Distinct ShcA signaling complexes influence breast tumor growth and resistance to tyrosine kinase inhibitors

Jacqueline R Ha

Faculty of Medicine

Division of Experimental Medicine

McGill University, Montreal

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Abstract

Aberrant activation of tyrosine kinase signaling networks is recognized as a key effector of breast cancer plasticity and therapeutic responsiveness. The functional redundancy and adaptability of the tyrosine kinome have hindered durable clinical responses to tyrosine kinase inhibitors. The commonality between most phospho-tyrosine signaling networks is their shared use of adaptor proteins to transduce mitogenic signals. ShcA is one such adaptor protein that is essential for breast cancer progression. ShcA employs two phospho-tyrosine binding domains (PTB and SH2 domains) and key phospho-tyrosine residues to promote mammary tumorigenesis. We previously demonstrated that the ShcA PTB domain is essential for breast tumor initiation downstream of receptor tyrosine kinases (RTKs), such as ErbB2, by activating the AKT/mTOR pathway. Using genetic and pharmacological approaches, we now show that the ShcA PTB domain dynamically controls signaling networks in breast cancer cells, not only to transduce tumorigenic signals downstream of RTKs (PTB-dependent), but also to create a negative feedback loop that prevents secondary activation of ShcA-SH2 driven complexes (PTB-independent) to activate Src family kinases, including Src and Fyn. This bifurcation of signaling complexes from distinct ShcA pools transduces non-redundant signals that integrate the AKT/mTOR and SFK pathways to cooperatively increase breast tumor growth and resistance to tyrosine kinase inhibitors, including Lapatinib and PP2. Breast tumors also rely on the ShcA PTB domain to bind numerous negative regulators to ensure that the strength and duration of pro-tumorigenic responses is tightly controlled. Recruitment of these negative regulators to the PTB domain of ShcA is dependent, in part, on serine phosphorylation, which results in the termination of ShcA-dependent signaling responses. We establish the first *in vivo* evidence of unique N-terminal regulatory elements, namely Serine 29 and a S29-W38 motif, upstream of the PTB domain, that control non-redundant processes that limit PTB-dependent and -independent ShcA complexes during breast tumorigenesis. Collectively, this study mechanistically dissects the interplay between diverse intracellular ShcA pools and their ability to influence the tyrosine kinome to affect breast tumor heterogeneity and therapeutic resistance.

Resume

L'activation aberrante des réseaux de signalisation de protéines-tyrosine kinase est reconnue comme un facteur déterminant de la plasticité du cancer du sein et aussi de sa sensibilisation thérapeutique. La redondance fonctionnelle et l'adaptabilité du kinome de la tyrosine ont entravé des réponses cliniques durables aux inhibiteurs de la tyrosine kinase. La plupart des réseaux de signalisation de la phospho-tyrosine ont en commun l'utilisation de protéines adaptatrices pour la transduction de signaux mitogéniques. ShcA est l'une de ces protéines adaptatrices essentielles à la progression du cancer du sein. ShcA utilise deux domaines de liaison à la phospho-tyrosine (domaines PTB et SH2) et des résidus clés de phospho-tyrosine pour favoriser la tumorigenèse mammaire. Précédemment, nous avons démontré que le domaine PTB de ShcA est essentiel pour l'initiation de la tumorigenèse du sein en aval de récepteurs tyrosine kinases (RTK), tels que ErbB2, en activant la voie de signalisation AKT / mTOR. En utilisant des approches génétiques et pharmacologiques, nous démontrons maintenant que le domaine PTB de ShcA contrôle, de manière dynamique, les réseaux de signalisation dans les cellules cancéreuses du sein. Ceci est non seulement pour transmettre les signaux tumorigènes en aval des RTK (dépendant de la PTB), mais également pour créer une boucle de rétroaction négative qui empêche l'activation secondaire de complexes commandés par le domaine SH2 de ShcA (indépendant du PTB) pour activer les kinases de la famille Src, y compris Src et Fyn. Cette bifurcation de complexes de signalisation provenant de réservoirs distincts de ShcA dans la cellule permet la transduction de signaux non redondants qui intègrent les voies AKT / mTOR et SFK, afin d'augmenter de manière coopérative la croissance des tumeurs mammaires et la résistance aux inhibiteurs de la tyrosine kinase, notamment le Lapatinib et la PP2. Les tumeurs du sein dépendent également du domaine PTB de ShcA pour lier de nombreux régulateurs négatifs, afin de s'assurer que la force et la durée des réponses pro-tumorigènes sont strictement contrôlées. Le recrutement de ces régulateurs négatifs dans le domaine PTB de ShcA dépend partiellement, de la phosphorylation de la sérine, ce qui entraîne la fin des réponses de signalisation dépendant de ShcA. Nous établissons la première preuve *in vivo* d'éléments régulateurs N-terminaux uniques, notamment, Serine 29 et un motif S29-W38, en amont du domaine PTB, qui contrôlent les processus non redondants qui limitent les complexes ShcA dépendants et indépendants du PTB pendant la phase tumorigenèse. Ensemble, cette étude dissèque mécaniquement l'interaction entre divers réservoirs de ShcA intracellulaires

et leur capacité d'influencer le kinome de la tyrosine, afin d'influencer l'hétérogénéité des tumeurs du sein et la résistance thérapeutique.

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Chapter 3.1 was published as an original research article:

> Ha, J.R., Ahn, R., Smith, H.W., Sabourin, V., Hébert, S., Cepeda Cañedo E., Im, Y.K., Kleinman, C.L., Muller, W.J., Ursini-Siegel, J. (2018). Integration of distinct ShcA signaling complexes promotes breast tumor growth and resistance to tyrosine kinase inhibitors. *Mol Cancer Res*, 16(5), 894-908.

Chapter 3.2 contains material to be included in a manuscript in preparation for publication as an original research article:

> Mechanistic and functional characterization of unique negative regulatory pathways of distinct ShcA signaling complexes during mammary tumorigenesis.

Publications that include work performed by JRH, but not included in the presentation of this dissertation:

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> Im, Y.K., La Selva, R., Gandini, V., Ha, J.R., Sabourin, V., Sonenberg, N., Pawson, T., Topisirovic, I., Ursini-Siegel, J. (2014). The ShcA Adaptor activates AKT signling to potentiate breast tumor angiogenesis by stimulating VEGF mRNA translation in a 4E-BP-dependent manner. *Oncogene*, 34(13), 1729-1735.
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> Ahn, R., Sabourin, V., Ha, J.R., Cory, S., Maric, G., Im, Y.K., Hardy, W.R., Zhao, H., Park, M., Hallett, M., Siegel, P.M., Pawson, T., Ursini-Siegel, J. (2013). The ShcA PTB domain functions as a biological sensor of phospho-tyrosine signaling during breast cancer progression. *Cancer Res*, 73(14), 4521-4532.

Contributions of the Authors

This study was designed, executed, and analyzed under the guidance of my supervisor, Dr. Josie Ursini-Siegel. The composition of this dissertation and presentation/design of the experimental figures were accomplished with the supervision of Dr. Ursini-Siegel. In addition, several collaborations were established to complete the work presented in this thesis. The contributions of the co-authors have been outlined below.

Conception and design of this study by Dr. Josie Ursini-Siegel and Jacqueline R. Ha was based on the published work of Ryuhjin Ahn and Young Kyuen Im. NIC $Src^{-/-}$ breast cancer cell lines were developed by Dr. Harvey W. Smith from transgenic mouse models generated by Dr. William J. Muller. Reagents for BioID were provided by Dr. Marc R. Fabian. PTPN12 antibody was provided by Dr. Michel L. Tremblay. Eduardo Cepeda Cañedo provided animals for the *in vivo* studies. Mammary fat pad injections were assisted by Ryuhjin Ahn and Valerie Sabourin. Immunohistochemistry of breast tumor samples were assisted by Valerie Sabourin. Biostatistics and computational analysis of bioinformatics data was performed by Steven Hébert and Dr. Claudia L. Kleinman.

Original Contributions to Knowledge

1. We provide the first evidence that the ShcA adaptor protein is a critical convergence point downstream of numerous tyrosine kinases and that perturbation of discrete ShcA-dependent signaling complexes significantly impacts breast tumor growth and therapeutic responsiveness. Specifically, we demonstrate that PTB-independent ShcA complexes serve to augment mammary tumorigenesis by increasing the activity of the Src and Fyn tyrosine kinases through the SH2 domain, while ShcA tyrosine phosphorylation sites are dispensable for these PTB-independent ShcA pools to amplify tumor growth. Finally, we establish that increased Src activation downstream of ShcA PTB-independent signaling complexes increases resistance to Lapatinib.

2. Through genetic and pharmacologic approaches, we establish a ShcA-dependent resistance mechanism underlying PTB-independent breast tumorigenesis. PTB-independent ShcA signaling complexes rely on the ShcA SH2 domain to activate Src signaling to augment mammary tumor growth. However, the loss of the ShcA/Src signaling axis from these PTB-independent pools enables the reprogramming of signal networks to increase their reliance on mTOR signaling.

3. Using a previously generated dataset identifying 100 differentially expressed genes that distinguished tumors that did or did not augment mitogenic signals from PTB-independent ShcA complexes, we stratify 1218 human breast cancers using publicly available TCGA RNA-seq platform. Primary human breast tumors display characteristic features observed in our model system, including reduced pY317-ShcA levels, elevated pY416-Src, pS235/6-rS6 levels, and increased Src, Fyn, and Lyn mRNA expression. Lastly, the genomic loss of PTPN12 is a characteristic of breast tumors that have engage PTB-independent ShcA complexes, establishing a possible relationship between this phosphatase and its role in restricting ShcA-dependent Src activation in human breast tumorigenesis.

4. We provide the first *in vivo* evidence evaluating the biological significance of unique regulatory elements within the ShcA N-terminal domain, including Serine 29 and a S29-W38 motif. We show that these two regulatory elements have non-overlapping mechanisms that control ShcA regulated tumorigenic responses.

Preface

This PhD dissertation is written in a traditional monograph-based format. The thesis is divided into eight sections:

1. Rationale of the study and comprehensive literature review
2. Materials and methods that encompass the total body of work
3. Results comprised of work that has been published as an original research article and is in preparation for publication
4. General discussion
5. Future directions and prospective studies
6. Overall summary and implications of the study
7. Comprehensive bibliography
8. Appendices of permissions and waivers

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List of Abbreviations

Term	Abbreviation
Antibody-dependent cellular cytotoxicity	ADCC
Aromatase inhibitor	AI
Bovine serum albumin	BSA
Central Collagen Homology 1	CH1
c-Jun N-terminal kinase	JNK
Copy number abberations	CNA
Copy number variants	CNV
CUB domain-containing protein 1	CDCP1
Cytokeratin	CK
DEP-domain-containing mTOR-interacting protein	Deptor
Dimethyl sulfoxide	DMSO
Disease free survival	DFS
Dual specificity phosphatase	DUSP
Ductal carcinoma in situ	DCIS
Dulbecco's modification of Eagle medium	DMEM
Empirical Cumulative Distribution Functions	ECDF
Epidermal growth factor	EGF
Epidermal growth factor receptor	EGFR
Epithelial to mesenchymal transition	EMT
Estrogen receptor	ER
Eukaryotic initiation factor 4E (eIF4E)-binding protein	4E-BP
Extracellular signal–regulated kinase	ERK
Fetal bovine serum	FBS
Fibroblast growth factor receptor	FGFR
Focal adhesion kinase	FAK
Growth Factor Receptor Network	GFRN
Heparin-binding EGF-like growth factor	HB-EGF
Human epidermal growth factor receptor	HER2
Immunohistochemistry	IHC
Insulin growth factor 1 receptor	IGF1R
Insulin receptor	InsR
Insulin receptor substrate	IRS
Internal ribosome entry site	IRES
IQ Motif Containing GTPase Activating Protein 1	IQGAP
Janus kinase	JAK
Mammalian lethal with Sec13 protein 8	mLST8
Mammary epithelial growth supplement	MEGS
Mammary tumour virus-long terminal repeat	MMTV-LTR
Matrix Metalloprotease 2	MMP2
Mechanistic target of rapamcyin	mTOR
Mitogen activated protein kinase	MAPK
Neu-internal ribosome entry site (IRES)-Cre	NIC
Overall survival	OS

Term	Abbreviation
p70 ribosomal S6 Kinase	S6K
Pathologic complete response	pCR
Patient-derived xenografts	PDX
Phosphatidylinositol 3,4,5-trisphosphate	PI(3,4,5)P3 or PIP_3
Phosphatidylinositol 4,5-bisphosphate	PI(4,5)P2 or PIP_2
Phosphoinositide 3-kinase	PI3K
Phosphoinositide-dependent protein kinase 1	PDK1
Phospholipase C gamma	PLCγ
Phospho-tyrosine	pTyr or pY
Phospho-tyrosine binding	PTB
Platelet Derived Growth Factor Receptor	PDGFR
Pleckstrin homology	PH
Polyomavirus Middle T antigen	PyV-MT
Polyvinylidene difluoride	PVDF
Progesterone receptor	PR
Progression free survival	PFS
Proline-rich AKT substrate 40 kDa	PRAS40
Protein kinase A	PKA
Protein kinase B	PKB
Protein kinase C	PKC
Protein phosphatase 2A	PP2A
Protein serine/threonine phosphatase	PSP
Protein tyrosine phosphatase	PTP
PTP—proline, glutamic acid, serine, and threonine rich	PTP-PEST
Ras homolog enriched in brain	Rheb
Reactive Oxygen Species	ROS
Receptor tyrosine kinase	RTK
Regulatory-associated protein of mTOR	Raptor
Reverse phase protein arrays	RPPA
Ribosomal protein S6	rS6
Selective estrogen receptor modulators	SERM
SH2 domain-containing inositol-5-phosphatase 2	SHIP2
Signal transducer and activator of transcription	STAT
Single sample gene set enrichment analysis	ssGSEA
Smooth muscle actin	SMA
Son of sevenless	Sos
Src family kinase	SFK
Src homology 1	SH1
Src homology 2	SH2
Src homology 3	SH3
Src homology 4	SH4
Stress-ativated protein kinase	SAPK
The Cancer Genome Atlas	TCGA
Transforming growth factor	TGF
Trastuzumab emtansine	T-DM1

Term	Abbreviation
Triple negative breast cancer	TNBC
Tuberous sclerosis proteins 1 and 2	TSC1/2
Tyrosine kinase inhibitors	TKI
Vascular endothelial growth factor	VEGF

Chapter 1 Introduction

1.1 Rationale of the Study

Phospho-tyrosine signaling networks have emerged as fundamental effectors and modulators of numerous biological processes including cell growth and survival, motility and invasion, and cellular differentiation. Indeed, the aberrant regulation of the tyrosine kinome is observed in human disease processes, including cancer initiation, progression, and metastasis. The success of specific tyrosine kinase inhibitors (TKIs) strongly validates the clinical relevance of tyrosine phosphorylation in cancer pathobiology. However, owing to the frequent activation, amplification, overexpression or mutation of numerous tyrosine kinases in cancer, and the significant redundancy within phospho-tyrosine signaling networks, the tyrosine kinome has become a source of tumor heterogeneity and a barrier to therapeutic efficacy. Since the stratification of breast cancers into diverse subtypes, receptor tyrosine kinases (RTKs) and downstream signaling intermediates including adaptors, cytoplasmic tyrosine kinases, and protein tyrosine phosphatases (PTPs) have proven to have a significant impact in shaping breast cancer plasticity, sensitivity to targeted therapies, and acquired resistance. Given the dynamic and adaptive regulation of the tyrosine kinome in cell growth and survival programs, understanding the contribution of phospho-tyrosine signaling networks in breast tumorigenesis is warranted.

The commonality between most phospho-tyrosine signaling networks is their shared use of adaptor proteins to transduce mitogenic signals. ShcA is one such adaptor protein that is a key convergence point downstream of RTKs and serves to integrate multiple signal transduction pathways dysregulated in breast cancer. ShcA contains two phospho-tyrosine binding motifs: a carboxy-terminal Src homology 2 (SH2) and an amino-terminal phospho-tyrosine binding (PTB) domain to facilitate its interactions with cellular partners, including Src and ErbB2, respectively. ShcA also contains three tyrosine phosphorylation sites [Y239/240 and Y317 (Y313 in mice)] that transduce Ras-dependent and -independent signals. Upon the activation of RTKs, ShcA binds the RTK through its PTB or SH2 domains, leading to phosphorylation of its tyrosine residues. This results in the transduction of Ras/ERK and PI3K/AKT mitogenic signaling pathways downstream of the ShcA phospho-tyrosine residues.

Transgenic mouse models have also reinforced the requirement for an intact ShcA PTB domain in ErbB2 driven breast cancer. Deletion of the five main tyrosine phosphorylation residues within

the cytoplasmic tail of ErbB2 compromises the ability of ErbB2 to induce mammary tumorigenesis [1]. However, reconstitution of the ShcA PTB domain binding site alone is sufficient to restore the kinetics and severity of breast tumor development [1].These studies identified ShcA as a strong inducer of mitogenic signaling. Alternatively, transgenic mouse models where ShcA can no longer engage the transforming oncogene through its PTB domain delays mammary tumor onset [2]. Paradoxically, once tumors form, the growth and angiogenic potential of these tumors are significantly increased relative to control mice. These studies suggest that fail safe mechanisms exist to limit aberrant ShcA signaling in breast cancer cells. Indeed, alongside its pro-tumorigenic properties, the ShcA PTB domain is also essential for signal termination. The ShcA PTB domain binds numerous negative regulators, including PTPN12 [3], SHIP2 [4], PTPε [5] and can sequester oncogenic effectors such as ERK [6]. While some of these interactions require the phospho-tyrosine binding pocket of the ShcA PTB domain (PTPN12, SHIP2), others are phospho-tyrosine independent (PTPε, ERK). The recruitment of negative regulators to the ShcA PTB domain is dependent, in part, on serine phosphorylation, which results in the termination of ShcA-dependent signaling responses and the subsequent shift in the ShcA interactome to favour cytoskeletal reorganization [7]. Specifically, the recruitment of PTPN12 has been associated with the phosphorylation of Serine 29 at the N-terminal of ShcA [7]. Serine 29 phosphorylation is dependent on a AGC kinase binding motif, RXXS/T (inclusive of Serine 29). Interestingly, Serine 29 is also part of a putative Src binding motif, mapped to a 10-amino acid region upstream of the ShcA PTB domain [8].

Given the duality of the ShcA PTB domain, the mechanism underlying its regulation and impact on mammary tumorigenesis has yet to be determined. Additionally, the biological impact of serine/threonine phosphorylation of ShcA PTB domain dependent interactions and signal transduction has not been thoroughly investigated. The **objective** of this body of work is to understand how phospho-tyrosine signaling pathways upstream and downstream of the ShcA PTB domain differentially regulate breast tumorigenesis. We **hypothesize** that the ShcA PTB domain functions as a biological sensor of RTK signaling to control breast cancer initiation, tumor growth, and therapeutic responsiveness.

1.2 Introduction to Literature Review

The human tyrosine kinome is an essential network that integrates and transduces diverse signaling pathways to affect various biological processes such as cell growth, survival, mobility, invasion, and differentiation. These signals are initiated by a large repertoire of signaling proteins including 58 receptor tyrosine kinases (belonging to 20 families), 34 cytoplasmic tyrosine kinases (belonging to 10 families), and 46 tyrosine phosphatases (comprising 18 families) that respond to extracellular stimuli from growth factors, cytokines, and extracellular matrix proteins [9]. Tyrosine phosphorylation serves three major functions [9]:

1. Modulate the inherent catalytic activity of effector molecules within the cell,
2. Create docking sites for adaptor proteins, which serve to bridge a variety of signaling molecules and coordinate intracellular signaling responses in both a phospho-tyrosine dependent and independent manner, and
3. Control the strength and duration of signaling responses within the phospho-tyrosine network.

Classical signaling pathways begin with activation of RTKs and downstream effector pathways that alter gene expression, protein function, and cytoskeletal organization. Generally, RTKs are activated through ligand-induced homo- or hetero-dimerization, which then facilitates the autophosphorylation of tyrosine residues in the kinase activation loop or juxtamembrane region of the RTK. These phosphorylated tyrosine residues create docking sites for adaptor proteins and other tyrosine kinases that contain Src Homology 2 (SH2) and/or Phospho-tyrosine Binding (PTB) domains, which then transduce signals downstream of RTKs. Protein tyrosine phosphatases (PTP) regulate phospho-tyrosine dependent signaling to ensure the stringent temporal and spatial control of cellular communication and response to signal input.

The importance of a tightly controlled tyrosine phosphorylation network can be exemplified by its deleterious effects in human disease, such as cancer. In breast cancer, the aberrant regulation of the tyrosine kinome has been implicated in all stages of mammary tumorigenesis including breast cancer initiation, progression, and metastasis. Indeed, through the molecular classification and stratification of breast cancers [10], fundamental molecular differences between tumor subtypes

have been identified including genetic drivers that correlate to sub-type specific tyrosine kinase signaling networks [11] as well as those that contribute to inter- and intra-tumoral heterogeneity. Gene expression analyses have also been essential for validating global signaling networks in the search for therapeutic targets and biomarker discovery. However, the efficacy of tyrosine kinase inhibitors (TKIs) in the clinic are limited due to intrinsic or acquired resistance. Breast tumors re-program their tyrosine kinome through aberrant activation of alternative tyrosine kinases with complementary or compensatory function [12]. As such, the inherent malleability of the tyrosine kinome is a significant barrier for durable therapeutic responses.

The following discussion will focus on the current understanding of phospho-tyrosine dependent signaling that is prevalent in mammary tumorigenesis, and the role of these signaling networks in establishing resistance to current therapy *in vitro* and *in vivo*.

1.3 Breast Cancer

Breast cancers are classified into 6 subtypes—normal like, luminal A, luminal B, HER2 enriched, claudin low, and basal-like, which center on differing histopathological parameters including estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (ErbB2/HER2) oncogene amplification, Ki-67 proliferation mark, and the level of claudin proteins [13, 14] (Figure 1).

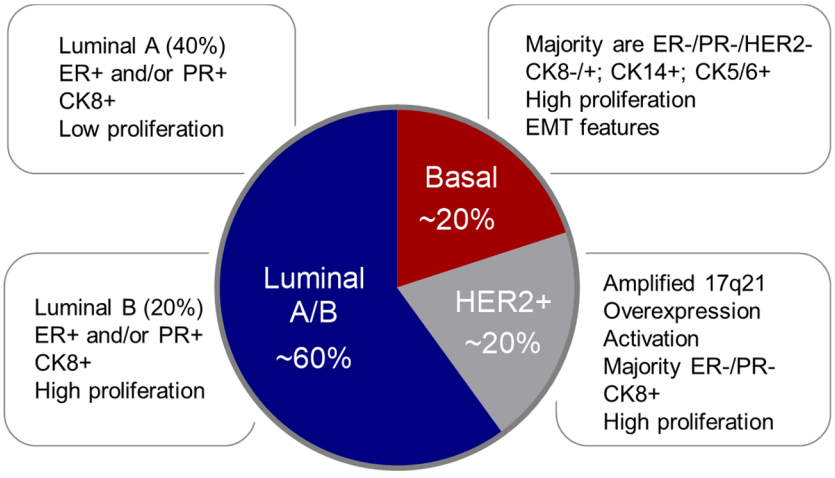


Figure 1: **Heterogeneity in breast cancer**. The percentages of individual breast cancer subtypes, as defined by intrinsic gene expression profiling, are shown. Salient features and markers characteristic of each subtype are indicated [9].

The adult mammary gland is a bilayer structure comprised of an inner layer of luminal epithelial cells lining the ducts and an outer layer of basally oriented myoepithelial cells contacting the basement membrane (Figure 2).

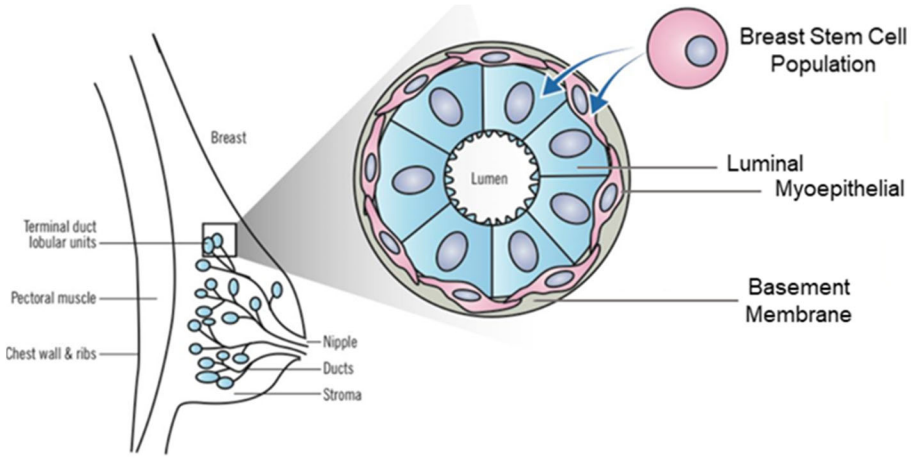


Figure 2: Schematic representation of the architecture of normal mammary epithelium. Retrieved and adapted from [15].

Luminal epithelial cells are characterized by the expression of cytokeratin (CK) 8 and 18, adherens (E-cadherin) and tight junctional (ZO-1, Claudins) proteins. Myoepithelial cells express CK5 and 14 and smooth muscle actin (SMA) [13, 14]. Luminal epithelial progenitors are the cell of origin for luminal and HER2+ breast cancers and retain CK8/18 and E-cadherin expression [16]. Basal breast cancers express CK14 and SMA, and, were previously thought to be restricted to the myoepithelial compartment [17]. Although literature has associated luminal and basal cells-of-origin for luminal and basal-like breast cancer, respectively, there is increasing evidence to suggest that both subtypes originate from the luminal epithelial lineage [18-21]. Studies that examined breast tumors arising in carriers of germline mutations in BRCA1 which have the basal-like phenotype [18, 22, 23], showed an increase in luminal progenitor numbers in breast tissue of BRCA1 mutation carriers and a correlation between the gene expression profile of normal human luminal progenitors and basal-like breast cancers [20]. Indeed, the luminal progenitor gene signature strongly associated with basal-like breast cancers while the basal/stem cell signature correlated to tumours which classified as Normal-like or Claudin-low [20]. It has therefore been proposed that loss of BRCA1 function in mammary stem cells results in tumor formation

associated with a block in luminal differentiation [18, 24-26]. These findings suggest that most basal-like breast cancers are derived from luminal intermediate cells and not from basal stem cells as was originally expected. In addition, seminal work from Curtis et al., 2012 demonstrated a novel classification of breast cancer according to joint clustering of copy number and gene expression data [27]. Using integrated genomic/transcriptomic analysis of 997 breast cancers, the authors find that 10 integrative clusters (IntClust 1–10) were categorized by well-defined copy number aberrations (CNA) which further divided the known instrinsic subtypes [27]. For example, subtype-specific *trans* [1] -acting aberrations modulated transcriptional changes, such as chromosome 5 deletion-associated cell cycle networks in basal cancers. Moreover, projecting the molecular profiles of these integrative subgroups onto signaling pathways also revealed CNA-expression landscapes that determined, IGF1R, KRAS and EGFR amplifications. These new subgroups warrant further investigation and have implications for better understanding therapeutic responses to targeted agents, particularly tyrosine kinases or phosphatases.

Luminal breast cancers are categorized by the overexpression of estrogen receptor (ER) and/or progesterone receptor (PR). ERα isoform accounts for 70% of all breasts cancer cases and are treated with endocrine therapy including selective estrogen receptor modulators (SERMs), such as tamoxifen, and the aromatase inhibitors (AI) letrozole, anastrozole, and exemestane [28]. SERMS are compounds that interact with ER in target organs as either ER agonists or antagonists [29], while AIs inhibit the enzyme, aromatase, to reduce the level of estrogen production [30]. Luminal A breast cancers are often ErbB2/HER2 negative, low Ki-67 by immunohistochemistry, and exhibit the best clinical outcome. Luminal B cancers are ER+ however, can be associated with ErbB2/HER2 positivity and high Ki-67. They are more aggressive and are associated with poor clinical outcome (Figure 1). The ErbB2/HER2 enriched subtype[2] is distinguished by the gene amplification of the ErbB2/HER2 oncogene within an amplicon of 11 genes [31, 32] (Figure 1). Targeting ErbB2/HER2 through trastuzumab, pertuzumab, trastuzumab emtansine (T-DM1), and lapatinib in routine clinical practice has had a dramatic effect on slowing disease progression and improving patient overall survival [9]. Unlike the luminal and ErbB2/HER2 subtypes, basal-like and/or triple negative breast cancers (ER-/PR-/HER2-) harbor a wide spectrum of chromosomal

[1] In this study, *trans* (distal) loci was defined as being outside a 3-megbases window surrounding the gene of interest, while c*is* (proximal) loci was defined as those within this window. Reference 27.
[2] Discussed in Chapter 1.5.1, Page 13.

amplifications, deletions and point mutations [33] and have been stratified into two groups based on molecular profiling studies: basal A and basal B [34] (Figure 1). Basal A tumors co-express luminal (CK8/18) and myoepithelial (CK14, SMA) markers. In contrast, Basal B tumors, also referred to as claudin-low, lack luminal epithelial markers, acquire stem cell features and express mesenchymal genes such as Vimentin, Snail1/2, Twist1/2 and ZEB1/2 [35]. Large-scale genome sequencing of basal-like/triple negative breast cancer (TNBC) tumors have not identified any therapeutically targetable oncogenic alterations. As such, chemotherapy and radiotherapy are still the mainstay of treatment for these cancers.

1.4 Breast Tumor Heterogeneity and the Tyrosine Kinome

Gene expression profiling and transcriptome analyses have provided systematic identification of driver and passenger genes that give rise to the molecular and functional diversity observed in breast tumor pathology. While stratification of breast cancer based on such analyses and biomarker assessment have been valuable to therapeutic decision-making, inter- and intra-tumoral heterogeneity remains a clinical challenge in patient treatment and outcome (Figure 3).

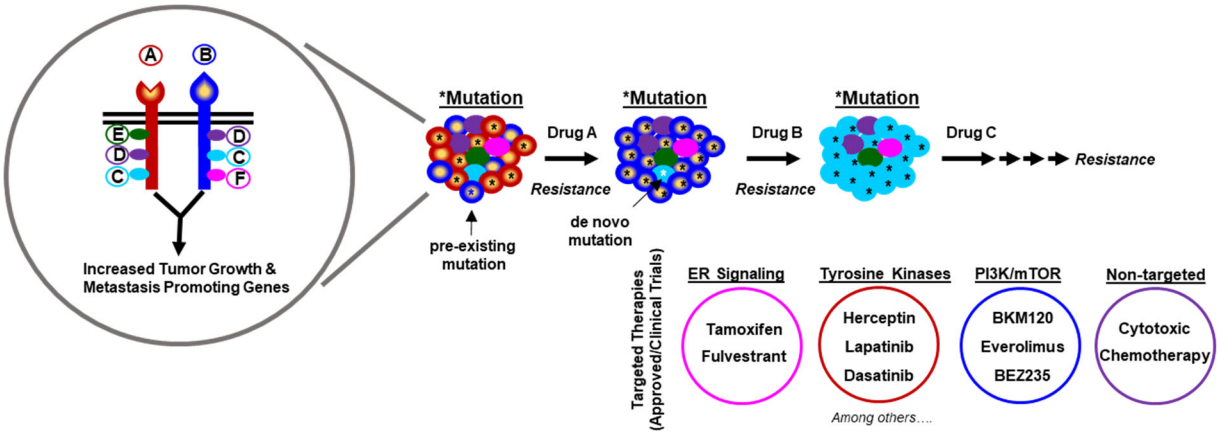


Figure 3: Schematic representation of the inherent malleability of the tyrosine kinome leading to therapeutic resistance and breast tumor heterogeneity. The efficacy of targeted therapies in the clinic are limited due to intrinsic or acquired resistance. Breast tumors have the capacity to re-program their tyrosine kinome through aberrant activation of alternative tyrosine kinases with complementary or compensatory function.

Luminal and basal cells are organized into branching ducts and maintain homeostasis within the mammary gland [36]. Based on the hierarchy of the mammary epithelium, the origins of inter- and intra-tumoral heterogeneity have been postulated by various models including the differentiation state of the initially transformed cell (cancer cell-of-origin), cancer cell plasticity, and the impact of the tumor microenvironment. Linear clonal evolution suggests that genetically distinct sub-clonal population of cells arise through intercellular genetic variation, that are then selected based on phenotypic advantages within a given tumor microenvironment [37]. If a clone fails to outcompete its predecessors, heterogeneity will be observed [37]. On the other hand, branched or parallel tumor evolution warrants that distinct subclones within a tumor bed evolve in parallel, leading to subclonal diversity [37, 38] and defined patterns of instability during tumor progression. For example, studies on breast cancer that assessed the temporal sequence of mutations [37, 39] found that mutational patterns were similar to germline mutations early in cancer development. However, later in tumor progression, there were significantly altered mutational patterns. This suggested that genomic landscapes can be altered over time and across the tumor bed.

Studies using whole-genome and exome single cell sequencing approaches, alongside single nuclei copy number profiling investigated the mutational evolution of ER+ and TNBC. These studies revealed many sub-clonal and *de novo* mutations, where no two tumor cells were genetically identical [40]. This suggested that point mutations evolved gradually over long periods of time, generating extensive clonal diversity. Interestingly, structural changes in DNA, such as amplification and deletions, were highly similar between the two subtypes. This implied that chromosome rearrangements were an early event, followed by stable clonal expansions to form the tumour mass [40]. As such, predictive biomarkers are seen to evolve throughout tumor progression. This is particularly relevant in metastatic dissemination, as selective pressures from treatment regimens as well as from the metastasis cascade itself, can enrich for aggressive clonal populations. In line with these observations, a pan-cancer analysis of various cancers reported that a single biopsy could accurately represent the driver gene mutations of a patient's untreated metastases [41].

Landmark studies by Koren et al., 2015 and Van Keymeulen et al., 2015 have also provided evidence that links an activating oncogenic event to the induction of multipotency in breast cancer

[42-44]. Specifically, these studies show that activating genetic mutations in the PIK3CA[3] gene, namely p110α^{H1047R}, causes the loss of lineage restriction and tumor heterogeneity from basal and luminal cells in the adult mouse mammary gland [43, 44]. These observations contrasted the previously held belief that basal-like breast cancers that express high levels of basal cell markers originated from transformed basal progenitor/stem cells and that luminal-type breast cancers with high level of luminal cell markers originated from luminal progenitors. Using lineage tracing[4] in transgenic mice, induction of p110α^{H1047R} luminal epithelial cells resulted in transcriptional profiles and markers of basal epithelial cells, while p110α^{H1047R} in basal epithelial cells gave rise to luminal epithelial signatures. As such, genetic mutation of PI3K was sufficient for the loss of lineage restriction by either luminal or basal epithelial cells. This is an extremely important development considering the high mutational rate of PI3K in all subtypes of breast cancer and the implications this may have on the efficacy of current treatment options.

Variations in subclonal architecture and phenotypic variations are also a consequence of altered signal transductions that shape the evolution of the tumor genome [37]. Clonally dominant genetic events such as ErbB2/HER2 amplification, are complicated by alternative stochastic events in gene expression, protein stability, transcriptional and epigenetic cues that feedback to compensate from therapeutic intervention. For example, while the majority of ErbB2/HER2-positive breast cancers show homogeneous patterns of ErbB2/HER2 amplification and protein overexpression, there is a subset of ErbB2/HER2-positive cancers that are mosaics with intermixed clones of breast cancer cells exhibiting different patterns of HER2 gene amplification and overexpression [45]. Work by Ng et al., 2015 illustrates that ErbB2/HER2 heterogeneous breast cancers are ER+ and predominantly TP53 mutated [45]. In addition, alterations in driver genes were restricted to the HER2-negative component, including amplification of PIK3CA or MYC. The authors note that it is not clear whether HER2 amplification was an early event and was subsequently lost in the HER2-negative sub-populations, or whether HER2 amplification was acquired in the HER2-positive components at a relatively late stage of tumorigenesis due to alterations in these driver

[3] PI3KCA gene encodes the p110α catalytic subunit of phosphoinositide3-kinases (PI3K), a lipid kinase that functions downstream of RTKs. P110α^{H1047R} is the most recurrent activating mutation causing constitutive PI3K signaling and the formation of heterogeneous mammary tumors. Reference 42.

[4] Lineage organization in PI3K-driven tumours was assessed using mice expressing Cre recombinase inducible transgenes with or without activating mutation of p110α driven by Lgr or K5 promoters (expressed by basal epithelial cells) or K8 promoter (expressed by luminal epithelial cell). Reference 43 and 44.

genes. This study provides insight into the temporal and spatial heterogeneity of driver oncogenes within a tumor and provides evidence that even targeted therapies such as trastuzumab may only be effective in a subset of cancer cells. This leaves untargeted cells free to adapt to such therapeutic interventions. For example, trastuzumab can increase the expression of ErbB3/HER3[5] as a resistance mechanism to ErbB2 inhibition [46]. On the other hand, ErbB3 can also maintain the luminal epithelium at the luminal progenitor stage *in vitro* and *in vivo* [47]. ErbB3 loss shifted gene expression patterns towards those of mammary basal cells/stem cells and limited differentiation of mammary epithelial cells along the luminal lineage. Moreover, breast tumors arising from the loss of ErbB3 increase the expression of the basal marker, CK5. This observation demonstrates that the re-activation and/or expression of alternative RTKs can direct cell fate decisions in cancers and impact mammary cell populations contributing to tumor heterogeneity [47].

Integrative network analysis of exomes and RNAi screening of luminal and basal subtypes have also demonstrated that a set of characteristic genes may regulate subtype-specific survival and proliferative signaling networks [11]. For example, AKT1, PIK3CA, and ESR1 are dominantly selected in luminal subtype-specific survival signaling whereas TP53 and SRC are dominant in the basal subtypes. A study by Rahman et al., 2017, assessed the Growth Factor Receptor Network (GFRN) in 1119 breast tumors from The Cancer Genome Atlas (TCGA) and across 55 breast cancer cell lines from the Integrative Cancer Biology Program (ICBP43) and estimated various signal transduction pathway activity signatures in relation to pro- and anti-apoptotic protein expression and drug responses in breast cancer cell lines [48]. Interestingly, there were two distinct signatures which included concurrent activation of the HER2, IGF1R, and AKT pathways or the concurrent activation of the EGFR, KRAS, RAF1, and BAD pathways. Pathway activation of HER2, IGF1R, and AKT was deemed the "survival phenotype", while activation of EGFR, KRAS, RAF1, and BAD as the "growth phenotype". Typically, when one set of pathways was active, the other set was inactive, indicating that each sample tends to have a dominant GFRN phenotype. Those breast tumors representing the "growth phenotype" were more sensitive to common chemotherapies and targeted therapies directed at EGFR and MEK. Alternatively, the "survival

[5] The ErbB3/ErbB2 (HER3/HER2) heterodimer is the most potent oncogenic ErbB signaling pair, which leads to the activation of PI3K/AKT and Ras/MAPK signaling cascade. Discussed in Chapter 1.5, Page 12.

phenotype" was more sensitive to drugs inhibiting HER2, PI3K, AKT, and mTOR, but more resistant to chemotherapies [48]. Additionally, Stuhlmiller et al., 2015 observed heterogeneity in kinome adaptation to lapatinib treatment in ErbB2/HER2 amplified cell lines [49]. Depending on the cell line studied, there was either reactivation, re-expression, or inhibition of certain kinase networks, including RTK networks comprised of ErbB receptor family members, MET, IGF1R, and FGFRs and non-RTKS involving JAK1, FAK1, and FRK and YES, and multiple kinases involved in cytoskeletal regulation that become upregulated after targeted RTK inhibition.

