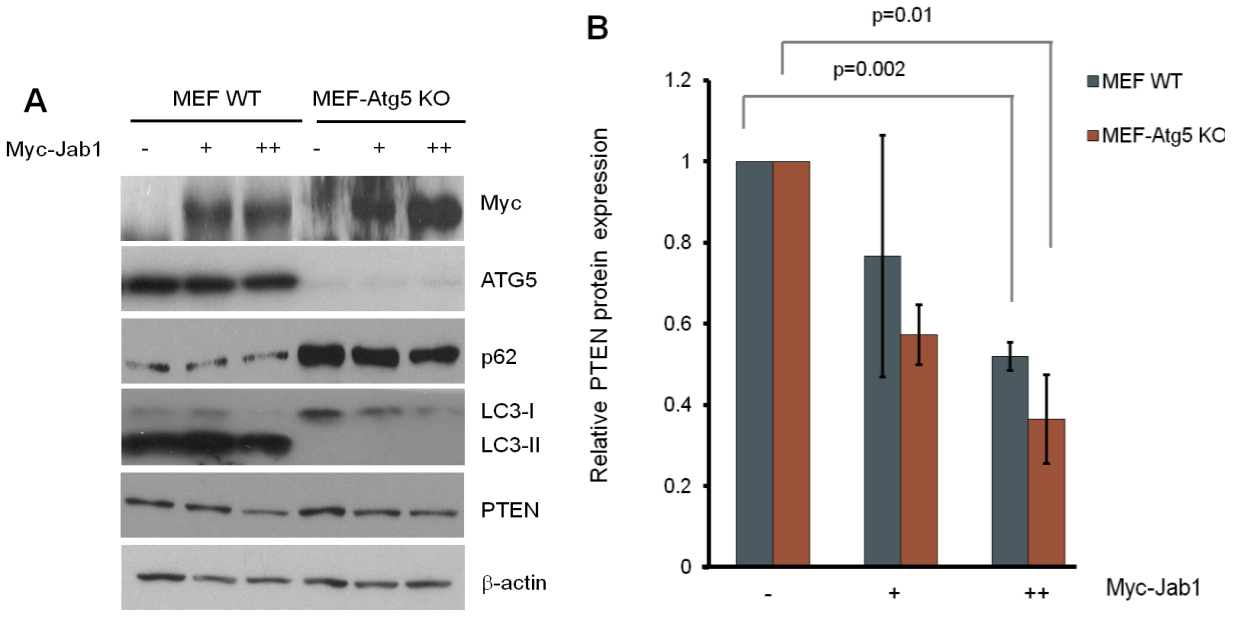
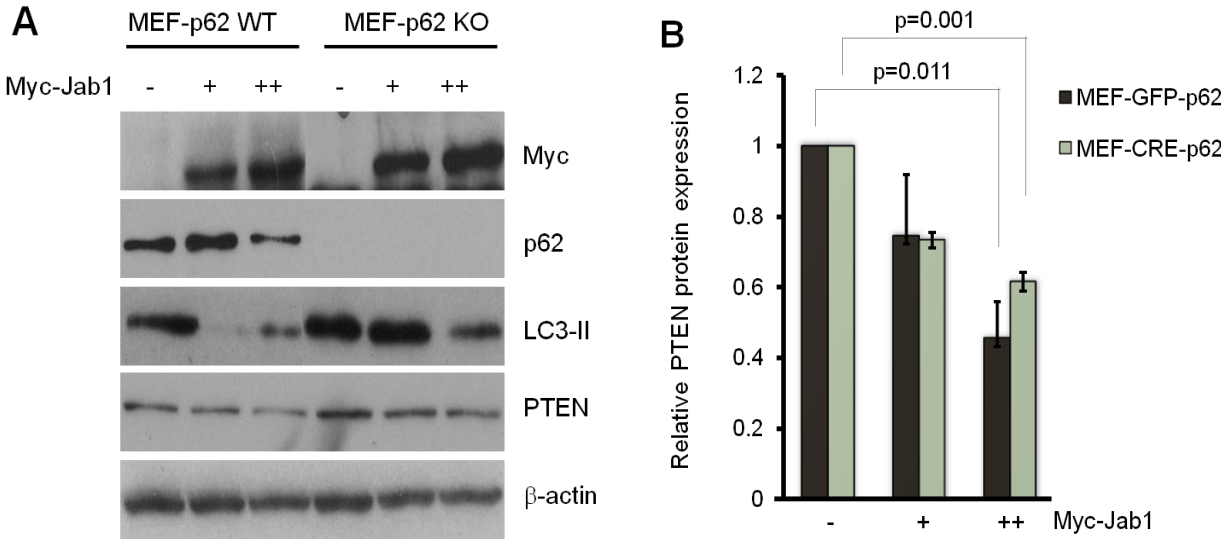


Figure 53. Jab1 facilitates PTEN degradation in MEF-p62 WT and MEF-p62 KO.

(A) Western blot showing protein expressions of autophagy markers (p62 and LC3) and PTEN in MEF-p62 WT and MEF-p62 KO cells transfected with increasing amounts of Myc-Jab1.

(B) Graph representing the means of PTEN protein expression levels from repeated experiments as described in **(A).** Experiments were repeated three times. The data were presented as means ± standard deviations. Differences between individual groups were analyzed using a t test. P values <0.05 were considered statistically significant.

Minus signs (-) indicate empty vector transfection.



4.3. PTEN stability is regulated through the CMA pathway

I then assessed the contribution of the CMA pathway in regulating PTEN degradation. The first step in the CMA pathway is the recognition of CMA substrates by Hsc70 through the KFERQ-like motifs in the substrates (table 4) (146). Using a screening program developed by a computational biologist at MD Anderson Cancer Center, I found that PTEN contained a KFERQ-like motif (330KDKAN334) in the C2-domain (Figure 54, top). The alignment of PTEN sequences of different species around this KDKAN motif was then generated using the Clustal Omega web server. As shown in Figure 54, bottom, the KDKAN motif was conserved across species, which suggests that this motif plays a critical role in PTEN function during evolution.

Furthermore, structural analysis using PyMOL program revealed that 330KDKAN334 motif is located on the surface of human PTEN and is solvent accessible, suggesting that this motif on PTEN is accessible by Hsc70 (Figure 55A and B).

Next, I examined the effects of silencing Hsc70 on PTEN protein expression. I found that LC3 level was not affected by Hsc70 silencing which implied that lysosomal function was not impaired. On the other hand, I showed that depletion of Hsc70 in BT474 cells led to an approximately 90% increase in PTEN protein levels (Figure 56). Taken together, my results provide preliminary finding that PTEN stability is regulated specifically through the CMA pathway in breast cancer cells.

Figure 54. The KDKAN motif is located in the C2 domain of PTEN and is evolutionarily conserved.