The progression of breast cancer is rarely dependent on mutually exclusive mechanisms, but rather a combination of several integrating components that contribute to cellular plasticity, genomic evolution, and fitness. Noticeably, aberrant phospho-tyrosine networks and their intrinsic adaptiveness are key to driving tumor growth and survival. Collectively, there is mounting evidence for an important interplay between mammary gland cell compartments and cell intrinsic cues that govern tumor heterogeneity and provide cells with a competitive advantage for cell growth and proliferation under various selection pressures.

1.5 Overview: Epidermal Growth Factor Receptor Family

The epidermal growth factor family of RTKs is comprised of several members including, EGFR (ErbB1, HER), ErbB2 (HER2), ErbB3 (HER3), and ErbB4 (HER4). Structurally, the EGFR family of RTKs are single chain transmembrane glycoproteins and are characterized by a **(1)** extracellular N-terminal ligand binding ectodomain containing leucine-rich subdomains L1 and L2, **(2)** cysteine-rich subdomain CR1 and CR2 containing the dimerization loop responsible for receptor-receptor interaction, **(3)** transmembrane domain, **(4)** short juxtamembrane domain, **(5)** tyrosine kinase domain, and **(6)** tyrosine-containing C-terminal tail [50]. The ErbB family either homo- or heterodimerize upon the binding of soluble ligands—altogether, comprising a total of 28 different combinations [51]. Uniquely, ErbB2 does not have a ligand-binding domain, while ErbB3 is catalytically inactive. ErbB2 is the preferred heterodimerization partner of other ErbB receptors and does not have a ligand. ErbB2 is 100-fold more potent in its transforming ability than EGFR, although the two receptors are 85% homologous [52-54]. ErbB2 is activated by overexpression, homodimerization, or by ligand-mediated stimulation of other ErbB receptors through heterodimerization [55]. In addition, alternative splicing of ErbB2 in the cysteine rich domain has

been demonstrated to be important for its transforming potential [56]. ErbB3 is the preferred binding partner of ErbB2 and ErbB2/ErbB3 heterodimers have the greatest transforming ability [57]. ErbB4 is regulated at the post-transcriptional level by alternative splicing to generate an RTK that does (exon 16) or does not (exon 15) contain the proteolytic cleavage site for two proteases, TACE and gamma-secretase [58]. Inclusion of exon 16 permits cleavage of the extracellular domain of ligand-activated ErbB4, releasing a soluble intracellular domain (4ICD), that can translocate to the nucleus where it regulates the expression of ERα target genes [59].

ErbB ligands are proteolytically processed and released as soluble molecules to engage the receptors [60]. EGFR binds EGF [61, 62], transforming growth factor-α (TGF-α) [63-65], and amphiregulin [65]. ErbB3 binds neuregulin-1[66-70], neuregulin-2 [71-74] and neuroglycan C [75]. ErbB4 is also able to bind neuregulin-1 and neuregulin-2, but exclusively engages neuregulin-3 [76], neuregulin-4 [77], and tomoregulin [78]. In addition, EGFR and ErbB4 can bind heparin-binding EGF-like growth factor (HB-EGF) [79], betacellulin [80, 81], epiregulin [82, 83] and epigen [84]. Upon extracellular ligand binding, ErbB RTK family members traditionally activate Ras/Mitogen activated protein kinase (MAPK), PI3K/AKT, Phospholipase C (PLC)γ1/PKC, Src, and Signal transducer activator of transcription (STAT) signaling pathways [85] (Figure 4).

1.5.1 ErbB2 in Breast Cancer

The ErbB2/HER2 enriched subtype accounts for approximately 15-20% of breast cancers [86] where amplification, overexpression, and alternative splicing of this RTK is the primary driver of tumor cell growth [49, 87]. DNA amplification of the ErbB2 locus, chromosome 17q12-21, leads to the consequent overexpression of ErbB2 [9]. It has been reported that the ERRα/PGC1β transcriptional axis works to control ErbB2 expression and amplify genes within the HER2 amplicon. ERRα competes with ERα for binding to the ErbB2 promoter, which relieves ERα-mediated repression of ErbB2 transcription to promote Tamoxifen resistance [88]. However, 15% of HER2+ breast tumors overexpress the receptor in the absence of gene amplification [89]. Genomic sequencing of 25 HER2+ (non-amplified) breast tumors identified recurrent mutations and in-frame deletions within the ErbB2 gene [9, 90]. The most frequent mutational “hotspots” within the ErbB2 locus include (1) the extracellular domain which facilitates receptor homo- or

hetero-dimerization and (2) the kinase domain itself, which possesses the catalytic activity of the enzyme. These mutations enhance ErbB2-mediated signal transduction and increase the tumorigenic potential of ErbB2, both *in vitro* and *in vivo* [9, 90]. ErbB2 can also be proteolytically cleaved of its N-terminal extracellular domain to form a p95 carboxy terminal fragment by ADAM10 metalloprotease [91, 92] or, from alternative splicing or alternative translation initiation [93-95]. p95HER2 homodimers form disulphide bridges leading to its constitutive activation and propagation of mitogenic signaling pathways including, PLCγ1, MAPK/ extracellular signal-regulated kinase 1/2 (ERK1/2), Src, AKT/protein kinase B (PKB), Janus kinase (JAK)/STAT, and stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) pathways [96-99]. Additionally, ErbB2 may also undergo alternative splicing to form an isoform encoding a 16-amino acid in-frame deletion in the juxtamembrane domain, denoted as Δ16ErbB2 [99]. Like p95Her2, Δ16ErbB2 is constitutively active and can form disulfide-bridged, ligand-independent homo dimers [56, 93].

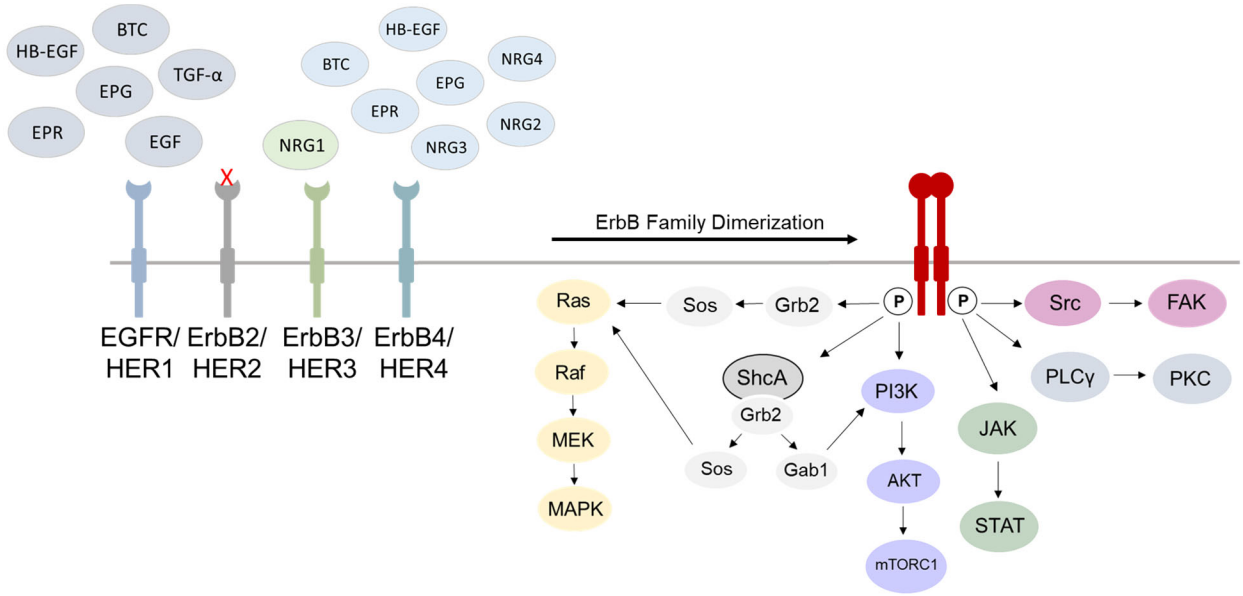


Figure 4: Schematic representation of the Erb family of RTKs, ligands, and signaling pathways. The epidermal growth factor family of RTKs is comprised of several members including, EGFR (ErbB1, HER), ErbB2 (HER2), ErbB3 (HER3), and ErbB4 (HER4). Upon extracellular ligand binding, ErbB RTK family members traditionally activate Ras/MAPK, PI3K/AKT, PLCγ1/PKC, Src, and STAT signaling pathways.

1.6 Overview: Non-RTKs in Breast Cancer

Since the identification of Src as a tyrosine kinase over 35 years ago, there has been a surge of scientific discovery demonstrating a fundamental role for aberrant tyrosine phosphorylation in numerous disease processes, including breast cancer initiation, progression, and metastatic spread. Signaling intermediates that act downstream of RTKs, including PI3K and Src, have SH2 domains or other phospho-tyrosine binding domains that allow specific interactions with phospho-tyrosine residues on activated RTKs. While RTKs serve to integrate extracellular stimuli, cytoplasmic tyrosine kinases are equally important to drive mitogenic signaling responses downstream of RTKs to affect breast tumorigenesis and response to therapy.

1.6.1 Src Family Kinases in Breast Cancer

The Src family kinases (SFKs) encompasses nine members: Src, Yes, Fyn, Fgr, Lyn, Hck, Lck, Yrk, and Blk. Briefly, the SFKs contain a conserved amino-terminal region that is essential for the interaction of myristic and/or palmitic fatty acids within the plasma membrane [100, 101]. This is then followed by the unique domain (SH4) comprised of 50–70 residues which are divergent among family members [102]. Next, are the SH3 and SH2 domains, a linker sequence, the kinase domain, and lastly, the C-terminal regulatory sequence [103] (Figure 5).

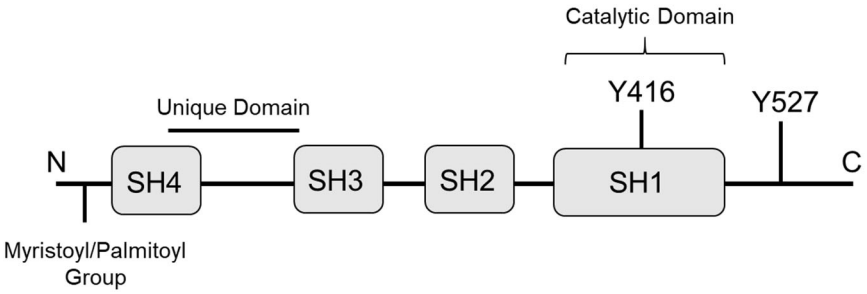


Figure 5: Schematic representation of the domain structure of SFKs. SFKs contain a conserved amino-terminal region essential for the interaction of myristic and/or palmitic fatty acids within the plasma membrane, Unique domain (SH4), SH3 and SH2 domains, a linker sequence, the kinase domain (SH1), and the C-terminal regulatory sequence.

The catalytic domain is necessary for the autophosphorylation of tyrosine (Y; Tyr) 416 for maximal enzymatic activity [104]. Within the C-terminal regulatory sequence is the auto-inhibitory Y527 residue, which is phosphorylated by Csk allowing for the intramolecular binding of the SH2 domain [105]. The SH3 domain interacts with proline residues of the linker sequence which stabilizes the SFK in an inactive conformation [106, 107]. The release of SFKs into its active form involves either the dephosphorylation of Y527 by protein tyrosine phosphatases, or the competition of an alternative SH2-domain containing protein with higher affinity for the phosphorylated Y527 residue [102] (Figure 6).

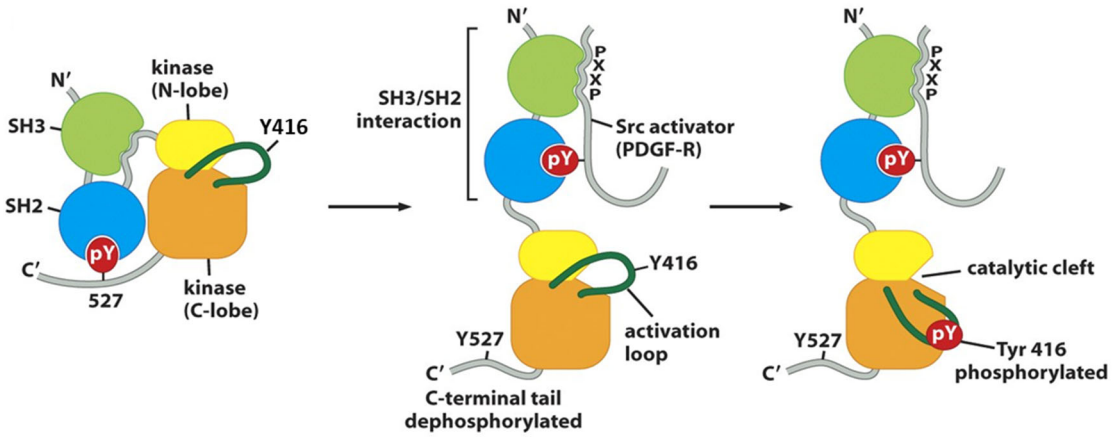


Figure 6: Schematic representation of the intramolecular structure of Src and its activation. Adapted from [108].

Src is considered the prototypical tyrosine kinase and the most well studied SFK family member in mammary tumorigenesis. Src was recognized as a key effector of breast cancer upon the observation that breast tumors increased Src kinase activity relative to normal breast tissue [109-111]. Indeed, examination of over 125 human breast tumor samples identified that more than 70% of breast tumor tissue contained Src kinase activity that was 2-50 fold greater than normal breast epithelium or immortalized mammary epithelial cells [110, 112-114]. However, unlike EGFR or ErbB2, the overexpression of Src alone is not sufficient to transform fibroblasts, *in vitro* or the mammary epithelium, *in vivo* [114-117]. Constitutive activation of Src in transgenic mice is weakly oncogenic, leading to the induction of hyperplasias [118]. These data suggest that Src may function to promote tumor growth by participating in or augmenting mitogenic signaling pathways

downstream of RTKs. In fact, mammary epithelial expression of either Polyomavirus Middle T antigen (PyV-MT)[6] [119] or ErbB2 [54] is sufficient to increase the activation of Src. Moreover, genetic and biochemical analyses of EGFR- and ErbB2-overexpressing mammary tumor cells demonstrate a direct interaction between Src and EGFR or ErbB2. EGFR/Src complex formation leads to the phosphorylation of Tyr845 and Tyr1101 of the RTK [117, 120] and increases phosphorylation of downstream EGFR effectors, including Src itself and MAPK [121, 122]. ErbB2/Src interactions are dependent on Tyr877 within the kinase domain of ErbB2 [54]. Interactions between ErbB2/Src leads to enhanced transforming potential and disruption of epithelial cell polarity [54, 123]. The significance of Src in ErbB2+ breast cancer will be extensively discussed throughout the following text and will not be further addressed here.

Early studies have also demonstrated that estrogen was able to activate many of the same effectors classically thought to be linked with EGFR signaling, of which, included Src. Indeed, global gene expression analyses of ER+ breast cancer that have adapted to estrogen deprivation demonstrated an upregulation of Src and FAK signaling pathways [124]. Src directly phosphorylates ERα on Y537, promoting its transactivation [125-127]. Conversely, ERα signaling can be attenuated using Src inhibitors [128]. In tamoxifen-resistant MCF-7 cells, the activation of Src is sufficient to promote cell invasion and motility [129]. Furthermore, the co-inhibition of Src and ER can impair breast cancer growth *in vitro* and *in vivo* [129-133]. Collectively, these studies demonstrated an essential role of Src and potentially other SFK family members in the luminal breast cancer subtype. Consistent with these observations, it has been shown that increased Lyn tyrosine kinase activity promotes cell proliferation and permits escape from anti-estrogen therapies [134]. Deep sequencing of resistant ERα tumors identified point mutations in the Lyn tyrosine kinase, which hyper-activated its kinase activity [134]. These mutations included a novel D189Y mutation that increased Lyn activity and the phosphorylation of known SFK targets including FAK, EGFR, HER3, and IRS1 [134]. The D189Y mutation resides within the SH2 domain of Lyn and is required for auto-inhibition of its tyrosine kinase activity by mediating an intra-molecular interaction with the tyrosine-phosphorylated Y507 residue in the cytoplasmic tail. Another study identified overexpression of the Fyn tyrosine kinase in Tamoxifen-resistant ERα+ luminal breast cancer cell lines [135]. Reduced Fyn expression, or treatment with a pan SFK inhibitor, Dasatinib,

[6] Discussed in Chapter 1.9.2, Page 35.

significantly decreased the proliferative potential of these breast cancer cell lines and re-sensitized them to Tamoxifen. In contrast, Fyn overexpression in parental cells was sufficient to reduce Tamoxifen sensitivity [135]. Interestingly, it was further identified that the subcellular localization of Fyn could determine the clinical outcome of ER+ breast cancer patients. Specifically, the presence of Fyn in the plasma membrane was associated with longer overall survival (OS) in ER+ early disease breast cancer patients, and longer progression free survival (PFS) in advanced disease patients [135]. Moreover, patients whose tumors showed only cytoplasmic or nuclear Fyn were found to have significantly shorter PFS [135]. Thus, for those ER+ breast tumors that have poor treatment outcome, the subcellular localization of Fyn, and possibly alternative SFKs such as Src, may serve as a biomarker in both early and advanced disease stages [135].

Aberrant activation of ERα and ErbB2 signaling in the luminal and HER2+ subtypes, respectively, provided therapeutic opportunities that have been exploited to significantly improve patient outcomes [9]. Unfortunately, basal/ TNBC are more pleiotropic with respect to the RTK repertoire which has limited targetable therapeutic strategies [9]. Phospho-proteomic analysis of luminal and basal breast cancer cell lines identified SFK signaling networks, including FAK and p130Cas, that were specifically engaged in basal breast cancer cell lines [136]. Lyn was a key component to this network. Indeed, reduced Lyn expression levels has been shown to decrease the migratory and invasive properties of independent basal cell lines [136]. These observations aligned with studies demonstrating that Lyn stimulates an epithelial-to-mesenchymal transition (EMT) and promotes the formation of breast cancer liver metastases from basal cell lines [137, 138]. In addition, Lyn was identified to phosphorylate PEAK1, a member of the NKF3 family of tyrosine kinases, which significantly increases the growth, invasion and EMT of immortalized, triple negative mammary epithelial cells [139].

1.6.2 PI3K/AKT Pathway in Breast Cancer

Another class of SH2 domain signaling molecules that associate with and are activated by RTKs are phosphoinositide 3-kinases (PI3K). The central role of the PI3K/AKT pathway is mediated by the activation of the PI3K heterodimer which belongs to the Class IA of PI3Ks [140]. The heterodimer consists of two subunits, the p85 regulatory subunit (PIK3R1) which regulates the activation of the p110 catalytic subunit (PIK3CA) in response to ligand-dependent and/or -

independent activation of RTKs [141, 142]. The p85 regulatory subunit contains two SH2 domains which bind pTyr-X-X-Met (YXXM) motifs within RTKs and associated binding proteins [140].

PI3Ks phosphorylate phosphatidylinositol 4,5 bisphosphate (PIP_2) to generate phosphatidylinositol 3,4,5-trisphosphate (PIP_3). Upon the formation of PIP_3, AKT is recruited to the plasma membrane through its pleckstrin homology (PH) domain. This leads to AKT phosphorylation at Threonine (T) 308 and Serine (S) 473 residues and subsequent activation of AKT substrates that regulate the inhibition of pro-apoptotic proteins, stimulate protein synthesis, and potentiate cell proliferation [142]. The phosphoinositide-dependent protein kinase 1 (PDK1) phosphorylates AKT at T308, which is required for AKT activity [143, 144]. Maximal activation of AKT occurs upon the phosphorylation of S473 by mechanistic target of rapamcyin (mTOR) complex 2 (mTORC2) [145]. The activation of PI3K/AKT pathway is classically known to be tightly controlled by the tumor suppressor gene, PTEN. PTEN dephosphorylates PI (3,4,5) P3 to PI (4,5) P2 [146, 147]. The loss of PTEN and activating mutations in PIK3CA are among the most common aberrations in human malignancies, including breast cancer [148, 149] (Figure 7).

In addition to its role in mediating its effects on cell survival and proliferation, AKT also induces dramatic effects on protein synthesis through its regulation of the mTOR pathway. The mTOR protein is a 289-kDa serine-threonine kinase that belongs to the PI3K-related kinase family [150]. Briefly, AKT phosphorylates and inactivates Tuberous sclerosis proteins 1 and 2 (TSC1/2) which acts as a GTPase activating protein for Ras homolog enriched in brain (Rheb). Rheb-GTP directly activates mTORC1 [151, 152]. mTORC1 consists of mTOR, a scaffolding protein RAPTOR (regulatory-associated protein of mTOR) and mLST8 (mammalian lethal with Sec13 protein 8), proline-rich AKT substrate 40 kDa (PRAS40); and DEP-domain-containing mTOR-interacting protein (Deptor) [150]. This complex phosphorylates eukaryotic initiation factor 4E (eIF4E)-binding protein (4E-BP) and p70 ribosomal S6 Kinase (S6K). 4E-BP phosphorylation inhibits its ability to bind and sequester the eIF4E mRNA cap-binding protein, thereby permitting the assembly of the cap-binding complex and subsequent translation initiation [142]. The activation of S6K acts on many substrates, including transcription factors, ribosomal protein S6, and other protein substrates involved in translation initiation and elongation [142].

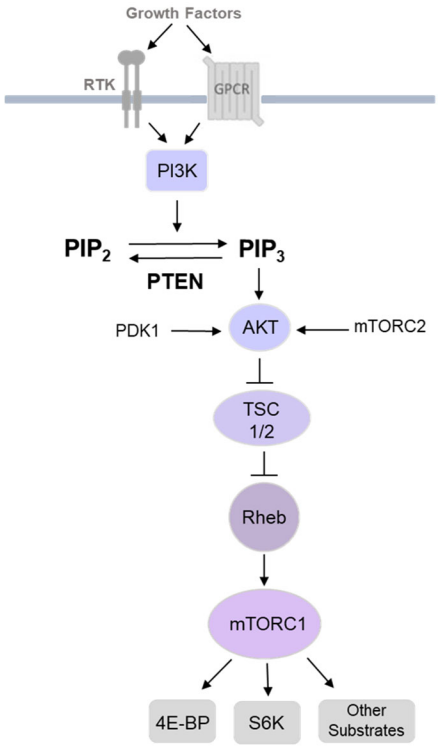


Figure 7: Simplified schematic representation of the PI3K/AKT/mTOR pathway. Adapted from [153].

Transgenic mouse models have been used to establish the essential role of the PI3K/AKT signaling pathway in mammary tumorigenesis. Expression of PyV-MT in the mammary epithelium results in the rapid development of multifocal metastatic mammary tumors [154], due to the ability of PyV-MT to associate with and activate, PI3K, Src, and the ShcA adaptor protein [119, 155-157]. Consistent with the importance of PI3K/AKT signaling for cell proliferation and survival, transgenic mice expressing a mutant PyV-MT uncoupled from the PI3K pathway (MMTV/MTY315/322F) develop extensive mammary gland hyperplasia that are highly apoptotic [158]. Co-expression of a constitutively active AKT (HA-PKB-308D473D or Akt-DD) is sufficient to dramatically reverse this phenotype—mammary tumorigenesis is accelerated due to reduced apoptotic cell death through the activation of the FKHR forkhead transcription factor and translational upregulation of cyclin D1 levels. Similarly, the activation of AKT alone induces ErbB2-mediated breast tumorigenesis, resulting in increased mammary epithelial cell proliferation and post-transcriptional upregulation of cyclin D1 [159]. These studies demonstrated the

importance of PI3K/AKT signaling in providing the necessary cues for cell survival in breast cancer.

Recent studies demonstrate that pharmacological PI3K inhibition increases signaling downstream of numerous RTKs (ErbB3, FGFR1, InsR, and IGF1R) as a compensatory mechanism to re-engage the PI3K/AKT pathway in luminal breast cancer cells [9, 160]. Indeed, autocrine InsR and IGF1R signaling is enriched in primary luminal breast cancers and rescues AKT signaling in Fulvestrant-resistant, ER+ breast cancers [161]. ErbB3 amplifies PI3K/AKT signaling owing to the presence of six binding sites for the p85 regulator subunit of PI3K in its cytoplasmic tail [162]. Interestingly, reduced ErbB3 expression in ErbB2+ luminal breast tumors is sufficient to sensitize them to pharmacological PI3K inhibitors [160]. RON, a member of the MET RTK family, undergoes alternative splicing to create an N-terminally short form (sfRON), which lacks most of the extracellular domain. This splicing event constitutively hyper-activates sfRON-mediated signaling, which leads to the phosphorylation of two tyrosine residues in the RON cytoplasmic tail. These residues reside within consensus binding sites for the SH2 domain of p85 regulatory subunit of PI3K allowing for increased PI3K signaling and breast cancer metastasis [163]. Combination treatment with RON tyrosine kinase inhibitors and PI3K inhibitors synergistically impairs tumor growth in ER+ patient-derived xenografts (PDXs), with or without an activating PIK3CA mutation, achieving durable responses following cessation of this combination therapy [164]. In line with these observations, the MET RTK also contributes to innate resistance to ErbB2-targeted therapies through the activation of the PI3K/AKT pathway. Decreased MET or ErbB2 expression is sufficient to reduce PI3K/AKT pathway activation [165].

1.7 Overview: Protein Tyrosine Phosphatases in Phospho-tyrosine Signaling

Tyrosine phosphorylation is balanced by protein tyrosine phosphatases, which can either upregulate or downregulate downstream signaling. Depending on the cellular context, the overall biological outcome hinges on specific phosphorylation events that either activate or inhibit downstream protein function.

Approximately 140 protein phosphatases have been identified. They are traditionally divided into two classes, protein serine/threonine phosphatases (PSPs) and protein tyrosine phosphatases (PTPs). PSPs include the PPP, PPM, and FCP/SCP families [166, 167]. The cysteine-based PTP

superfamily includes approximately 100 members [168, 169] grouped into classical PTPs and dual specificity phosphatases (DUSPs). Classical PTPs play critical roles in tyrosine kinase signaling [170], whereas DUSPs can dephosphorylate tyrosine or serine/threonine residues. Some DUSPs function as lipid or glycogen phosphatases. The EYA family represents a small collection of phosphatases with an aspartate-based catalytic domain [168, 171].

PTPs have diverse protein substrate and tissue expression profiles in cancer and contribute to the regulation of various cell growth and survival programs. Tyrosine-specific PTPs are highly selective enzymes, attributable to a unique signature motif within the catalytic domain, [I/V]HCSXGXGR[S/T]G, referred to as the PTP signature motif [172, 173]. Additional levels of substrate specificity are facilitated through the non-catalytic N- and C-terminal segments important for subcellular distribution, and, substrate recognition and binding. The conserved cysteine residue in the catalytic domain is essential for catalysis. However, under conditions of oxidative stress, the cysteine is oxidized causing a conformational change in PTPs and subsequent inactivation of enzymatic activity. A comprehensive study conducted by Karisch et al., 2011 identified and quantified the expression of PTPs (PTPome) and the oxidized counterpart of the PTPome (oxPTPome) [174]. Interestingly, Karisch et al., 2011 discovered an unanticipated complexity in PTP oxidation by exogenous and endogenous reactive oxygen species (ROS) in normal and neoplastic cells [174]. Indeed, cancer cells often produce high levels of ROS which could decrease basal PTP activity and enhance tyrosyl phosphorylation [175]. Moreover, RTK activation leads to transient H_2O_2 production, which is required for full RTK phosphorylation and downstream signaling [176]. Depending on the cancer studied, PTPs differ in their relative sensitivity to oxidation. Moreover, global ROS does not correlate with PTP oxidation profiles across cancers[7] [172].

1.7.1 PTPN12/PTP-PEST

PTPN12, also referred to as PTP-PEST (PTP—proline, glutamic acid, serine, and threonine rich), is a ubiquitously expressed 120 kDa cytosolic PTP located at chromosome 7q11.23. Genetic deletion of PTPN12 in the mouse germline is embryonically lethal. PTPN12 is essential for the

[7] The impact oxPTPome may have in the progression of breast cancer is yet to be determined. The possibility that phospho-tyrosine interactome of breast cancer subtypes may differentially alter PTP oxidation represents a new layer of complexity in signal transduction and to the cancer phenotype. Reference 172.

accurate control and precise balance of cell adhesion, morphology, and development [177]. Indeed, embryo fibroblasts derived from mice devoid of PTPN12 exhibit increases in cell spreading and are defective in cell motility [177, 178]. As such, PTPN12 is primarily identified for its role in cytoskeletal rearrangement including processes involved in cell migration, cell spreading, and cell division [177-182].

Structurally, PTPN12 contains a conserved N-terminal catalytic domain [183] and a carboxyl-terminal tail comprising several PEST-rich regions that are essential for substrate and/or adaptor protein interactions. This includes a non-canonical NPLH motif that engages ShcA in a phospho-tyrosine-independent manner [3] (Figure 8).

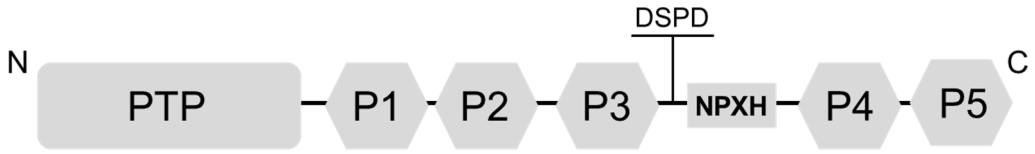


Figure 8: Schematic representation of murine PTPN12. PTPN12 contains a N-terminal catalytic domain (PTP), five proline rich regions (P1-P5), a NPXH motif, and caspase dependent cleavage site (DSPD). Adapted from [184].

To date, approximately 18 PTPN12 substrates have been identified, including HER2, FAK, PYK2, PSTPIP, WASP, p130Cas, paxillin, catenin, c-Abl, ArgBP2, p190RhoGAP, RhoGDI, cell adhesion kinase beta (CAKb), and Rho GTPase [185]. PTPN12 is phosphorylated by cyclic AMP-dependent protein kinase (PKA) and protein kinase C (PKC) at Ser 39 and Ser 435; phosphorylation of Serine 39 decreases PTPN12 affinity for its substrates [186]. Ras dependent activation of ERK also phosphorylates PTPN12 at Serine 571, causing the recruitment of PIN1 and the isomerization of PTPN12 [187]. This facilitates the interaction of PTPN12 with FAK and the subsequent dephosphorylation of FAK at Y397 [188] to regulate focal adhesion dynamics. PTPN12 dependent cell migration is coordinated through its regulation of Rac1/Vav2, RhoA/p190RhoGAP, and the subcellular localization and phosphorylation status of Rho GDP dissociation inhibitor 1 (RhoGDI1) [189]. PTPN12 has also been shown to be directly cleaved by caspase-3 on its ^{549}DSPD motif. This produces an N-terminal fragment with increased catalytic activity [184, 190]. Interestingly, this cleavage event enhances the interaction between PTPN12 and paxillin, which facilitates cellular detachment [191]. Altogether, these data establish PTPN12

as a key player in regulating cellular morphology, migration, and adhesion in programmed cell death.

1.7.1.1 PTPN12 in Breast Cancer

Mutations affecting PTPN12 catalytic activity have been found in several breast cancer cell lines and verified in primary breast tumors [192]. Specifically, three amino acid substitutions, V322I and T573A which elevates phosphatase activity and E709K which reduces its catalytic activity, were identified in the PTPN12 non-catalytic carboxyl domain in breast cancer and kidney tumours [192]. V322I and T573A mutations were found to enhance PTPN12 phosphatase activity, while mutation of E709K had the opposite effect. Inactivating mutations also include H230Y, which is particularly enriched in TNBC, but not in other subtypes [193].

In addition to the propensity of breast tumors to modulate the activity of PTPN12, analysis of primary breast tumor samples revealed that 22.6% of breast cancers exhibit PTPN12 deletion [193], which supported the hypothesis that PTPN12 is a tumor suppressor. Accordingly, Sun et al., 2011 demonstrated that decreased PTPN12 expression can enhance anchorage-independent growth and loss of acinar structure in human mammary epithelial cells [193]. These effects were mediated by the consequent increase in tyrosine phosphorylation of RTKs, including ErBB2, EGFR, and PDGFR. Indeed, assessment of RTKs in human TNBCs as well as PDXs, revealed that PTPN12-regulated RTKs, MET, PDGFRβ, EGFR and HER2 were broadly expressed [194]. To further corroborate these finding, restoring PTPN12 expression alone can downregulate the phosphorylation of each of these endogenous RTKs as well as their downstream effectors in TNBC models [194]. Moreover, PTPN12/MET and PTPN12/PDGFRβ interactions were rapidly enhanced following ligand stimulation [194]. Similarly, PTPN12 has been shown to interact with ErbB2 [3, 193, 195] to influence proliferative and migratory signal outputs of downstream effector proteins, including ShcA [7]. This suggested that PTPN12 is recruited to activated receptors to limit the duration or extent of RTK signaling. Collectively, these mechanistic data suggest that PTPN12 may function as a negative regulator of several proto-oncogenic RTKs in breast cancer.

The tumor suppressive properties of PTPN12 were also independently identified by Li et al., 2015 using ErbB2-driven mammary epithelium-specific PTPN12 deficient transgenic mouse models of breast cancer [196]. Breast tumors arising from mice deficient in PTPN12 had rapid tumor onset,

and increased tumor outgrowth. These mice also had an increased frequency of lung metastases. These tumors were also enhanced in Cas, Pyk2, and paxillin tyrosine phosphorylation. The invasive phenotype of these tumor cells was corrected with the inhibition of Pyk2, a cytoplasmic tyrosine kinase that regulates migration. Furthermore, depletion of PTPN12 decreased the susceptibility of these cells to anoikis. Interestingly, ErbB2+ breast tumors devoid of PTPN12 partially acquired markers of basal-type breast cancer and EMT while losing markers of luminal-type breast cancer. Specifically, PTPN12-deficient tumors and cell lines increased the expression of SMA and CK5, decreased CK8, and increased expression of RNA encoding Zeb1 and Zeb2, N-cadherin, cyclin D2, and matrix metalloprotease 2 (MMP-2) [196]. Collectively, these studies support the role of PTPN12 as a tumor suppressor in breast cancer progression and that it is a key mediator of transforming breast cancer into a more aggressive phenotype. Interestingly, loss of PTPN12 expression is most frequently observed in TNBCs, in contrast to HER2 amplified tumors which rarely exhibit loss of PTPN12. The near-mutual exclusivity of HER2 amplification and PTPN12 loss suggests that there is redundancy between these two events and that they are part of the same pathway [193].

Alternatively, the above studies are contrary to Harris et al., 2014 where PTPN12 expression was reported to be increased in TNBC [197]. Analysis of two breast cancer mRNA datasets established that high levels of PTPN12 mRNA correlate with less favorable prognosis. Harris et al., 2014 reported that PTPN12 protects cells against aberrant ROS accumulation and death induced by oxidative stress. Cells lacking PTPN12 were defective in their ability to activate FOXO1/3a, transcription factors required for the upregulation of several antioxidant genes. This was due to the hyperactivation of PDK1. PDK1 inactivates FOXO1/3a by phosphorylation and compromises the intracellular antioxidant response. Provided the most recent evidence of PTPN12 in breast cancer tumorigenesis, whether re-activation or inhibition of PTPN12 may be of benefit is still under debate and requires further investigation.

1.8 Breast Cancer Therapies and the Adaptive Kinome

1.8.1 Brief Overview

The frequent activation, amplification, overexpression, or point mutation of tyrosine kinases is a major component of tumor heterogeneity and has made disease outcome difficult to predict within

each subtype. As a result, therapies that aim to target downstream signal transduction pathways have become the standard of care or have entered clinical trials. To date, tyrosine kinase inhibitors (TKIs) have been shown to improve patient outcome, however, the response to these therapies are short-lived due to intrinsic or acquired resistance. Indeed, simple exposure of tyrosine kinase-addicted breast cancer cell lines to one or more growth factor ligands for a given RTK is sufficient to reverse sensitivity to TKIs [9, 198]. Moreover, cellular reprogramming of the adaptive kinome in response to anti-ErbB2 therapies demonstrates the redundancy within RTK signaling pathways to overcome the therapeutic response [49].

The luminal subtype, characterized by the expression of ER, is treated with hormonal therapy such as Tamoxifen. Next generation sequencing has provided the mutational spectrum of ER+ breast tumors including genetic alterations in ErbB2, ErbB3, PDGFRA, EphA7, DDR1, MET, and c-KIT. ER+ tumors are also frequently dysregulated in PIK3CA and/or AKT activity, independently of RTKs, either by amplification or mutational activation [30]. Although hormone therapy has been a milestone in the treatment of this subtype, multiple interactions of the ER to growth factor and kinase signaling pathways, including EGFR/HER2 receptor family, IGFR, MAPK/ERK, and PI3K pathways remains a barrier for long term therapeutic responses [28].

TNBCs, lacking the expression of ER, PR, and HER2/ERBB2, often overexpress one or more RTKs or non-RTKs, including MET, EGFR, EphA2 FGFR, NGFR, c-KIT, Lyn, and FAK [9, 136, 199, 200]. Despite having multiple kinases and substrates as potential therapeutic targets, there is no single oncogenic driver in basal-like breast cancers. As such, effective targeted therapies are still very limited, and chemotherapy is considered the standard of care.

Although the luminal and basal subtypes have some degree of variable drug responses, targeted therapeutics in HER2+ breast cancer has been largely successful. Specifically, the monoclonal antibody trastuzumab, and the ATP-competitive EGFR/ErbB2 inhibitor lapatinib, dampen downstream MAPK/ERK and PI3K/AKT pathways [28, 49]. However, the initial response to therapy can be short-lived due to the presence of activating mutations in PIK3CA, upregulation of other RTKs, and dysregulation of signaling intermediates such as non-RTKs and adaptor proteins that circumvent the inhibitory effects of these therapies [201].

1.8.2 HER2/ErbB2 Breast Cancer and Anti-HER2 Therapy

Standard of care ErbB2-targeted therapies, which include Lapatinib, Trastuzumab, Pertuzumab, and Trastuzumab emtansine (T-DM1) have had a dramatic effect on slowing disease progression and improving overall patient survival in women with HER2+ disease. Trastuzumab and Pertuzumab are monoclonal antibodies that bind distinct regions within the ErbB2 extracellular domain and block tumor progression mediated by this receptor. Their mechanism of action includes blocking HER2-dependent signal transduction and increasing antibody-dependent cellular cytotoxicity (ADCC) [202]. Patients with ErbB2/HER2 amplification or overexpression experience substantial improvement with trastuzumab therapy. The pivotal clinical trial published by Slamon et al., 2001 provided the first definitive evidence of the efficacy of anti-HER2 agents. In this study, chemotherapy and trastuzumab was shown to improve time to progression to 7.4 months from 4.6 months. Overall survival (OS) improved to 25.1 months from 20.3 months with the addition of trastuzumab alone, and 1-year survival was 33% with trastuzumab compared with 22% with chemotherapy alone [203]. Interestingly, pertuzumab can increase the responsiveness of HER2+ breast cancers to trastuzumab [204-206]. The addition of pertuzumab to first line treatment with trastuzumab and taxane increased median survival of more than a year for patients with HER2+ metastatic breast cancer [207].

Lapatinib serves as an additional therapeutic strategy in treating HER2+ breast cancers in combination with trastuzumab—combination therapy with trastuzumab and lapatinib has synergistic effects as both intracellular and extracellular HER2 domains are targeted [208, 209]. Indeed, dual blockage was able to improve PFS in women with metastatic HER2+ breast cancer [209, 210], and also improved pathologic complete response (pCR) rate in locally advanced HER2+ breast cancer patients who received neoadjuvant chemotherapy [209, 211, 212]. Unfortunately, acquired resistance to lapatinib often develops in patients who initially responded to therapy by inducing FOXO3a and ER-regulated genes. This has brought on the notion that lapatinib mediated resistance is in part, facilitated by a transition from HER2 to ER dependent cell survival signals [209, 213].

Trastuzumab emtansine (also known as T-DM1) was developed to deliver site directed cytotoxic agents to the tumor site and thereby reduce systemic side effects. T-DM1 is usually a second line

of treatment for patients who have progressed since trastuzumab therapy [209, 214, 215]. T-DM1 significantly improved PFS and OS in women who had previously received trastuzumab and a taxane for the treatment of metastatic disease [214, 216]. Although most patients will develop resistance to this agent, the mechanisms associated with this phenomenon are not well understood. Since the efficacy of T-DM1 is dependent on its accumulation within the cell cytoplasm to evoke cell death [209, 217], studies have speculated that the mechanism of T-DM1 resistance is due to low HER2 expression, poor internalization of the HER2/TDM1complexes, defective intracellular and endosomal trafficking of the HER2/T-DM1 complex, defective lysosomal degradation of T-DM1, or increased expression of drug efflux pumps [209, 218].

1.8.3 Mechanisms of Resistance to Anti-HER2 Therapies

Although ErbB2-targeted therapies are effective in most early stage HER2+ tumors, a subset of patients experience relapse due to the presence of intrinsic or acquired resistance. Moreover, these ErbB2 inhibitors do not produce robust and durable responses in women with metastatic HER2+ disease. The mechanisms underlying resistance to HER2 targeted therapies are pleiotropic and can result from the emergence of treatment-induced resistant sub-clones caused by the activation of compensatory signaling nodes [9]. Studies have shown that ligand stimulation of ErbB3 can confer resistance to trastuzumab-sensitive cells, while the inhibition of ErbB3 activation can arrest proliferation [205, 219, 220]. Activation of IGFIR signaling perpetuates Ras/MAPK and PI3K/AKT pathways which is also seen as a mechanism for trastuzumab resistance. Indeed, cell lines that co-express IGFIR and ErbB2 are less responsive to trastuzumab [221], and inhibition of IGFIR is sufficient to re-sensitize resistant cells to trastuzumab treatment [222]. The MET RTK is also heterogeneously expressed in HER2+ breast tumors [9, 165] and together, can sustain PI3K/AKT signal transduction and protect breast cancer cells from ErbB2 inhibition [9, 165]. Similarly, EphA2 RTK is overexpressed in Trastuzumab resistant primary human breast cancers and relies on Src-dependent EphA2 phosphorylation and the subsequent activation of PI3K/AKT signaling. EphA2 expression levels positively correlated with poor DFS and OS in these patients [9, 223]. Inhibition of EphA2 was sufficient to re-sensitize mammary tumors to Trastuzumab treatment, *in vivo* [9, 223].

Approximately 30% of all HER2+ breast cancers express the p95ErbB2 C-terminal fragment—these tumors are resistant to trastuzumab by virtue of the fact that the antibody epitope resides within the region of the extracellular domain that is lacking in p95ErbB2 [224]. The presence of p95ErbB2 has been associated with reduced 5-year DFS and lower response rates to trastuzumab compared with patients expressing full-length ErbB2 [209, 225]. However, preclinical models have demonstrated that these tumors are sensitive to lapatinib. Lapatinib inhibited p95ErbB2 phosphorylation and reduced downstream AKT and MAPK phosphorylation, resulting in cell growth inhibition [209, 225]. Trastuzumab has also been shown to increase the expression of ErbB4, its proteolytic cleavage, and the nuclear localization of the 4ICD fragment. Nuclear 4ICD is considered an independent prognostic marker of poor responsiveness to trastuzumab monotherapy [9, 226]. Functional studies show that inhibition of ErbB4 expression (via shRNAs), ErbB4 signaling (using pharmacological ErbB4 inhibitors), or ErbB4 cleavage (via gamma-secretase inhibitors) re-sensitizes HER2-amplified tumors to trastuzumab treatment [9, 226].

1.8.4 Src Tyrosine Kinase in Trastuzumab Resistance

The capacity of ErbB2 to perform its potent transforming activity is due to its ability to associate with key downstream oncogenic pathways. Primary human breast cancer specimens illustrate that approximately 70% of tyrosine kinase activity is due to Src in human breast cancer, especially for those tumors that are expressing activated ErbB2 [227]. This increase in Src activity is due to the ability of ErbB2 to form stable complexes with Src in a SH2 domain dependent manner [123, 227-231]. Complex formation is restricted to the ErbB2 receptor kinase domain (an interaction centered around Y877) [123, 228, 229]. Breast carcinoma cells that were devoid only of ErbB2, and no other ErbB family members were defective in cell invasion, disrupted in cell polarity and cell-cell junctions in a MAPK-dependent manner [232, 233]. Indeed, Src enhances E-cadherin and integrin signaling which in turn, activates various other signaling networks [234].

Given the immense role of Src in mammary tumorigenesis, studies have established Src as a key signaling node for trastuzumab resistance in HER2+ breast cancers. A seminal investigation by Zhang et al., 2011 demonstrated that breast cancers become resistant to trastuzumab through the overexpression of other RTKs including IGF1R, EGFR or through the loss of PTEN, and increasing the activation of Src [235]. Conversely, pharmacological inhibition of Src activity or

Src expression levels through RNAi mediated approaches reduced the activation of numerous tumorigenic signaling pathways, including EGFR, ErbB3, and AKT, in HER2-amplified breast cancer cells and re-sensitized mammary tumors to trastuzumab therapy, *in vivo* [235]. Mechanistically, this was due to the ability of PTEN to interact with Src and dephosphorylate it at Y416, the activating phosphorylation site that increases the kinase activity of Src. Thus, in the context of Trastuzumab resistant cells that have lost PTEN, leaves Src uninhibited to execute a feed forward signal cascade that promotes the activation of EGFR, ErbB2, and ErbB3, which in turn, activates Src [235, 236]. In addition, CUB domain-containing protein 1 (CDCP1), a large transmembrane glycoprotein with five tyrosine phosphorylation sites in the cytoplasmic tail, is frequently co-expressed with HER2 and interacts with activated ErbB2/ErbB3 heterodimers to facilitate ErbB2/Src binding and enhance both ErbB2 and Src activity to confer trastuzumab resistance in HER2-amplified breast cancers [237]. Furthermore, transforming growth factor (TGF)-β binds to HER2 and integrins, leading to Src activation [238]. Specifically, β1 integrin increases resistance to lapatinib and/or trastuzumab through the upregulation of FAK and Src kinases. Moreover, β1 integrin is associated with poor OS in patients with early-stage breast cancer [239]. As such, β1 integrin is now a predictive indicator for patients with intrinsic resistance to trastuzumab [240]. Despite these data, phase II clinical trial of Dasatinib (an SFK inhibitor) in breast cancer patients with advanced HER2+breast cancer showed little survival benefit [241]. This again highlights the genetic complexity that is characteristic of HER2+ disease and supports the utilization of combination therapies with SFK inhibitors and HER2-neutralizing therapies and/or PI3K/AKT inhibitors.

1.8.5 PI3K/AKT Pathway in Trastuzumab Resistance

Hyperactivation of the PI3K/AKT/mTOR signaling pathway through mutational activation of PIK3CA or the loss of PTEN is a mechanism by which HER2+ breast cancers can acquire resistance to anti-HER2 therapies. The loss of PTEN is observed in 20%–25% of HER2+ breast cancers [242-245], while activating mutations of PIK3CA occurs in approximately 25% of primary breast cancers that are positive for PTEN expression [242, 245]. The assessment of both PIK3CA and PTEN status in primary tumor samples is considered a predictive biomarker for disease progression after trastuzumab therapy in HER2 amplified breast tumors. Generally, multiple studies have demonstrated a clear association of PIK3CA mutation and lower pCR, irrespective of

treatment [209, 246, 247]. Mutations in the catalytic (E454K) and kinase domains (H1047R) of the p110α subunit of PI3K increases resistance to both trastuzumab and lapatinib [246, 248, 249]. Consistent with this data, HER2+/PIK3CA^{H1047R} transgenic mouse models are resistant to trastuzumab/lapatinib and trastuzumab/pertuzumab combination therapy [250]. The addition of a PI3K inhibitor to trastuzumab/lapatinib or trastuzumab/pertuzumab therapy reverses the resistance to the anti-HER2 combinations and results in tumor regression [250-252].

Despite the potential of PI3K inhibitors as an addition to standard of care combination therapy, the ability of breast tumors to re-engage and activate the ErbB family of receptors, particularly ErbB3 poses a barrier to anti-HER2 therapy. The upregulation of ErbB3 expression has been observed in tumors treated with anti-HER2 therapy [46]. Increased ErbB3 expression is an attempt to reinforce ErbB2/ErbB3 heterodimers and re-engage the PI3K signaling pathway. While ErbB2 predominantly induces the Ras and AKT signaling pathways, ErbB3 primarily recruits the regulator p85 subunit of PI3K to amplify mitogenic signal transduction [46, 162, 253, 254]; the propensity for ErbB3 to do so is owed to the presence of six binding sites for the p85 regulator subunit of PI3K in its cytoplasmic tail [162]. In addition, resistance to anti-HER2 therapy can also be mediated by neuregulin b1 (NRG), the ligand of ErbB3 [209]. Specifically, NRG can trigger the formation of ErbB2/ErbB3 heterodimers and activate downstream PI3K/AKT signaling [219]. Indeed, reducing ErbB3 expression is sufficient to sensitize ErbB2+ luminal breast tumors to pharmacological PI3K inhibitors [160] and to overcome resistance to trastuzumab [255].

Inhibition of downstream effectors including, mTOR is also a therapeutic strategy that has the potential to circumvent trastuzumab resistance. Effectively, a dual mTORC1/mTORC2 inhibitor (INK-128) was shown to increase the anti-tumor activity of HER2-targeted therapies in Trastuzumab-resistant breast cancer cells and in a PDX from a patient that relapsed following Trastuzumab treatment [256]. Consistently, clinical trials that examined the addition of everolimus (an mTORC1-specific inhibitor) prolonged PFS from 5.78 months to 7 months in hormone receptor negative, HER2+ breast cancer patients [257]. Mechanistically, trastuzumab decreases the formation of the eIF4F complex by increasing the formation of eIF4E/4E-BP complexes, which sequesters eIF4E away from the 5'mRNA cap [9, 258]. Moreover, eIF4E overexpression is sufficient to increase mRNA translation and increase trastuzumab resistance in breast cancer cells, both *in vitro* and *in vivo* [9, 258].

1.9 Mouse Models of ErbB2 Driven Breast Cancer

Over the past thirty years, transgenic mouse models have become useful tools to study the tissue specific effects of oncogene induced transformation by growth factor receptors and downstream intracellular signaling pathways. To assess the genetic requirements and the direct role of activated oncogenes in the mammary epithelium, transgenic mouse models were developed by fusing the mouse mammary tumour virus-long terminal repeat (MMTV-LTR) promoter to one of several oncogenes and introducing these constructs to the genomes of mice [259-262]. The MMTV is a retrovirus that is associated with the development of mammary carcinomas in infected mice [263]. MMTV-LTR drives the expression of transgenes in alveolar luminal epithelial cells during all stages of mammary gland differentiation [264, 265]. The MMTV-LTR is progesterone and dihydrotestosterone responsive but is not induced by estrogen [266]. Early studies using the MMTV/c-myc and MMTV/v-Ha-ras transgenic models demonstrated that these mice eventually develop mammary adenocarcinomas. However, MMTV/c-myc and MMTV/v-Ha-ras expression alone or in combination was not sufficient to efficiently transform mammary epithelial cells, resulting in tumors that were stochastic in nature. This suggested that there was a step-wise progression of events required for full malignant transformation of the mammary epithelium [262].

Given the direct clinical correlates of the overexpression and/or amplification of ErbB2 in breast cancer [86, 262, 267-269], the need for a model to assess the step-wise progression of mammary tumorigenesis was required. The NEU and MMTV Polyomavirus Middle T Antigen (PyV-MT) transgenic mouse models provided the first direct evidence of the potent effects of RTKs in mammary tumorigenesis [262].

1.9.1 Neu/ErbB2

The Neu oncogene was initially isolated from a chemically induced rat neuroblastoma [270], which encoded 185 kDa protein with close homology to EGFR and other members of the tyrosine kinase class of proto-oncogenes [271]. A single amino acid substitution in the transmembrane domain (V664E, Neu-NT) of the Neu receptor results in its constitutive activation [272]. Expression of Neu-NT under the transcriptional control of the MMTV promoter in the mouse mammary gland results in the early induction of hyperplastic and dysplastic nodules and eventual

formation of adenocarcinoma (5-7 months) [262]. This model demonstrated that activated Neu was sufficient for mammary gland transformation within a single step [262].

While the constitutive activation of the Neu receptor was sufficient to induce mammary carcinogenesis in this model, this did not represent human breast tumors which overexpressed or amplified wildtype ErbB2. Moreover, clinical specimens revealed no apparent activating mutations in ErbB2 that rendered it constitutively active [273, 274]. This led to the development of the MMTV/NEU proto-oncogene (unactivated) transgenic mouse model [275]. In contrast to those tumors expressing activated Neu, expression of 'unactivated' Neu was not sufficient for mammary tumorigenesis. However, after a variable yet long latency period (7 months), animals developed focal mammary tumors and metastatic lung disease. Interestingly, these tumors displayed high levels of intrinsic Neu tyrosine kinase activity and *de novo* tyrosine phosphorylation of cellular proteins [275]. Further characterization of mammary glands revealed that at least 65% of the mammary tumors arising from these strains carried somatic mutations in the transgene [274], specifically in-frame deletions of 7 to 12 amino acids in the extracellular region [274] and/or mutations affecting highly conserved cysteine residues, causing constitutive formation of disulfide bond-mediated receptor dimers [276]. To supplement these findings, the MMTV/Neu-NDL transgenic mouse model which included these in-frame deletions (NDL1 and NDL2) within the Neu transgene, demonstrated accelerated mammary tumor initiation alongside the presence of point mutations that targeted cysteine residues [56]. Indeed, in human breast cancer, ErbB2 undergoes alternative splicing to generate the Δ16ErbB2 spliced isoform capable of ligand independent homodimerization [9, 56, 93]. Moreover, Δ16ErbB2 expression is sufficient to induce primary tumor growth and lung metastases, and, aggressive multi-focal mammary tumors in xenograft [99] and transgenic [277] mouse models, respectively. Mammary tumors arising from both these tumor models were molecularly distinct from wild-type ErbB2 expressing tumors; specifically, these tumors expressed both basal and luminal cytokeratins, while wild-type ErbB2 expressing mammary tumors were positive for luminal cytokeratins [278]. Altogether, these studies demonstrate that mutational activation of the Neu proto-oncogene was crucial for mammary tumor induction in transgenic mice [279].

A limitation of the activated Neu transgenic mouse models is that the expression of Neu is under the control of a strong viral promoter. Moreover, due to the random integration of multiple copies

of the transgene within the genome (causing variable transgene expression between mouse models), data interpretation was considered confounded and also, brought into question the physiological relevance of these models in human breast cancer [279]. To amend these variables, a transgenic mouse model that places Neu-NT under the control of the endogenous mouse ErbB2 promoter was developed[8] [279, 280]. Expression of Neu-NT under these conditions was not sufficient for rapid mammary tumor formation—these animals formed focal tumors after a long latency period. Strikingly, 85% of the mammary tumors had amplified copies (2–22 copies) of the activated Neu allele relative to the wild-type allele and expressed elevated levels of Neu transcript and protein [280]. Thus, this model demonstrated that, like human HER2+ breast tumors, mammary tumorigenesis required the amplification and elevated expression of the Neu oncogene.

1.9.1.1 Elucidation of Phospho-tyrosine Networks using ErbB2 Breast Cancer Models

Transgenic mouse models have also been instrumental in elucidating the downstream signaling pathways involved in mammary tumor initiation and progression, including the activation of principle effectors of ErbB2-dependent mammary tumorigenesis, ShcA and Grb2.

To systematically address the role of tyrosine phosphorylation in Neu-mediated transformation in breast tumorigenesis, a Neu/ErbB2 mutant, NT-NYPD[9], that harbor mutations on specific tyrosine autophosphorylation sites (sites A through E) within the regulatory region demonstrated the importance of phospho-tyrosine signaling networks in mammary tumor induction [1, 253, 281]. Transgenic animals expressing NT-NYPD failed to activate Ras/ERK and PI3K/AKT signaling pathways and inefficiently transformed the mammary epithelium. This suggested that breast tumor initiation was dependent on the engagement of Ras and PI3K [1, 281]. Previous studies have also shown that MMTV/Neu-NT and MMTV/Neu expressing mammary tumors have elevated Src tyrosine kinase activity [227, 229]. To corroborate these findings, NYPD mutants retained the capacity to couple to SFKs [1], suggesting that the activation of SFKs was associated with mammary tumorigenesis downstream of RTKs [227-229].

[8] The Neu transgene is silenced by an upstream loxP flanked neomycin cassette and mammary-specific expression of NEU-NT is achieved by crossing these animals with MMTV–Cre transgenic mice (MMTV–CreFlneo–NEU-NT mice). Reference 279 and 280.
[9] Tyr to Phe mutation at position YA-1028 (site A), YB-1144 (site B), YC-1201 (site C), YD-1226/1227 (site D), YE-1253 (site E). Reference 1, 253, and 281.

To dissect the functional significance of these phospho-tyrosine residues, a series of restoration mutants were generated for each autophosphorylation site (site A-E) of the Neu receptor. Site B-E independently transduced transforming signals, whereas site A repressed transforming signals from the receptor [253]. Further characterization of the sequence motifs surrounding these sites confirmed YXNX and NLYY motifs[10] that corresponded to Grb2 and ShcA binding, respectively. Grb2 associated directly with Y1144 (site B; YB) and indirectly through tyrosine-phosphorylated Shc proteins at Y1226/1227 (site D; YD) [253]. The expression of either YB or YD alone was capable of transformation, however, only YB was able to develop efficient metastatic disease [1]. YD and YB strains coupled to both the ERK and AKT signaling pathway and upregulated ErbB3 [1].

1.9.2 Polyomavirus Middle T Antigen (PyV-MT)

Another potent oncogene that serves to parallel the initiation and progression of mammary tumorigenesis is the Polyomavirus (PyV) Middle T antigen (MT) (PyV-MT) [154]. PyV-MT is a 421-residue polypeptide with a molecular mass of 55 kDa [282]. PyV-MT requires a 22-residue stretch of hydrophobic amino acids located near the C-terminus to associate to the cell membrane[11] [283] (Figure 9). PyV-MT has no enzymatic activity and asserts its transforming ability by mimicking constitutively active RTKs. PyV-MT associates with proto-oncogenes to modulate activities of cellular proteins involved in proliferation [284], including SFKs (Src, Yes, and Fyn) [53, 86, 154, 261, 285, 286], p85 subunit of PI3K [287, 288] , protein phosphatase 2A (PP2A) [289-291] , YAP [292, 293], ShcA [156, 284], PLCγ1 [293, 294], and 14-3-3 adaptor proteins [158, 295]. PyV-MT engages SFKs directly (185-210aa of PyV-MT) or indirectly through its interaction with PP2A. PP2A association is dependent on an approximately 190 amino acid stretch found at the N-terminus of PyV-MT, which includes cysteine (CXCXXC) motifs [296]. PP2A serves as a scaffold to recruit SFKs to the cellular membrane and phosphorylate PyV-MT at three specific tyrosine residues: Y-315, -250, -322 [297-299]. These sites enable the recruitment of

[10] Replacing conserved asparagine residues within Grb2 (Asn 1144) or ShcA (Tyr 1226/1227) binding motifs on Neu impairs the transforming abilities of these add back mutants.
[11] This region is functionally essential for PyV-MT to localize to the cell membrane. Deletion of this region abrogates its ability to transform as well as bind to several signaling molecules.

PI3K[12], ShcA[13], and PLCγ1[14], respectively. The PyV-MT-associated PI3K, PLCγ1 and ShcA are in turn, tyrosine phosphorylated by Src, resulting in stimulation of PI3K and PLCγ-1 activity and the subsequent interaction between ShcA and Grb2 [156, 284]. This results in the permanent activation of ERK1/2, AKT [300], and AP1 family of transcription factors [301, 302], and increased c-myc transcription [303]. Transgenic models directing PyV-MT to the mammary epithelium is sufficient for mammary tumor initiation [154, 273] and the development of highly fibrotic multifocal mammary adenocarcinoma and secondary metastatic lung tumors [154].

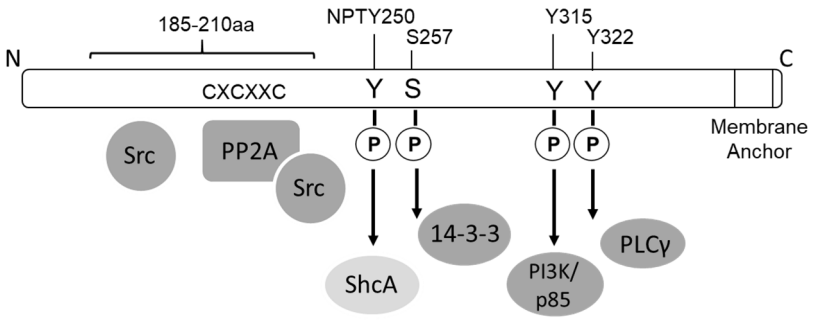


Figure 9: Schematic representation of the structure of PyV-MT and its interactors. PyV-MT is a 421-amino acid polypeptide with no catalytic activity. PyV-MT contains a C-terminal hydrophobic region, necessary for membrane association. PyV-MT associates with various proto-oncogenes to modulate activities of cellular proteins involved in cellular proliferation including, PI3K, Src, PP2A, 14-3-3 and ShcA adaptor proteins, p85 regulatory subunit of PI3K, and PLCγ.

1.9.2.1 Elucidation of Phospho-tyrosine Networks using PyV-MT Mouse Models

To assess the biological effects of PyV-MT driven mammary tumorigenesis in the context of ShcA and PI3K dependent signaling, transgenic models that have decoupled these proteins from PyV-MT have been developed [158]. The mammary epithelium of these mice express a mutant lacking either the ShcA binding site (MT-Y250F) or the PI3K binding sites (MT-Y315/322F) under the transcriptional control of the MMTV promoter-enhancer. Both MT-Y250F and MT-Y315/322F strains showed delayed mammary tumor onset and epithelial hyperplasia [158]. Hyperplastic lesions arising from MT-Y250F and MT-Y315/322F mammary tissue revealed sustained Src

[12] SH2 domain of the p85 regulator subunit prefers the pYMXM sequence consistent with the pY315MPM of PyV-MT. However, tyrosine residues 322 has also been implicated. Reference 158 and 284.
[13] ShcA binds to PyV-MT in an NPTY-dependent manner. Cell transformation by PyV-MT requires its association with p52ShcA, but not p46ShcA, via the PTB domain. Reference 156.
[14] PLCγ1 binds to a YDLI motif at Y322. Reference 294.

activation, suggesting that the delayed tumor onset was not due to the inability of PyV-MT to complex with or activate Src [158]. Consistent with these observations, Src activity is required for the rapid induction of MMTV/MT mammary tumors and metastasis [304]. Germline deletion of Src inhibits mammary ductal outgrowth [305] and rarely results in the induction of tumors [306], but does give rise to mammary epithelial hyperplasias [118]. It has also been observed that alternative SFKs do compensate to initiate mammary tumorigenesis in the absence of Src. Studies have demonstrated that the deletion of Yes in MMTV/ PyV-MT transgenic mice results in mammary tumors that are histologically indistinguishable from MMTV/PyV-MT control tumors. Fyn, on the other hand, did not display increased tyrosine kinase activity in fibroblasts or in PyV-MT induced mammary tumors [158]. In addition, MT-Y315/322F tumors exhibit elevated rates of apoptosis, establishing the PyV-MT/PI3K interaction as a necessary signal for cell survival. Interestingly, in 7% of the tumors arising from MT-Y250F strains (uncoupled from ShcA), the mutant PyV-MT re-acquired the capacity to bind ShcA through somatic mutations within the transgene [158]. This reversion also contributed to higher frequencies of lung metastases. This suggested that there is a great deal of selective pressure to sustain ShcA dependent signal transductions during PyV-MT driven mammary tumorigenesis and metastatic progression.

Given the propensity of MMTV/PyV-MT tumors to re-acquire ShcA signaling for mammary tumorigenesis, these observations suggested a requisite role for adaptor proteins for breast tumor induction. To explore the role of ShcA in PyV-MT driven mammary tumorigenesis, mice co-expressing ShcA or Grb2 and mutant MT-Y250F in the mammary epithelium were generated. Co-expression of MT-Y250F with either ShcA (MMTV/ShcA/MT-Y250F) or Grb2 (MMTV/Grb2/MT-Y250F) resulted in a dramatic acceleration of mammary tumorigenesis compared to parental mutant PyV-MT controls [307]. In contrast to the MMTV/MT-Y250F strain, which developed mammary tumors with an average latency of 111 days, the MMTV/ShcA/MT-Y250F and MMTV/Grb2/MT-Y250F mice developed mammary tumors with average latencies of 94 and 80 days, respectively [307]. The observed accelerated tumor phenotype was in part, due to the increased expression levels of ShcA or Grb2 and the compounded effects of ErbB3 and ErbB2 activation [162, 254]. Incidentally, ErbB3 is also overexpressed in both invasive and non-invasive HER2+ human breast tumors [308]. Therefore, these observations suggest that elevated levels of the Grb2 or ShcA adapter protein alone can accelerate mammary tumor progression and sensitize the mammary epithelial cell to growth factor receptor signaling [307].

PyV-MT does not contain a direct Grb2 binding site. As such, the ShcA interacting motif of PyV-MT is the only known sequence to recruit Grb2 into the PyV-MT complex [156, 284]. Given that Grb2 and ShcA were equally able to initiate tumorigenesis, a study by Nicholson et al., 1994, investigated whether Grb2 bound directly to an activated receptor was equivalent to Grb2 associated via ShcA [309]. Inventively, the ShcA associated sequence of PyV-MT was replaced by either a Grb2-binding motif from the mouse EGFR or the Y313 region of ShcA and cellular transformation was assessed *in vitro*. Both these sequences were able to re-instate Grb2 binding to approximately wild-type PyV-MT levels, but neither mutated PyV-MT could fully restore transforming capacity, alone or in combination. However, replacing mutant PyV-MT with the ShcA Y239/240 region was more effective than Y313 in cellular transformation. This study identified that ShcA Y239/240 and Grb2 interactions as an essential signaling event downstream of PyV-MT and further reinforced that ShcA, and likely no other PTB domain-containing proteins, was responsible for this phenotype [309].

1.10 Overview: Signaling Domains in Phospho-tyrosine Signaling

The tyrosine phosphorylation of RTKs enables the recruitment and activation of cellular proteins. This is mediated through the engagement of Src Homology 2 (SH2) and Phospho-Tyrosine Binding (PTB) domain containing effector proteins that recognize distinct and specific motifs surrounding the phospho-tyrosine residue. Upon their engagement with an activated RTK, additional effector proteins with SH2, SH3 (Src homology 3), PTB and PH (Pleckstrin Homology) domains are recruited to the activated receptor and transduce intracellular biochemical signals, including canonical Ras/MAPK and PI3K/AKT pathways which results in cell cycle progression, proliferation and survival.

SH2 and PTB domains are present in a diverse set of proteins and usually in combination with other catalytic and non-catalytic domains. Since the discovery of these domains, over 120 SH2 containing and 60 PTB containing proteins have been identified [310, 311].

1.10.1 SH2 Domain

The SH2 domain was first described by Sadowski et al., 1986, where an approximately 100 amino acid sequence in v-Fps/Fes oncoprotein was discovered to be necessary for cellular transformation

[312]. Based on its homology, the SH2 domain was name after the corresponding region in Src family and Abl cytoplasmic tyrosine kinases [313]. Generally, the affinity of an SH2 domain will depend on the amino acid sequence carboxyl to the phospho-tyrosine residue [314]. Within the positively charged binding pocket, a critical Arginine (Arg) residue (within a very highly conserved FLVR motif) [310, 315, 316] and amino acids +1 to +6 C-terminal to phospho-tyrosine residue [317-319] ensures specificity. Such binding specificity dictates whether a signaling effector is recruited to a given RTK or scaffold protein. This, in turn, determines which pathway(s) is activated downstream of specific phospho-tyrosine containing proteins. For example, the SH2 domain of Grb2 preferentially binds pYXNX motifs (where X represents any amino acid) present among its interacting partners including the ShcA proteins. Grb2's preference for Asparagine (Asn) at the +2 position is mediated by a Tryptophan (W) residue, W121, located in the SH2 domain. Indeed, mutation of this position (W121T) displays weak binding to pYXNX motifs [320]. A Leucine (L) or Proline (P) residue at position +3 (xx-pY-xx[L/P]x) is strongly preferred by Crk SH2 domains. SH2 domain of SFKs prefer a pYEEI motif whereas the SH2 domains from PI3K or PLCγ prefer pYϕXϕ (where ϕ represents a hydrophobic side chain) [311, 317][15].

1.10.2 PTB Domain

PTB domains are found in scaffold proteins that often contain additional modular domains and motifs for multiprotein complex formation [321].The PTB domain was independently discovered in ShcA [322, 323] and insulin receptor substrate 1 (IRS-1) [324] adaptor proteins. Although functionally similar to SH2 domains, a notable feature of the PTB domain is that it binds amino acids that are amino-terminal to the phospho-tyrosine residue [314]. Since the discovery of PTB domains, structural comparisons have grouped PTB domains into 3 broad families: IRS-1/Dok-like, ShcA-like, and Dab-like [325]. The NPXY motif is common among PTB domain substrates and is considered the canonical binding motif for PTB domain containing proteins. The presence of an Asn at position -3, and Pro at position -2 (relative to tyrosine at position 0) within an NPXY motif is necessary for specificity and high affinity binding [326].

[15] For a complete list of SH2 domain containing proteins and SH2 domain sequence specificities see Reference 311.

PTB domains vary in their dependence on the phosphorylation of the NPXY tyrosine to engage its interactors. The majority of PTB domains, especially those of the Dab and Numb like family, bind irrespective of ligand phosphorylation or preferentially recognize unphosphorylated ligands [321]. Members of the ShcA and IRS-1/Dok families bind with higher affinity to phosphorylated motifs and therefore, are considered as the primary scaffolds represented in both normal and oncogenic RTK signaling. However, even canonical ShcA/IRS-1/Dok like PTB domains have exceptions and can bind proteins independent of tyrosine phosphorylation. For example, binding of ShcA to the carboxyl terminal of PTPN12 depends on the ^{599}NPLH^{602} sequence motif that closely resembles the ShcA canonical binding consensus motif NPXY [3]. The histidine residue at position 0 serves as a surrogate to tyrosine and is thought to be post-translationally modified for high affinity binding to ShcA proteins [3]. Additional non-canonical interactors of ShcA include cytoskeletal regulator IQGAP1 (IQ motif-containing GTPase-activating protein 1) [327], PP2A [328], and PTPε [5]. Interestingly, the ShcA PTB domain is also very similar to the plekstrin homology (PH) domain [329]. The ShcA PTB domain is capable of recognizing phospholipids (although at a lower affinity), in a stereospecific manner [330]. Thus, the ShcA PTB domain has dual functions capable of membrane localization and receptor interaction through a single domain.

1.11 ShcA Adaptor Protein

The physical and functional interplay of SH2 and PTB domain containing proteins are considered molecular switches that control the temporal and spatial activation of RTKs and provide a degree of specificity for downstream signal transduction. Receptor and cytoplasmic tyrosine kinases must recruit adaptor proteins to propagate oncogenic signals. These adaptor proteins are a requisite step of RTK signaling and are universally engaged across all breast cancer subtypes. Although adaptor proteins themselves lack intrinsic catalytic activity, they contain numerous phospho-tyrosine binding domains and motifs (including SH2 and PTB domains) to assemble large macromolecular complexes and integrate signals based on binding specificity, subcellular localization, and proximity to its binding partners. Such signal integration is essential to support a multitude of biological processes including proliferation, survival, migration, invasion, and tumor/stromal crosstalk.

ShcA is classically identified as a prototypical adaptor protein, linking upstream RTK activation to several downstream signal transduction pathways. The ability of ShcA to form multiple phospho-tyrosine dependent complexes through its various domains and motifs has deemed it an essential molecule that regulates numerous cellular processes including migration, invasion, angiogenesis, and, cell proliferation and survival. The mammalian ShcA gene (located on chromosome 1q21.3) contains two tandem promoters that encodes three proteins, p46ShcA, p52ShcA, and p66ShcA. p46ShcA, and p52ShcA are produced through alternative translation initiation, resulting in an additional 45 amino acids extension on the amino terminus of p52ShcA. p66ShcA is generated from a separate transcript through different promoter usage and is the longest isoform [9]. Concurrent disruption of p66ShcA, p52ShcA and p46ShcA results in embryonic lethality by E11.5 (embryonic day 11.5) in mice [331]. However, animals with the specific deletion of p66ShcA experience a 30% increase in lifespan [332]. This is due to the involvement of p66ShcA in cellular oxidative stress, rendering it as a gene implicated in the aging process [332].

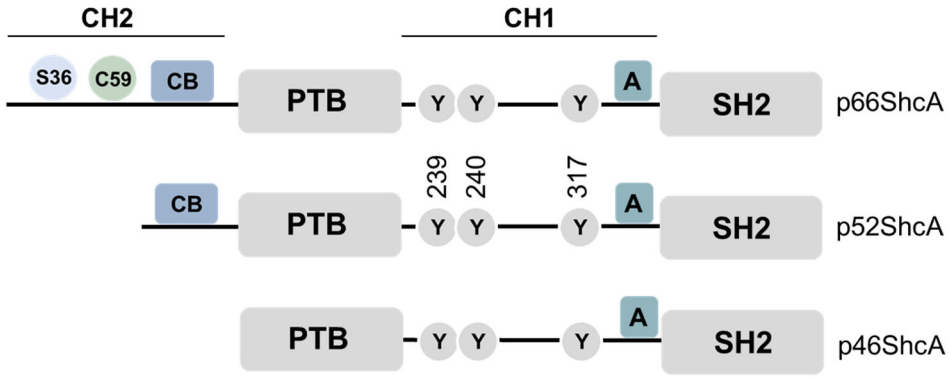


Figure 10: Schematic representation of the ShcA isoforms, p66ShcA, p52ShcA, p46ShcA. ShcA isoforms share a common modular structure, including the PTB and SH2 domains, and CH1 region which houses ShcA tyrosine residues 239, 240, and 317 (313 in mice). The CH2 region is unique to p66ShcA and contains key residues for oxidative stress response (Serine 36), oligomerization (Cysteine 59), and cytochrome C binding (CB). The CB region is also present in p52ShcA but absent in p46ShcA. The adaptin binding motif (A) is conserved in all isoforms.

Structurally, ShcA contains two phospho-tyrosine binding motifs including an amino-terminal PTB domain and a carboxy-terminal SH2 domain. The central collagen homology 1 (CH1) domain houses three tyrosine phosphorylation sites at residues 239/240 and 317 (tyrosine 313 in mice). All three isoforms are identical throughout these regions. Unique to p66ShcA, is the N-terminal CH2 domain, which is essential for the oxidative stress response. Specifically, the CH2 domain houses a critical Serine 36 residue which undergoes phosphorylation by stress kinases [332] and localization to the mitochondria [333-335], a Cysteine 59 residue that mediates the tetramerization of p66ShcA [336] , and a cytochrome C binding (CB) region that is important to generate ROS [333]. p52ShcA also contains a CB region, while p46ShcA is devoid of this site [333, 337] (Figure 10).

p46ShcA and p52ShcA are ubiquitously expressed within the cell and are the primary isoforms identified for transducing pro-mitogenic and anchorage-dependent growth signaling downstream of RTKs [338]. p66ShcA has differential expression patterns in select cell types and can be epigenetically repressed through cytosine methylation and histone deacetylation [339]. Contrary to p46/p52ShcA, p66ShcA inhibits receptor tyrosine kinase signaling, causes programed cell death, promotes differentiation [333, 340, 341], and permits anoikis [342]. From this point forward, the focus of this discussion will be on p46/p52ShcA isoforms and collectively identified as ShcA.

1.11.1 ShcA Driven Signaling Responses

Upon growth factor stimulation, ShcA is recruited to activated RTKs through its PTB or SH2 domain, leading to its phosphorylation at tyrosine residues 239, 240, and 317 within the CH1 domain [326, 343-345]. ShcA phospho-tyrosines become docking sites for other SH3 and SH2 containing proteins. This includes Grb2/Sos complexes, which results in the activation of Ras-MAPK pathway [338, 343] or Grb2/Gab1 interactions that activate the PI3K/AKT pathway [346]. The ShcA CH1 region is also enriched in glycine and proline residues [338] which form Pro-Xaa-Xaa-Pro motifs that bind the SH3 domains of cellular proteins (Figure 11).

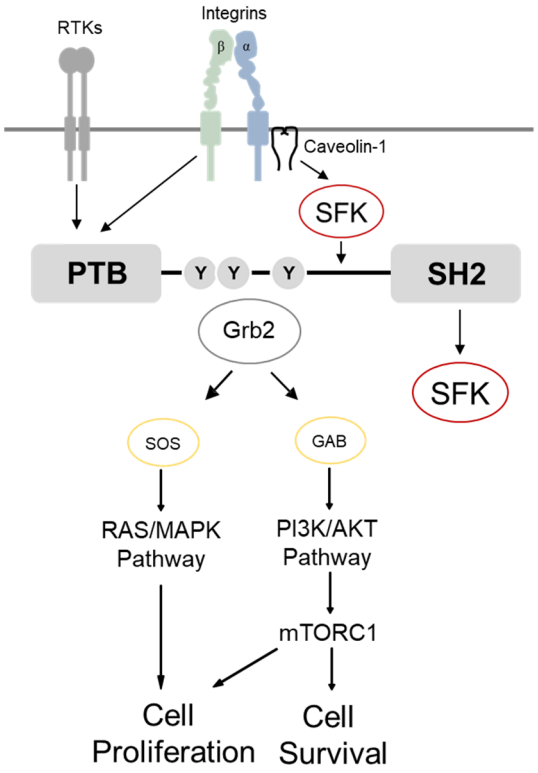


Figure 11: Schematic representation of ShcA dependent signaling. Upon growth factor stimulation, ShcA is recruited to activated RTKs through its PTB or SH2 domain, leading to its phosphorylation at tyrosine residues 239, 240, and 317 within the CH1 domain. ShcA phospho-tyrosines become docking sites for other SH3 and SH2 containing proteins. This includes Grb2/Sos complexes which results in the activation of Ras-MAPK pathway or Grb2/Gab1 interactions that activate the PI3K/AKT pathway. The ShcA CH1 region is enriched in glycine and proline residues which enable the interaction of SH3 containing proteins. The SH2 domain of ShcA can also engage non-RTKs, including the SFK family members.