(Top) Schematic representing PTEN structure.

(Bottom) Sequence alignment around the KDKAN motif (highlighted with light brown) in PTEN collected from six species. All PTEN sequences were obtained from the Uniprot database. The alignment was generated using the Clustal Omega web server. Identification and alignment of the KDKAN motif were performed by Lu Chen, MD Anderson Cancer Center, Houston, TX.

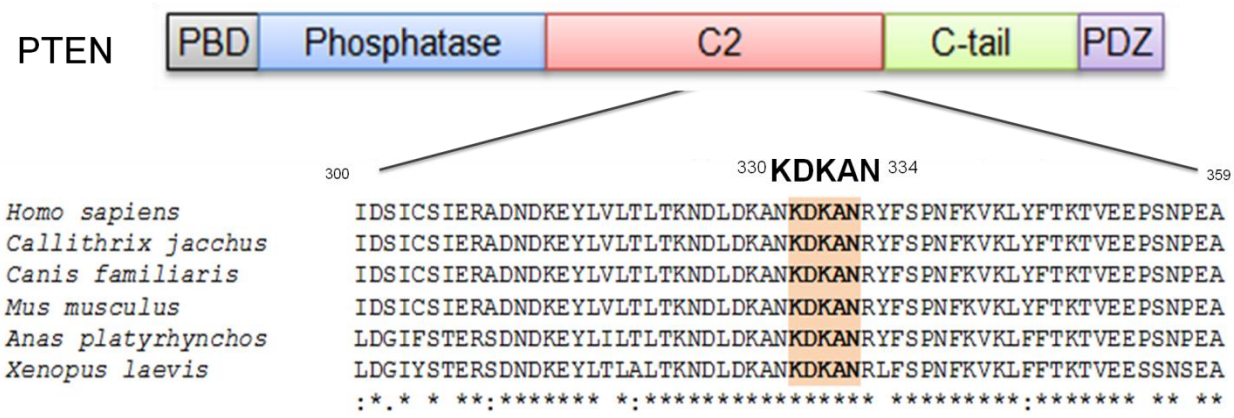


Figure 55. The KDKAN motif locates on the surface of PTEN protein and is solvent accessible.

(**Left**) The 3D structure of human PTEN (protein data bank identifier 1D5R) built by PyMOL. The KDKAN motif in the C2 domain of PTEN was represented with sticks and mesh.

(**Right**) Close-up of the KDKAN motif interface in human PTEN.

The structure of PTEN was generated by Lu Chen, MD Anderson Cancer Center, Houston, TX.

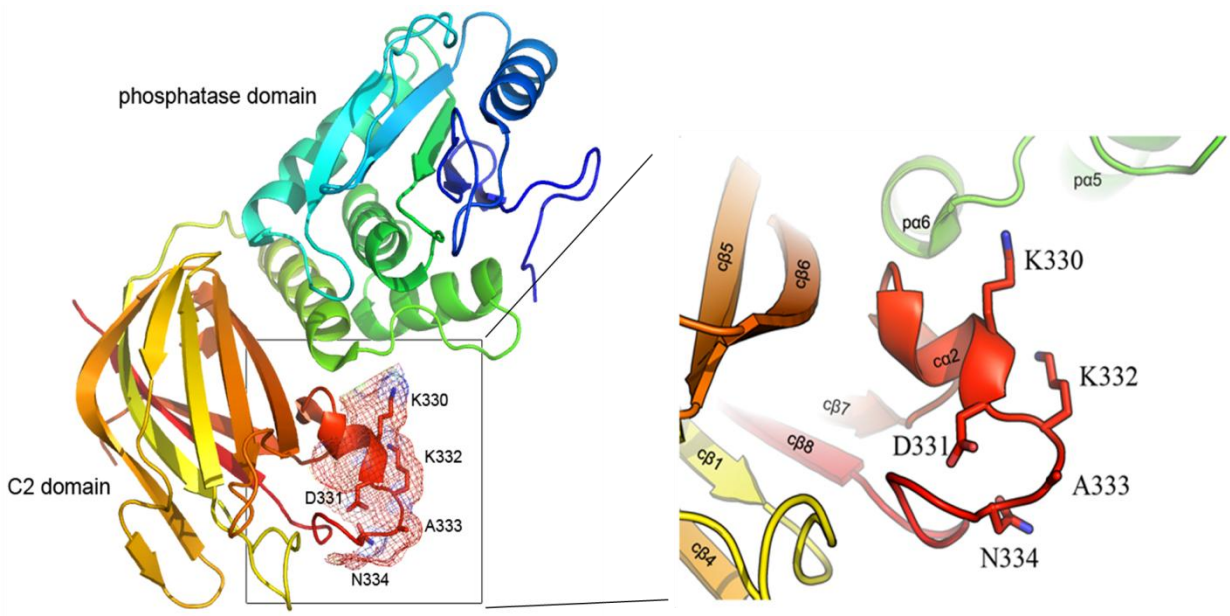
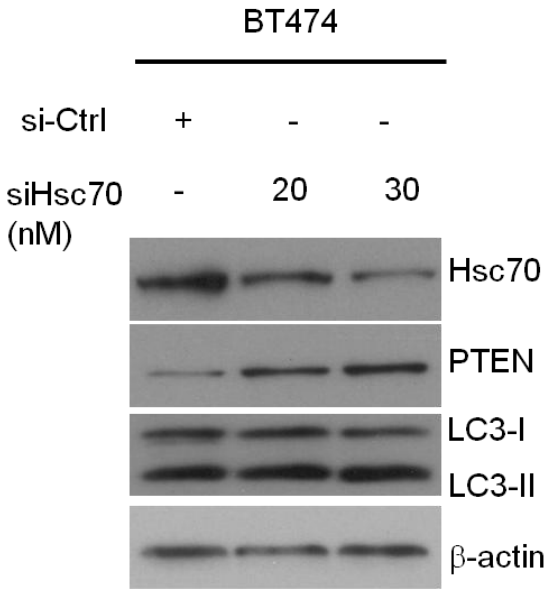


Figure 56. Depletion of Hsc70 increases PTEN protein expression level.

Western blot showing changes in PTEN protein levels in BT474 cells transfected with either siCtrl or increasing amounts of siHsc70.



CONCLUSION

The studies in this chapter provide significant insight into the molecular mechanism by which Jab1 regulates PTEN expression in breast cancer cells.

Firstly, I demonstrated that Jab1 did not affect PTEN mRNA expression (Figure 37). Instead, Jab1 bound to PTEN and facilitated PTEN protein degradation (Figures 38 and 39). Secondly, I found that deletion of the C-terminal end of Jab1 abolished the ability of Jab1 to degrade PTEN while deletion of the other domains (MPN, N-terminal, and JBD domains) increased Jab1 abilities to associate with PTEN (Figures 39-41). I further demonstrated that the changes in their associations with PTEN were independent of their sub-cellular localization and/or protein expressions (Figure 42).