1.11.1.1 ShcA SH2 Domain

Since ShcA possessed no catalytic activity, it was set apart from other SH2 containing proteins including catalytically active Src, PLCγ1, and PI3K. The ShcA SH2 domain was first isolated from a human cDNA screen and was found to share high amino acid sequence homology to Src (60%), Crk (57%), and c-fes (63.3%) [338]. Early studies identified ShcA as a direct interactor of activated RTKs (EGFR [338], PDGFRβ [347] , FGFR1 [348] and FGFR2 [349]) through its SH2 domain, resulting in the phosphorylation of ShcA and consequent transformation of fibroblasts. Specifically, Y992 of EGFR was found to be a minor ShcA SH2-domain-binding site, whereas Y1148 was a secondary motif that facilitated interaction with both the PTB and SH2 domains of

ShcA [350-352]. Several tyrosine binding sites on PDGFRβ, Y579, Y740, Y751, Y771, bound the ShcA SH2 domain, however with variable and low affinity [347]. Additionally, Y766 of FGFR, to some extent, facilitated the interaction of the ShcA SH2 domain *in vitro* [348].

Evidence from these early studies also determined that ShcA was highly tyrosine phosphorylated in v-Src [353], v-fps [353] , PDGFR [347], Neu [354], and InsR [355] transformed cells, which then led to the investigation of potential cytoplasmic tyrosine kinases that may facilitate ShcA phosphorylation. SFKs, Src [349] and Lyn [356] were able to engage the ShcA SH2 domain. In both cases, ShcA/Src and ShcA/Lyn interactions increased the phosphorylation of ShcA as well as SFK tyrosine kinase activity [8, 356]. Interestingly, Src and Lyn interactions were dependent on the availability of phosphatidylinositol 4,5-bisphosphate (PI(4,5)P2) and PI3K, respectively [356, 357]. The presence of PI(4,5)P2 stimulated the phosphorylation ShcA by Src, while immunoprecipitates of ShcA/Lyn contained activated PI3K and correlated with Lyn and ShcA tyrosine phosphorylation. The presence of PI3K in this multimeric complex may be explained by the ability of the ShcA SH2 domain to recruit 14-3-3 adaptor proteins and indirectly engage the p85 regulator subunit of PI3K [358].

1.11.1.2 ShcA PTB Domain

The ShcA PTB domain is primarily identified as an initiator of phospho-tyrosine signaling downstream of RTKs and non-RTKs. These interactions include RTKs of the ErbB and Trk families, FGFR, IGFR, InsR, RET, VEGFR, and a number of other signaling and adhesion proteins (amyloid precursor protein, interleukin receptors, integrins, and low-density lipoprotein receptors) [321]. The ShcA PTB domain recognizes the tyrosine phosphorylated residue surrounded by the motif Asn-Pro-X-pTyr (NPXY) with isoleucine or a similar hydrophobic amino acid (ψX) at position -5 relative to the phospho-tyrosine at position 0 [321]. Ligand recognition by the ShcA PTB domain also depends on a conserved Arginine 175 at the C-terminal of the ShcA PTB domain [326]. Unlike SH2 domains, PTB domains share low sequence homology amongst themselves and exhibit extremely high ligand binding selectivity. For example, the PTB domain of IRS-1 does not bind NPXpY peptides derived from known ShcA binding sites in Trk receptors and EGFR [359-361].

A subset of integrin receptors are capable of transducing signals through the ShcA adaptor protein, including laminin (α6β4) [362, 363], collagen (α2β1) [364], collagen/laminin (α1β1), fibronectin (α5β1) [365], and vitronectin (αvβ3) [337, 364, 365]. Integrins are transmembrane receptors that bind to extracellular matrix ligands and facilitate interactions between the cytoskeleton and cytoplasmic signaling molecules. This allows for the transmission of events occurring at the plasma membrane to effect gene expression. Integrins are a family of 24 transmembrane heterodimers generated from a combination of 18 α-integrin and 8 β-integrin subunits [366]. Heterodimerization of beta and alpha subunits results in the activation of FAK, which undergoes autophosphorylation, to then engage Src or Fyn. The FAK-SFK complex regulates focal adhesion assembly/disassembly and the inhibition of apoptosis [367].

The direct engagement of ShcA to tyrosine phosphorylated β4 is through both the ShcA SH2 and PTB domain. On the other hand, recruitment of ShcA to β1 and α_v integrin is indirect and mediated through the alpha subunit within caveolin-1. Caveolin-1 links integrins to Fyn tyrosine kinase (which may be substituted with Yes, Lck, or Lyn) and facilitates the recruitment of ShcA through its SH3 domain [368]. ShcA is then phosphorylated to transduces ERK dependent signaling and promote cell cycle progression. Integrins that are not able to recruit ShcA leads to an exit from the cell cycle, and consequent cell death [368]. This highlights the importance of the trimeric ShcA/FAK/SFK complex in integrin mediated cell survival.

In addition to its pro-tumorigenic properties, the ShcA PTB domain is also essential for signal termination. This property of ShcA ensures that the strength and duration of pro-tumorigenic responses is tightly controlled. The ShcA PTB domain binds numerous negative regulators, including PTPN12 [3], SHIP2 [4], PTPε [5] and has the ability to sequester ERK [6]. While some of these interactions require the phospho-tyrosine binding pocket of the ShcA PTB domain (PTPN12, SHIP2), others are phospho-tyrosine independent (PTPε, ERK). For example, ErbB2 and the protein tyrosine phosphatase, PTPε, compete to bind the ShcA-PTB domain. Recruitment of ShcA via its PTB domain to the ErbB2 receptor protects ShcA from PTPε-mediated dephosphorylation and consequent dampening of downstream signal transductions [5]. Alternatively, Suen et al., 2013 identified a ShcA dependent control mechanism that prevents aberrant signal transduction by ERK and inhibits its recruitment to the MAPK pathway [6]. ShcA/ERK interactions occur through a non-canonical binding interface on the ShcA PTB domain

(RRRKPCSRPLS, residues 97–107) in non-stimulated cells and are dissociated upon stimulation by EGFR. In the context of oncogenic signaling, increased ShcA recruitment to phospho-tyrosine binding sites through its PTB domain increases the level of free ERK, and consequently, the amplification of MAPK signal transductions [6]. Additionally, PTPN12 binds the ShcA PTB domain through a non-canonical NPXH motif upon serine phosphorylation of ShcA [7]. These works identify the central role of ShcA in controlling signal output in stimulated and non-stimulated conditions. Moreover, they suggest that a dynamic signaling network surrounds ShcA, where tyrosine phosphorylation can monitor the state of growth factor stimulation and determine the temporal activation and deactivation of signal transduction.

Interestingly, the ShcA PTB domains adopts a similar scaffold to the PH domain, which is known to bind to acidic phospholipids and localize proteins to the plasma membrane [369-371]. Specifically, the ShcA PTB domain engages PI(4,5)P2 and PI(4)P in a stereospecific manner [330, 357]. Recognition of phospholipid ligands are determined by three critical amino acids found within the PTB domain, R112, K116, K139 [330]. Mutation of these residues, R112Q, K116A, K139A, abrogates ShcA PTB domain interactions with phospholipids and its localization to the plasma membrane, without affecting phospho-tyrosine dependent interactions. The ability to engage phospholipids was essential for membrane localization and receptor activation to maximize ShcA phosphorylation. Thus, the duality of the PTB domain may serve two step-wise functions: (1) phospholipid mediated membrane localization of ShcA and (2) interaction of ShcA to the activated receptor [330].

The phosphorylation of ShcA on its tyrosine residues is considered an early event and is essential for the subsequent release of ShcA SH2 domain for ligand binding [372]. Unphosphorylated ShcA remains in a resting state where the SH2 domain is occluded from engaging its interactors, while the PTB domain is free to bind RTKs and Ins-1,4,5,-P3 (but not Ins-1,3,5-P3) for membrane localization [372]. PI(4,5)P2 was shown to stimulate the phosphorylation of pY239/240 ShcA by Src [373], an identified ShcA SH2 domain interactor. The addition of wortmannin or LY294002 (both inhibitors of PI3K) inhibits the phosphorylation of ShcA, but also increases the activation of Src. This indicates that the coordinated efforts of PI3K or PtdIns 3,4,5-trisphosphate and Src can function as an upstream positive regulator of ShcA tyrosine phosphorylation [373].

1.11.1.3 ShcA CH1 Domain

The ShcA CH1 domain is a 145-amino acid stretch (233-277aa) with 50% homology to human α1 collagen [338, 374, 375]. The collagen homologous region is rich in glycine (10.3%) and proline (20%) where 50% and 65% of proline and glycine, respectively, are conserved [338] . The dense proline and glycine stretches contribute to Pro-Xaa-Xaa-Pro motifs that bind SH3 domains of cellular kinases such as Src, Fyn and Lyn [368, 376], although there exists isoform specific restrictions (specifically for p66ShcA and p46ShcA) [376]. The most well characterized feature of the CH1 domain is the presence of tyrosine residues, tyrosine 239/240 and 317 (313 in the mouse), essential for downstream ShcA dependent signal transductions. Three short stretches of conserved amino acids are found within the CH1 domain. This includes two that are centered around the 239/240 tyrosine residues (YYNS/DΦPXKXPP) [344]. The second, surrounding tyrosine 317 lies within a YVNV consensus sequence [344]. The third conserved site is associated with the adaptin interacting motif (amino acids 346–355), which contributes to the ability of Shc proteins to associate with endosomes [377, 378].

The recruitment of ShcA to activated RTKs results in its phosphorylation on tyrosine residues Y239/240 and Y317. The phosphorylation of ShcA, provides optimal binding sites for the SH2 domain of Grb2 [379]. Grb2 is comprised of an SH3-SH2-SH3 signaling module where the N-terminal SH3 and C-terminal SH3 facilitate the engagement of Son of sevenless (Sos), a guanine nucleotide exchange factor of Ras or Gab1 adaptor protein to the inner membrane to activate Ras/ERK and PI3K/AKT signaling pathways, respectively [320, 343]. High affinity binding of Grb2 requires the phosphorylation of tyrosine 317 [379]. Early studies have demonstrated that the retention of only the ShcA Y317/SH2 domain fusion protein was sufficient for ShcA phosphorylation, Grb2 engagement, and induction of cellular transformation. However, further analyses elucidated that the Grb2 SH2 domain can also bind Y230/240 with 3-fold higher affinity when compared to Y317 alone. Specifically, it was Y239, that was required to bind Grb2, while Y240 was dispensable [344]. Given the binding specificities of Grb2 to activated ShcA phospho-tyrosines, this indicated that there were non-redundant roles of ShcA phospho-tyrosines in downstream signal transductions. Indeed, Y239/240 has been shown to be important for c-myc activation and the prevention of apoptosis, while Y317 induced transcriptional activation of c-fos [380]. Moreover, allosteric communication between the SH3 domains of Grb2 ensures that Sos1

solely binds to its N-terminal SH3 domain and Gab1 to its C-terminal SH3 [381]. As many RTKs can directly bind Grb2, while others rely on ShcA to indirectly recruit Grb2 via its CH1 domain, the association of these two adaptor proteins can be seen to fulfill both redundant and non-redundant biological functions depending on the cellular context.

Previous studies have identified a ShcA phosphorylation-dependent gating mechanism whereby tyrosine phosphorylation of ShcA induces a conformational change, which opens the SH2 domain to increase its ability to bind ligands. For example, the ShcA SH2 domain has been shown to interact with the cadherins and CEACAM1, the latter of which dampened MAPK activity in response to growth factor stimulation [372, 382, 383]. George et al., 2008 suggests that one outcome of this gating process could be the fine tuning of MAPK or PI3K/AKT signaling pathways that run in parallel; for example, stimulating activity through complex formation with Grb2 and down regulating activity through interactions with proteins at the SH2 domain. Altogether, these data suggest that ShcA tyrosine phosphorylation is capable of modulating temporal and spatial control of ShcA signal transductions.

1.11.2 Serine and Threonine Phosphorylation of ShcA

The phosphorylation of ShcA at its various serine and threonine residues has only recently come to the forefront of ShcA dependent signal transduction. The first evidence of ShcA serine phosphorylation was demonstrated by 2D-electrophoresis of phosphoamino acids from fibroblasts treated with insulin [355]. A rapid 10-fold increase in ShcA tyrosine phosphorylation was observed, while serine phosphorylation increased in a linear fashion [355]. Following this study, inducers of the serine/threonine kinase PKC[16], namely phorbol 12-myristate 13-acetate (PMA) treatment [384] or 12-O-tetradecanoylphorbol-13-acetate (TPA), were found to affect the interaction of ShcA with PTPN12. In particular, the activation of PKC resulted in the phosphorylation of Serine 29 [385] and also increased the interaction of PTPN12 to ShcA 8-10 fold [384]. Interestingly, PKCε was found to be an interactor of ShcA upon EGF stimulation [7].

[16] PKC family of proteins are serine/threonine kinases comprised of 10 isoforms (PKC α, βI, βII, γ, δ, ε, η, θ, ζ, λ/ι). Activation of PKC traditionally involves g protein coupled receptors. Classically, PKC responds to the second messengers, calcium (Ca2+) and diacylglycerol (DAG) which activates PKC. Reference 423.

The seminal work by Zheng et al., 2013 mechanistically elucidated the role of key serine and threonine residues that become phosphorylated upon EGF stimulation, specifically Serine 29, Threonine 214, and Serine 335 in ShcA dependent signal transduction. In this study, the recruitment and stabilization of the PTPN12/ShcA interaction was confirmed to be through Serine 29. Specifically, upon EGF stimulation, ShcA is tyrosine phosphorylated, leading to the engagement of Grb2 complexes. This leads to the activation of Ras/MAPK and PI3K/AKT pathways. Signal transduction is then rapidly antagonized by the phosphorylation of Serine 29. This phosphorylation event is thought to be mediated by AKT through the AGC kinase motif, RXXS/T (inclusive of ShcA Serine 29), resulting in the recruitment and stabilization of PTPN12 to the ShcA PTB domain [7]. ShcA is subsequently displaced from EGFR, dephosphorylated at its Grb2 binding NPXY motifs, and replaced by SgK269 pseudokinase which binds the ShcA PTB domain. SgK269 then brings in serine/threonine phosphatases to increase cytoskeletal reorganization and trafficking through non-SH2 dependent proteins [7]. This study was the first to mechanistically identify serine phosphorylation as an additional regulatory component in ShcA dependent pathway activation following growth factor stimulation [7].

Serine 29 has also been implicated in the recruitment of Src to the N-terminus of ShcA[17]. A minimal N-terminal segment, encompassing Serine 29 upstream of the ShcA PTB domain is capable of binding Src [8]. p46ShcA which lacks the first 45 amino acids found in p52ShcA, could neither interact with Src [8], nor PTPN12 [384]. Amino acid residues 40-49 (which overlaps with the PTB domain) was specifically required to activate Src. The activation of Src was dependent on Methionine (M) 46—mutation of M46P abrogated the ability of ShcA to activate Src [8].

Threonine 214 phosphorylation is dependent on the presence of Grb2 and feedback phosphorylation of ERK, upon EGF stimulation [7]. Recently, three threonine residues on ShcA (T214, T276 and T407) were found to be contained within the ERK consensus substrate sequence (S/T-P) [386]. Constitutive phosphorylation of T214, T276 and T407 collectively increased the activation of ERK and sustained the activation of AKT, independent of growth factor stimulation. Interestingly, the activation of T214, T276, and T407 increased the engagement of the 14-3-3ζ

[17] *In vitro* studies using a syntetic peptide comprising residues 410-428 of Src named the IDA (Inter-DFG-APE)-Src peptide helped to identify this region between conserved amino acid sequence motif, DFG (Asp-Phe-Gly) and APE (Ala-Pro-Glu) of the Src protein kinase domain. This region is required to engage ShcA at its amino terminus. Reference 8.

adaptor protein which has previously been described to engage the ShcA SH2 domain to upregulate PI3K signaling [358, 386]. Thus, this poses an intriguing alternative for ShcA signal transductions, as threonine phosphorylation of ShcA becomes an alternative route to activate AKT signaling independent of RTKs.

The kinase that phosphorylates Serine 335 has yet to be determined [7]. However, its phosphorylation is correlated with the engagement of cytoskeletal remodeling proteins, implicating its role in controlling cell morphology, movement and proliferation [7]. Substitution of Serine 335 was able to decrease the binding of ShcA binding partners, which suggested that this site is also important for the stabilization of ShcA signaling complexes [7].

1.11.3 ShcA Dependent Mammary Tumorigenesis

Clinical and genetic evidence supports an essential role for ShcA in breast cancer initiation and metastatic progression. In human breast cancers, 28 genes, including ShcA, reside within an amplicon (1q21-23) that is found in approximately 15% of all breast cancers and is enriched in the basal subtype and in luminal/p53 negative breast cancers [9, 387, 388]. Moreover, total ShcA protein levels are enriched in HER2 and basal breast cancers [389] and high Y317 ShcA phosphorylation is a predictor of nodal status, disease stage, and relapse in breast cancer patients [390, 391].

In vitro and *in vivo* studies have been instrumental in identifying the mechanisms underlying ShcA dependent breast cancer tumorigenesis and have found it to be a key protein that can modulate the landscape of breast cancer pathology. Complete deletion of ShcA from mammary epithelial cells expressing an intact ErbB2 receptor results in the dramatic impairment of tumor formation (9% penetrance) [392]. Moreover, ErbB2 driven mammary tumorigenesis is significantly impaired upon the mutation of the ShcA consensus binding site of ErbB2; tumor formation is restored by expressing ErbB2 that retained a functional ShcA-binding site [1, 56]. These results indicate that ShcA is necessary and sufficient to initiate mammary tumorigenesis [392].

The generation of transgenic mice harboring loss-of-function mutations in the PTB domain, SH2 domain, or tyrosine phosphorylation sites of ShcA [393] have been powerful tools for detailed structural and functional analyses of this adaptor protein in breast cancer. Using these models, we

and others have revealed non-overlapping roles for each ShcA functional domain during breast cancer progression [2, 392]. The importance of ShcA phospho-tyrosine dependent signaling downstream of RTKs was first demonstrated in MMTV/PyV-MT transgenic mice expressing targeted phospho-deficient ShcA harbouring phenylalanine substitutions of the Y313 or Y239/240 phospho-tyrosine residues (ShcA313F, ShcA2F or ShcA3F) [393]. The ShcA tyrosines were found to play critical and non-redundant roles in the early stages of mammary tumorigenesis. Tumor outgrowth required the retention of Y313 phosphorylation within the stromal compartment for tumor cell survival, whereas Y239/240 was necessary for angiogenesis in a cell autonomous manner [392]. Mutation of either the Y313 or Y239/240 phosphorylation sites in ShcA was also sufficient to impair ErbB2-induced tumor angiogenesis, however abrogation of all three phospho-tyrosine sites significantly reduced angiogenesis, in part, through the regulation of VEGF production [392]. This supported previous findings that recruitment of ShcA to ErbB2 receptor was required to induce VEGF expression and enhance angiogenesis [394]. Im et al., 2015 further corroborated these findings using breast cancer cells expressing a tyrosine phosphorylation-defective ShcA mutant. Specifically, ShcA-dependent activation of AKT, but not the Ras/MAPK pathway, induced VEGF production by increasing VEGF mRNA translation downstream of the ShcA phospho-tyrosine residues in a 4E-BP dependent manner [395].

In addition to regulating tumor cell survival, proliferation and angiogenesis, both MMTV/PyV-MT transgenic mice and ErbB2 expressing breast cancer cells have also demonstrated that the ShcA tyrosine residues are necessary for the metastasis of breast cancer cells from the primary site [392]. ShcA is an important mediator in synergising migratory and invasive effects observed downstream of the TGFβ and ErbB2 signaling pathways. Diminished ShcA expression is sufficient to ablate TGFβ induced motility in ErbB2 expressing breast cancer cells [396]. Migration and invasion require the ShcA PTB domain and the association of Y239/240 of ShcA with Crk adaptor proteins [397]. On the other hand, MMTV/PyV-MT transgenic mice expressing a targeted ShcA allele harboring a point mutation in the SH2 domain (R397K) rendering ShcA unable to engage in SH2-dependent interactions, delays tumor onset, but also impairs the development of lung metastasis and the number and size of metastatic lesions compared to PyV-MT controls [358]. Moreover, ShcA mutants debilitated in SH2 driven signaling were more susceptible to apoptosis, implicating the SH2 domain in cell survival. The observed phenotype was dependent on the activation of the AKT pathway, primarily through the interaction of 14-3-3 adaptor proteins and

the p85 regulatory subunit of PI3K to the ShcA SH2 domain [358]. The interplay between the SH2 domain and phospho-tyrosine residues of ShcA is in accordance with the observation that tyrosine phosphorylation of ShcA induces a conformational change, which renders the SH2 domain able to participate in phospho-tyrosine dependent interactions [372]. Alternatively, MMTV/PyV-MT transgenic mouse models expressing one ShcA allele that cannot engage in PTB-dependent interactions due to a point mutation in the ShcA PTB domain (R175Q), ablated phospho-tyrosine dependent PyV-MT/ShcA interactions and delayed tumor onset [2]. Despite the inability of the ShcA PTB domain to bind the transforming oncogene, tumors that arose from these mice paradoxically, were accelerated in their growth rate relative to wild-type ShcA controls. These tumors hyperactivated an autocrine loop involving the fibronectin receptor, resulting in increased activation of Src kinase and FAK, which in turn laterally activated RTKs including PDGFR, MET, and FGFR in a ShcA SH2 domain dependent manner. This study provided the first *in vivo* evidence of the tumor suppressive properties of the ShcA PTB domain and its ability to limit SH2 domain driven mitogenic responses in mammary tumorigenesis.

While canonical ShcA signal transductions have focused on the ShcA tyrosine phosphorylation sites as the primary engagement point for mitogenic signal transductions downstream of RTKs, the impact of distinct ShcA complexes recruited to the ShcA PTB and SH2 domains have been overlooked. The following work will examine discrete ShcA-dependent signaling complexes emanating from the ShcA PTB and SH2 domain and mechanistically dissect the interplay between distinct ShcA pools in mammary tumorigenesis and therapeutic resistance.

Chapter 2 Materials and Methods

2.1 Cell Culture

NMuMG-NeuNT, an ErbB2-transformed mammary tumor cell line, was generated and cultured as previously described [392]. NMuMG-NeuNT cell lines were stably transfected with a pMSCV/Hygromycin expression vector (Clontech Cat: 634401) subcloned with cDNAs expressing: (1) wild-type ShcA ($ShcA^{WT}$), (2) a ShcA mutant harbouring an arginine to glutamine substitution at amino acid 175 in the phospho-tyrosine binding pocket of the PTB domain of ShcA (PTB^{MUT}), (3) a PTB^{MUT} harbouring tyrosine to phenylalanine substitution at amino acids 239, 240, and 313 (PTB^{MUT}/3F), (4) a PTB^{MUT} with an arginine to lysine substitution at amino acid 397 in the phospho-tyrosine binding pocket of the SH2 domain ($PTB/SH2^{MUT}$), (5) a ShcA mutant harbouring a serine to alanine substitution at amino acid 29 (S29A; $ShcA^{S29A}$), (6) a ShcA mutant with a Serine 29-Tryptophan 38 (S29-W38) deletion ($ShcA^{\Delta 10}$), (7) a PTB^{MUT} harbouring a S29A subsitition ($PTB^{MUT/S29A}$), or (8) a PTB^{MUT} with a S29-W38 deletion ($PTB^{MUT/\Delta 10}$). All ShcA constructs are C-terminally tagged with a 3X-FLAG epitope. Four ShcA-expressing clones were pooled for each cell line, which were maintained in 0.5 mg/ml Hygromycin (Wisent Bioproducts Cat: 450-141-WL). NIC/$Src^{-/-}$ cell lines were established from MMTV/Neu-internal ribosome entry site (IRES)-Cre (NIC) mammary tumors, in which both c-Src alleles have been deleted by Cre-mediated excision ([54, 398, 399]. Culture conditions for these cells have also been previously described [54, 398, 399]. NIC/$Src^{-/-}$ cells were stably transfected with a pQCXIB expression vector (Addgene Cat: 22266) subcloned with Flag-tagged $ShcA^{WT}$ or PTB^{MUT} described above. Transfected cells were selected with 8 μg/ml Blasticidin (Wisent Bioproducts Cat: 400-190-EM). Cell lines represent pools of 6 expressing clones. All cell lines were routinely screened for mycoplasma contamination using a Mycoprobe Mycoplasma Detection Kit (R&D Systems Cat: CUL001B), at least once per month or one day prior to any mammary fat pad injection.

2.2 Cell Line Authentication

NMuMG cells were purchased from ATCC and NMuMG-NeuNT cell lines (ErbB2 transformed) were generated from tumor explants and cultured as previously described [392]. NIC/$Src^{-/-}$ and PyV-MT cell lines were generated and cultured as previously described [54, 233, 399, 400]. Experiments performed on cell lines were passaged no more than 8 times per month.

2.3 CRISPR/Cas9 Genome Editing

Sequences targeting murine c-Src, Fyn, and Lyn were determined by the CRISPR Design Tool (http://crispr.mit.edu): c-Src 5'-GAGCCCTCGGAAAACGTGCACGG-3', Fyn 5'-GGGACCCTACGCACGAGAGGAGG-3', Lyn 5'-GTTCGGTCAGTATTACGTACTGG-3'. The gBlocks (Integrated DNA Technologies) containing U6 promoter, the designed target sequence, gRNA scaffold and termination signal were subcloned into the pQCXIB expression vector. CRISPR guide sequences were stably transfected into NMuMG-NeuNT Flag-tagged ShcA and ShcA mutant expressing cells and selected with 8μg/ml Blasticidin. Loss of c-Src, Fyn, or Lyn expression was verified by immunoblot analysis. Cell lines represent pools of 3-4 clonal populations.

2.4 Pharmacological Inhibitors

Cell lines were treated with media containing dimethyl sulfoxide (DMSO; Bioshop Cat: DMS666), PP2 pan-Src Family Kinase inhibitor (2 μM; Sigma Aldrich Cat: P0042), Lapatinib Ditosylate EGFR and HER2/ErbB2 inhibitor (500nM; Selleckchem Cat: S1028), or Torin1 mTOR inhibitor (50nM; Tocris Cat: 4247).

2.5 Immunoblot Analysis

Whole-cell lysates were generated from cell lines or flash frozen tumor tissue in PLCγ cell lysis buffer (50mM HEPES [pH 7.5], 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1 mM EGTA, 1.5 mM $MgCl_2$, 10mM sodium orthovanadate, 10 mM NaF, and 1 μg/ml Chymostatin, 2 μg/ml Antipain, 2 μg/ml Leupeptin, 1μg/ml Pepstatin, 2 μg/ml Aprotinin) as previously described [392]. Lysates were separated by SDS-PAGE, transferred onto polyvinylidene difluoride (PVDF) membranes, blocked in 3% Bovine Serum Albumin (BSA; Bioshop Cat: ALB001.500), and probed with antibodies listed in Table 1. For studies involving inhibitors, cell lines were seeded into full culture media and allowed to attached overnight. The following day, media was changed to 2.5% FBS DMEM for a minimum of 12 hours. Cell were then treated with 2.5% FBS DMEM containing inhibitors. Whole-cell lysates were collected seven-hours post treatment. Densitometric analysis was conducted using Image J software.

Epitope	Catalogue Number	Company	Dilution
Phospho-AKT (S473)	9271	Cell Signaling Technologies	1:1000
AKT	9272	Cell Signaling Technologies	1:1000
Phospho-4E-BP1 (S65)	9456	Cell Signaling Technologies	1:1000
4E-BP1	9452	Cell Signaling Technologies	1:1000
Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204)	9101	Cell Signaling Technologies	1:2000
P44/42 MAPK (Erk1/2)	9102	Cell Signaling Technologies	1:2000
Phospho-FAK (Tyr576/577)	3281	Cell Signaling Technologies	1:1000
FAK	06-543	Millipore	1:1000
Flag	F1804	Sigma Aldrich	1:1000
Fyn	4023	Cell Signaling Technologies	1:1000
Grb2	3972	Cell Signaling Technologies	1:1000
Phospho-Her2/ErbB2 (Tyr1221/1222)	2243	Cell Signaling Technologies	1:1000
Lyn	2796	Cell Signaling Technologies	1:1000
Neu/ErbB2	Sc-284	Santa Cruz	1:5000
PTPN12	-	Gift from Dr. Michel L. Tremblay	1:5000
Phospho-p70 S6 Kinase (Thr389)	9205	Cell Signaling Technologies	1:1000
P70 S6 Kinase	2708	Cell Signaling Technologies	1:1000
Phospho-S6 Ribosomal Protein (Ser240/244)	2215	Cell Signaling Technologies	1:1000
S6 Ribosomal Protein	2217	Cell Signaling Technologies	1:1000
Phospho-Shc (Tyr239/240)	2434	Cell Signaling Technologies	1:1000
Shc	06-203	Millipore	1:2000
ShcBP1	ab184467	Abcam	1:1000
SHIP2	AF5389	R&D Systems	1:1000
Phospho-Src Family Kinase (Tyr416)	2101	Cell Signaling Technologies	1:1000
Src	05-184	Millipore	1:1000
Tubulin	T5168	Sigma Aldrich	1:5000

Table 1: Antibodies used for immunoblot analyses.

2.6 Immunoprecipitation

Cell lines or tumor lysates were lysed with cytoplasmic extraction lysis buffer (10 mM Tris [pH 8.4], 140 mM NaCl, 1.5 mM $MgCl_2$, 0.5% NP40, 1 mM DTT, 10mM sodium orthovanadate, 10 mM NaF, and 1 µg/ml Chymostatin, 2 µg/ml Antipain, 2 µg/ml Leupeptin, 1 µg/ml Pepstatin, 2 µg/ml Aprotinin). Cytoplasmic extracts were mixed with 30 µl bed volume of a 1:1 ratio of protein G-Sepharose 4 Fast Flow (GE Health-care Cat: 17-0618-02)–cytoplasmic extraction lysis buffer

solution. Flag-tagged ShcA was immunoprecipitated with flag-specific antibodies (1:250; Sigma Aldrich Cat: F1804) at 4°C overnight. Beads were subsequently washed three times with cytoplasmic extraction lysis buffer (without protease and phosphatase inhibitors). Complexes were eluted with SDS Page Loading Buffer (4X Tris-HCL/SDS [pH 6.8], 3% Glycerol, 10%SDS, 12 mg bromophenol blue) at 95°C for 8 minutes.

2.7 BioID

Flag-tagged ShcA and ShcA mutant cDNA was subcloned into pcDNA3.1(-) MycBirA-R118G (a gift from Dr. Marc R. Fabian) and subsequently subcloned into the pQCXIB expression vector. N-terminal MycBirA-tagged ShcA wild-type, ShcA mutants, and MycBirA-only vector control was expressed in MMTV Middle T antigen mammary epithelial cells or NMuMG-NeuNT breast cancer cell lines and selected with Blasticidin. Cells were seeded into in complete media. The following day, media was changed to 0.5% FBS DMEM for a minimum of 12 hours and then pulsed with 50 µM Biotin (Sigma-Aldrich Cat: B4501-1G) in 10% FBS for 24 hours. Cells were harvested by trypsinization and collected by centrifugation at 250 x g for 5 minutes. Whole cell lysates were generated using RIPA buffer (1% Triton X-100, 50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.1% SDS) supplemented with 50U Benzonase (Millipore Cat: 70664-3), 0.5% Sodium Deoxycholate (Sigma Aldrich Cat: D6750), and phosphatase and protease inhibitors. Lysates were then sonicated two times at 50% amplitude for 10 seconds on ice and subsequently centrifuged at 16 000 x g for 30 minutes at 4°C. Biotinylated proteins were pulled down using 30 µl bed volume of 1:1 Avidin Agarose beads (Thermo Scientific Cat: 20219)-RIPA buffer solution, rotating for 3-4 hours at 4°C. Beads were collected by centrifugation at 3000 rpm for 1 minute at 4°C and then washed three times with RIPA Buffer (without protease and phosphatase inhibitors). Proteins were eluted using 2x Laemmli buffer (4% SDS, 20% Glycerol, 120mM Tris-HCl [pH 6.8]) at 95°C for 8 minutes. Eluates were characterized by immunoblot analysis.

2.8 Clonogenic Assay

For each cell line, 50 cells were seeded into 12-well plates and treated with various inhibitors 24 hours later. Inhibitors were replenished every two days over a 10-day period. Cells were fixed with 10% Buffered Formalin (VWR Cat: 16004-128) for 20 minutes at room temperature.

Subsequently, each well was washed with distilled water and then stained with 0.1% Crystal Violet/20% Methanol solution for 30 minutes. Plates were then rinsed with water and dried overnight. Plates were scanned using the Oxford Optronix GelCount™ system. Data was analyzed using positive pixel count algorithms with Aperio ImageScope software.

2.9 Soft Agar Assay

NMuMG-NeuNT - $1.5x10^4$ cells were plated into 1.5 ml of 0.4% Agar (Bioshop Cat: AGR001.500)/ 20% Fetal Bovine Serum (FBS; Wisent Bioproducts Cat: 080-150) Dulbecco's Modified Eagle's Medium (DMEM; Wisent Bioproducts Cat: 319-005-CL) over a layer of 2ml 0.6% Agar/20% FBS DMEM in 6-well plates. Cells were treated with either full culture media or media containing inhibitor (DMSO, PP2, or Torin1). To account for the volume of agar, inhibitor concentrations were adjusted to 6 μM PP2 and 150 nM Torin1 to obtain an approximate final concentration of 2 μM and 50 nM, respectively. For inhibitor studies, media containing inhibitors or DMSO was replenished every 3 days and monitored over a 10-day period. NIC/$Src^{-/-}$ - $1.5x10^4$ cells were plated into 1.5 ml of 0.4% Agar/ 10% FBS DMEM supplemented with Mammary Epithelial Growth Supplement (MEGS; 3 ng/ml Human Epidermal Growth Factor (Invitrogen Cat: PHG0311), 0.5 mg/ml Hydrocortisone (Wisent Bioproducts Cat: 511-002-UG), 5 mg/ml Insulin, 0.4%v/v Bovine Pituitary Extract (Wisent Bioproducts Cat: 002-011-IL)) over a layer of 2ml 0.6% Agar/10% FBS DMEM supplemented with MEGS in 6-well plates and monitored for 10 days. Images of four fields per well were acquired by an inverted light microscope using Infinity Capture software. The number of colonies and size of foci were analyzed using Aperio ImageScope software.

2.10 Mammary Fat Pad Injection

$5x10^4$ NMuMG-NeuNT or $25x10^4$ NIC-$Src^{-/-}$ wild-type or mutant ShcA expressing breast cancer cells were injected into the fourth mammary fat pad of 8-10-week old SCID-Beige female mice. Animals were monitored for tumor initiation and tumor outgrowth every two days using caliper measurements. Breast tumors were fixed in 10% Buffered Formalin, embedded in Optimal Cutting Temperature medium (OCT; VWR Cat: CA95057-838), or frozen in liquid nitrogen. All animal studies were approved by the Animal Resources Council at McGill University and comply with guidelines set by the Canadian Council of Animal Care.

2.11 Immunohistochemistry

Paraffin or OCT embedded breast tumor sections were stained with primary antibodies and staining conditions listed in Table 2. Slides were scanned using a Aperio ScanScope XT Digital Slide Scanner and data was analyzed using positive pixel count or nuclear algorithms (Aperio ImageScope).

Epitope	Catalogue Number	Company	Dilution	Tissue	Antigen Retrieval
Cleaved caspase 3	9661	Cell Signaling Technologies	1:250	Paraffin	Sodium Citrate Buffer[18]
Ki67	ab15580	Abcam	1:500	Paraffin	Sodium Citrate Buffer
Phospho-S6 Ribosomal Protein (Ser240/244)	2215	Cell Signaling Technologies	1:2000	Paraffin	Sodium Citrate Buffer
Phospho-Src Family (Tyr416)	2101	Cell Signaling Technologies	1:200	Paraffin	TE Buffer[19]

Table 2: Antibodies used for immunohistochemistry.

2.12 Bioinformatics

All gene signatures were projected across 1218 human breast cancers from TCGA dataset using single sample gene set enrichment analysis (ssGSEA) as described before [401]. Briefly, a score is defined to represent the degree of enrichment of a given gene set in a sample: gene expression values for each sample are rank-normalized, and an enrichment score is produced using the Empirical Cumulative Distribution Functions (ECDF) of genes, with the final score computed by integrating the difference between a weighted ECDF of genes in the signature and the ECDF of the remaining genes [401]. This calculation is repeated for each signature and each sample in the dataset. To compute ssGSEA scores, we used the GenePattern software implementation from the Broad Institute, ssGSEAProjection (v6) [402]. Spearman correlations between each signature and

[18] Composition of Sodium Citrate Buffer (1X): 10mM Sodium Citrate (2.94g Tris-sodium citrate (dihydrate) in 1000ml distilled water with 0.05% Tween20. pH adjusted to 6.0 with 1N HCl.
[19] Composition of TE Buffer (1X): 1.21g Tris and 0.37g EDTA in 1000ml distilled water with 0.05% Tween 20. pH adjusted to 9.0.

expression values of specific genes (Src, Fyn and Lyn) were then computed. For visualization purposes, patients were ranked-ordered and stratified in quartiles, and the mean expression value for each gene and each quartile was computed. By keeping the patients in the same quartiles, we then interrogated relative pY317-ShcA, pY416-Src and pS235/6-rS6 levels in these human breast tumors using the TCGA RPPA breast cancer datasets (n=747). Finally, for tumors in which we had both RNA-Seq and RPPA data, we also interrogated copy number levels for SHC1 (ShcA), PTPN12 (PTPN12) and INPPL1 (SHIP2) using the TCGA copy number breast cancer dataset.

2.13 Statistical Analysis

Unless otherwise indicated, all *in vitro* studies were carried out with three biological replicates and with six technical replicates per experimental group. Data was normalized to the control groups as appropriate. For the *in vivo* studies, mammary fat pad injections were performed with at least 4-5 age-matched mice inoculated with breast cancer cells in both mammary fat pads (n=8-10 breast tumors per cohort). The following statistical analyses were used throughout the study. Chapter 3.1: Two-tailed, unpaired student's *t* test (Figures 6B-E, 7B-G, 8A-D, 12B-E, 14A, 14C, 17); One-way ANOVA with a Tukey's multiple comparisons test (Figures 1E-F, 2A-C, 3A-C, 4A, 4C, 5A, 9, 10, 12F, 13A-B, 15A-D); Two-way ANOVA with a Tukey's multiple comparisons test (Figures 5B, 11B-C, 16A-B). Chapter 3.2: Two-tailed, unpaired student's *t* test (Figures 1A-B, 4A-B, 6A-B, 7A-B, 8A-B, 14F); One-way ANOVA with a Tukey's multiple comparisons test (Figures 3A-B, 4C, 5A-B, 6C-D, 7C-D, 8C, 9A-B, 10, 11C-D, 12, 13B-D, 14C).

Chapter 3 Results

3.1 Integration of distinct ShcA signaling complexes promotes breast tumor growth and tyrosine kinase inhibitor resistance.

3.1.1 Introduction

Aberrant phospho-tyrosine signaling networks have been demonstrated to be essential drivers of breast cancer progression. These networks not only promote tumor initiation but are also essential for uncontrolled tumor growth and metastatic spread [9]. The emergence of specific breast cancer subtypes reinforced intense research to better understand how tyrosine kinases contribute to breast tumor heterogeneity and response to therapy. For example, HER2+ breast cancer is an aggressive disease driven by the dysregulated activation of the HER2/ErbB2 receptor tyrosine kinase (RTK) [262, 403]. Introduction of Trastuzumab, a HER2-targeted therapy, into clinical practice has significantly improved patient outcome for HER2+ patients. Unfortunately, therapeutic responses are often short lived due to the expression of numerous receptor and non-receptor tyrosine kinases, including EGFR, ErbB3, Met, Lyn, and Src [49, 136, 139, 165, 235, 255, 404]. In this regard, the ability of HER2+ breast cancer cells to re-program their tyrosine kinome confers Trastuzumab resistance and also confounds the identification of effective targeted therapies. Thus, tyrosine kinases serve an essential role in establishing breast cancer heterogeneity and therapeutic sensitivity.

Adaptor proteins serve as critical intermediaries that transduce signals downstream of tyrosine kinases by integrating multimeric signaling complexes. Although many adaptor proteins lack intrinsic catalytic activity, they have the unique ability to spatially and temporally control the strength and duration of mitogenic responses downstream of tyrosine kinases. The ShcA adaptor protein is one such protein found downstream of ErbB2 that promotes breast cancer initiation [392]. Specifically, ShcA possesses two phospho-tyrosine binding domains (PTB, SH2) and three tyrosine phosphorylation sites (Y239/Y240/Y313) to transduce phospho-tyrosine dependent signals [405]. Key RTKs that are activated in HER2+ breast tumors, including ErbB2, EGFR, and ErbB3, bind the ShcA PTB domain to transduce highly dynamic signaling networks, leading to the phosphorylation of its tyrosine residues in the CH1 domain (Y239/Y240/Y313). These tyrosine phosphorylation residues create docking sites for Grb2/Sos and Grb2/Gab1 complexes, which activate the Ras/ERK and PI3K/AKT pathways, respectively [343, 406].

In addition to its pro-tumorigenic properties, the ShcA PTB domain is also necessary for signal termination. Several studies have established the ShcA PTB domain as a temporal switch to control EGFR signaling networks by facilitating the delayed recruitment of PTPN12 to terminate ShcA-coupled mitogenic responses [7]. Indeed, the ShcA PTB domain binds numerous negative regulators, including PTPN12, SHIP2, PTPε and can sequester oncogenic effectors such as ERK, in a constitutive or phospho-tyrosine dependent manner [4-6]. This corroborates previous *in vivo* studies, which demonstrated that the loss of PTB-driven ShcA signaling not only delayed breast tumor initiation but also paradoxically, potentiated subsequent tumor growth [2].

We previously show that PTB-dependent signaling complexes promote breast cancer growth by increasing AKT/mTOR signaling downstream of the ShcA tyrosine phosphorylation sites [395]. Additionally, we provided evidence that the ShcA PTB domain dynamically controls signaling networks in breast cancer cells, not only to transduce tumorigenic signals downstream of RTKs (PTB-dependent), such as ErbB2, but also to create negative feedback loops that prevents secondary activation of ShcA-SH2 driven complexes (PTB-independent), which are uncoupled from the transforming oncogene [2]. Given the absolute requirement for ShcA signaling in ErbB2+ breast cancer progression, whether and how unique PTB-dependent and -independent signaling complexes converge to promote mammary tumorigenesis is unknown. The overall aims of this study include:

1. To determine whether PTB-independent ShcA tumorigeneisis is dependent on the hyperactivation of ShcA signaling.

2. To identify ShcA interactors that contribute to amplified tumor growth in breast tumors that engage PTB-independent ShcA complexes.

3.1.2 PTB-independent ShcA pools require the ShcA phospho-tyrosine sites and an intact SH2 domain to potentiate mammary tumorigenesis, *in vitro*.

To model the consequence of deregulated ShcA signaling from PTB-independent ShcA pools, we employed a loss of function ShcA mutant (R175Q) in the phospho-tyrosine pocket of the PTB domain (PTB^{MUT}) [2, 393]. We then proceeded to either mutate all three ShcA tyrosine phosphorylation sites to phenylalanine residues (PTB^{MUT}/3F) or introduced a loss of function

mutation (R397K) in the phospho-tyrosine binding pocket of the SH2 domain (PTB/SH2^{MUT}), in the context of the PTB mutant (Figure 1A). Subsequently, FLAG-tagged constructs expressing the ShcA mutants were stably expressed in ErbB2-expressing breast cancer cells (Figure 1B). As controls, FLAG-tagged ShcA alleles harbouring a wild-type PTB domain (ShcA^{WT}) or the loss of function PTB mutant (PTB^{MUT}) were also stably expressed (Figure 1B). In effect, ectopic expression of a ShcA PTB^{MUT} allele in breast cancer cells that endogenously express wild-type ShcA creates two distinct intracellular pools of ShcA that independently (1) transduce oncogenic signals downstream from RTKs, including ErbB2, (ShcA; PTB-dependent) and (2) are unable to bind both RTKs and negative regulators (PTB^{MUT}; PTB-independent) allowing for the amplification of pro-tumorigenic signals (Figure 1A).

We first confirmed that an intact PTB domain is required for ShcA to engage in PTB-dependent interactions, including ErbB2, PTPN12 and SHIP2 [3, 4, 7, 321, 385] (Figure 1C). Second, SH2-dependent interactions with ShcBP1 [407] were selectively abrogated in PTB/SH2^{MUT}-expressing cells (Figure 1C). Lastly, mutation of the ShcA tyrosine phosphorylation sites ablated Grb2 binding (PTB^{MUT} vs. PTB^{MUT}/3F) (Figure 1D) [343]. These results confirm the specificity of the mutants generated.

To mechanistically define how these PTB-independent ShcA pools (PTB^{MUT}) promote breast tumor growth, we first tested the requirement of either phospho-tyrosine- and/or SH2-mediated ShcA signaling for tumor growth in soft agar. Interestingly, PTB^{MUT} expressing breast cancer cells had a reduced capacity to form colonies in soft agar relative to ShcA^{WT} controls (Figure 1E). This corroborated previous observations, which defined an important role for the ShcA PTB domain in ErbB2-driven tumor growth [1, 2, 321]. However, once formed, PTB^{MUT}-expressing foci were significantly larger than ShcA^{WT} controls (Figure 1F), validating several studies demonstrating that the ShcA PTB domain restricts tumor growth when ShcA is uncoupled from oncogenes such as ErbB2 [2, 4, 5, 385]. Interestingly, the tumorigenic potential from these PTB-independent ShcA pools was significantly debilitated in both PTB^{MUT}/3F and PTB/SH2^{MUT}-expressing cells (Figure 1F). This indicated that signals emanating from both ShcA phospho-tyrosine residues and SH2 domain are important for breast tumor growth, *in vitro*.

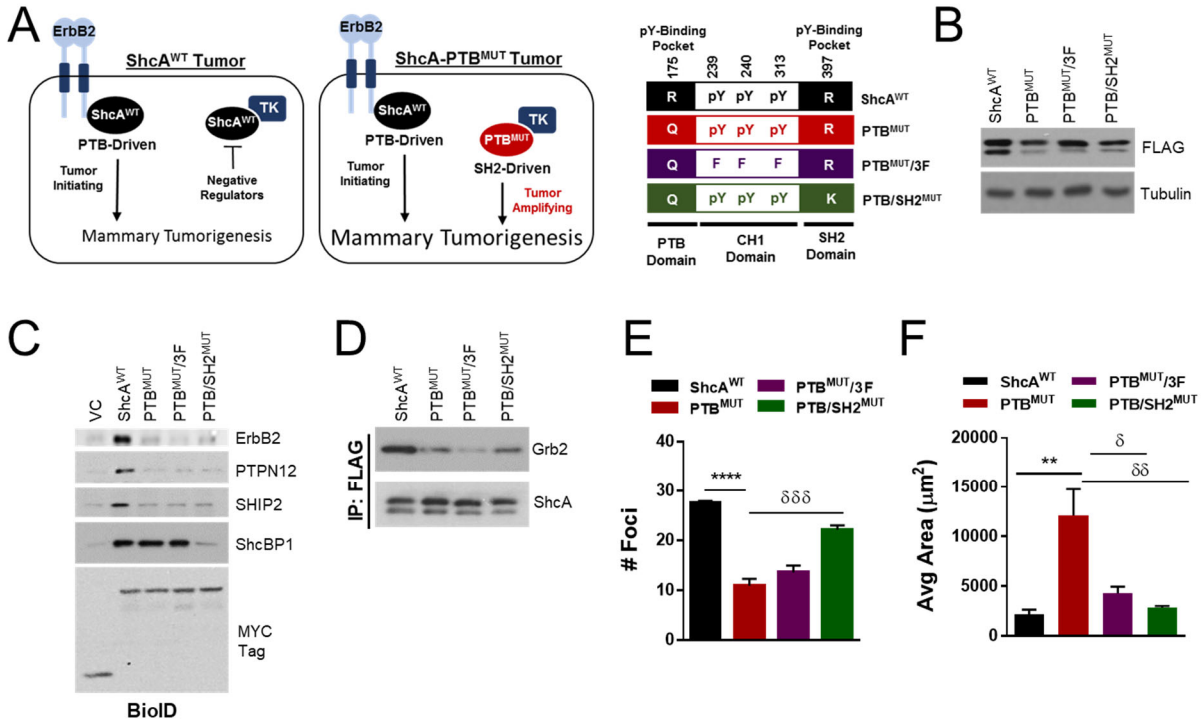


Figure 1: PTB-independent ShcA pools require a functional SH2 domain and tyrosine phosphorylation sites to promote mammary tumorigenesis, *in vitro*. (A) Schematic diagram illustrating how the ShcA PTB domain coordinates distinct intracellular signaling complexes that initiate and amplify mammary tumorigenesis in ErbB2-driven breast cancers. Schematic representation of ShcA alleles employed in this study is also shown. **(B)** Immunoblot analysis characterizing the expression of FLAG-tagged PTB domain mutant ShcA alleles in ErbB2 driven mammary epithelial cell lines (NMuMG-NeuNT). **(C)** MMTV Middle T antigen mammary epithelial cells expressing indicated Myc-BirA fusion proteins were subjected to BioID analysis. Biotinylated proteins were probed using the indicated antibodies by immunoblot analysis. **(D)** Grb2 interactions assessed by the immunoprecipitation of FLAG-tagged PTB domain mutant ShcA alleles expressed in NMuMG-NeuNT cells. **(E)** Number and **(F)** average area of foci formed in a soft agar assay from the indicated cell lines. The data is representative of three independent experiments (means ± SEM). ShcA^{WT} vs PTB^{MUT}: $^{**}P<0.01$, $^{****}P<0.0001$; PTB^{MUT} vs PTB^{MUT}/3F or PTB/SH2^{MUT}: $^{\delta}P<0.05$, $^{\delta\delta}P<0.01$, $^{\delta\delta\delta}P<0.001$.

3.1.3 PTB-independent ShcA pools require a functional SH2 domain to potentiate mammary tumorigenesis, *in vivo*.

We next investigated whether PTB-uncoupled ShcA pools (PTB^{MUT}) relied on the tyrosine phosphorylation sites and/or SH2 domain to amplify ErbB2-driven mammary tumor growth, *in vivo*. As expected, ShcA signaling from PTB-independent complexes (PTB^{MUT}) significantly accelerated tumor growth relative to $ShcA^{WT}$ controls (Figure 2A). Surprisingly, we observed that the ShcA tyrosine phosphorylation sites were dispensable for the enhanced tumor growth observed from PTB-independent ShcA complexes (PTB^{MUT} vs. PTB^{MUT}/3F). Rather, loss of SH2-dependent signaling from these ShcA pools ($PTB/SH2^{MUT}$) profoundly delayed breast tumor onset (Figure 2A). To better understand the mechanism underlying this phenotype, we performed Ki67 and Cleaved Caspase 3 immunohistochemical (IHC) staining on these tumors as markers of cell proliferation and apoptosis, respectively. At the experimental endpoint, we did not observe appreciable differences in the degree of cell proliferation or apoptosis in $ShcA^{WT}$, PTB^{MUT} and PTB^{MUT}/3F expressing tumors. However, we did observe that $PTB/SH2^{MUT}$-expressing tumors had the greatest proliferative potential, which was counteracted by a significantly increased apoptotic rate (Figures 2B-C). This suggests that loss of SH2-mediated signaling from PTB-uncoupled ShcA complexes initiates an apoptotic response, leading to a compensatory amplification of mitogenic responses to permit breast tumor growth.

3.1.4 PTB-independent ShcA pools simultaneously increase mTOR signaling and Src activity.

We and others established that PTB-dependent signaling complexes ($ShcA^{WT}$) promote breast cancer growth by increasing AKT/mTOR signaling downstream of the ShcA tyrosine phosphorylation sites [395]. Moreover, our previous work has also demonstrated that that PTB-uncoupled ShcA signaling complexes (PTB^{MUT}) activate Src in ErbB2-positive breast cancer cells [2]. Here, we confirmed this observation and further show that these PTB-uncoupled ShcA pools required an intact SH2 domain, and not the tyrosine phosphorylation sites, to activate Src (Figure 3A). Tumor-amplifying ShcA pools (PTB^{MUT}) had also upregulated mTOR signaling, as evidenced by modestly elevated pS65-4EBP1 levels and a more pronounced increase in T389-S6K phosphorylation, compared to $ShcA^{WT}$ controls (Figure 3A). This increase in mTOR signaling

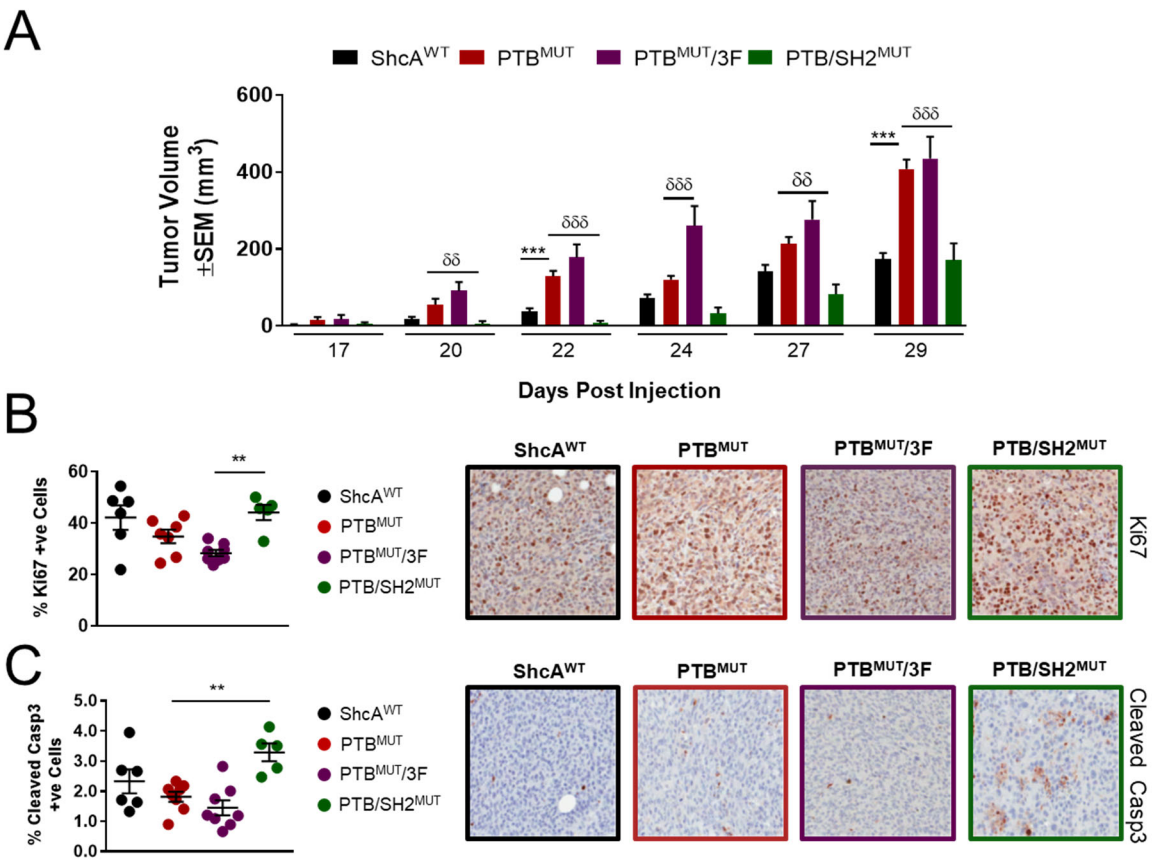


Figure 2: PTB-independent ShcA pools require a functional SH2 domain to potentiate mammary tumorigenesis, *in vivo*. (A) Mammary fat pad injection of the indicated cell lines into immunodeficient mice. The data is shown as average tumor volume (mm^3) ± SEM and is representative of 14 tumors per group. $ShcA^{WT}$ vs PTB^{MUT}: ****P<0.001*; PTB^{MUT} vs PTB^{MUT}/3F or $PTB/SH2^{MUT}$: $^{\delta\delta}P<0.01$, $^{\delta\delta\delta}P<0.001$. Immunohistochemical staining of mammary tumors using **(B)** Ki67 and **(C)** cleaved Casp3. The data depicts the average positively stained cells ± SEM and is representative of 6-8 tumors per group. ***P<0.01*.

was dependent on the ShcA tyrosine phosphorylation sites (Figure 3A). Interestingly, AKT activation was relatively unchanged, irrespective of the mutational status of these PTB-uncoupled ShcA signaling complexes (Figure 3A). Thus, mTOR and Src activation bifurcates downstream of PTB-independent ShcA pools through the ShcA tyrosine phosphorylation sites and SH2 domain, respectively.

To further examine how perturbation of ShcA-driven signaling complexes impacted breast tumor growth *in vivo*, we performed IHC analyses using pS240/244-ribosomal S6 (pS240/244-rS6) and pY416-Src specific antibodies. We show that pS240/244-rS6 levels are selectively reduced in PTB^{MUT}/3F tumors (Figure 3B), corroborating an important role for ShcA tyrosine phosphorylation in promoting mTOR signaling. Given that PTB^{MUT} and PTB^{MUT}/3F expressing tumors display comparable growth rates *in vivo* (Figure 2A), these data suggest that mTOR activation is not primarily responsible for the increased tumorigenic potential of PTB-uncoupled ShcA pools (Figures 3B). On the other hand, pY416-Src levels were profoundly elevated in PTB^{MUT} and PTB^{MUT}/3F expressing tumors relative to $ShcA^{WT}$ controls (Figure 3C). This is consistent with our *in vitro* studies showing that increased tumor growth was associated with a ShcA SH2-dependent increase in Src activity (Figure 3A). Unexpectedly, IHC analyses of endpoint tumors demonstrate that Src signaling was completely restored in PTB/$SH2^{MUT}$ tumors (Figure 3C) despite having severely impaired growth potential *in vivo* (Figure 2A). These results demonstrate that the ability of PTB-uncoupled ShcA pools to accelerate mammary tumorigenesis was strictly dependent on a functional SH2 domain. Moreover, when faced with loss of SH2-mediated signaling from PTB-independent ShcA complexes, there were significant selective pressures to re-acquire Src activity to permit eventual tumor growth.

3.1.5 ShcA tyrosine phosphorylation of PTB-independent ShcA pools requires Src activation.

Once activated, ErbB2 recruits and phosphorylates ShcA in a PTB-dependent manner [56, 281]. We now demonstrate that PTB-independent ShcA pools (PTB^{MUT}) were also tyrosine phosphorylated, albeit to a significantly lower extent (~3 fold) relative to PTB-dependent ShcA complexes ($ShcA^{WT}$) (Figure 4A). Given that numerous Src family kinases (SFK) have been

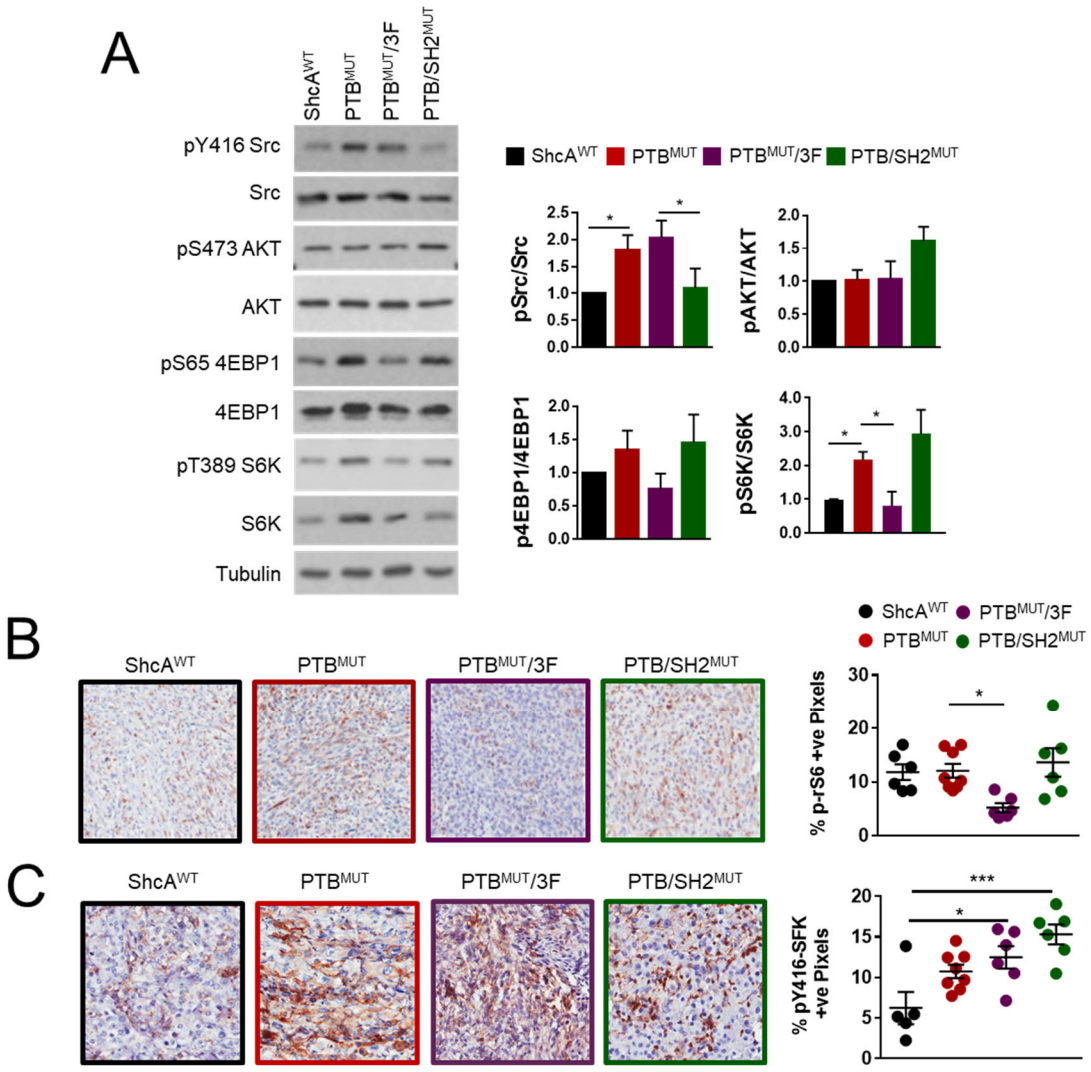


Figure 3: PTB-independent ShcA pools employ distinct domains to hyperactivate mTOR and Src signaling. (A) Immunoblot analysis with the indicated antibodies in ErbB2-driven breast cancer cells expressing specified FLAG-tagged ShcA alleles. The data is representative of three independent experiments (means ± SEM). **P<0.05*. **(B) and (C)** Immunohistochemical staining of tumors using pS240/244-rS6 and pY416-Src specific antibodies, respectively. The data depicts the average positively stained pixels ± SEM is representative of 6-8 tumors per group. **P<0.05, ***P<0.001*.

demonstrated to bind ShcA, both in a constitutive and phospho-tyrosine inducible manner [349, 356, 368, 408], we employed a pan SFK inhibitor (PP2) at sub-optimal doses (2 μM) to test whether PTB-independent ShcA pools are more reliant on SFKs to phosphorylate ShcA (Figure 4A). We confirmed that there was SFK inhibition at the reported dose (Figure 4B). SFK inhibition did not impact ShcA tyrosine phosphorylation from PTB-coupled complexes ($ShcA^{WT}$) but attenuated ShcA phospho-tyrosine levels from PTB-uncoupled ShcA pools (PTB^{MUT}) relative to their respective DMSO controls (Figure 4A). Additionally, PP2 inhibition of SFKs at a higher dose (20 μM) completely abrogated ShcA tyrosine phosphorylation, specifically from PTB-independent ShcA complexes (Figure 4C). As expected, a ShcA mutant lacking the tyrosine phosphorylation sites (PTB^{MUT}/3F) was not phosphorylated, both in control and PP2-treated breast cancer cells (Figure 4A, 4C). Remarkably, ShcA pools that cannot engage in PTB- or SH2-driven interactions (PTB/$SH2^{MUT}$) were residually tyrosine phosphorylated in an SFK-dependent manner (Figure 4A, 4C). This highlights an accessory role for constitutive ShcA/SFK interactions in transducing phospho-tyrosine driven ShcA signals. Combined, these results suggest that PTB-uncoupled ShcA pools are dependent on SFKs to transduce signals downstream of the ShcA tyrosine phosphorylation sites.

Given that the ShcA SH2 domain is required to activate Src from PTB-independent signaling complexes, we next interrogated whether they rely on an intact SH2 domain to bind SFKs. Unexpectedly, the ShcA SH2 domain is dispensable for Src, Fyn or Lyn recruitment to PTB-uncoupled ShcA pools (PTB^{MUT} vs PTB/$SH2^{MUT}$) (Figure 4D). Paradoxically, tumor amplifying ShcA pools with an intact SH2 domain (PTB^{MUT}) exhibited the lowest level of SFK binding, relative to $ShcA^{WT}$ controls (Figure 4D), despite displaying a 2-fold increase in Src activation (Figure 3A). Collectively, these results suggest that the ability of tumor amplifying ShcA pools to activate Src required a functional SH2 domain but was uncoupled from direct SFK recruitment to ShcA signaling complexes.

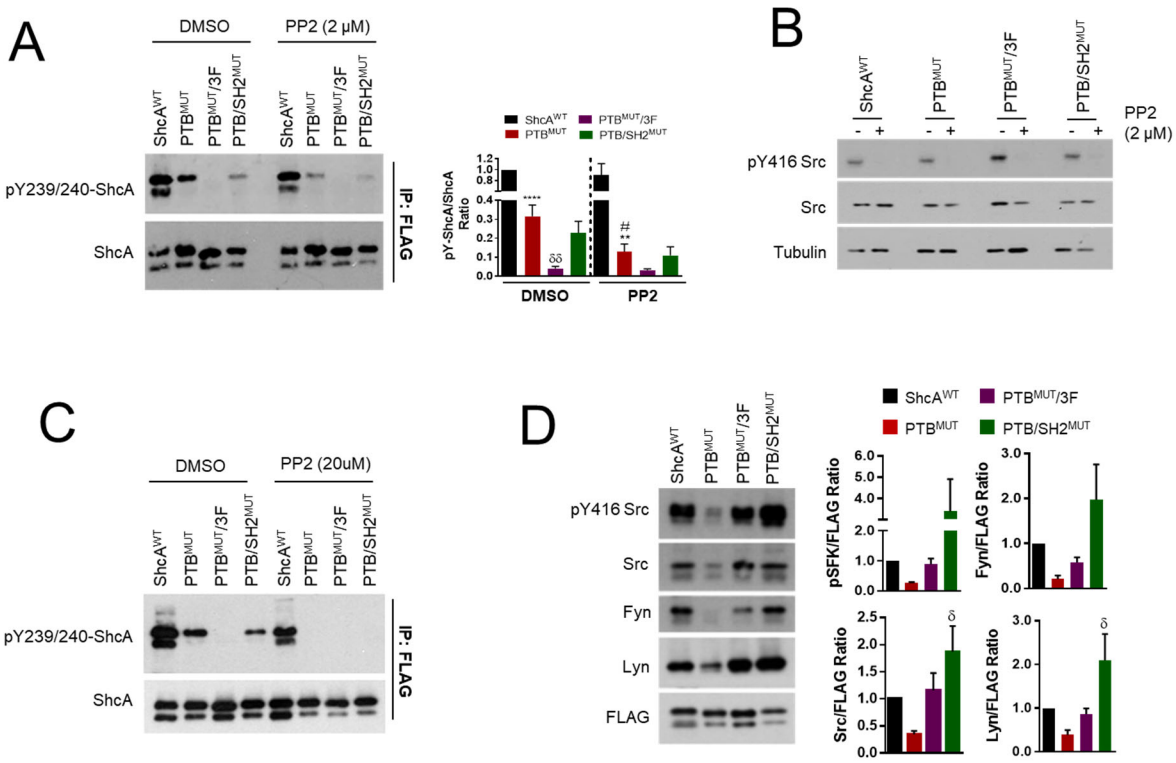


Figure 4: ShcA tyrosine phosphorylation of PTB-independent ShcA pools requires Src activation. (A) FLAG immunoprecipitates were probed with pY239/240-ShcA or ShcA-specific antibodies by immunoblot analysis. Both DMSO and PP2 (2 μM)-treated cells were assayed. The barplot represents densitometric quantification of three independent experiments (means ± SEM) using Image J software. One-way ANOVA (DMSO or PP2)—ShcA^{WT} vs PTB^{MUT}: $^{**}P<0.01$, $^{****}P<0.0001$; PTB^{MUT} vs PTB^{MUT}/3F: $^{\delta\delta}P<0.01$; PTB^{MUT} (DMSO) vs. PTB^{MUT} (PP2): $^{\#}P<0.05$. **(B)** Immunoblot analysis of indicated cell lines confirming Src activity inhibition using PP2 (2 μM) and vehicle alone (DMSO) at seven hours. **(C)** Immunoblot analysis of ShcA tyrosine phosphorylation (pY239/240-ShcA) using immunoprecipitates of FLAG-tagged ShcA^{WT}, PTB^{MUT}, PTB^{MUT}/3F, and PTB/SH2^{MUT} upon treatment of DMSO and PP2 (20 μM). **(D)** FLAG immunoprecipitates from indicated cell lines were probed with pY416-Src, Src, Fyn, Lyn, and Flag specific antibodies via immunoblot analysis. The barplot represents densitometric quantification of three independent experiments (means ± SEM) using Image J software. PTB^{MUT} vs PTB/SH2^{MUT}: $^{\delta}P<0.05$.

3.1.6 PTB-independent ShcA pools rely on both the SH2 domain and tyrosine phosphorylation sites to mediate Lapatinib resistance.

ErbB2-expressing breast cancer cells are predominately reliant on ErbB2/ShcA complexes for their tumorigenic potential, in part, through the ability of these cells to suppress ErbB2-uncoupled mitogenic signals in a PTB-dependent manner ($ShcA^{WT}$) [2]. Given the ability of PTB-independent ShcA signaling complexes to coordinately activate SFKs in ErbB2-positive breast cancer cells, we next assessed whether their hyperactivation alters the sensitivity of breast cancer cells to pharmacological inhibition of ErbB2 and EGFR (lapatinib), both of which bind the ShcA PTB domain. Recall that PTB^{MUT}-expressing cells retain the ability to engage both PTB-independent ShcA complexes alongside PTB-dependent interactions from endogenous ShcA alleles. Inhibition of ErbB2 signaling was confirmed—phosphorylated ErbB2 levels were severely diminished in all lapatinib-treated cells (Figure 5A). Using a clonogenic assay, we demonstrate that increased PTB-independent ShcA signaling promoted lapatinib resistance in ErbB2-transformed breast cancer cells (Figure 5B). Upon further investigation, we found that the ERK and AKT pathways remained elevated in lapatinib-treated PTB^{MUT}-expressing cells compared to $ShcA^{WT}$ controls (Figure 5A). The loss of either phospho-tyrosine (PTB^{MUT}/3F)- or SH2 (PTB/$SH2^{MUT}$)-driven signaling from these PTB-independent ShcA pools reversed lapatinib resistance (Figure 5B) and impaired ERK and AKT/mTOR signaling (Figure 5A). Moreover, ErbB2 inhibition had no impact on Src activation in any cell line tested (Figure 5A). Combined, these data suggest that increased signaling from PTB-independent ShcA pools rely on both the SH2 domain and tyrosine phosphorylation sites to retain ERK and AKT activation in response to pharmacological ErbB2/EGFR inhibition, which contributed to Lapatinib resistance.

3.1.7 The increased tumorigenic potential of PTB-independent ShcA signaling complexes requires Src tyrosine kinase.

Although these studies identify an essential role for the ShcA-SH2 domain in promoting mammary tumorigenesis from PTB-independent signaling complexes, they do not demonstrate a causal role for Src in this phenotype. To test this, we ectopically expressed $ShcA^{WT}$ and PTB^{MUT} alleles in ErbB2 transformed mammary tumors established from transgenic mice lacking Src (NIC/$Src^{-/-}$) in the mammary epithelial compartment (Figure 6A) [399] . As previously described, PTB-

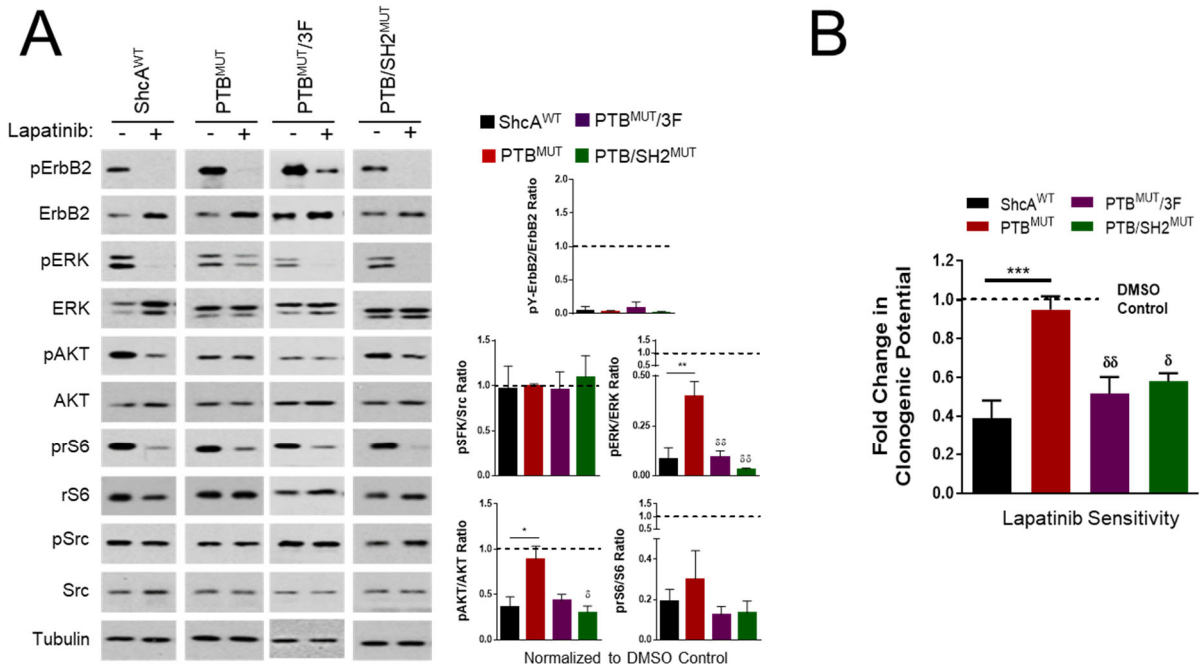


Figure 5: PTB-independent ShcA pools rely on both the SH2 domain and tyrosine phosphorylation sites to mediate Lapatinib resistance. (A) Immunoblot analysis of indicated cell lines confirming ErbB2 activity inhibition using vehicle alone (DMSO) or sub-optimal doses of Lapatinib (0.5 μM) for seven hours. Whole cell lysates were characterized by immunoblot analysis with indicated antibodies. Densitometric quantification of the immunoblots was performed using Image J software. The data is representative of three independent experiments (means ± SEM). ShcA^{WT} vs. PTB^{MUT}: *$P<0.05$, **$P<0.01$. PTB^{MUT} vs PTB^{MUT}/3F or PTB/SH2^{MUT}: $^{\delta}P<0.05$, $^{\delta\delta}P<0.01$. **(B)** Clonogenic assays of specified cell lines treated with DMSO and Lapatinib (0.5 μM). The data is depicted as a fold change in viability relative to DMSO control and is representative of three independent experiments (means ± SEM). ShcA^{WT} vs PTB^{MUT}: ***$P<0.001$; PTB^{MUT} vs PTB^{MUT}/3F or PTB/SH2^{MUT}: $^{\delta}P<0.05$, $^{\delta\delta}P<0.01$.

independent ShcA pools were significantly hypo-phosphorylated relative to ShcA^{WT} controls in Src-deficient cells (Figure 6B). In the absence of Src, mammary tumor onset and growth were comparable between ShcA^{WT} and PTB^{MUT} expressing cells, *in vivo* (Figure 6C). Additionally, there was no appreciable change in number or average area of foci in soft agar, *in vitro* (Figure 6D). We next performed IHC analyses of endpoint tumors and found that pY416-SFK levels were comparable between ShcA^{WT} and PTB^{MUT} expressing mammary tumors (Figure 6E). Moreover, IHC staining further revealed that both Src-deficient ShcA^{WT} and PTB^{MUT} breast tumors displayed comparable rates of proliferation and apoptosis (Figures 6E). These results suggest that the ability of PTB-independent ShcA pools to promote mammary tumorigenesis required Src. Alternatively, pS240/244-rS6 levels in ShcA-PTB^{MUT}-expressing mammary tumors were reduced (Figure 6E), which did not correlate with their tumorigenic potential (Figure 2A). This confirms our previous observation that mTOR signaling is not likely a major driver of mitogenic signals downstream of PTB-independent ShcA complexes.

Given that mammary tumors derived from NIC/Src$^{-/-}$ mice emerged in the absence of Src, it is possible that they significantly re-wired their kinome in response to Src loss. To confirm that these adaptive mechanisms did not account for the inability of PTB-uncoupled ShcA complexes to augment mammary tumor growth in a Src-deficient background, we used CRISPR/Cas9 genome editing to delete Src (Src-CR) from ShcA^{WT} and PTB^{MUT}-expressing, ErbB2-transformed (ErbB2/Src$^{+/+}$) breast cancer cells (Figure 7A). While Src was dispensable for the growth of ShcA^{WT} mammary tumors (Figure 7B), Src deletion impaired the ability of PTB-independent ShcA pools (PTB^{MUT}) to promote mammary tumorigenesis (Figure 7C). IHC analysis of pY416-SFK confirmed that both ShcA^{WT}/Src-CR and PTB^{MUT}/Src-CR tumors were debilitated in SFK activation relative to their respective vector controls (Figures 7D). Diminished tumor growth in PTB^{MUT}/Src-CR tumors was also associated with reduced pS240/244-rS6 levels while Src was dispensable for rS6 phosphorylation in ShcA^{WT} mammary tumors (Figures 7E). Moreover, at the experimental endpoint, proliferation and apoptosis were comparable in all groups, irrespective of the presence or absence of Src (Figure 7F-G). Thus, using two independent model systems, we show that loss of Src signaling from PTB-independent ShcA pools debilitated mTOR signaling (Figure 6E, 7E).