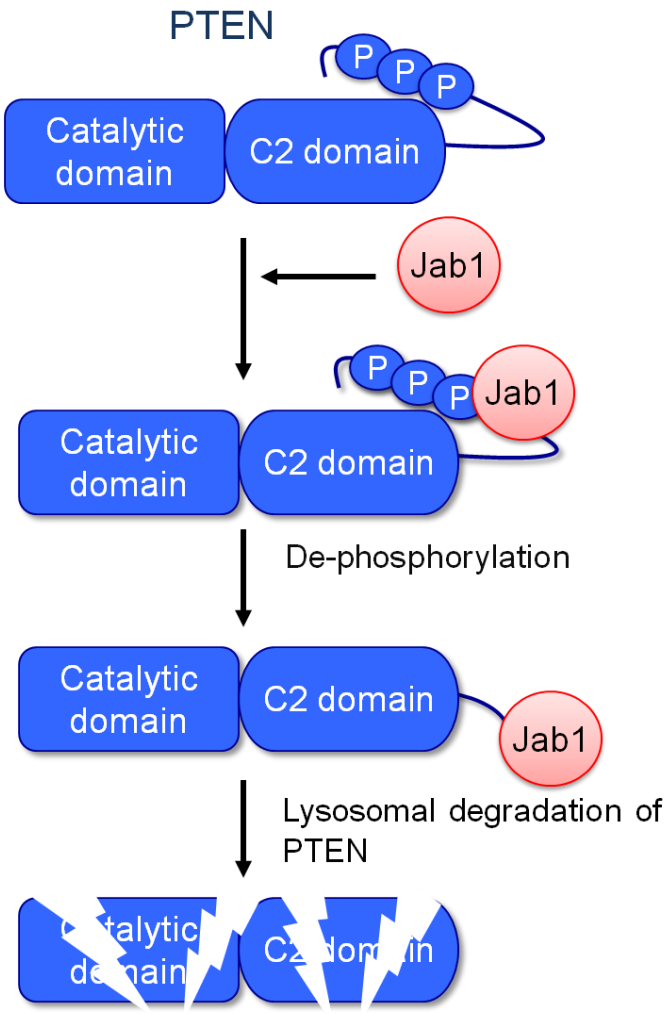
Further, I showed that compared to WT PTEN, the de-phosphorylated PTEN at the cluster of S380/T382/T383 (PTEN-3A) was degraded faster and preferentially associated with Jab1 (Figures 43 and 44). Several studies have identified that this phosphorylation cluster regulates PTEN conformational changes and PTEN stability. In another words, PTEN-3A is in the “open” conformation and less stable than “closed” WT PTEN (78, 80). Therefore, I propose a model in which PTEN is de-phosphorylated at this cluster and exposes its C-tail, which makes it more accessible for Jab1 to associate with and facilitating PTEN degradation (Figure 57).

Furthermore, I showed that blocking the 26S proteasome resulted in the accumulation of PTEN poly-ubiquitination (Figure 45), which is consistent with previous studies (92, 94, 95). However, I found that in the presence of Jab1, proteasome inhibitors did not prevent the degradation or the reduction in poly-ubiquitination of PTEN (Figures 46-48). Importantly, I demonstrated that PTEN poly-ubiquitination was

predominantly linked at K63 chains rather than at K48 chains (Figure 49). As mentioned in chapter 1, while proteins tagged with K48-linked poly-ubiquitin were target for proteasomal degradation, proteins with K63-linked chain were not recognized by proteasome system but are degraded by the lysosomal proteolytic pathway (143, 158). Consistence with these studies, I showed that inhibition of lysosomal activity but not proteasomal activity prevented PTEN destruction due to over-expression of Jab1 in breast cancer cells (Figure 50). Using chemical blockage and genetic inhibition of macro-autophagy, I demonstrated that macro-autophagy did not play a dominant role in translocating PTEN to the lysosome for degradation (Figures 51-53). Rather, I provided preliminary data that CMA regulated PTEN degradation (Figures 54-56).

Taking all these results together, I conclude that Jab1 interacts with PTEN protein and facilitates PTEN degradation through the lysosomal proteolytic pathway (Figure 57).

Figure 57. Proposed model in which the de-phosphorylation of PTEN at Serine380/Threonine382/383 leads to the degradation of PTEN by Jab1.



CHAPTER 5: THE ROLE OF JAB1 IN CONFERRING TRASTUZUMAB RESISTANCE IN HER2-POSITIVE BREAST CANCER

RATIONALE

The loss of PTEN and the rapid degradation of p27 are the two key mechanisms proposed to contribute to the resistance to trastuzumab (62). As mentioned in chapter 1, over-expression of Jab1 accelerated p27 degradation in breast cancer (114). In Chapters 3 and 4, I demonstrated that Jab1 was negatively correlated with PTEN in HER2+ breast cancer cells and in breast tumor specimens. Specifically, I showed that up-regulation of Jab1 facilitated PTEN degradation while depletion of Jab1 restored PTEN levels in HER2+ breast cancer lines. Therefore, *the goal of the studies in this chapter is to determine the role of Jab1 in trastuzumab resistance in HER2+ breast cancer.*