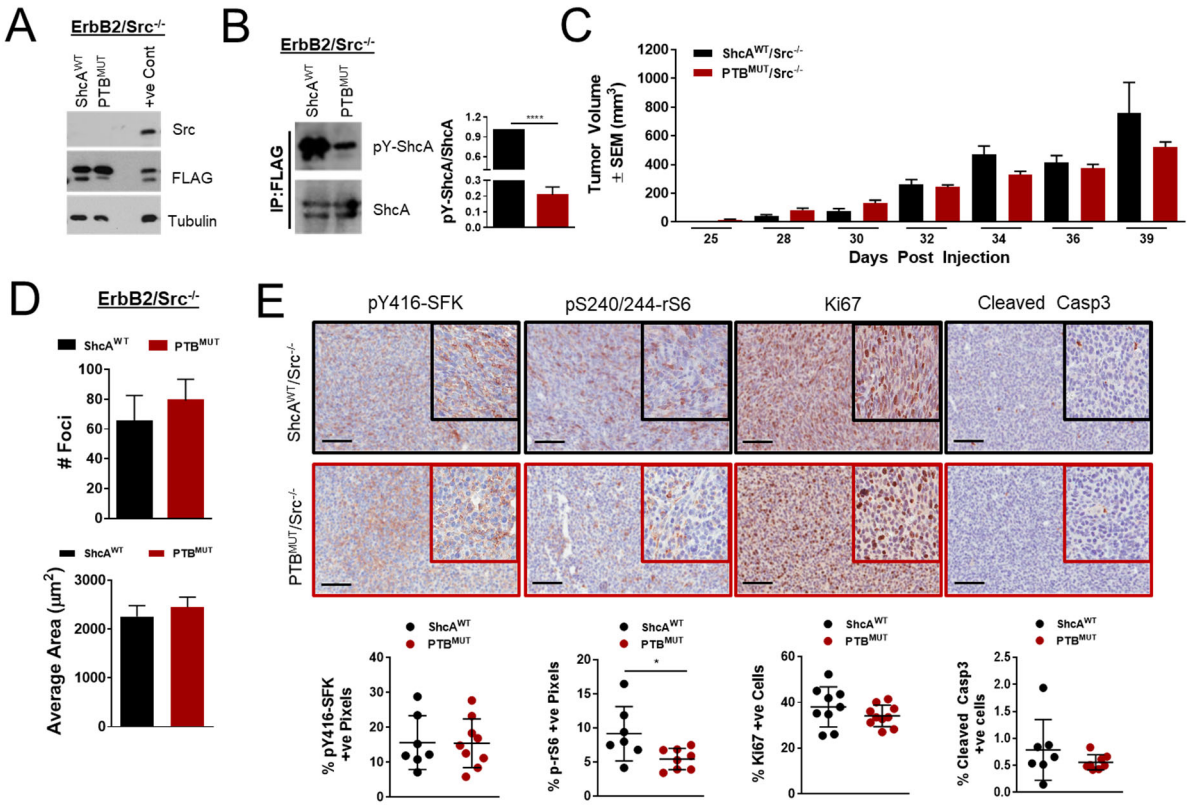
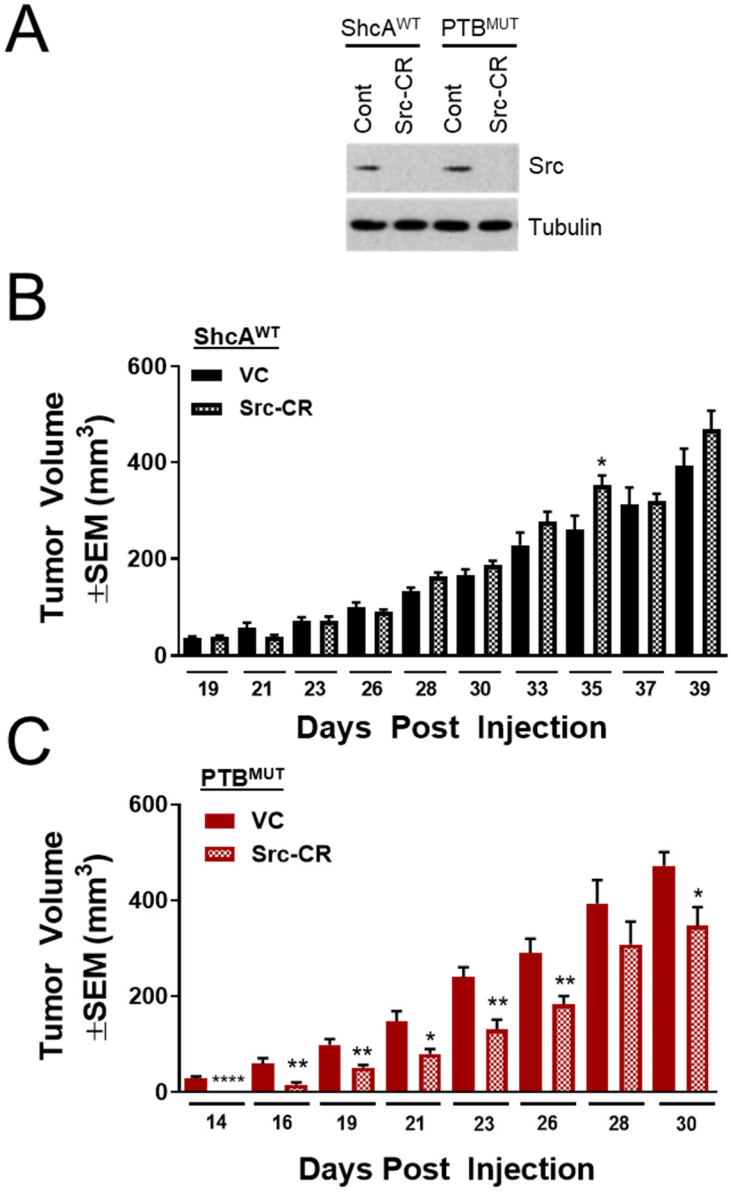


Figure 6: PTB-independent ShcA pools cannot accelerate tumor growth in the absence of Src. (A) NIC/Src$^{-/-}$ (ErbB2/Src$^{-/-}$) mammary tumor cells were engineered to express FLAG-tagged ShcA^{WT} or PTB^{MUT} alleles. **(B)** FLAG immunoprecipitates from indicated cell lines were probed with pY239/240-ShcA or ShcA specific antibodies via immunoblot analysis. The barplot represents densitometric quantification of three independent experiments (means ± SEM) using Image J software. ****$P<0.0001$. **(C)** Mammary fat pad injection of indicated cell lines into immunodeficient mice. The data is shown as average tumor volume (mm^3) ± SEM and is representative of 10 tumors per group. **(D)** Number and average area of foci formed of ErbB2/ Src$^{-/-}$ ShcA and PTB^{MUT} cells in a soft agar assay. The data is representative of three independent experiments (means ± SEM). **(E)** Immunohistochemical staining of mammary tumors using pY416-SFK, pS240/244-rS6**,** Ki67, and cleaved Casp3 specific antibodies. The data depicts the average positively stained cells or pixels ± SEM and is representative of 7-9 tumors per group. *$P<0.05$. Scale bar = 50 microns.


A
ShcA^WT
PTB^MUT
Cont
Src-CR
Cont
Src-CR
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Tubulin
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VC
Src-CR
Tumor Volume ±SEM (mm^3)
600
400
200
0
19
21
23
26
28
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Days Post Injection
C
PTB^MUT
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Tumor Volume ±SEM (mm^3)
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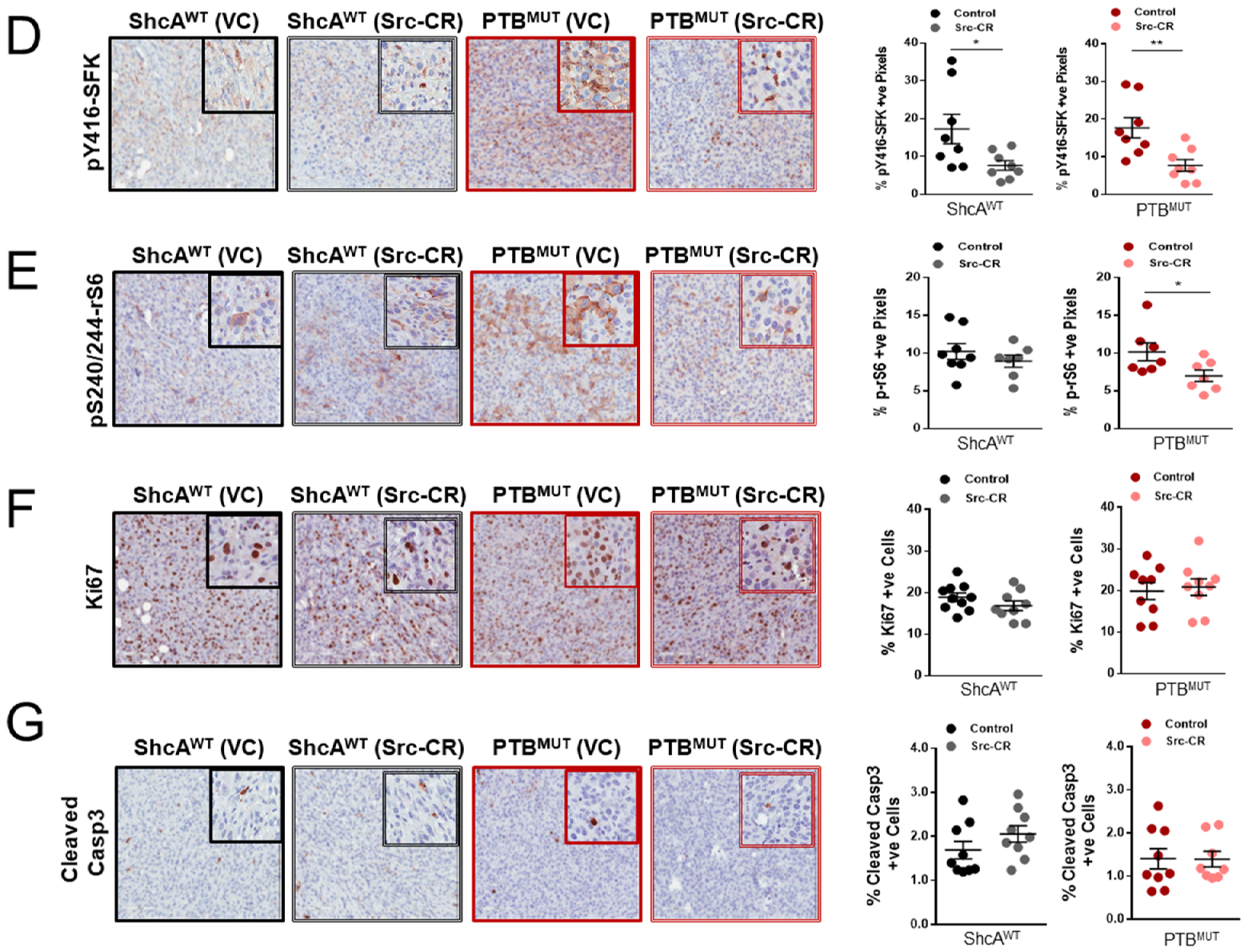
D
ShcA^WT (VC)
ShcA^WT (Src-CR)
PTB^MUT (VC)
PTB^MUT (Src-CR)
pY416-SFK
Control
Src-CR
% pY416-SFK +ve Pixels
ShcA^WT
PTB^MUT
E
pS240/244-rS6
% p-rS6 +ve Pixels
F
Ki67
% Ki67 +ve Cells
G
Cleaved Casp3
% Cleaved Casp3 +ve Cells

Figure 7: PTB-independent ShcA pools require Src to promote mammary tumorigenesis. (A) Src was deleted from ErbB2 mammary epithelial cell line (NMuMG-NeuNT) from the indicated ShcA expressing cell lines using CRISPR/Cas9 genomic editing. Immunoblot analysis of vector control and Src-CRISPR (CR) cell lines using Src- and Tubulin-specific antibodies. Vector control and Src-CR of NMuMG-NeuNT **(B)** $ShcA^{WT}$ and **(C)** PTB^{MUT} cells lines were injected into the mammary fat pads of immunodeficient mice. The data depicts the average tumor volumes (mm^3) ± SEM and is representative of 9-10 tumors per group. **P<0.05, **P<0.01, ****P<0.0001*. Immunohistochemical staining of **(D)** pY416-Src, **(E)** pS240/244-rS6, **(F)** Ki67, and **(G)** cleaved Casp3 specific antibodies in $ShcA^{WT}$ and PTB^{MUT} expressing NMuMG-NeuNT mammary tumors at endpoint. The data depicts the average positively stained cells or pixels ± SEM is representative of 8-10 tumors per group. **P<0.05, **P<0.01*.

3.1.8 PTB-independent signaling complexes become co-dependent on mitogenic signals emanating from both ErbB2 and alternative SFKs in the absence of Src.

Given that SFK activity was required to phosphorylate the ShcA tyrosine phosphorylation sites from PTB-independent ShcA pools (Figure 4A), we next assessed whether Src deletion would impact the level of ShcA tyrosine phosphorylation. Tyrosine phosphorylation of PTB-dependent ShcA signaling complexes was unaffected by Src deletion ($ShcA^{WT}$ vs. $ShcA^{WT}$/Src-CR) (Figure 8A). On the other hand, Src deletion severely reduced, but did not ablate, ShcA tyrosine phosphorylation from PTB-independent complexes (PTB^{MUT} vs. PTB^{MUT}/Src-CR) (Figure 8B). These data further indicated that Src, and potentially other SFKs selectively promote ShcA tyrosine phosphorylation in PTB-independent signaling complexes.

We previously established that increased SH2-driven ShcA signaling from PTB-independent complexes contribute to lapatinib resistance, presumably through increased Src activation (Figure 5B). Thus, we next examined whether Src deficiency impacted lapatinib sensitivity in mammary tumors that can (PTB^{MUT}) or cannot ($ShcA^{WT}$) activate ShcA signaling from PTB-independent complexes. As expected, Src deficiency had no impact on the sensitivity of $ShcA^{WT}$ tumors to lapatinib (Figure 8C) but profoundly sensitized PTB^{MUT}-expressing tumors to this ErbB2 inhibitor (Figure 8D). This suggests that in the absence of Src, tumors with increased signaling from PTB-independent ShcA complexes are more reliant on canonical ErbB2 signaling to transduce mitogenic stimuli. On the other hand, Src deficiency minimally impacted sensitivity to PP2, both in $ShcA^{WT}$- and PTB^{MUT}-expressing cells (Figure 8C-D), indicating that Src is likely the primary SFK that transduce mitogenic signals in ErbB2-transformed breast cancer cells *in vitro*. Additionally, while Src deficiency resulted in a 3-fold increase in the sensitivity of PTB^{MUT}-expressing cells to lapatinib treatment, co-incubation with lapatinib and PP2 resulted in a 22-fold decrease in cell viability (Figure 8D). This was contrasted by $ShcA^{WT}$ expressing breast cancer cells in which the effect of the Lapatinib/PP2 combination treatment was comparable, irrespective of Src status (Figure 8C). These results suggest that in the absence of Src, breast tumors which activate PTB-independent signaling complexes become co-dependent on mitogenic signals emanating both from ErbB2 and other SFKs. Thus far, our data demonstrates that PTB-dependent ShcA signaling complexes ($ShcA^{WT}$) not only serve to initiate mammary tumorigenesis downstream of ErbB2, but also to dampen tumor growth through interactions with negative

regulator(s) of mitogenic signaling. Moreover, co-expression of a ShcA allele lacking a functional PTB domain leads to the hyperactivation of mitogenic signaling from PTB-independent ShcA pools and the consequent acceleration of tumor growth (PTB^{MUT}) [2]. We now establish that these PTB-independent ShcA pools rely heavily on the ShcA SH2 domain and downstream Src signaling to potentiate mammary tumorigenesis.

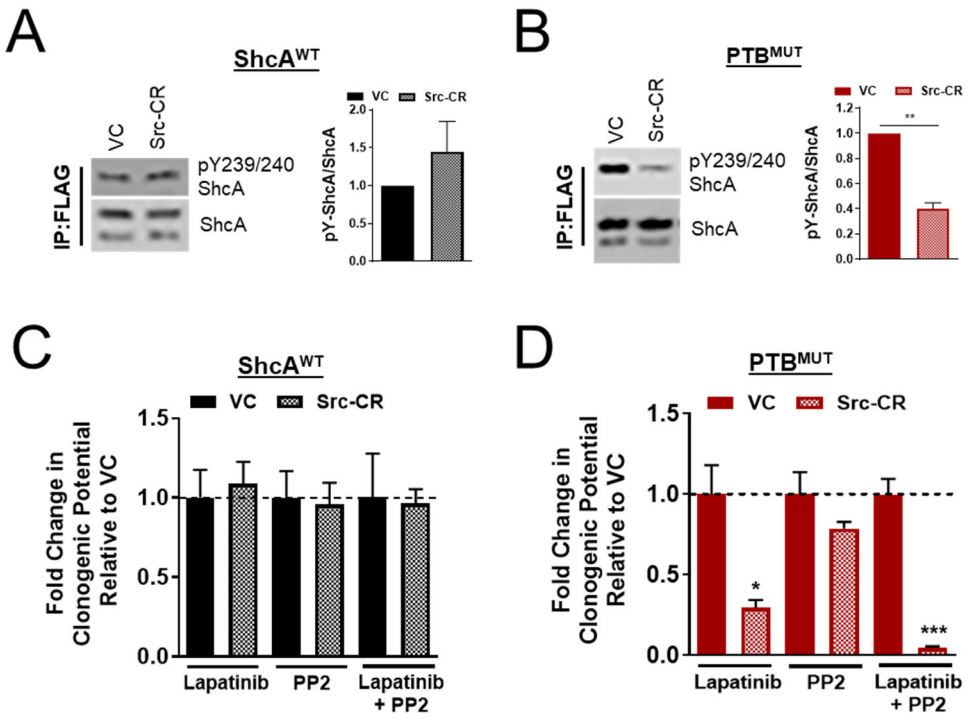

Figure 8: PTB-independent signaling complexes become co-dependent on mitogenic signals emanating both from ErbB2 and alternative SFKs in the absence of Src. FLAG immunoprecipitates from vector control (VC) and **(A)** $ShcA^{WT}$/Src-CR or **(B)** PTB^{MUT}/Src-CR were probed with pY239/240-ShcA or ShcA-specific antibodies via immunoblot analysis. The graph represents densitometric quantification of three independent experiments (means ± SEM) using Image J software. ***P<0.01*. Clonogenic assay of vector control (VC) and **(C)** $ShcA^{WT}$/Src-CR or **(D)** PTB^{MUT}/Src-CR treated with DMSO, Lapatinib (0.5 μM), PP2 (2 μM) alone, or in combination. The data is shown as fold change in viability relative to DMSO control and is representative of three independent experiments (means ± SEM). **P<0.05,* ****P<0.001*.

3.1.9 Fyn cooperates with Src to increase the tumorigenic potential of PTB-independent ShcA signaling complexes.

To assess the importance of other SFKs downstream of PTB-independent ShcA complexes, we used CRISPR/Cas9 genomic editing to delete Fyn (Fyn-CR) or Lyn (Lyn-CR) from ErbB2-transformed breast cancer cells (Figure 9A-B). We did not observe a compensatory increase in Src levels when either Fyn or Lyn were deleted in cells that can (PTB^{MUT}) or cannot ($ShcA^{WT}$) engage these PTB-uncoupled ShcA pools. By the same token, Src deletion did not appreciably alter Fyn or Lyn expression levels in these cells (Figure 9A-B). Moreover, while pY416-SFK and pY576/7-FAK levels (Src specific phosphorylation site) were significantly reduced in $ShcA^{WT}$/Src-CR and PTB^{MUT}/Src-CR expressing cells, SFK activation was unaltered by Fyn or Lyn loss in either cell line (Figure 9A-B). This suggests that Fyn and Lyn may play secondary roles in supporting the ability of PTB-independent ShcA pools to promote tumor growth. To test this, we examined the transformative potential of Src-, Fyn-, or Lyn-deficient breast cancer cells in a soft agar assay. In support of our *in vivo* study (Figure 7B), Src deletion impaired tumor initiation (number of foci) in breast cancer cells that retained a functional PTB domain ($ShcA^{WT}$) but was dispensable for tumor growth (average area of foci) (Figure 10A-B). In contrast, neither Fyn nor Lyn deletion appreciably altered the transforming potential of $ShcA^{WT}$ breast cancer cells (Figure 10A-B). In contrast, Src loss in breast cancer cells that hyperactivate PTB-independent ShcA complexes (PTB^{MUT}) significantly reduced both the number and size of foci formed (Figure 10C-D). This validated our *in vivo* observations (Figures 6 and 7C), where Src was necessary for the ability of these PTB-independent ShcA pools to augment mammary tumor growth. However, Fyn deletion in PTB^{MUT} expressing breast cancer cells did not impact foci formation but reduced their growth potential (~2 fold) relative to vector controls. Lyn, on the other hand, was dispensable for the transforming potential of ErbB2-driven breast tumors, irrespective of whether they had engaged PTB-independent ShcA complexes (Figure 10C-D). These data suggest that Src cooperates with Fyn downstream of PTB-independent ShcA pools to increase the tumorigenic potential of ErbB2+ breast cancers. To test this, we assessed how Src deletion, in combination with, SFK inhibition (2 μM PP2) impacted the tumorigenic potential of $ShcA^{WT}$ and PTB^{MUT} expressing cells. We first confirmed that SFK inhibition was achieved using suboptimal doses of PP2 (2 μM) (Figure 11A). As expected, SFK inhibition had minimal impact on the number or size of foci formed from beast

cancer cells that cannot amplify signaling downstream from PTB-independent ShcA pools ($ShcA^{WT}$) (Figure 11B). Similarly, Src deletion did not appreciably alter the tumorigenic potential of $ShcA^{WT}$ expressing cells but sensitized them to pharmacological SFK inhibition (Figure 11B). These data further support the observation that breast cancer cells preferentially rely on ErbB2/ShcA signaling complexes and are less dependent on SFKs. In this regard, we previously established that the ErbB2/ShcA signaling axis predominately activates AKT/mTOR signaling to promote mammary tumorigenesis [395]. Consistent with this observation, $ShcA^{WT}$ breast tumors were exquisitely sensitive to sub-optimal doses of mTOR inhibitor, Torin1 (50nM) (Figure 11A) in a soft agar assay (Figure 11B). Torin1 significantly reduced both the number and size of foci formed in these cells, independent of Src (Figure 11B). Moreover, the tumorigenic potential of breast cancer cells engaging PTB-independent ShcA signaling complexes (PTB^{MUT}) were similarly sensitive to Torin1 (Figure 11C). This highlights an important role for mTOR signaling in ErbB2+ breast cancers, irrespective of whether they transduce signals from PTB-independent ShcA pools. However, sub-optimal doses of SFK inhibitor, PP2, had no impact on the tumor forming potential of PTB^{MUT} expressing cells but did attenuate the growth of these foci (Figure 11C). In contrast, Src deletion, was sufficient to decrease both the number and size of foci formed by PTB^{MUT} expressing cells (Figure 10C-D) and further sensitized them to SFK inhibition by PP2 (Figure 11C). These data suggest that Src is principally responsible for the increased tumorigenic potential induced by PTB-independent ShcA complexes but that other SFKs, including Fyn play a cooperative role in this process.

3.1.10 Summary

Collectively, we show that PTB-independent complexes serve to augment mammary tumorigenesis by increasing the activity of the Src and Fyn tyrosine kinases through the SH2 domain. Surprisingly, the ShcA tyrosine phosphorylation sites are dispensable for the ability of these PTB-independent ShcA pools to amplify tumor growth, *in vivo*. Finally, we establish that increased Src activation downstream from ShcA PTB-independent signaling complexes increases resistance to Lapatinib, a dual EGFR/ErbB2 inhibitor. Taken together, these results provide the first evidence that the ShcA adaptor protein is a critical convergence point downstream of numerous tyrosine kinases and that perturbation of discrete ShcA-dependent signaling complexes significantly impacts breast tumor growth and therapeutic responsiveness.

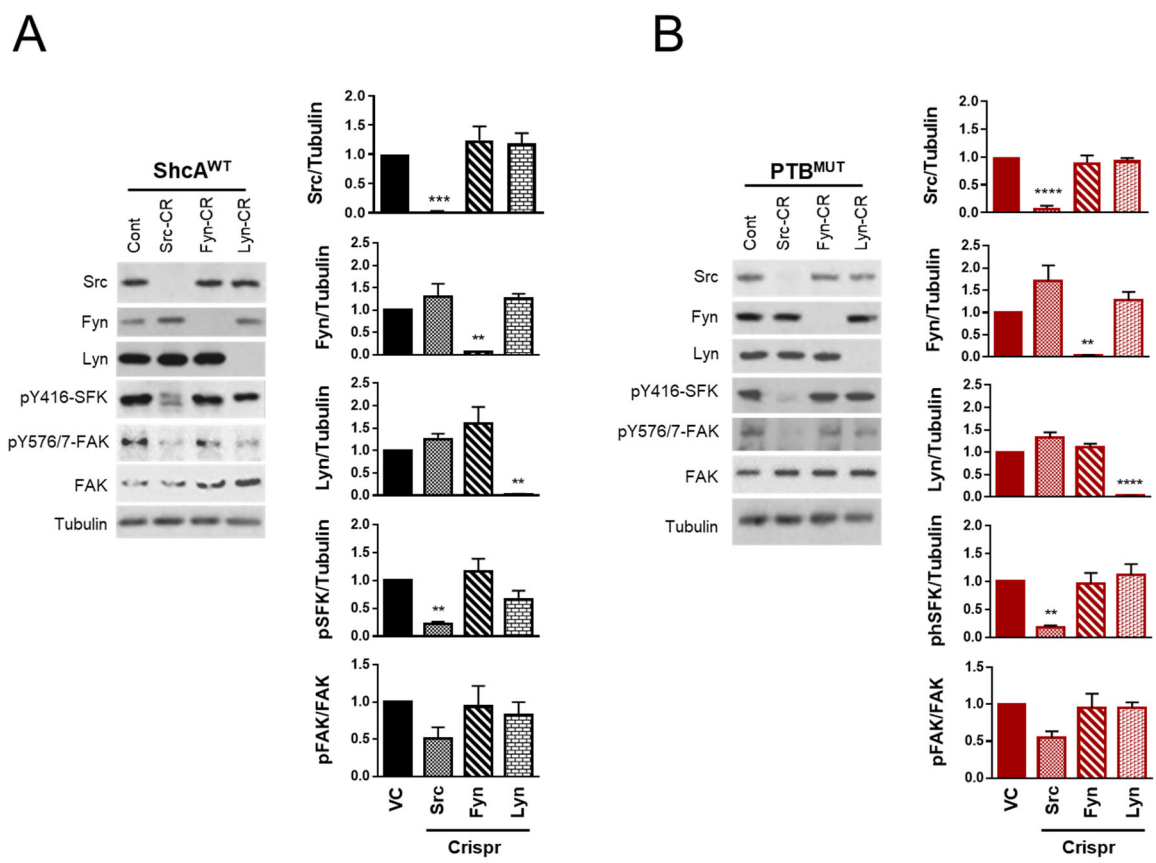

Figure 9: Immunoblot blot analysis of whole cell lysates from **(A)** ShcA^{WT} and **(B)** PTB^{MUT} cell lines upon deletion of Src, Fyn or Lyn using CRISPR/Cas9 genomic editing (Src-CR, Fyn-CR, Lyn-CR). Densitometric quantification was performed using Image J software. The barplots are representative of three independent experiments (means ± SEM). ***P<0.01, ****P<0.001, *****P<0.0001*.

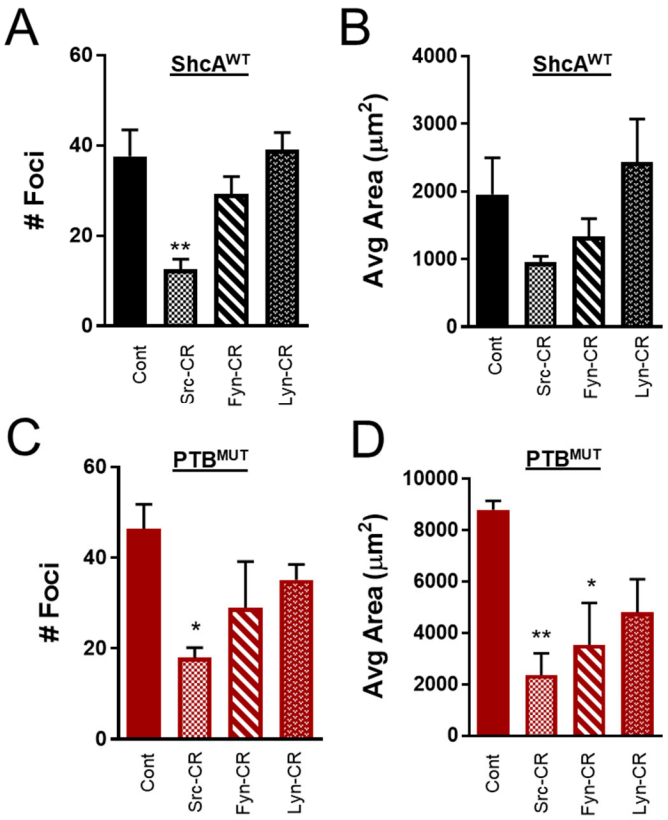

Figure 10: Fyn cooperates with Src to increase the tumorigenic potential of PTB-independent ShcA signaling complexes. (A, C) Number and **(B, D)** average area of foci formed in a soft agar assay from specified cell lines. The data is representative of three independent experiments (means ± SEM). **P<0.05, **P<0.01*.

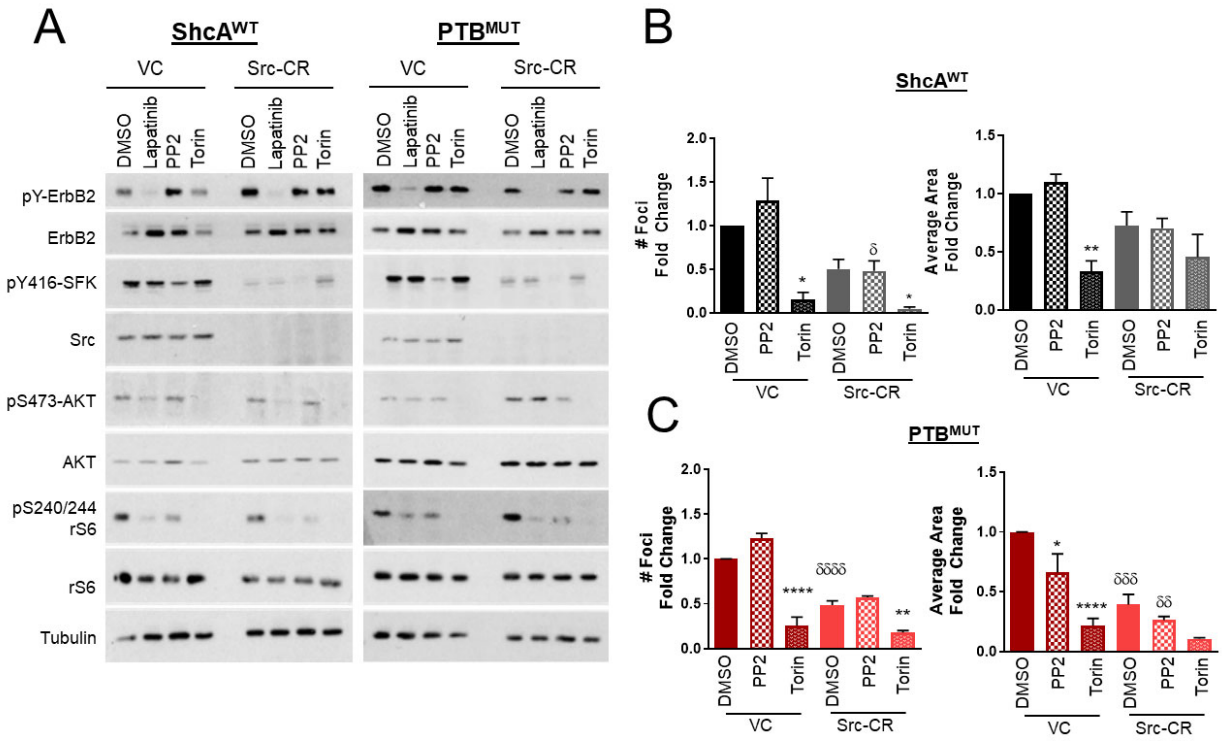

Figure 11: Breast tumors that engage PTB-dependent or -independent pools are sensitive to mTOR inhibition. (A) Immunoblot analysis of whole cell lysates of vector control (VC) and ShcA^{WT}/Src-CR or PTB^{MUT}/Src-CR treated with either vehicle alone (DMSO) and Lapatinib (0.5 μM), PP2 (2 μM), or Torin1 (50 nM). The fold change in number and average area of foci formed in soft agar of vector control (VC) and **(B)** ShcA^{WT}/Src-CR or **(C)** PTB^{MUT}/Src-CR relative to DMSO controls in the absence (DMSO) or presence of PP2 (2 μM) or Torin1 (50 nM). The data is representative of three independent experiments (means ± SEM). DMSO vs PP2 or Torin1 treatment: *$P<0.05$, **$P<0.01$, ****$P<0.0001$. VC versus Src-CR: $^{\delta}P<0.05$, $^{\delta\delta}P<0.01$, $^{\delta\delta\delta}P<0.001$, $^{\delta\delta\delta\delta}P<0.0001$.

3.1.11 PTB-independent ShcA signaling complexes rely on the ShcA SH2 domain to activate Src signaling to augment mammary tumor growth.

It is widely accepted that the ShcA PTB domain, which binds NPXY motifs, controls the fate of ShcA-coupled signaling complexes downstream of RTKs. However, an underappreciated, yet equally important aspect of ShcA dependent signaling arises from its SH2 domain. Recall that we establish that mutant ShcA allele lacking both a functional PTB domain and the tyrosine phosphorylation sites (PTB^{MUT}/3F) can still engage ShcBP1, an SH2-specific ShcA interactor, and robustly potentiate tumor growth *in vivo*. Furthermore, the ShcA SH2 domain has been found to be critical for breast cancer outgrowth and survival through the activation of the AKT pathway [358]. However, we also demonstrate ErbB2+ breast cancer cells that have disengaged from both PTB and SH2 coupled signaling complexes (PTB/$SH2^{MUT}$) still have the propensity for tumor initiation and outgrowth, despite the long latency (Figure 2A). Thus far, our data suggests that breast tumors require a functional ShcA SH2 domain to activate Src downstream PTB-independent ShcA signaling complexes (Figure 3A). Given that Src deficiency reduces tumor growth mediated by these PTB-uncoupled ShcA pools (Figures 6-7), we next interrogated the impact of Src loss in breast tumors that lack a functional SH2 domain from tumor-amplifying ShcA complexes (PTB/$SH2^{MUT}$).

3.1.11.1 PTB-independent complexes require the SH2 domain to engage Fyn for breast tumor growth upon the loss of Src.

We first ectopically expressed PTB/$SH2^{MUT}$ alleles in Src deficient (NIC/$Src^{-/-}$ and ErbB2/Src-CR) cells and assessed mammary tumor growth *in vivo* (Figure 12A-B). The loss of SH2-driven ShcA signaling did not ablate the ability of NIC/$Src^{-/-}$ breast cancer cells to form tumors *in vivo* (Figure 12B). Moreover, while a non-functional SH2 domain from these PTB-independent ShcA pools impaired the growth potential of ErbB2+ breast tumors (Figure 2A), Src deletion (Src-CR) surprisingly accelerated tumor growth (Figure 12D). This indicated that in the absence of Src and SH2 signaling from these PTB-uncoupled ShcA pools, secondary adaptive responses are likely required to facilitate breast tumor growth. Given that Fyn cooperates with Src to amplify the tumorigenic potential of PTB-independent signaling complexes (Figures 10), we interrogated whether simultaneous Src deficiency and a lack of SH2-driven ShcA signaling from these

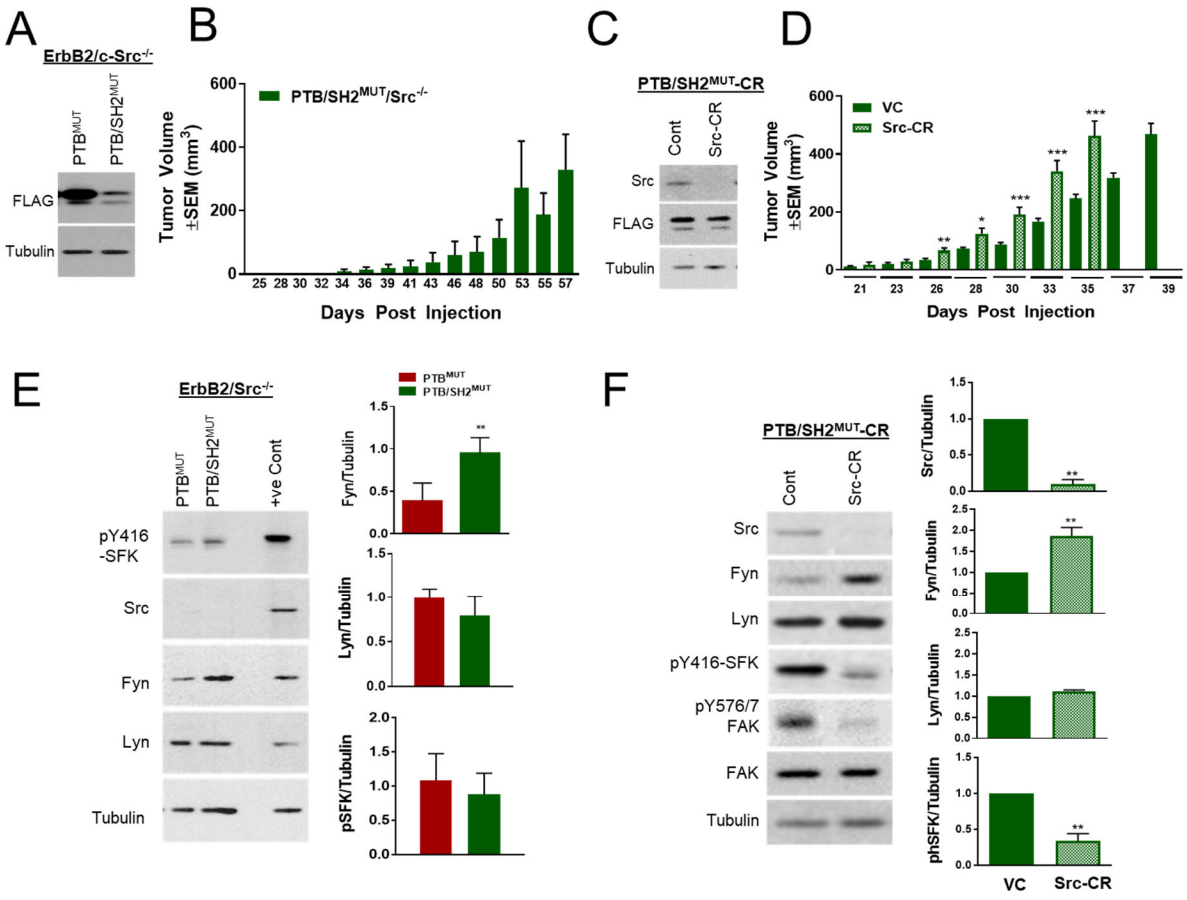

Figure 12: PTB-independent complexes require the SH2 domain to engage Fyn for breast tumor growth upon the loss of Src. (A) Immunoblot characterization of FLAG-tagged PTB/SH2^{MUT} ShcA expression in NIC/Src$^{-/-}$ (ErbB2/Src$^{-/-}$) mammary tumor cells. **(B)** Mammary fat pad injection of NIC/Src$^{-/-}$ PTB/SH2^{MUT} expressing cell lines into immunodeficient mice. The data is shown as an average tumor volume (mm^3) ± SEM and is representative of 10 tumors per group. **(C)** Immunoblot blot analysis of whole cell lysates characterizing the expression of FLAG-tagged PTB/SH2^{MUT} cell lines upon deletion of Src using CRISPR/Cas9 genomic editing. **(D)** Vector control (VC) and Src-CR of NMuMG-NeuNT PTB/SH2^{MUT} expressing cells lines injected into the mammary fat pads of immunodeficient mice. The data depicts the average tumor volumes (mm^3) ± SEM and is representative of 9-10 tumors per group. *$P<0.05$, **$P<0.01$, ***$P<0.001$. **(E)** Immunoblot analysis of the indicated NIC/Src$^{-/-}$ cell lines using Src, Fyn, Lyn and Tubulin specific antibodies. The positive control represents lysates from NMuMG-NeuNT cells. Densitometry was performed with Image J software. The data is representative of four independent experiments (means ± SEM). **$P<0.01$. **(F)** Immunoblot blot analysis of whole cell lysates of NMuMG-NeuNT PTB/SH2^{MUT} cells upon CRISPR/Cas9 deletion of Src with specified antibodies. The barplots are representative of three independent experiments (means ± SEM). **$P<0.01$.

complexes deregulated Fyn levels. Indeed, Src deficiency in two independent models (NIC/Src$^{-/-}$; ErbB2/Src-CR) significantly upregulated Fyn expression when PTB-independent ShcA pools could no longer signal through the SH2 domain (Figure 12E-F). Lyn expression levels, on the other hand, were minimally impacted in these cells (Figure 12E-F). Despite this fact, the compensatory increase in Fyn expression levels did not correlate with increased pY416-SFK levels in Src-deficient PTB/SH2^{MUT} cancer cells, both *in vitro* and *in vivo* (Figures 12E-F, 13A-B). This further emphasizes the requirement of Src as a key tyrosine kinase downstream of the ShcA SH2 domain in PTB-independent ShcA complexes.