RESULTS

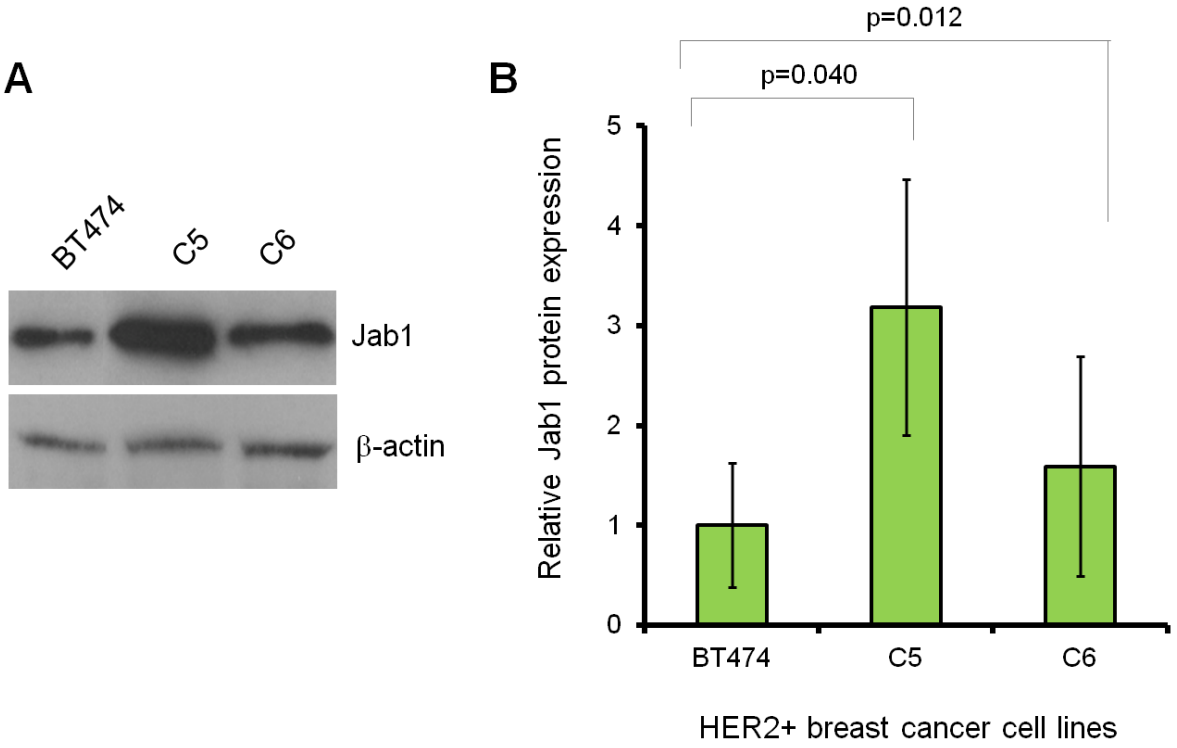
1. Jab1 protein is up-regulated in HER2-positive breast cancer lines that are resistant to trastuzumab

To understand the role of Jab1 in responses of HER2+ breast cancer cells to trastuzumab, I first compared the level of Jab1 protein expression in trastuzumab-responsive and trastuzumab-resistant cells. I found that protein levels of Jab1 was lower in trastuzumab-sensitive BT474 cells compared to C5 and C6 cells that were

unresponsive to trastuzumab (Figure 58A). I also noticed that Jab1 protein was significantly elevated in C5 cells compared to Jab1 levels in BT474 and C6 cells (Figure 58A). The quantitative data from repeated experiments confirm my finding that Jab1 is over-expressed in HER2+ breast cancer cells that are resistant to trastuzumab (Figure 58B).

Figure 58. Trastuzumab-resistant cells show higher protein expressions of Jab1 compared to trastuzumab-sensitive cells.

(A) Western blot comparing protein expressions of Jab1 in BT474, C5 and C6 lines. **(B)** Graph representing the means of Jab1 protein levels from repeated experiments as described in **(A).** Experiments were repeated three times. The data were presented as means ± standard deviations. Differences between individual groups were analyzed using a t test. P values <0.05 were considered statistically significant.



2. Alterations in expression levels of Jab1 mediate the responses of HER2-positive breast cancer cells to trastuzumab

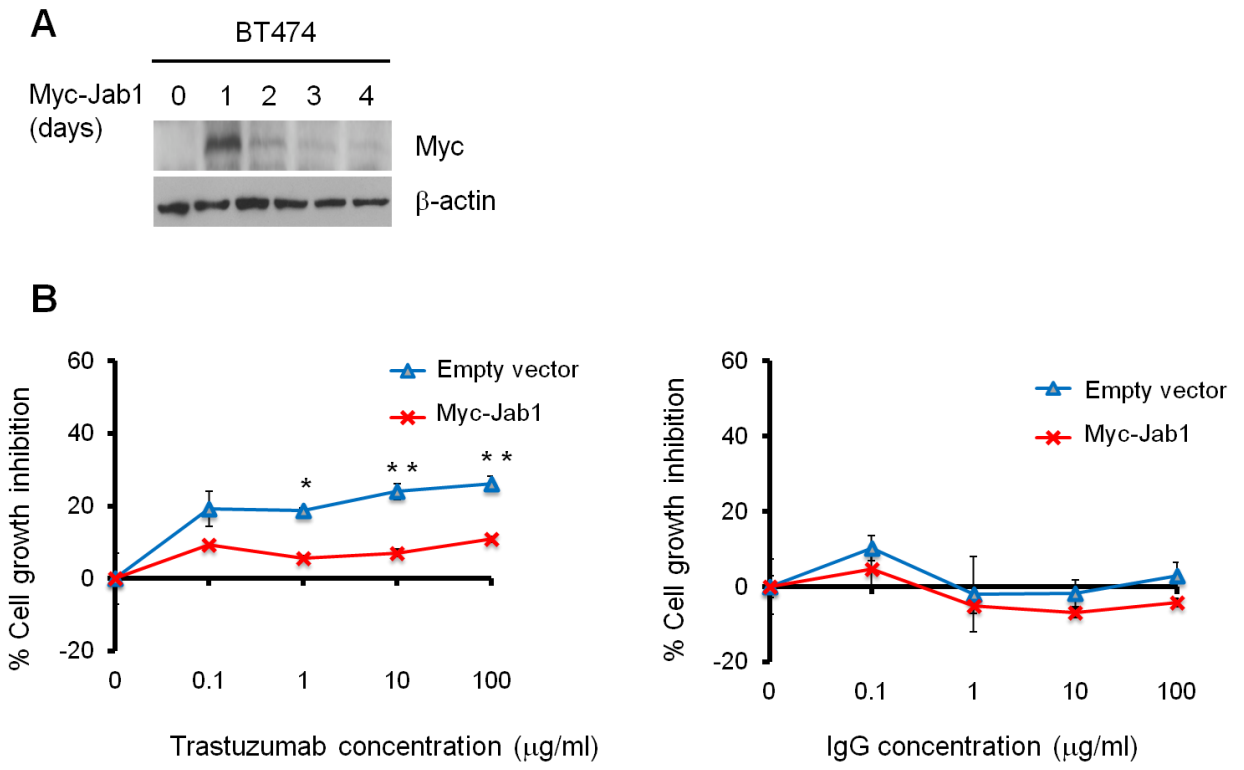
2.1. Over-expression of Jab1 confers breast cancer cells resistance to trastuzumab

I showed that over-expression of Jab1 down-regulated PTEN and that Jab1 was up-regulated in HER2+ breast cancer cells unresponsive to trastuzumab. Next, I identified the effects of Jab1 over-expression on the responses of HER2+ breast cancer cells to trastuzumab treatment. I introduced exogenous Myc-Jab1 into BT747 cells then treated the cells with either trastuzumab or IgG. The BT474 cells transfected with empty vector was used as controls. The expression of Myc-Jab1 was confirmed by western blot. After 5 days of incubation, the cell proliferation was measured using MTS assay. As shown in Figure 59A, though the Myc-Jab1 protein levels were decreased by days, expression of Myc-Jab1 was able to be detected at the day of 5 of the experiment. Further, I showed that over-expression of Jab1 reduced cells' responses to trastuzumab about 10% compared with the responses of the control BT474 cells (Figure 59B, left graph). IgG showed similar non-inhibitory effects on both control BT474 cells and Myc-Jab1 transfected cells (Figure 59B, right graph). Together, these findings indicate that Jab1 over-expression confers BT474 cells resistance to trastuzumab.

Figure 59. Over-expression of Jab1 antagonizes trastuzumab inhibitory effects in BT474 cells.

(A) Western blot showing protein levels of Myc-Jab1 in BT474 for different days.

(B) Growth rates of BT474 cells transfected with either empty vector or Myc-Jab1 and treated with increasing concentrations of trastuzumab (left graph) or control IgG (right graph). Experiments were repeated three times. The data were presented as means ± standard deviations. Differences between individual groups were analyzed using a t test. P values <0.05 were considered statistically significant, which were represented by the asterisk (*).



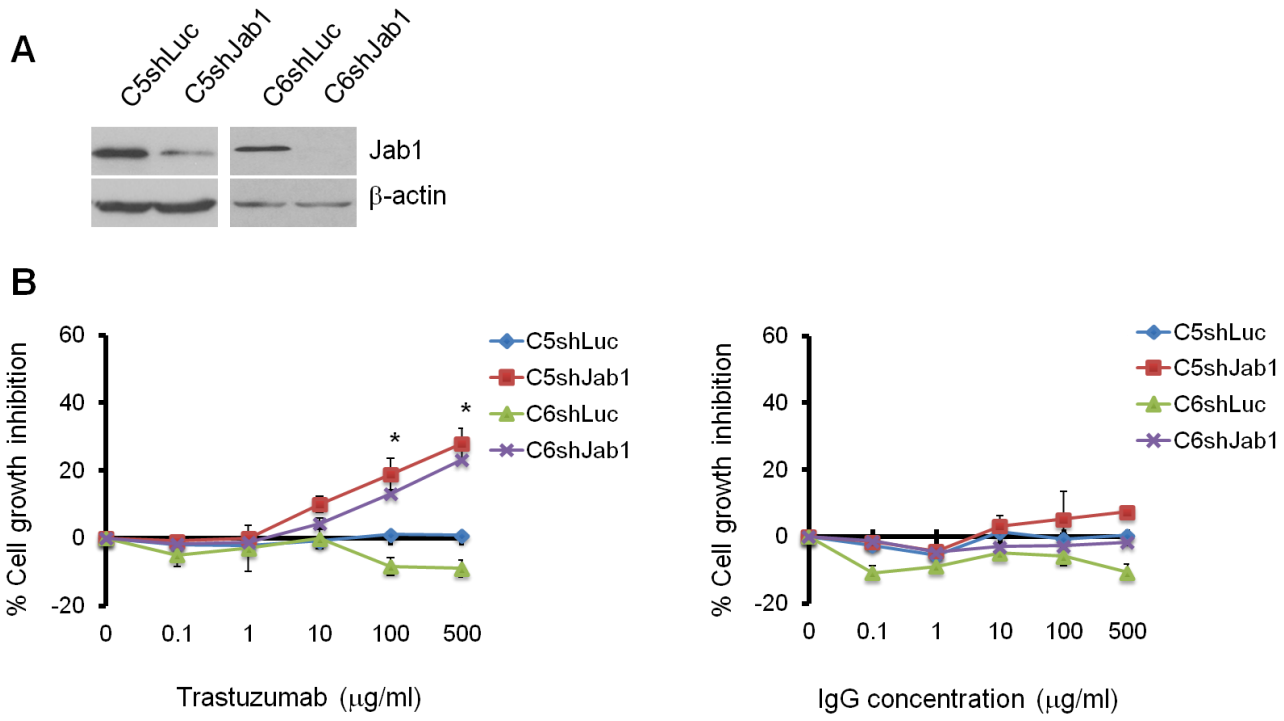
2.2. Silencing Jab1 increases trastuzumab inhibitory effect

If activation of PTEN and p27 are the keys to the therapeutic action of trastuzumab and if inhibition of Jab1 significantly restores PTEN and p27 levels, cancer cells that have lost Jab1 should be more responsive to this drug. To test this hypothesis, I treated the stable Jab1 knockdown C5 and C6 cells with either trastuzumab or IgG. The C5shLuc and C6shLuc lines were used as controls. The expression of Jab1 was confirmed by western blot. After 5 days, the cell proliferation was measured using MTS assay. I showed that the protein expressions of Jab1 were barely detected in C5shJab1 and C6shJab1 compared to C5shLuc and C6shLuc, respectively (Figure 60A). As shown in Figure 60B, the left graph, at the concentration of 100 μg/ml, trastuzumab started to have inhibitory effect on the proliferation of C5shJab1 and C6shJab1 (red and purple lines, respectively) but not C5shLuc and C6shLuc (blue and green lines). IgG did not show any effects on any of the four cell lines (Figure 60B, right graph). These results suggest that depletion of Jab1 increases the sensitivities to trastuzumab treatment in HER2+ breast cancer cells.

Figure 60. Depletion of Jab1 increases trastuzumab inhibitory effects in C5 and C6 cells.

(A) Western blot showing expression levels of Jab1 protein in pairs C5shLuc/C5shJab1 and C6shLuc/C6shJab1 lines.

(B) Growth rates of C5shLuc/C5shJab1 and C6shLuc/C6shJab1 cells treated with increasing concentrations of trastuzumab (left graph) or control IgG (right graph). Experiments were repeated three times. The data were presented as means ± standard deviations. Differences between individual groups were analyzed using a t test. P values <0.05 were considered statistically significant, which were represented by the asterisk (*).



CONCLUSION

In this chapter, I elucidated the role of Jab1 in mediating responses of HER2+ breast cancer cells to trastuzumab.

I found that in the cells that were refractory to trastuzumab, Jab1 protein was significantly up-regulated compared with the trastuzumab-responsive cells (Figure 58). My studies also demonstrated that silencing Jab1 in these resistant cells enhanced inhibitory effects of trastuzumab on the cell growth (Figure 60). Conversely, introducing Jab1 into trastuzumab-sensitive cells conferred the cells resistance to this drug (Figure 59).

Together, my results indicate that Jab1 positively contributes to trastuzumab resistance in HER2+ breast cancer cells.

CHAPTER 6: DISCUSSION

My studies in chapter 3 demonstrated that in HER2+ breast cancer, silencing Jab1 led to increase in PTEN protein expression and phosphatase function. My studies further showed that high Jab1 expression was associated with low PTEN expression in human breast tumor specimens. My subsequent studies in chapter 4 elucidated that Jab1 mediated the post-translational regulation of PTEN through the lysosomal proteolytic pathway. Consistent with the role of Jab1 in facilitating PTEN degradation and reducing PTEN function, my studies in Chapter 5 demonstrated that silencing Jab1 sensitized trastuzumab-resistant cells to the drug. In constrast, over-expressing Jab1 in trastuzumab-sensitive cells reduced the cells response to trastuzumab's inhibitory effects.