3.1.11.2 Loss of Src signaling downstream of the SH2 domain in PTB-independent ShcA complexes increases the dependency of ErbB2-driven breast tumors on mTOR signaling.

To further assess the mechanism underlying this phenotype, we performed IHC analyses of endpoint tumors. Src-deficient PTB/SH2^{MUT} tumors (NIC/Src$^{-/-}$; ErbB2/Src-CR), which lack of SH2-driven signaling from PTB-independent ShcA pools (PTB/SH2^{MUT}), collectively upregulated pS240/244-rS6 levels in mammary tumors and increased the proliferative index compared to SH2-proficient controls (Figure 13A-B). Thus, in the absence of Src and a functional SH2 domain, PTB-independent ShcA pools exhibit a compensatory increase in mTOR signaling to facilitate eventual tumor growth. In addition, the hyperactivation of mTOR signaling in Src-deficient cancer cells is not associated with increased ShcA phospho-tyrosine levels compared to Src-proficient controls (Figure 14A). The activation of ShcA phospho-tyrosine residues remained unchanged regardless of the presence or absence of Src. Given that ShcA-PTB/SH2^{MUT} can retain the activation of its phospho-tyrosines in the absence of Src to comparable levels of vector control, it is yet to be determined the activating tyrosine kinase that is sustaining ShcA tyrosine phosphorylation independent of the ShcA-SH2 domain. Taken together, these observations reinforce the importance of mTOR signaling in ErbB2-driven breast cancer, particularly in response to Src inhibition, and demonstrate the significant plasticity of mammary tumors to re-program their kinomes.

Given this observed hyperactivation of mTOR signaling, we tested whether Src deficient breast tumors that lack the ability to engage PTB-independent ShcA signaling, become more reliant on

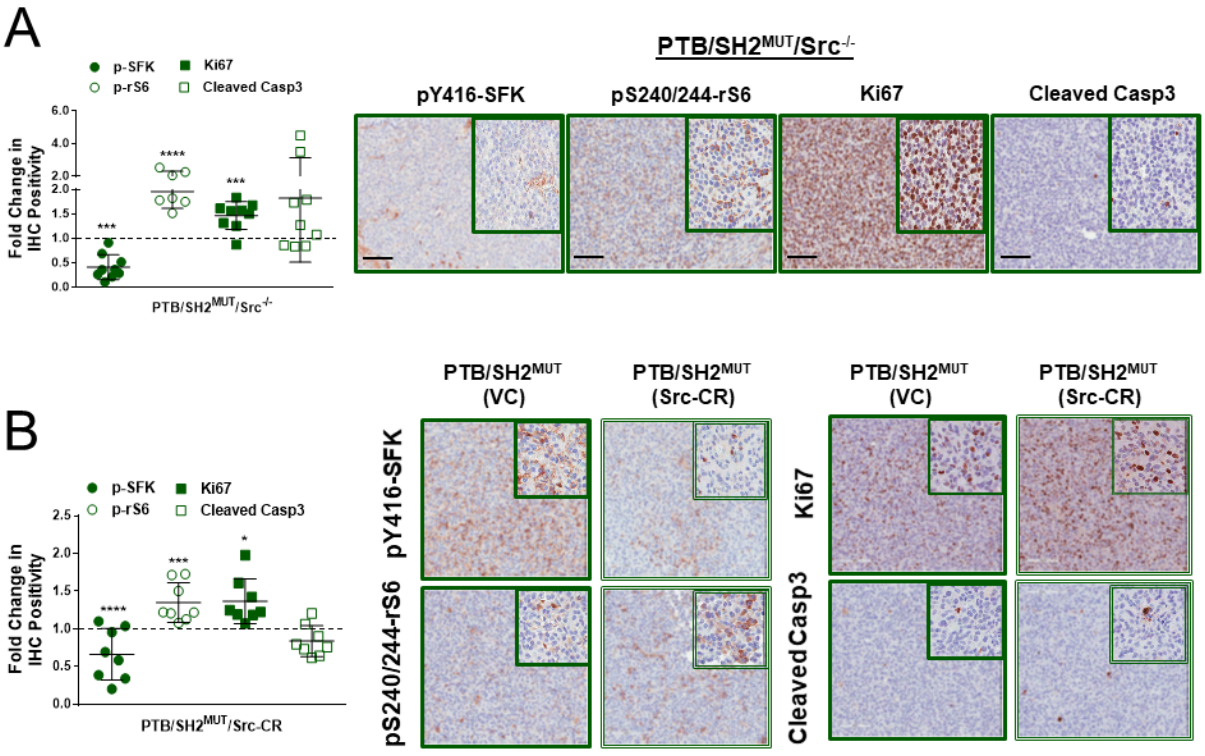

Figure 13: Loss of Src signaling downstream of the SH2 domain in PTB-independent ShcA complexes increases the dependency of ErbB2-driven breast tumors on mTOR signaling. Immunohistochemical staining of **(A)** NIC/Src$^{-/-}$ PTB/SH2^{MUT} and **(B)** vector control and Src-CR NMuMG-NeuNT PTB/SH2^{MUT} mammary tumors using pY416-SFK, Ki67, cleaved Casp3 and pS240/244-rS6 specific antibodies. The data depicts the fold change positively stained cells or pixels ± SEM and is representative of 7-9 tumors per group. *$P<0.05$, ***$P<0.001$, ****$P<0.0001$.

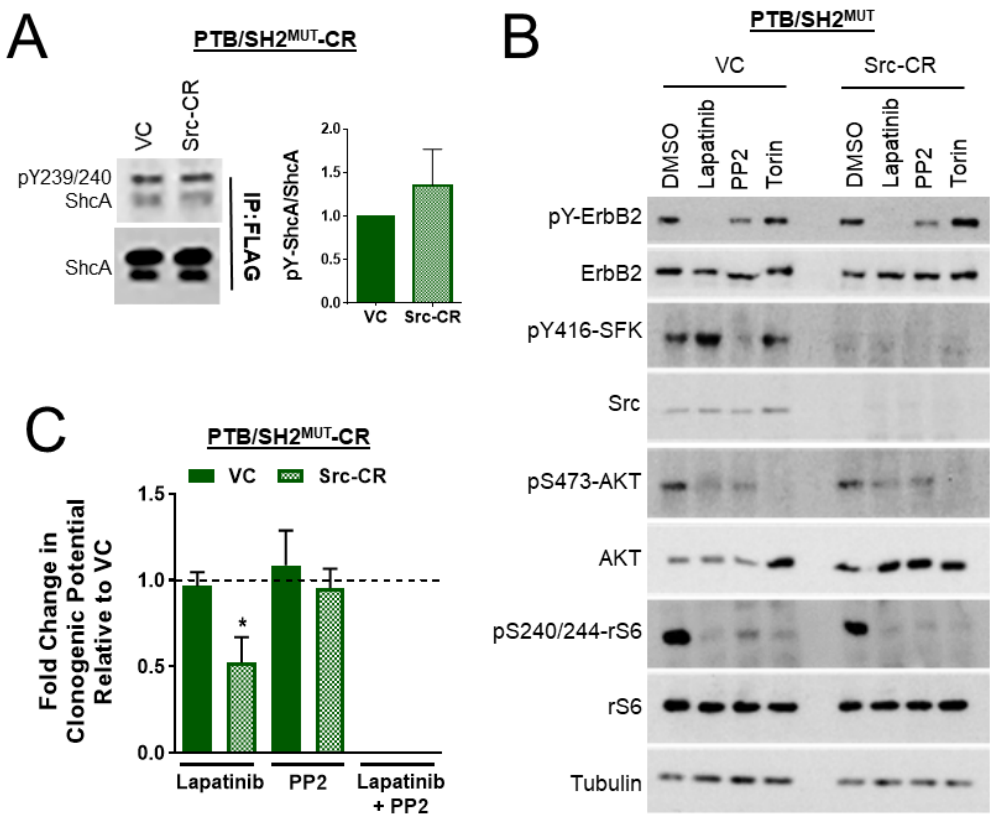

Figure 14: Hyperactivation of mTOR signaling upon the loss of Src is not associated with increased ShcA phospho-tyrosine levels. (A) FLAG immunoprecipitates from NMuMG-NeuNT PTB/SH2^{MUT} vector control (VC) and Src-CR cell lines probed with pY239/240-ShcA or ShcA specific antibodies by immunoblot analysis. The barplot represents densitometric quantification of three independent experiments (means ± SEM) using Image J software. **(B)** Whole cell lysates of PTB/SH2^{MUT} vector control (VC) and Src-CR expressing breast cancer cell lines (NMuMG-NeuNT) treated with either vehicle alone (DMSO) and Lapatinib (0.5 µM), or DMSO, PP2 (2 µM), Torin1 (50 nM), respectively were characterized by immunoblot analysis with indicated antibodies. **(C)** Clonogenic assays of specified cell lines treated with DMSO, Lapatinib (0.5 µM), PP2 (2 µM) alone, or in combination. The data is shown as fold change in viability relative to DMSO control and is representative of three independent experiments (means ± SEM). **P<0.05*.

PTB-driven ErbB2/ShcA signals. Using clonogenic assay, we treated Src proficient and deficient ErbB2+ positive cell lines to suboptimal doses of Lapatinib (0.5 μM), PP2 (2 μM), or in combination (Figure 14B). Src deletion sensitized PTB/SH2^{MUT} expressing cells to Lapatinib, demonstrating an increased reliance on ErbB2/ShcA signaling complexes (Figure 14C). The growth potential of these cells was also insensitive to SFK inhibition (PP2) (Figure 14C). This is consistent with our observations that Src is the primary kinase involved in the pro-tumorigenic growth response in PTB-independent breast tumors. However, simultaneous ErbB2 and SFK inhibition (Lapatinib and PP2) completely ablated the viability of PTB/SH2^{MUT} breast cancer cells (Figure 14C), suggesting a potential accessory role for Fyn or Lyn in Src-deficient cells. To test this, Fyn and Lyn were also stably deleted from PTB/SH2^{MUT} expressing cells using CRISPR/Cas9 genomic editing (Figure 15A). Interestingly, Fyn deletion reciprocally increased Src expression levels in PTB/SH2^{MUT} expressing cells, again highlighting the adaptive responses triggered in ErbB2-driven breast tumors to retain SFK function (Figure 15A). To address whether these perturbations in SFK levels were biologically significant, we assessed the impact of Src, Fyn or Lyn deficiency on the transforming potential of these cells in a soft agar assay. As observed in our *in vivo* study (Figure 12D), deletion of Src in PTB/SH2^{MUT} expressing breast cancer cells significantly accelerated tumor growth, but not focus formation, relative to Src proficient controls (Figure 15B-C). However, neither the loss of Fyn or Lyn significantly altered the tumorigenic potential of these PTB/SH2^{MUT} expressing cells relative to vector controls (Figure 15B-C). Thus, despite their upregulation, these SFKs do not significantly contribute to the transforming potential of mammary tumors that cannot transduce ShcA-SH2-coupled signals from PTB-independent ShcA pools (PTB/SH2^{MUT}). Rather, the observed compensatory activation of mTOR signaling likely sustained mammary tumor growth in these cells. To test this, we examined the sensitivity of both Src-proficient and Src-deficient PTB/SH2^{MUT}-expressing cells to mTOR inhibition (Torin1) (Figure 14B) in a soft agar assay (Figure 16A-B). As expected, the transforming potential of these cells was insensitive to pharmacological (PP2) or genetic (Src-CR) Src inhibition in a soft agar assay (Figure 16A-B). In contrast, loss of functional ShcA-SH2 domain from PTB-independent ShcA signaling complexes rendered these cancer cells exquisitely sensitive to Torin1, in a Src-independent manner (Figure 16A-B). These data suggest that loss of the ShcA/Src signaling axis from these ErbB2-independent pools re-programs signaling networks to increase their reliance on mTOR signaling.

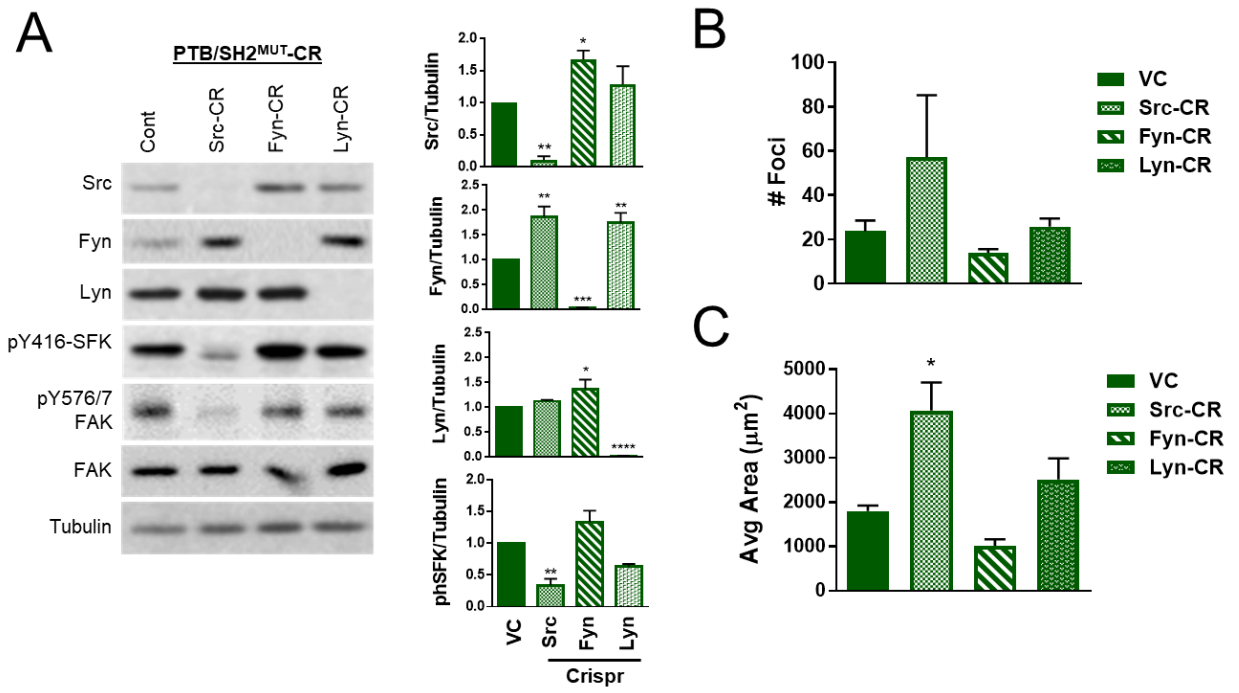

Figure 15: Fyn deletion reciprocally increases Src expression levels in PTB/SH2^{MUT} expressing cells. (A) Immunoblot blot analysis of whole cell lysates from PTB/SH2^{MUT} cell lines upon deletion of Src, Fyn or Lyn using CRISPR/Cas9 genomic editing, presented as a continuation of Figure 12F. The barplots represent densitometric quantification of three independent experiments (means ± SEM) using Image J software. **P<0.05 **P<0.01, ***P<0.001, ****P<0.0001*. **(B)** Number and **(C)** average area of foci formed in a soft agar assay from the specified cell lines. The data is representative of three independent experiments (means ± SEM). **P<0.05*.

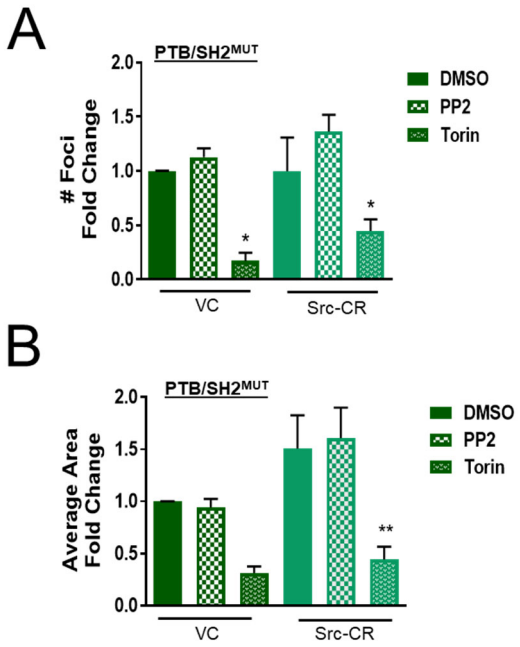

Figure 16: Activation of mTOR signaling sustains mammary tumor growth in breast tumors that have lossed a functional ShcA SH2 domain. The fold change in the **(A)** number and **(B)** average area of foci formed in soft agar of indicated cell lines relative to DMSO controls in the absence (DMSO) or presence of PP2 (2 μM) or Torin1 (50 nM). The data is representative of three independent experiments (means ± SEM). DMSO vs PP2 or Torin1 treatment: **P<0.05, **P<0.01*.

3.1.11.3 Summary

These data demonstrated that PTB-independent ShcA signaling complexes rely on the ShcA SH2 domain to activate Src signaling in order to augment mammary tumor growth. In addition, our results highlight that the loss of the ShcA/Src signaling axis from these ErbB2-independent pools re-programs signaling networks to increase their reliance on mTOR signaling.

3.1.12 A PTB^{MUT} gene signature is associated with increased mTOR and Src family kinase activation in human breast cancers.

To date, there is no evidence for any point mutations in ShcA. Therefore, our data would suggest that the loss or inactivation of genes that negatively regulate ShcA would amplify both PTB-dependent and independent ShcA signaling complexes. Corroborating our findings, clinical evidence has shown inactivating mutations within PTPN12 catalytic domains and its deletion in in human primary breast cancers [192]. Moreover, the loss of PTPN12 in luminal epithelium significantly accelerates tumor initiation, enhances tumor outgrowth and increase the plasticity of ErbB2 tumors to favour EMT like features in ErbB2 driven transgenic mouse models [196]. Further supporting our model system, patients with high levels of activated Src showed lower clinical response, a higher progressive disease and shorter overall survival rates after Trastuzumab treatment relative to patients with low activated Src [235]. Overall, Src protein levels and activity are increased 18-39% in human breast cancers compared to normal breast tissue [409]. Moreover, aberrant activation of the PI3K/AKT/mTORC1 pathway is also a mechanism of resistance correlated with shorter progression free survival in Trastuzumab treated patients [242].

To validate our experimental model, we utilized a previously generated dataset identifying 100 differentially expressed genes that distinguished tumors that did (PTB^{MUT}) or did not ($ShcA^{WT}$) augment mitogenic signals from PTB-independent ShcA complexes. Specifically, 36 differentially expressed genes (> 2.5-fold differentially expressed – Table 1) [2] were used to stratify 1218 tumors human breast cancers using the publicly available The Cancer Genome Atlas (TCGA) RNA-seq dataset.

Gene Symbol	Fold Change (PTB^{MUT} vs $ShcA^{WT}$)
Csf2rb	6.522660545
Gpnmb	5.475667086
Mgp	5.393142522
Csf2rb2	3.895808321
Pde4a	3.876175063
Ramp3	3.530536214
Ccl5	3.525238438
Mmp2	3.51726029
Slc6a15	3.318082851
Itgbl1	3.314886845
Serpina3m	3.150817458
Dab2	3.003560992
Gem	2.998507309
Ntrk3	2.970200127
Klhl30	2.90018819
Gpr30	2.889246652
Gm8995	2.884281968
Fgfr1	2.857278378
Smoc2	2.829555388
Ncf4	2.810290864
Grem1	2.765065609
Aldoc	2.691214764
Pappa2	2.690323035
Ccl7	2.658461672
Xlr4b	2.650232582
Gm10439	2.62439132
Rsad2	2.624247622
Angpt1	2.59225709
Sgcd	2.587565476
Cxcl16	2.576341126
Phex	2.574882893
Chrnd	2.572310432
Tns1	2.572267247
Amhr2	2.533613446
Gpr146	2.511758817
Ifitm3	2.507990489

Table 1: List of 36 differentially expressed genes (>2.5 fold relative to $ShcA^{WT}$ gene signature) comprising the ShcA-PTB^{MUT}-like gene signature (GEO GSE41718).

We performed single sample gene set enrichment analysis (ssGSEA), a computational method that calculates the absolute degree of enrichment of a gene set in individual samples [401]. Breast cancer patients were stratified into four quartiles based on the degree to which they most resemble a PTB^{MUT} gene signature (quartile 1: most $ShcA^{WT}$-like/PTB-dependent vs. quartile 4: most PTB^{MUT}-like/PTB-independent). These signatures represented gene expression changes that are correlated with increased signaling from PTB-independent ShcA pools. We first examined relative Src, Fyn and Lyn transcript levels within each quartile. We show that the acquisition of a PTB^{MUT}-like gene signature is associated with a modest but significant increase in Src mRNA levels (1.14-fold increase between 1^{st} and 4^{th} quartiles). In contrast Fyn, and Lyn mRNA levels are much more significantly increased in PTB^{MUT}-like human breast tumors compared to those that are most like $ShcA^{WT}$-like breast tumors (1.37-fold and 1.47-fold increase respectively between 1^{st} and 4^{th} quartiles) (Figure 17A). We also took advantage of the fact that the TCGA database contains RPPA data for 747 of these patients (n=171:1^{st} quartile; n=164: 2^{nd} quartile=; n=196: 3^{rd} quartile=; n=216 4^{th} quartile). We demonstrate that PTB^{MUT}-like breast tumors display significantly reduced pY317-ShcA levels coincident with elevated pY416-Src and pS235/6-rS6 levels compared to $ShcA^{WT}$-like breast tumors (Figure 17B). These results support a clinically-relevant role for PTB-independent ShcA complexes in hyperactivating Src and mTOR signaling, independently of the ShcA tyrosine phosphorylation sites, to increase the tumorigenic potential of human breast cancers.

3.1.12.1 PTB^{MUT} gene signature is associated with the loss of PTPN12 in human breast cancers.

Finally, as previously described, several negative regulators, particularly PTPN12 and SHIP2, have been shown to bind the PTB domain of ShcA to dampen ShcA-driven mitogenic signaling [3, 4, 7, 321, 385]. Copy number analyses recently showed that the ShcA gene, residing within the genomic region 1q21-1q23, which is amplified in a subset of human breast cancers [388]. Given that we could identify a subset of breast tumors that coordinately overexpress genes associated with a PTB^{MUT}-like signature alongside paralleled increases in SFK expression and activation (Figure 17A-B), we reasoned that these cancers were able to hyperactivate PTB-independent ShcA signaling complexes, either through genomic amplification of the ShcA gene and/or genomic loss of one or more PTB-dependent negative regulators.

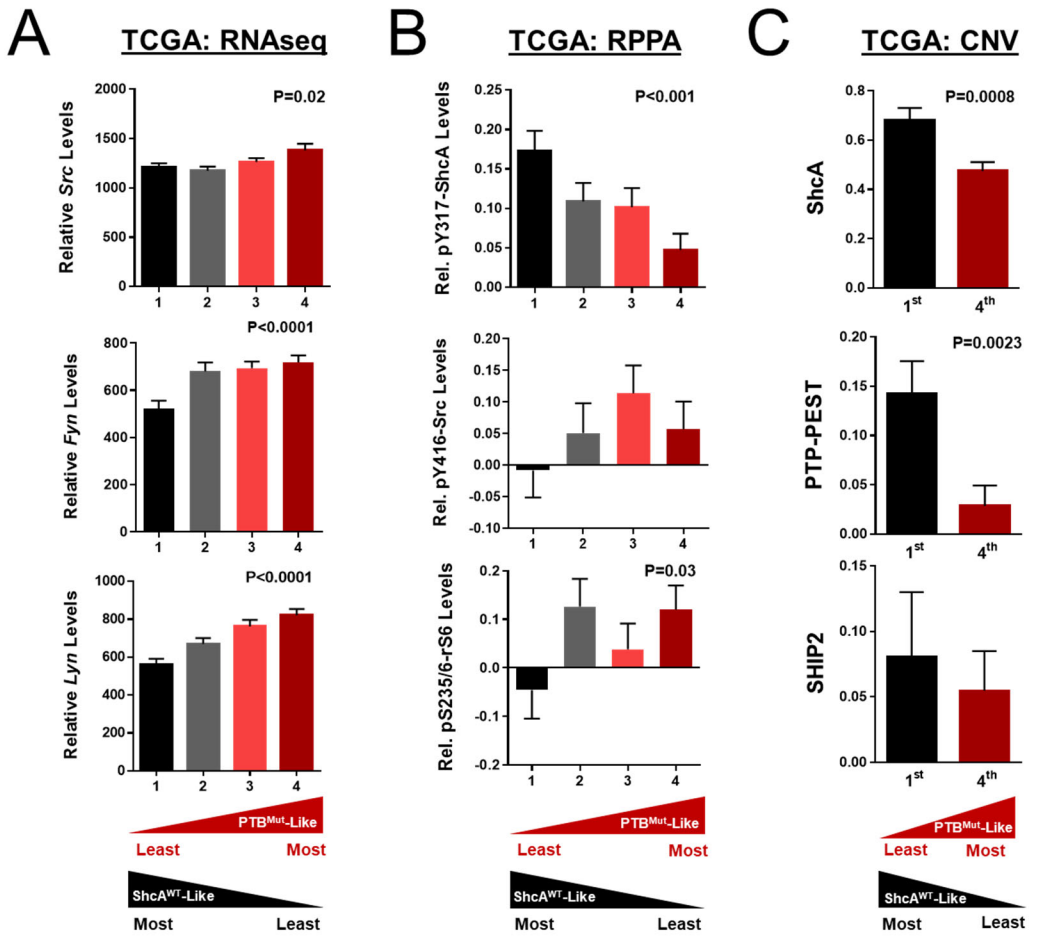

Figure 17: A PTB^{MUT} gene signature is associated with increased mTOR and Src family kinase activation in human breast cancers. Primary tumors from the TCGA RNA-seq dataset (n=1218) were equally stratified into four quartiles based on a gene expression signature (GEO GSE41718) associated with a PTB^{MUT} phenotype [2]. **(A)** The average Src, Fyn and Lyn transcript levels were evaluated in each quartile. **(B)** For a subset of these breast tumors (n=747), RPPA data is also available: 1st quartile (n=171); 2nd quartile (n=164); 3rd quartile (n=196); 4th quartile (n=216). The relative pY317ShcA, pY416-Src and pS235/236-rS6 levels in each quartile are shown. The data is represented as average expression levels ± SEM. Statistical analysis was performed by comparing the 1st and 4th quartiles. **(C)** Copy number variants (CNV) of ShcA, PTPN12, and SHIP2 in human primary tumors stratified into the 1st (n=171) and 4th quartile (n=216).

To test this, we asked whether copy number variations of ShcA, PTPN12 or SHIP2 were associated with tumors that most closely resemble a $ShcA^{WT}$-like or PTB^{MUT}-like gene signature. Interestingly, ShcA amplification was enriched in $ShcA^{WT}$-like breast tumors, suggesting that ShcA overexpression did not contribute to the development of these PTB-independent ShcA signaling complexes (Figure 17C). In contrast, PTPN12, and not SHIP2, genomic loss was specifically enriched in PTB^{MUT}-like breast tumors (Figure 16C), suggesting a possible relationship for PTPN12 in limiting the aberrant activation of ShcA-driven Src activation in human breast cancers.

3.1.12.2 Summary

In accordance with our model system, we establish that PTB^{MUT}-like primary human breast tumors display significantly reduced pY317-ShcA levels coincident with elevated pY416-Src and pS235/6-rS6 levels compared to $ShcA^{WT}$-like breast tumors. Moreover, breast tumors that acquire a PTB^{MUT} like signature are associated with an increase in SFKs, Src, Fyn, and Lyn mRNA expression. These results provide significant evidence of a clinically relevant association of PTB-driven ShcA complexes in modulating transcriptional and signal transduction events that regulate SFK and AKT/mTOR mitogenic activity. Lastly, genomic loss of PTPN12 is categorically observed in PTB^{MUT}-like breast tumors, establishing a possible relationship between this phosphatase and its role in restricting ShcA dependent Src activation in human breast tumorigenesis.

3.2 Mechanistic and functional characterization of unique negative regulatory pathways of distinct ShcA signaling complexes during mammary tumorigenesis.

3.2.1 Introduction

ShcA is an essential scaffold protein that transmits activating signals downstream of receptor and non-receptor tyrosine kinases. By virtue of its two phospho-tyrosine binding domains, PTB and SH2 domain, ShcA is capable of forming various multimeric complexes to transduce mitogenic signaling through both phospho-tyrosine dependent and independent mechanisms. The ShcA PTB domain is identified as the primary initiator of phospho-tyrosine signaling downstream of most RTKs found in breast cancer, including ErbB2. Specifically, the ShcA PTB domain binding site of ErbB2 has been mapped to the consensus NPXY motif (Y1226/7) [281]. Indeed, transgenic mouse models have reinforced the requirement for an intact ShcA PTB domain in ErbB2 driven breast cancer. Deletion of the five main tyrosine phosphorylation residues within the cytoplasmic tail of ErbB2 compromises the ability of ErbB2 to induce mammary tumorigenesis [1]. However, reconstitution of the ShcA binding site alone restored the kinetics and severity of breast tumor development [1].

Although RTK signaling is predominantly executed through tyrosine phosphorylation, the activation of RTKs can also robustly increase the serine/threonine phosphorylation of various proteins. This coordinated activation of phospho-tyrosine and phosphoserine/threonine networks result in both positive and negative feedback loops to tightly control mitogenic signaling. For example, growth factor stimulation leads to the recruitment of Grb2/Sos complexes to phosphorylated RTKs or phosphoproteins including ShcA, and the subsequent serine/threonine phosphorylation of Sos1 by MAPK [410]. Paradoxically, Sos1 phosphorylation also results in an autoinhibitory effect, by disrupting its association with Grb2 and ShcA and, consequently the RTKs at the plasma membrane [410-412]. Alternatively, ERK phosphorylation of Gab1 can enhances the recruitment of the p85 regulatory subunit of PI3K to Gab1, resulting in an increase in PI3K and ERK signaling [386, 413]. Finally, ShcA/ERK interactions occur through a non-canonical binding interface on the ShcA PTB domain in non-stimulated cells thus sequestering ERK and restricting MAPK signaling. Upon growth factor stimulation, ERK dissociates from ShcA, leading to the amplification of MAPK signal transduction [6].

Seminal work by the Pawson laboratory elucidated the role of key serine and threonine residues that become phosphorylated within ShcA upon EGF stimulation, specifically Serine 29, Threonine 214, and Serine 335 [7]. Phosphorylation of Serine 29 is thought to be mediated by AKT through the AGC kinase motif, RXXS/T (inclusive of ShcA Serine 29), while Threonine 214 phosphorylation is ERK dependent. The kinase that phosphorylates Serine 335 has yet to be identified. Thus, the very signals downstream of ShcA, including AKT and ERK, are those that feedback to further regulate ShcA signal transduction. This work identified a dynamic and integrated phospho-tyrosine and phosphoserine/threonine signaling interactome that was coordinated through ShcA to temporally control RTK signaling [7].

Thus far, our data demonstrates that distinct intracellular ShcA pools influence the activation of diverse signal transduction pathways. First, we have shown that ErbB2-expressing breast cancer cells are predominately reliant on ErbB2/ShcA complexes (PTB-dependent; $ShcA^{WT}$) for their tumorigenic potential, in part, by activating the AKT/mTOR pathway downstream of the ShcA phospho-tyrosine residues [2]. Second, we established that PTB-independent ShcA complexes (PTB^{MUT}) shift to rely on the SH2 domain to activate multiple Src family kinases (SFK), including Src and Fyn to cooperatively accelerate and amplify breast tumor growth [414]. The ability of these distinct ShcA pools to dynamically transduce non-redundant signals suggests that primary breast cancers can acquire a PTB^{MUT} phenotype by losing a negative regulator of the PTB domain. A unique property of the ShcA PTB domain is its ability to bind negative regulators. The recruitment of negative regulators to the ShcA-PTB domain depends, in part, on serine phosphorylation. Specifically, the phosphorylation of Serine 29 is required for the stabilization of PTPN12/ShcA interactions. Upon EGF stimulation, ShcA is tyrosine phosphorylated, leading to the engagement of Grb2 complexes. Signal transduction is then rapidly antagonized by the phosphorylation of Serine 29 and subsequent recruitment of PTPN12. Interestingly, Serine 29 is also part of a putative Src binding motif, mapped to a 10-amino acid region upstream of the ShcA PTB domain [8].

Given the requirement for ShcA signaling in ErbB2+ breast cancer progression [1, 392], how these unique Serine 29 dependent motifs regulate PTB-dependent and PTB-independent ShcA complexes in breast tumorigenesis is currently unknown and remains to be investigated. Although there is evidence that serine/threonine phosphorylation of ShcA regulates the signaling potential

of the adaptor protein, the mechanism is still poorly understood. Given the potential interplay between SFKs, AKT, and PTPN12 at the N-terminal of ShcA, the **objective** of this study is to understand how these signaling pathways upstream of the ShcA PTB domain differentially regulate mammary tumorigenesis.

3.2.2 PTB-dependent ShcA complexes require a 10-amino acid region, encompassing Serine 29 of ShcA, to negatively regulate breast tumor growth, *in vivo*.

To model the biological significance of Serine 29 in ShcA dependent breast tumorigenesis, we generated mutant ShcA alleles that can no longer be phosphorylated at Serine 29 (S29A) or a 10-amino acid (S29-W38) deletion (Δ10) spanning the putative N-terminal Src binding site, in the context of PTB-dependent ($ShcA^{WT}$) and PTB-independent ShcA pools (PTB^{MUT}) (Figure 1A). PTB-dependent ShcA mutant alleles retain a functional PTB domain to engage RTKs and its negative regulator(s), while impeding Serine 29 dependent interactions [7, 385]. On the other hand, PTB-independent ShcA complexes have lost the ability to engage RTKs and its negative regulator(s) through the phospho-tyrosine binding pocket of the PTB domain, including PTPN12 [3, 326, 414]. We first stably expressed FLAG-tagged $ShcA^{WT}$, $ShcA^{S29A}$, $ShcA^{\Delta 10}$, $PTB^{MUT,}$ $PTB^{MUT/S29A}$, and $PTB^{MUT/\Delta 10}$ alleles in ErbB2-transformed breast cancer cells (Figure 1B-C). Subsequently, we investigated whether PTB-coupled ($ShcA^{WT}$) and -uncoupled ShcA (PTB^{MUT}) pools relied on Serine 29 phosphorylation or the S29-W38 motif within the N-terminal domain to regulate ErbB2-driven mammary tumor growth, *in vivo*.

The loss of Serine 29 phosphorylation ($ShcA^{S29A}$) accelerated breast tumor growth 1.5-fold, while the loss of the S29-W38 motif ($ShcA^{\Delta 10}$) further increased tumor growth potential by 2.5-fold at the experimental endpoint, relative to $ShcA^{WT}$ controls (Figure 2A). These data demonstrated that PTB-dependent ShcA complexes required Serine 29 phosphorylation and the 10-amino acid region encompassing Serine 29, to negatively regulate breast tumor growth. We similarly assessed the impact of Serine 29 loss and S29-W38 deletion in PTB-independent ShcA complexes (PTB^{MUT}), *in vivo*. In the context of PTB-independent ShcA pools, the loss of Serine 29 phosphorylation, accelerated tumor growth 2.0-fold, relative to PTB^{MUT} controls at the experimental endpoint (Figure 2B). This data provided evidence that Serine 29 phosphorylation had additional tumor suppressive properties independent of the ShcA PTB domain. In contrast, the loss of the S29-W38

motif from PTB-independent ShcA pools ($PTB^{MUT/\Delta 10}$) had no significant impact on tumor growth (Figure 2B). These data suggest that the tumor suppressive properties imparted by this 10-amino acid region was dependent on a functional PTB domain. Provided that $ShcA^{\Delta 10}$ expressing tumors (which can still engage interactors through its PTB domain) have accelerated tumor growth *in vivo* (Figure 2A), we can posit that there exists an interplay between the PTB domain and this 10-amino acid region that works cooperatively to suppress tumorigenesis. On the other hand, the increased growth potential of $PTB^{MUT/S29A}$ breast tumors suggested that Serine 29 imparts a fundamentally different growth inhibitory property that is independent of the ShcA PTB domain (Figure 2A-B) and thus, the engagement of its negative regulators.

To better understand the mechanisms underlying these phenotypes, we performed Ki67 and Cleaved Caspase 3 immunohistochemical (IHC) staining on these tumors as markers of cell proliferation and apoptosis, respectively (Figure 3A-B). At the experimental endpoint, both $ShcA^{S29A}$ and $ShcA^{\Delta 10}$ expressing breast tumors had increased proliferative potential, relative to $ShcA^{WT}$ controls (Figure 3A). These results reinforced that Serine 29 and the S29-W38 motif of ShcA transduces negative regulatory signals that limit mitogenic signaling. However, while $ShcA^{\Delta 10}$ breast tumors were relatively unchanged in their apoptotic response, breast tumors that engaged $ShcA^{S29A}$ PTB-dependent pools displayed increased levels of apoptosis (Figure 3B). This suggested that there are selective pressures in place for breast tumors expressing $ShcA^{S29A}$ to engage in mitogenic signal responses and simultaneously overcome a heightened apoptotic response for tumor growth. Altogether these findings support that independent N-terminal regions of PTB-dependent ShcA pools, Serine 29 phosphorylation and the S29-W38 motif, negatively regulate breast tumorigenesis.

3.2.3 Increased tumor growth mediated by the loss of Serine 29 phosphorylation and S29-W38 motif of PTB-dependent ShcA pools is independent of ShcA tyrosine phosphorylation.