1. Jab1 negatively regulates protein expression of PTEN in HER2-positive breast cancer

PTEN mutations and its role as a negative regulator of PI3K signaling have been extensively studied (86). However, relatively little is known about the pathway(s) controlling PTEN stability and activity. My studies in chapter 3 and chapter 4 expand on this field by demonstrating that Jab1 negatively regulates PTEN stability, thus modifying PTEN lipid and tyrosine phosphatase functions.

In chapter 3, I showed that silencing Jab1 up-regulated PTEN in HER2+ breast cancer cells that were either sensitive or resistant to trastuzumab (Figures 26, 28, and 29). I also found that up-regulation of PTEN due to Jab1 silencing was more significant

in trastuzumab-resistant cells compared to trastuzumab-sensitive cells (Figures 27 and 28). My studies in chapter 5 further clarified these differences by identifying that protein levels of Jab1 were at least 2-fold higher in these resistant cells compared with the sensitive cells (Figure 58). As the Jab1 protein is higher in the resistant cells, it is expected that the silencing Jab1 would create more significant effects in these cells. The finding that the degree of increased PTEN due to Jab1 knockdown depends on Jab1 expression levels also suggest that targeting Jab1 is more beneficial in the cells over-expressing Jab1 compared with the cells having low level of Jab1.

Two independent studies have shown that NEDD4-1 protein is negatively correlated with PTEN protein and that NEDD4-1 degrades PTEN protein in non-small cell lung cancer (91, 92). Later, Maddika and colleagues (2011) by using an unbiased approach (tandem affinity-purification), demonstrated that WWP2 interacted with PTEN and that over-expression of WWP2 facilitated PTEN degradation. However, this group could not verify that inhibition of NEDD4-1 led to increased PTEN protein expression (94). In my studies in chapter 3, the correlations of mRNA levels of WWP2 and NEDD4-1 with PTEN protein in invasive breast carcinoma in the TCGA BRCA dataset did not support that WWP2 and NEDD4-1 were negatively correlated with PTEN protein expression. Instead, I showed that WWP2 mRNA and PTEN protein were not clinically correlated whereas NEDD4-1 mRNA was positively correlated with PTEN protein level in human breast tumors (Figure 31A and B, respectively). There are two possible explanations: (1) PTEN degradation mediated by NEDD4-1 is cell-type dependent and (2) mRNA expressions of NEDD4-1 and WWP2 do not correlated with their protein expression levels. Given the protein expressions of NEDD4-1 and WWP2 are not available in TCPA BRCA dataset, further studies are needed to confirm the

clinical correlation of these two proteins with PTEN levels. On the other hand, I demonstrated that Jab1 mRNA was the only to show the negative correlation with protein expression of PTEN in clinical specimens of invasive breast carcinoma (Figure 30A). I also showed that in TCPA BRCA dataset, PTEN protein levels were significantly lower in the clinical specimens with high Jab1 protein expressions (Figure 30B). Taking these findings together, my results strongly argue that high expression of Jab1 is causally linked to low expression of PTEN in human breast carcinoma.

I further demonstrated that this elevation in PTEN protein due to Jab1 knockdown was accompanied by increases in the ability of PTEN to de-phosphorylate PIP3 and p-Src-Y416 (Figures 32-34). This increase in PTEN phosphatase activity suggests an increase in the amount of functional PTEN present in the cells. As mentioned in chapter 1, PTEN protein down-regulation was common in breast cancer and was associated with tumor progression and resistance to targeted therapies such as trastuzumab (98, 99). Growing evidence also supports that subtle changes in the dose of PTEN can have profound effects on tumor susceptibility (101, 102). My findings, therefore, suggest a therapeutic implication for targeting Jab1 in order to restore PTEN expression and function and thus, improve the effectiveness of breast cancer therapies. My studies in chapter 5 provided preliminary evidence for the therapeutic application of inhibiting Jab1 by demonstrating that depletion of Jab1 enhanced trastuzumab inhibitory effects in the cells resistant to this drug (Figures 58 and 60).

My studies in chapter 4 further showed that PTEN and Jab1 physically interacted and that the C-terminal end of Jab1 was required for its association with PTEN and for subsequent PTEN degradation (Figures 38-41). In contrast, deletion at

the MPN, N-terminal or JBD domains of Jab1 markedly increased interactions between Jab1 and PTEN proteins (Figure 39). It was possible that different mutations resulted in the changes in protein expression or subcellular localizations which may affect protein-protein interaction. However, I showed that all WT and mutant Jab1 isoforms showed similar protein levels and expressed in the cytoplasm. The results on one hand suggest that the C-terminal end of Jab1 likely represents the docking region where PTEN interacts with Jab1. On the other hand, the finding imply that the deletion at the MPN, N-terminal or JBD domains leads to changes in Jab1 conformation which makes it more accessible to PTEN.

Several studies have proposed that there was a steady-state switch between the “open” and “closed” conformations of PTEN, which mediates protein-protein interaction (78, 80, 88, 90). These groups also found that the phosphorylated PTEN at the C-terminal tail, specifically at S380, T382, and T383, was predominantly in a “closed” conformation, which stabilized PTEN (Figure 13) (78, 80, 88). In contrast, the un-phosphorylated PTEN (PTEN-3A) was predominantly in an “open” conformation and less stable. Similarly, my studies in chapter 4 demonstrated that “closed” WT PTEN was more stable than “open” PTEN-3A (Figure 43). Interestingly, I demonstrated that Jab1 associated with both WT PTEN and PTEN-3A. Additionally, PTEN-3A showed two-fold higher binding to Jab1 which resulted in faster degradation of PTEN-3A by Jab1 compared to WT PTEN (Figures 43 and 44). I predicted that the nonphosphorylated/ “open” conformation of PTEN makes it more accessible to Jab1, resulting in the rapid degradation of PTEN by Jab1 (Figure 57). To verify the model, future work is needed to compare the kinetics of PTEN and PTEN-3A in association with Jab1.

In conclusion, my studies in chapter 3 and chapter 4 demonstrate that Jab1 regulates PTEN stability and function through its interaction with PTEN

2. Jab1 facilitates PTEN degradation through the lysosomal proteolytic pathway

A number of models have proposed that the UPS controls PTEN stability (86). Recently, four independent studies comprehensively showed that PTEN ubiquitination was accumulated in the presence of proteasome inhibitors. These studies also identified that addition of proteasome inhibitors restored PTEN protein expression (91, 92, 94, 95). My findings in chapter 4 showed that PTEN poly-ubiquitination was markedly increased when 26S proteasome was inhibited by MG-132 (Figure 45), which is consistent with these studies. However, I observed that in the presence of Jab1, blocking 26S proteasomal activity failed to prevent the accumulation in PTEN poly-ubiquitination (Figure 48). Additionally, over-expressing Jab1 led to gradual decrease in PTEN protein levels in breast cancer cells, even in the presence of proteasome inhibitors (Figures 46 and 47). My findings supported that the stability of PTEN *per se* was regulated by the UPS in a normal condition. However, in breast cancer cells with over-expressed Jab1, there was an alternative mechanism that regulated PTEN protein degradation.

In eukaryote cells, besides the UPS, proteins can be degraded through the lysosomal proteolytic pathway (143). A well-studied signal for determining the pathway that degrades a protein is through its poly-ubiquitin chains. Among them, K48- and K63- linked chains have been reported to mediate protein degradation (155, 159). Precisely, several groups have consistently showed that K48-linked poly-ubiquitin

targets a protein for proteasomal degradation while K63-linked chains represent signal for lysosomal degradation (160-162, 186). My studies in chapter 4 found that PTEN poly-ubiquitination was predominantly linked at the K63 rather than the K48 (Figure 49). This result implied that PTEN destruction was mediated through the lysosomal pathway. My studies later validated this hypothesis by showing that blocking the activity of lysosomal proteases impaired the ability of Jab1 to facilitate PTEN degradation. In addition, when Jab1 was up-regulated, lysosomal inhibitors significantly increased PTEN levels (Figure 50). These findings indicate that lysosomal inhibitors potentially restore PTEN protein levels in breast cancer cells with high levels of Jab1.

In mammalian cells, there are three pathways that deliver a protein for lysosomal degradation, macro-autophagy, CMA, and micro-autophagy (129, 143). Among them, macro-autophagy and CMA have been experimentally reported to crosstalk with the UPS to regulate protein degradation. In my studies in chapter 4, I showed that pharmacological inhibition of macro-autophagy did not show any effect on PTEN protein expression. Previous studies demonstrated that targeted manipulation of macro-autophagy (such as ATG5 or p62 knockdown) impaired protein degradation (130, 134). In my studies, I found that neither ATG5 nor p62 knockdown prevented PTEN degradation induced by Jab1 over-expression (Figures 52 and 53). These findings indicate that macro-autophagy does not play a critical role in the Jab1-mediated PTEN degradation.

Instead, I identified that PTEN contained the KDKAN motif (Figure 54) which is a recognition motif for CMA degradation (148). I also showed that this motif was solvent accessible (Figure 55) which suggests that this motif can be recognized by the CMA mediator. In the CMA process, Hsc70 was the only protein that can bind to the CMA

recognition motif on a substrate and translocate it to the lysosome for destruction (150, 153). In my studies, I demonstrated that depletion of Hsc70 significantly up-regulated PTEN protein expressions in breast cancer cells without affecting lysosomal function (Figures 56).

Moreover, it was demonstrated that a reduction in macro-autophay could promote CMA activity (178). My data previously suggested that inhibition of the CMA rather than macro-autophagy was effective in restoring PTEN expression. My findings therefore support that the activation of CMA due to inhibition of macro-autophagy results in effective degradation of PTEN protein. As CMA is a comprehensive multi-process and that 30% of cellular proteins contains the CMA motif (144, 147), further studies are required to confirm that PTEN is a *bona fide* CMA substrate (table 4). Also, whether Jab1 regulates PTEN expression through the CMA pathway is a critical question to be address.

It was previously shown that proteasome inhibitors prevented PTEN degradation in breast cancer cells and that there was crosstalk between the UPS, macro-autophagy, and CMA (86, 144). My results in general demonstrated that in the presence of Jab1, blocking the lysosome, but not proteasomal activity, prevented PTEN degradation and that CMA rather than macro-autophagy may regulate PTEN degradation. My unexpected findings raise important questions regarding the exact pathway mediating PTEN degradation. For instance, in what contexts is PTEN degraded through the UPS or through the CMA? One possible explanation for my finding is that Jab1 over-expression together with proteasome or macro-autophagy inhibitors triggers cellular stress, which activates maximal CMA activity and subsequently leads to rapid degradation of PTEN. Therefore, it is also important to

clarify how Jab1 and CMA co-oporate to regulate PTEN stability and function in breast cancer.

Taken together, my findings demonstrate that the lysosomal proteolytic pathway is the dominant mechanism by which Jab1 controls PTEN stability in HER2+ breast cancer. I speculate that selective inhibition of lysosomal enzymes in combination with targeting the Jab1-PTEN binding domain would effectively restore PTEN levels which are frequently reduced in breast cancer. This would subsequently decrease p-AKT and p-Src levels and also increase responsiveness of breast cancers to targeted therapy including trastuzumab.

3. Jab1 over-expression confers breast cancer cells resistance to trastuzumab

It is well-studied that the loss of PTEN and the constitutive active AKT and Src play pivotal role in trastuzumab resistance (98, 99, 106). In chapter 3 and 4, I demonstrated that over-expressing Jab1 led to significant reduction in PTEN protein expression and increases in active AKT and Src. Also, I found that PTEN is required for Jab1 to mediate p-Src levels. Other studies of our group showed that PTEN is also required for Jab1 to mediate p-AKT levels (unpublished data). In my studies in Chapter 5, I showed that Jab1 protein was significantly up-regulated in the cells that were refractory to trastuzumab compared with the trastuzumab-responsive cells (Figure 58). In addition, over-expression of Jab1 was significantly correlated with the poor response rate to trastuzumab (Figure 59). Consistently, I demonstrated that silencing Jab1 in these resistant cells enhanced inhibitory effects of trastuzumab (Figure 60), which is consistent with the functional effects of increased PTEN expression and function due to

Jab1 depletion. These results imply that Jab1 confers trastuzumab resistance through regulating PTEN expression and function. Yet further studies are needed to confirm the effects of PTEN in trastuzumab resistance mediated by Jab1.

In general, my findings provide *in vitro* evidence that inhibition of Jab1 or of Jab1-PTEN interaction could overcome resistance to trastuzumab. Moreover, I speculate that the elevation of Jab1 together with the down-regulation of PTEN can serve as predictive markers for the response to trastuzumab in HER2+ breast cancer cells.

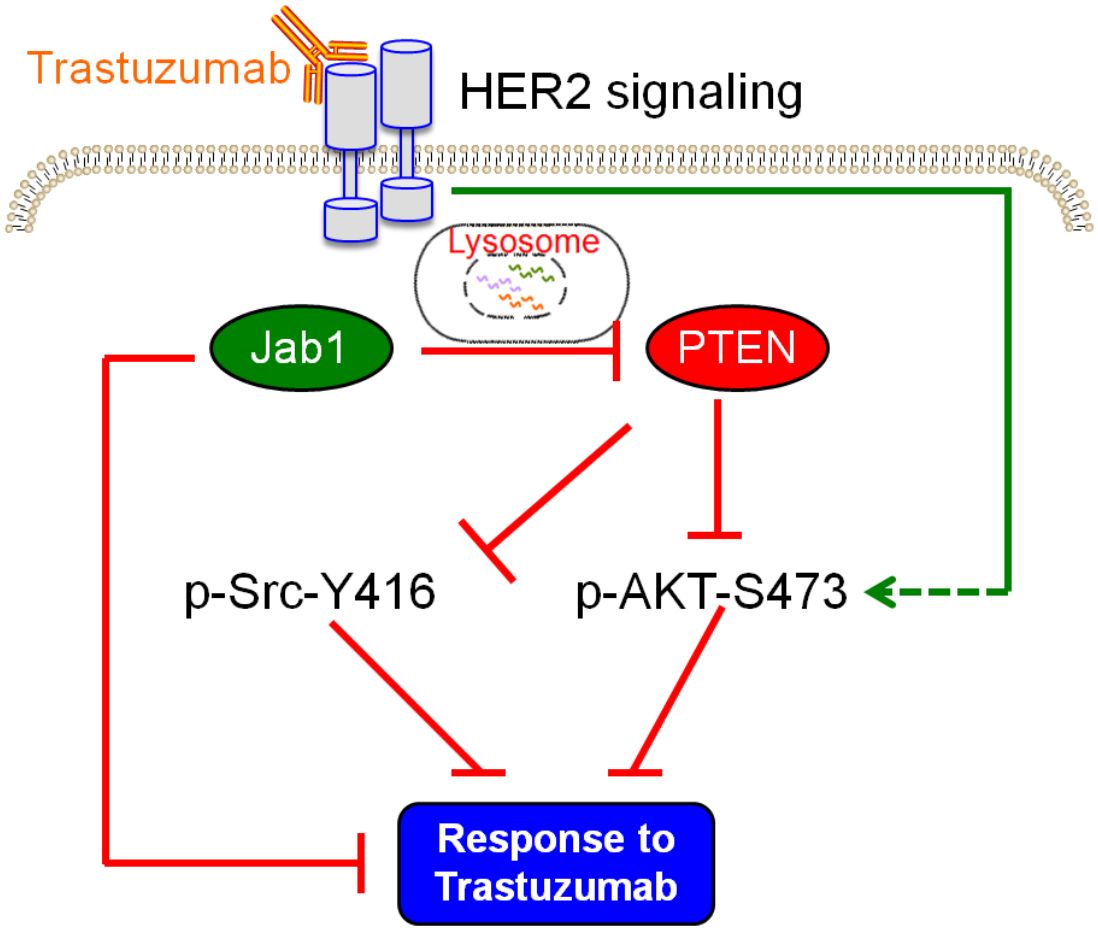
4. Conclusion and future directions

4.1. Conclusion

My studies support a model in which high expression of Jab1 in HER2+ breast cancer antagonizes the inhibitory effects of trastuzumas and promotes PTEN degradation through the lysosomal proteolytic pathway (Figure 61). PTEN down-regulation mediated by Jab1 in turn increases p-Src-Y416 and p-AKT-S473 levels which also contribute to the resistance to trastuzumab (Figure 61).

Figure 61. Proposed model in which Jab1 negatively regulates PTEN protein and confers resistance to trastuzumab in HER2-positive breast cancer.

Red lines indicate the inhibition and green lines indicate the activation.



4.2. Future directions

- To demonstrate that Jab1 facilitates PTEN degradation through the CMA pathway

My studies in chapter 4 indicated that PTEN contains the CMA-recognition motif and that inhibition of a CMA mediator (Hsc70) prevented PTEN degradation in breast cancer cells (Figures 55-57). As discussed in chapter 1 (table 4), to confirm that PTEN is an authentic CMA substrate, the next steps are to identify (1) whether PTEN physically interacts with Hsc70 and lysosomal membrane LAMP-2A and (2) whether mutated PTEN at the KDKAN motif can still be recognized by Hsc70 or LAMP-2A.

Furthermore, if Jab1 mediates PTEN degradation via the CMA pathway, selective inhibition of this pathway should block PTEN degradation induced by Jab1. As mentioned in chapter 1, the most selective way to block CMA was to silence LAMP-2A and/or Hsc70, since selective chemical inhibitors for CMA are not currently available (144). Therefore, to confirm that Jab1 degrades PTEN through the CMA, I will knockdown either Hs70 or LAMP-2A in breast cancer cells and compare PTEN protein levels in the presence or absence of Jab1 over-expression.

In general, the completion of these studies will solidify the finding that Jab1 facilitates PTEN degradation through the CMA pathway in breast cancer cells.

- To identify whether PTEN is required for the resistance to trastuzumab mediated by Jab1 over-expression

My studies in chapter 3 and chapter 5 demonstrated that inhibition of Jab1 increased PTEN protein level and sensitized trastuzumab-resistant cells to the treatment of this drug. As discussed in chapter 1, up-regulation of PTEN alone also enhanced trastuzumab's inhibitory effects on the cell proliferation. To identify whether PTEN is required for Jab1 to mediate cells' responses to trastuzumab, I will stably silence Jab1 in HER2+ breast cancer cells that lack PTEN protein expression or have non-functional PTEN. MTS assay will then be used to evaluate how these conditions affect responses of the cells to trastuzumab treatment. If PTEN is required for Jab1 to mediate trastuzumab resistance, trastuzumab will show no effect on the proliferation of the PTEN-deficient cells regardless of Jab1 status.

In general, the completion of these studies will provide insights into how Jab1 modulates the resistance to trastuzumab in HER2+ breast cancer cells. Clinically speaking, these findings will help guide the selection of patients for the treatment with trastuzumab based on PTEN and Jab1 levels.

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Publications

2014 **Vu T**, Sliwkowski MX and Claret FX. Personalized drug combinations to overcome trastuzumab resistance in HER2-positive breast cancer. *Biochim Biophys Acta.* 2014 Jul 25. PMID: 25065528

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Academic Awards and Honors

2014 Student Oral Presentation Award – First Place. Experimental Therapeutics Academic Program Retreat 2014, MD Anderson Cancer Center, Houston, Texas, US

2013 Student Oral Presentation Award – First Place. Experimental Therapeutics Academic Program Retreat 2013, MD Anderson Cancer Center, Houston, Texas, US

2013 Poster Presentation Award – First Place. 10th Annual Vietnam Education Foundation Fellows and Scholar Conference, Tallahassee, Florida, US

2012 Student Oral Presentation Award – Third Place. Experimental Therapeutics Academic Program Retreat 2012, MD Anderson Cancer Center, Houston, Texas, US