One of the most striking differences between PTB-coupled ($ShcA^{WT}$) and PTB-uncoupled (PTB^{MUT}) ShcA pools lies in their use of the ShcA tyrosine phosphorylation sites to promote tumor growth. Upon the formation of ErbB2/ShcA complexes ($ShcA^{WT}$), the phosphorylation of ShcA tyrosine residues is one requisite step to activate AKT/mTOR signaling [395, 406]. Although ShcA

tyrosine phosphorylation may also activate AKT/mTOR signaling from PTB-independent ShcA complexes (PTB^{MUT}), these sites are dispensable for mammary tumor growth, *in vivo* [414]. Studies have also shown that Serine 29 dependent recruitment of the negative regulator, PTPN12 antagonizes pro-mitogenic EGFR signaling to dampen ShcA-dependent signaling downstream of its phospho-tyrosine residues [7]. Moreover, we and others have identified Src as one tyrosine kinase responsible for the phosphorylation of the ShcA phospho-tyrosine sites [414]. As such, we proceeded to characterize ShcA tyrosine phosphorylation levels using immunoprecipitation of FLAG-tagged $ShcA^{WT}$ or PTB^{MUT} alleles in the context of either the loss of Serine 29 phosphorylation or the S29-W38 motif. PTB-dependent ShcA pools ($ShcA^{WT}$) displayed no appreciable change in ShcA phospho-tyrosine levels across all mutations (Figure 4A-B). Accordingly, breast cancer cell lines that expressed $ShcA^{S29A}$ and $ShcA^{\Delta 10}$ collectively showed no alterations in basal ERK and AKT/mTOR signaling *in vitro* (Figure 4C), suggesting that ShcA signaling downstream of its phospho-tyrosine residues remains intact but did not contribute to the increased tumorigenic response observed *in vivo* [395, 414].

To confirm these observations, we further assessed alterations in ShcA phospho-tyrosine activation using materials obtained from endpoint breast tumors (Figure 5A-B). Corroborating our *in vitro* data, there was no significant change in ShcA tyrosine phosphorylation levels in breast tumors expressing $ShcA^{S29A}$ and $ShcA^{\Delta 10}$ alleles, compared to $ShcA^{WT}$ controls (Figure 5A-B). IHC analysis further confirmed that downstream AKT/mTOR signaling, as measured by rS6 phosphorylation (pS240/244), remained constant across breast tumors regardless of mutational status (Figure 5C). Collectively, our observations suggest that PTB-dependent ShcA complexes that no longer engage Serine 29 or the S29-W38 motif, accelerate tumor growth due to mitogenic signaling that are independent of the ShcA phospho-tyrosine residues. These results were unexpected as early studies identified that the loss of Serine 29 increased ShcA tyrosine phosphorylation [385]. However, in support of our results, recent *in vivo* studies where PTPN12 has been lost from the mammary epithelium have demonstrated that ShcA phosphorylation remains unchanged, despite ShcA having lost a negative regulator [196].

Next, we wanted to confirm our previous report that breast tumors that engage PTB-independent ShcA complexes were indeed uncoupled from the ShcA phospho-tyrosine residues. Using immunoprecipitation of FLAG-tagged PTB^{MUT} alleles, we found that ShcA phospho-tyrosine

levels were unchanged in $PTB^{MUT/S29A}$ compared to PTB^{MUT} controls (Figure 6A), which did not correlate with the increased tumor growth observed *in vivo* (Figure 2B). Unexpectedly, $PTB^{MUT/\Delta 10}$ expressing breast tumors, which were relatively unchanged in tumor growth (Figure 2B), had hyperactivated ShcA phospho-tyrosine residues relative to PTB^{MUT} controls (Figure 6B). We also observed that basal ERK and AKT/mTOR signaling in PTB^{MUT} expressing tumors was unchanged, irrespective of the mutational status (Figure 6C-D). Provided that both $ShcA^{S29A}$ (PTB-dependent) and $PTB^{MUT/S29A}$ (PTB-independent) expressing breast tumors were both accelerated in tumor growth despite having no alterations in the activation of ShcA phospho-tyrosine residues, suggested that Serine 29 possessed additional tumor suppressive properties that regulate both PTB-coupled and -uncoupled ShcA pools independent of the ShcA phospho-tyrosine sites. In the context of $PTB^{MUT/\Delta 10}$ expressing breast tumors, the hyperactivation of the ShcA phospho-tyrosine residues imply that there was a propensity for these breast tumors to re-engage the ShcA phospho-tyrosine sites to amplify ShcA-dependent signaling. However, given that there is no appreciable change in tumor growth kinetics upon the loss of the S29-W38 motif (Figure 2B), this proposed that ShcA tyrosine phosphorylation was uncoupled from the tumorigenic response. Altogether, these data show that PTB-dependent and -independent ShcA complexes no longer rely on the ShcA phospho-tyrosine residues to sustain tumor growth upon the loss of Serine 29 phosphorylation and the S29-W38 motif.

3.2.4 Increased tumor growth upon the loss of Serine 29 phosphorylation is not dependent on Src recruitment or activation.

We have demonstrated that the loss of Serine 29 phosphorylation increases tumor growth in both PTB-dependent and -independent ShcA expressing breast tumors (Figure 2A-B). This indicated that the growth inhibitory properties of Serine 29 phosphorylation were independent of a functional PTB domain (PTB-coupled vs PTB-uncoupled). Additionally, accelerated tumor growth was independent of the ShcA tyrosine phosphorylation residues and associated with no apparent alterations in downstream ShcA-dependent signaling, including ERK or AKT/mTOR activation (Figure 4 and Figure 5). Studies have demonstrated that Serine 29 phosphorylation is necessary to shift ShcA-dependent signaling from pro-mitogenic/survival signaling to a form that stimulates cytoskeletal reorganization in a phospho-tyrosine independent manner [7]. Given these results, we assessed whether the loss of Serine 29 phosphorylation affected the ability of PTB-dependent

ShcA pools to recruit and activate Src. Co-immunoprecipitation of FLAG-tagged $ShcA^{WT}$ and $ShcA^{S29A}$ alleles demonstrated that Src recruitment was comparable between these two groups (Figure 7A). Correspondingly, basal levels of Src and FAK activation remained unchanged, *in vitro* (Figure 7C). IHC analyses of endpoint breast tumors also illustrated that Src activation remain unchanged in breast tumors engaging $ShcA^{S29A}$ complexes relative to $ShcA^{WT}$ controls, *in vivo* (Figure 7D). These results collectively establish that the tumorigenic response observed upon the loss of Serine 29 phosphorylation was independent of Src recruitment or activation.

Recall that our previous *in vivo* studies demonstrate that the loss of PTB-driven ShcA signaling (PTB^{MUT}) not only delayed breast tumor initiation but also paradoxically, potentiated subsequent tumor growth [2] through the hyperactivation of Src [414]. Indeed, we show that the loss of a functional PTB domain (PTB^{MUT}) alone, is sufficient to increase Src activation by 2-fold [2, 414]. Yet, PTB-independent ShcA pools exhibited the lowest level of SFK binding [414]. These results demonstrated that the ability of PTB-independent ShcA pools to activate Src was uncoupled from direct SFK recruitment to ShcA signaling complexes. Interestingly, PTB-independent ShcA pools that have lost the ability to phosphorylate Serine 29, increased Src recruitment 20-fold compared to PTB^{MUT} alone (Figure 8A). However, this increase in Src recruitment did not correlate with the activation of SFKs, or involve the upregulation of alternative SFKs, including Fyn and Lyn (Figure 8C). These data corresponded to previous reports which show that germline deletion of PTPN12, a PTB domain dependent negative regulator of ShcA, did not alter Src phosphorylation levels, even though downstream effectors of integrin signaling (Cas, Pyk2) were hyper-phosphorylated [196]. As such, we establish that Src recruitment was uncoupled to Src activation in PTB-independent ShcA complexes that have lost Serine 29 regulation. Thus, we propose that Serine 29 differentially regulates Src recruitment and activation within different pools of ShcA. Specifically, Serine 29 phosphorylation of PTB-coupled ShcA complexes regulates tumor growth independent of Src recruitment or Src activation. However, for those breast tumors that have engaged PTB-independent ShcA pools, Serine 29 phosphorylation seems to play a role in recruiting Src but is uncoupled from requiring further Src activation through Serine 29 dependent complexes.

3.2.5 Loss of Serine 29 phosphorylation in PTB-dependent breast tumors increases resistance to SFK inhibition.

We next addressed whether the loss of Serine 29 phosphorylation within breast tumors that engaged PTB-dependent ShcA complexes shifted to rely on alterative signaling pathways to sustain tumor growth. In particular, we wanted to confirm that the increased tumor growth observed for $ShcA^{S29A}$ breast tumors (Figure 2A) was indeed, independent of SFK activation. To do so, we assessed the clonogenic capacity of these breast tumors upon treatment with suboptimal doses of a pan SFK inhibitor, PP2 (2 μM). We first established that there was inhibition of SFK activity and its substrate FAK upon PP2 treatment (Figure 9A). SFK inhibition significantly reduced the ability of $ShcA^{WT}$ breast cancer cells to form colonies (number of foci) (Figure 9B-C), however had marginal effects on colony outgrowth (Figure 9B and 9D). This reinforced our previous results which demonstrated that breast tumors engaging PTB-dependent $ShcA^{WT}$ pools heavily relied on downstream ShcA phospho-tyrosine signaling for tumor outgrowth [395, 414]. Surprisingly, breast cancer cells expressing $ShcA^{S29A}$ were highly resistant to SFK inhibition (Figure 9B-D). Colony formation and outgrowth remained relatively unchanged compared to DMSO controls upon PP2 treatment (Figure 9C-D). We also observed no alterations in ShcA-dependent mitogenic signaling as measured by ERK and AKT/mTOR activity for both $ShcA^{WT}$ and $ShcA^{S29A}$ breast cancer cells relative DMSO controls (Figure 10). Altogether these results suggested that Src no longer played a role in the initiation (number of foci) or growth potential (average area) of breast cancer cells engaging in PTB-dependent $ShcA^{S29A}$ complexes. Moreover, these data indicated that PTB-coupled ShcA pools were able to transduce alternative signals downstream of ShcA, independent of Src activation, to accelerate tumor growth upon the loss of Serine 29 phosphorylation.

Given the ability of PTB-independent ShcA (PTB^{MUT}) signaling complexes to coordinately hyperactivate SFKs in ErbB2-positive breast cancer cells, we next assessed whether the loss of Serine 29 phosphorylation would alter the sensitivity of breast cancer cells that have engaged PTB-independent ShcA complexes to SFK inhibition. It is important to highlight here, that ectopic expression of PTB^{MUT} in breast cancer cells that endogenously express wild-type ShcA creates a ShcA pool that can independently transduce oncogenic signals downstream RTKs, including ErbB2, (ShcA; PTB-dependent) and a secondary pool, incapable of binding to RTKs and negative

regulators (PTB^{MUT}). This PTB-independent pool (PTB^{MUT}) allows for the amplification of pro-tumorigenic signals through SH2 domain dependent activation of SFKs to accelerate tumor growth. Thus, breast tumor initiation is mediated by signals emanating from PTB-coupled ShcA pools, while accelerated tumor outgrowth is largely contingent upon PTB-independent signaling complexes [414]. Upon SFK inhibition (Figure 11A), there was no appreciable change to the number of colonies formed regardless of the loss of Serine 29 phosphorylation (Figure 11B-C). However, SFK inhibition did reduce the average area of the colonies by ~2-fold in both PTB^{MUT} and $PTB^{MUT/S29A}$ expressing breast cancer cells, relative to their respective DMSO controls (Figure 11D). This reinforced our previous work that the hyperactivation of SFKs is fundamental to the mechanism underlying PTB- independent breast tumorigenesis. Moreover, these data demonstrated that the differential increase in tumor growth observed for $PTB^{MUT/S29A}$ breast tumors (Figure 2B), did not involve Src activation.

Altogether, we see that upon the loss of Serine 29, PTB-dependent breast tumors have no change in Src binding and activity, and, are completely resistant to SFK inhibition by PP2. On the other hand, for PTB^{MUT} breast tumors, which are characteristically amplified in SFK activity, the loss of Serine 29 phosphorylation is sufficient to increase the engagement of Src but does not further sensitize them to Src inhibition relative to PTB^{MUT} controls. These data highlighted that the growth inhibitory signaling arising from either PTB-coupled or -uncoupled Serine 29 complexes were independent of Src and had non-overlapping mechanisms to control tumor growth.

3.2.6 Loss of Serine 29 phosphorylation from PTB-dependent ShcA pools increases Lapatinib resistance.

Thus far, we have established that Serine 29 phosphorylation is tumor suppressive in the context of PTB-dependent ShcA complexes (Figure 2A). Loss of Serine 29 is sufficient to increase tumor growth, yet mechanistically, was not associated with mitogenic signaling downstream of the ShcA phospho-tyrosine residues or the recruitment and activation of Src. Given that Serine 29 phosphorylation of ShcA has been shown to be through a negative feedback loop that involves AKT downstream of the ShcA phospho-tyrosine residues [7], we next wanted to assess the contribution of ErbB2 activation on Serine 29 phosphorylation in PTB-dependent ShcA complexes. To do so, we used a clonogenic assay to characterize the sensitivity of breast cancer

cells expressing the loss of function ShcA^{S29A} mutant to pharmacological inhibition of ErbB2 and EGFR (lapatinib). We first demonstrate that there is complete inhibition of ErbB2 activation upon lapatinib treatment at suboptimal doses (0.5 μM) relative to DMSO controls (Figure 12). Unexpectedly, ShcA^{S29A} expressing cells were highly resistant to ErbB2/EGFR inhibition (Figure 13A-C). Upon further examination, we found that ERK and mTOR signaling pathways remained elevated in lapatinib treated ShcA^{S29A} expressing cells compared to ShcA^{WT} controls (Figure 12). Correspondingly, ShcA^{S29A} expressing breast cancer cells increased the activation of ShcA phospho-tyrosine residues relative to lapatinib treated ShcA^{WT} controls (Figure 13D). This suggested that there was a re-engagement of the ShcA phospho-tyrosine residues to sustain ERK and mTOR signaling upon ErbB2 inhibition. Recall, PTB-independent (PTB^{MUT}) ShcA breast tumors are highly resistant to lapatinib [414]. PTB-independent breast tumors can also retain ERK and AKT/mTOR activation in response to ErbB2/EGFR inhibition [414]. In these PTB-uncoupled breast tumors, resistance to Lapatinib was an adaptive response that required both the ShcA phospho-tyrosine residues and the SH2 domain. However, given that ShcA^{S29A} expressing breast cancer cells still retain a functional PTB domain, this suggests that the tumor suppressive effects of Serine 29 lie upstream of the ShcA PTB domain and independent of ErbB2 activation. Alternatively, these results also imply that lapatinib resistance caused by the loss of Serine 29 phosphorylation may be performed by other RTKs or non-RTKs that independently increase ERK and AKT/mTOR signaling.

3.2.7 Loss of Serine 29 phosphorylation in ShcA reduces but does not ablate PTPN12 interaction.

To date, PTPN12 is the only putative negative regulator that has been associated with both Serine 29 phosphorylation and the ShcA PTB domain [7, 384, 385]. Accordingly, it was necessary to identify whether these unique biological phenotypes may be due to the differential engagement of PTPN12. We used a proximity-dependent biotin identification (BioID) proteomics approach to biochemically screen and identify the level of PTPN12 engagement of FLAG-tagged ShcA^{WT}, ShcA^{S29A}, PTB^{MUT}, and PTB$^{MUT/S29A}$ from ErbB2-transformed breast cancer cell lines (Figure 14A). We first show that ShcA^{WT} breast tumors across all mutations express ErbB2 (Figure 14B) and engage ErbB2 (Figure 14C). This is expected as these ShcA mutant alleles still retain a functional PTB domain. We also confirm that breast cancer cells that express any variation of the

loss of function PTB^{MUT} allele express ErbB2 and PTPN12 (Figure 14B). We also validate that PTB-independent ShcA complexes (PTB^{MUT}) across all mutations have lost the ability to engage ErbB2 and PTPN12 (Figure 14D-E). These observations are expected as ErbB2 and PTPN12 require a functional ShcA PTB domain for complete interaction [3, 323]. In the context of PTB-dependent pools, the loss of Serine 29 phosphorylation (ShcA^{S29A}) reduced but did not abrogate PTPN12 recruitment (Figure 14C). These results corroborated previous reports that highlight the phosphorylation of Serine 29 as a requisite step in stabilizing ShcA/PTPN12 interactions [7, 385]. However, the increased growth rate of PTB$^{MUT/S29A}$ cannot be explained by the loss of PTPN12 engagement alone (Figure 2A). This is evident from the observations that PTB$^{MUT/S29A}$ complexes which have lost the ability to engage PTPN12 (Figure 14D), are still able to accelerate tumor growth *in vivo* (Figure 2B). These data suggest that Serine 29 phosphorylation differentially regulates distinct pools of ShcA (PTB-coupled vs PTB-uncoupled) and highlights an unidentified and novel regulatory network that is coordinated by the phosphorylation of Serine 29.

3.2.8 Loss of S29-W38 motif in PTB-dependent ShcA pools increases tumor growth by hyperactivating Src, *in vivo*.

Having demonstrated that the S29-W38 within the N-terminal of ShcA increases tumorigenesis, we recognized this accelerated growth phenotype mirrored that of breast tumors that engaged PTB-independent ShcA complexes (PTB^{MUT}) [2, 414]. Indeed, ShcA-PTB^{MUT} expressing breast tumors are also accelerated in their tumor growth kinetics and uncoupled from mitogenic signals emanating from the ShcA phospho-tyrosine residues [414]. Mechanistically, we determined that PTB^{MUT} breast tumors predominately rely on the ShcA SH2 domain to activate SFKs, Src and Fyn to sustain and amplify tumor growth [414]. However, it has been reported that the S29-W38 motif of ShcA is also required to recruit Src and increase its activation [8]. This site-specific interaction between the ShcA amino terminus and the catalytic domain of Src was necessary to activate Src [8].Therefore, we addressed whether the increased tumor growth observed in ShcA$^{\Delta 10}$ breast tumors (Figure 2A) was dependent on Src engagement and/or activity.

Corroborating previous evidence, we demonstrate that the loss of the S29-W38 motif significantly reduces but does not abolish the ability of ShcA to bind Src (Figure 7B). We expected this partial reduction in Src engagement as the Src SH3 domain has also been established to interact with

ShcA CH1 proline rich regions [368] as well as the ShcA SH2 domain [349]. However, the fact that there is a significant reduction in Src binding upon S29-W38 deletion suggests that this region is a major site of Src engagement. Altogether, these data validated previous reports [8], but also provided evidence that the growth advantage imparted by the loss of S29-W38 region (Figure 2A) was independent of Src recruitment. On the other hand, we observed that the levels of Src recruitment did not correlate with SFK activation. Indeed, there was no appreciable change in Src activation upon the loss of the S29-W38 motif, *in vitro* (Figure 7C). Unexpectedly, IHC analysis of $ShcA^{\Delta 10}$ breast tumors at experimental endpoint demonstrated that these tumors had hyperactivated Src, *in vivo* (Figure 7D). This suggested that despite the inability to effectively recruit Src, $ShcA^{\Delta 10}$ breast tumors had established alternative mechanisms to further activate Src for tumor growth. These results provided evidence that the S29-W38 motif of ShcA played a role in limiting Src activation during breast tumorigenesis independent of the ShcA phospho-tyrosine residues (Figure 4B and 5B).

We next wanted to determine if the N-terminal regulation of PTB-independent ShcA complexes (PTB^{MUT}) affected Src recruitment. Interestingly, Src engagement is significantly increased in $PTB^{MUT/\Delta 10}$ mutants (5-fold) relative to PTB^{MUT} controls (Figure 8B), while steady state Src activation and levels remained unchanged (Figure 8C). Thus, in the context of $PTB^{MUT/\Delta 10}$ expressing breast tumors, Src recruitment and activation seem to be uncoupled to the observed tumor growth phenotype (Figure 2B). Given that PTB^{MUT} breast tumors already have elevated Src activation [414], these data suggest that further hyperactivating Src does not increase the tumorigenic potential from PTB-independent ShcA complexes that have lost the S29-W38 motif. As we do not see any appreciable changes to tumor growth in $PTB^{MUT/\Delta 10}$ breast tumors relative to PTB^{MUT} controls, these data emphasize that the S29-S38 motif and PTB domain cooperate to restrict mitogenic signaling during breast tumorigenesis.

3.2.9 PTB-dependent ShcA pools require Src activation to sustain tumor growth potential upon the loss of the S29-W38 motif.

Thus far, the data demonstrates that the loss of S29-W38 motif in PTB-dependent ShcA complexes can increase tumor growth through the hyperactivation of Src, *in vivo*. This led us to examine whether the S29-W38 region altered the sensitivity of breast tumors to SFK inhibition. We used

clonogenic assays to treat ShcA^{WT} and ShcA$^{\Delta 10}$ expressing breast cancer cells with suboptimal doses of SFK inhibitor PP2 (2 μM) and assessed the number and size of colonies formed (Figure 9A-B). SFK inhibition was able to reduce colony formation (number of foci) of both ShcA^{WT} and ShcA$^{\Delta 10}$ breast cancer cells. However, colony growth potential (average area) was significantly reduced for only ShcA$^{\Delta 10}$ expressing cells (Figure 9C-D). These data suggested that both the initiation and outgrowth of ShcA$^{\Delta 10}$ colonies were highly dependent on SFK activation. In addition, PP2 treatment caused no apparent alterations in ERK or AKT signaling for both ShcA^{WT} and ShcA$^{\Delta 10}$ expressing breast cancer cells compared to DMSO controls (Figure 10). Rather, SFK inhibition was able to concomitantly reduce rS6 phosphorylation of ShcA$^{\Delta 10}$ expressing cells (Figure 10). This indicated that the S29-W38 motif played a role in regulating Src dependent activation of mTOR signaling from PTB-dependent ShcA pools.

We next assessed whether the S29-W38 motif would similarly alter the sensitivity of PTB-independent breast cancer cells to pharmacological inhibition by PP2 at suboptimal doses (2 μM) (Figure 11A). As expected and demonstrated previously, SFK inhibition did not impact the number of colonies formed regardless of the loss of S29-W38 region (Figure 11B-C). However, SFK inhibition was able to reduce the average area of the colonies by ~2-fold in both PTB^{MUT} and PTB$^{MUT/\Delta 10}$ expressing foci, relative to their respective DMSO controls (Figure 11D). This reinforced our previous work that the hyperactivation of SFKs is fundamental to the mechanism underlying PTB- independent breast tumorigenesis, but also demonstrated that PTB^{MUT} breast tumors are no longer contingent upon the presence of the S29-W38 region for sustained tumor growth. Taken together, these data highlight the dichotomy between breast tumors that have either engaged PTB-dependent or -independent ShcA complexes. Although PTB-dependent ShcA pools (ShcA^{WT}) do not rely on Src for tumor outgrowth, the loss of the S29-W38 region from PTB-dependent ShcA pools (ShcA$^{\Delta 10}$) is sufficient to increase the dependence of these tumors on Src (Figure 9C-D). This suggests that the S29-W38 region plays a significant role in PTB-dependent ShcA pools to restrict the hyperactivation of Src and prevent the transition of these breast tumors to those that have engaged PTB-independent ShcA complexes that are capable of re-wiring mitogenic signaling networks to hyperactivate SFKs.

3.2.10 Loss of S29-W38 motif in PTB-dependent ShcA pools sensitizes breast tumors to Lapatinib.

We have since established that the S29-W38 region is able to limit the growth of breast tumors that engage PTB-dependent ShcA complexes. It is also apparent, that ShcA$^{\Delta 10}$ expressing breast tumors parallel breast tumors that have engaged PTB-independent ShcA complexes. This includes the ability of these tumors to accelerate tumor growth, hyperactivate Src, and uncouple itself from mitogenic signaling downstream of ShcA phospho-tyrosine residues. A defining feature of PTB-independent ShcA breast tumors is their heightened resistance to lapatinib. This response is dependent on the re-engagement of ERK and AKT/mTOR activity, and also the presence of Src. The loss of Src alone is able to re-sensitize PTB-independent breast tumors to lapatinib [414]. Our data suggests that ShcA$^{\Delta 10}$ breast tumors are also reliant on Src activity in its capacity to sustain mTOR signaling (Figure 10). This implied that Src and mTOR signaling work cooperate to amplify tumor growth in the context of S29-W38 motif loss. However, since ShcA$^{\Delta 10}$ mutant alleles still retain a functional PTB domain, whether these mitogenic signals stem from ErbB2/ShcA complexes or from alterative complexes involving the S29-W38 region remained to be identified. As such, we used a clonogenic assay to assess the impact of S29-W38 deletion on lapatinib sensitivity in ErbB2+ breast cancer cells engaging PTB-dependent ShcA pools. We first demonstrate that there is complete inhibition of ErbB2 activation upon lapatinib treatment at suboptimal doses (0.5 μM) relative to DMSO controls (Figure 12). Lapatinib inhibition reduced the number and area of colonies formed in both ShcA^{WT} and ShcA$^{\Delta 10}$ expressing breast cancer cells (Figure 13A-C). These results demonstrate that ShcA$^{\Delta 10}$ expressing tumors not only rely on SFK activation for tumor growth (Figure 9) but are also dependent on mitogenic signals emanating from ErbB2. Accordingly, both ShcA^{WT} and ShcA$^{\Delta 10}$ expressing breast cancer cells decreased ERK and mTOR signaling upon ErbB2 inhibition, however, AKT activation remained unchanged relative DMSO controls (Figure 12). Decreased mitogenic signaling also corresponded to a comparable decrease in ShcA tyrosine phosphorylation (Figure 13D). Altogether, these results indicated that ShcA$^{\Delta 10}$ complexes transduce signals that are dependent on both ErbB2 and SFK activation. Given that the loss of the S29-W38 region can sensitize these tumors to SFK and ErbB2 inhibition, this suggests that there is a cooperative effect between SFK and ErbB2 activation to accelerate tumor growth. Moreover, these data propose that signals emanating from PTB-driven ErbB2/ShcA (PTB domain dependent) and S29-W38 complexes integrate to control mammary tumor growth.

3.2.11 S29-W38 motif independently regulates PTPN12 engagement to ShcA.

Our data demonstrates that the S29-W38 region of the ShcA amino terminus has growth inhibitory properties that negatively regulate breast tumorigenesis. The loss of this 10-amino acid region is sufficient to accelerate tumor growth that parallels breast tumors that have engaged PTB-independent ShcA pools (Figure 14F). This suggested that the S29-W38 region can engage negative regulators to limit the hyperactivation of ShcA signaling complexes, which may be mirrored by those tumors that engage PTB-independent ShcA pools. Indeed, we have identified an inverse correlation between a PTB-independent ShcA gene signature and PTPN12 copy number levels in human breast cancers [414]. Moreover, several studies have reported that the loss of either the ShcA/PTPN12 interaction [7] or loss of PTPN12 alone [196] is able to potentiate breast tumorigenesis. Accordingly, we proceeded with BioID analysis to identify the level of PTPN12 engagement of FLAG-tagged ShcA^{WT}, ShcA$^{\Delta 10}$, PTB^{MUT}, and, PTB$^{MUT/\Delta 10}$ from ErbB2-transformed breast cancer cell lines (Figure 14A). The most striking observations was that the loss of the 10-amino acid region alone (ShcA$^{\Delta 10}$) was able to abolish PTPN12 interactions, reflecting the interactions observed for PTB^{MUT} expressing breast tumors (which lack a functional PTB domain) (Figure 14C and 14E). Given that both PTB^{MUT} and ShcA$^{\Delta 10}$ breast tumors are relatively paralleled in tumor growth potential (Figure 14F), these results provide further evidence that PTPN12 may indeed be the negative regulator of ShcA, requiring the S29-W38 motif to bind ShcA. However, it does not preclude other novel negative regulators that may attenuate PTB domain dependent tumorigenesis. Given our data, the S29-W38 motif seems to possess unique properties that determine the engagement of ShcA interacting proteins and that regulate downstream signaling networks during breast tumorigenesis.

3.2.12 Summary

This work provides the first *in vivo* evidence that evaluates the biological significance of unique regulatory elements within the ShcA N-terminal domain, including Serine 29 and a S29-W38 motif. We show that Serine 29 phosphorylation of both PTB-dependent or -independent ShcA complexes is necessary to negatively regulate tumor growth. This data highlights that Serine 29 controls an unidentified mitogenic signaling network(s) that is independent of the ShcA PTB domain. Finally, we demonstrate that ErbB2+ breast cancers require the ShcA S29-W38 motif to

recruit negative regulator(s) to the PTB domain to restrain Src activation and prevent the acquisition of a PTB-independent phenotype.

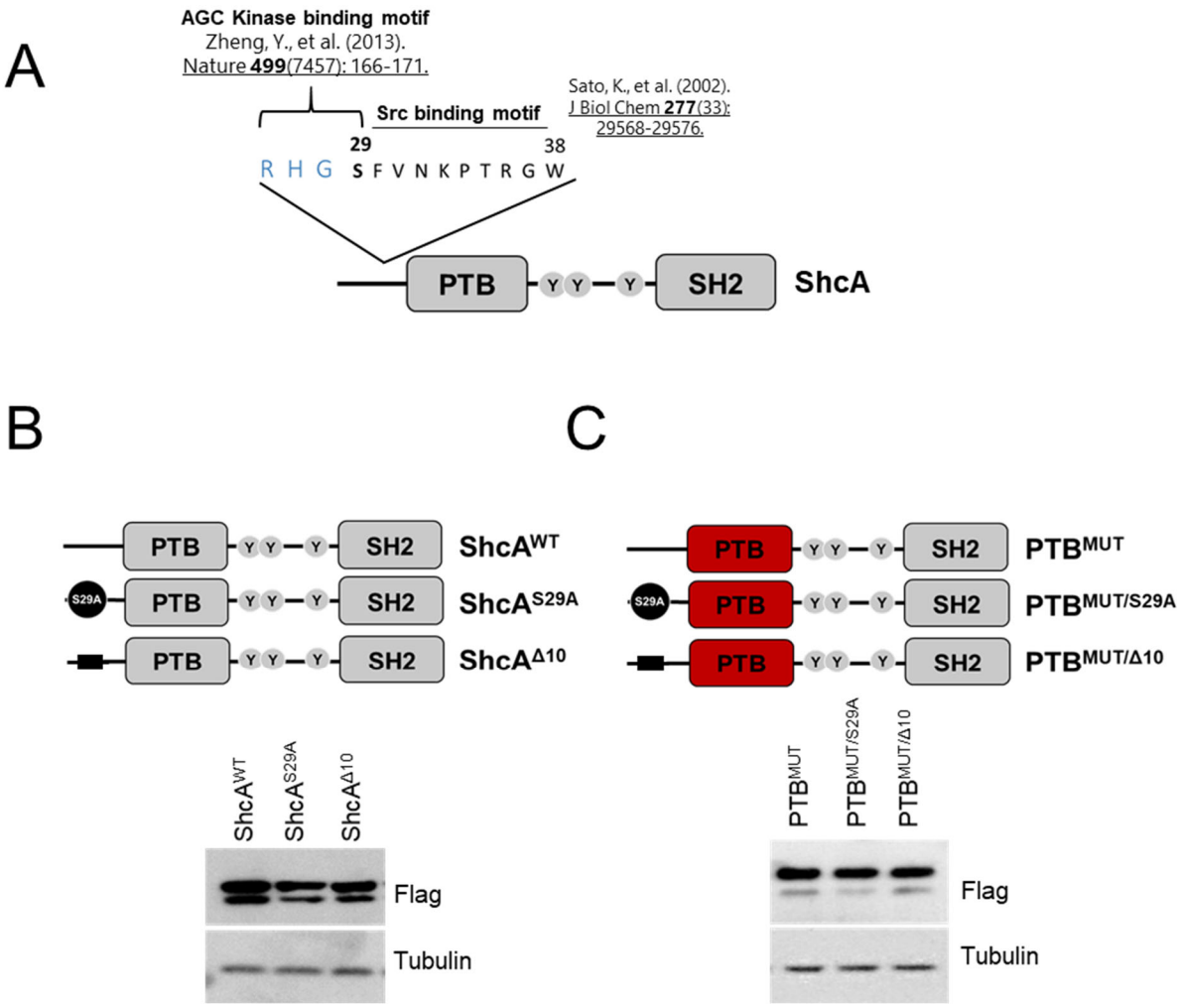

Figure 1: Serine 29 encompasses an AGC Kinase and Src binding motif. (A) Schematic representation of the location of AGC Kinase binding motif and Src binding motif within ShcA. **(B)** Schematic representation and immunoblot analysis characterizing the expression of FLAG-tagged $ShcA^{WT}$, $ShcA^{S29A}$ and $ShcA^{\Delta 10}$ alleles expressed in ErbB2-transformed mammary epithelial cell lines (NMuMG-NeuNT) using Flag antibody. **(C)** Schematic representation and immunoblot analysis characterizing the expression of FLAG-tagged PTB^{MUT}, $PTB^{MUT/S29A}$ and $PTB^{MUT/\Delta 10}$ alleles expressed in NMuMG-NeuNT using Flag antibody.

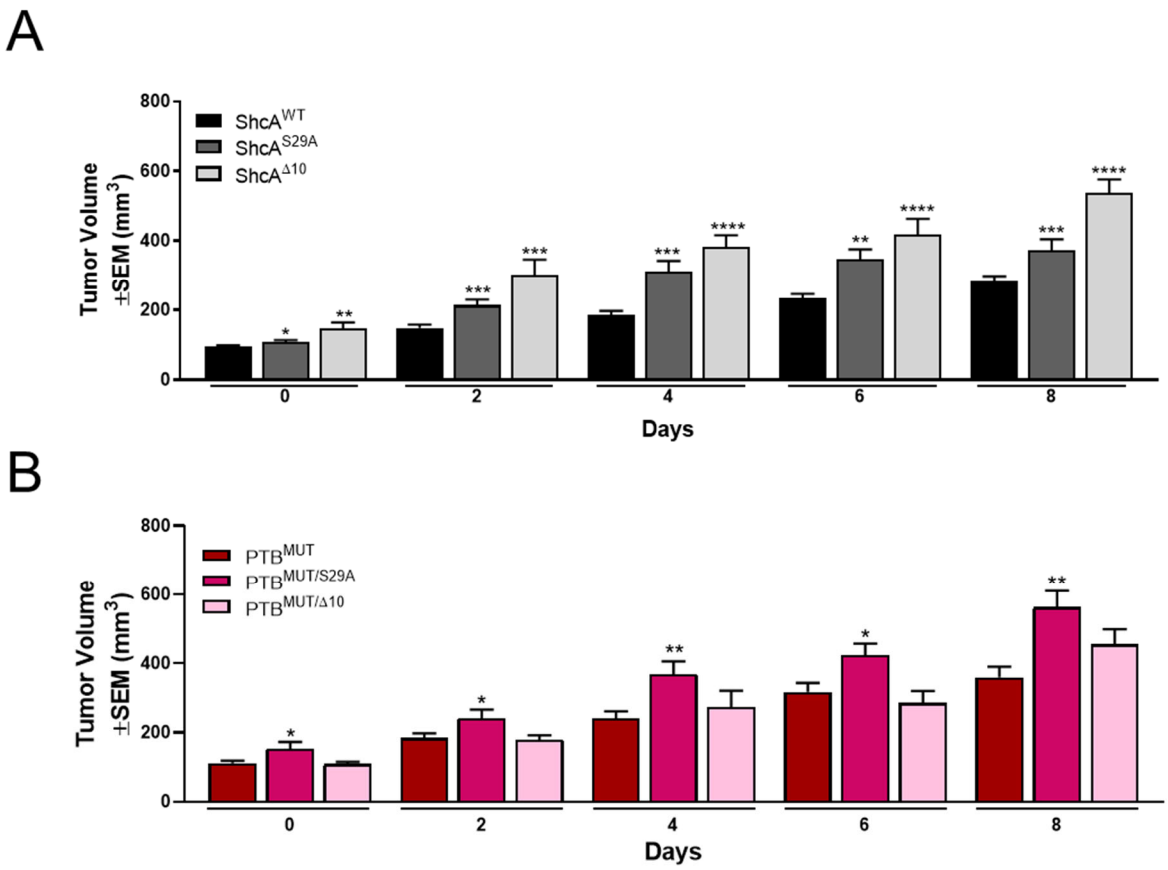

Figure 2: PTB-dependent ShcA complexes require Serine 29 and S29-W38 motif to negatively regulate breast tumor growth, *in vivo*. (A) Tumor growth curves of $ShcA^{WT}$, $ShcA^{S29A}$, and $ShcA^{\Delta 10}$ expressing ErbB2+ breast cancer cell lines (NMuMG-NeuNT) injected into the mammary fat pad of immunodeficient mice. The data is shown as an average tumor volume ± SEM (mm^3) adjusted to a volume of 100 mm^3 on Day 0 and represents $ShcA^{WT}$ n=28, $ShcA^{S29A}$ n=24, $ShcA^{\Delta 10}$ n=18, per group. $ShcA^{WT}$ vs. $ShcA^{S29A}$ or $ShcA^{\Delta 10}$: *$P<0.05$, **$P<0.01$, ***$P<0.001$, ****$P<0.0001$. **(B)** Tumor growth curves of PTB^{MUT}, $PTB^{MUT/S29A}$ and $PTB^{MUT/\Delta 10}$ expressing ErbB2+ breast cancer cell lines (NMuMG-NeuNT) injected into the mammary fat pad of immunodeficient mice. The data is shown as an average tumor volume ± SEM (mm^3) adjusted to a volume of 100 mm^3 on Day 0 and represents PTB^{MUT} n=23, $PTB^{MUT/S29A}$ n=13, $PTB^{MUT/\Delta 10}$ n=12 per group. PTB^{MUT} vs. $PTB^{MUT/S29A}$: *$P<0.05$, **$P<0.01$.

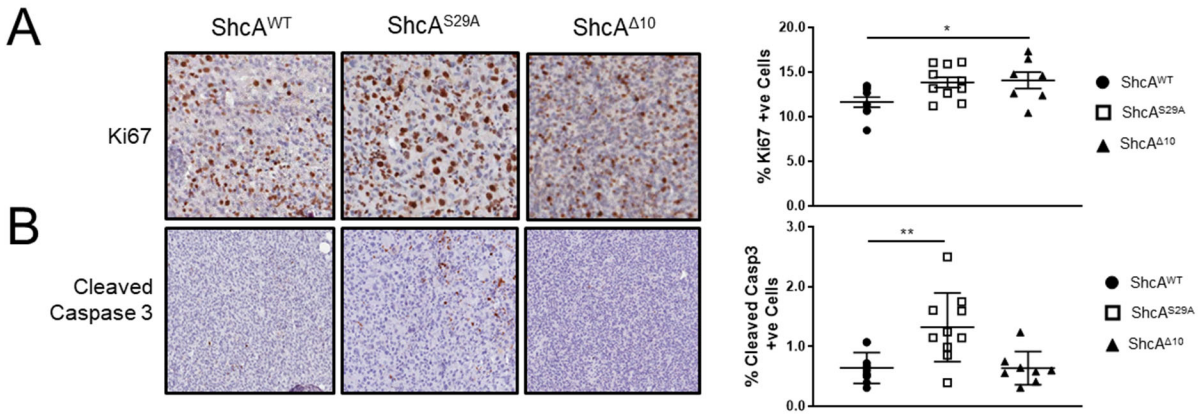

Figure 3: Immunohistochemical staining of mammary tumors using **(A)** Ki67 and **(B)** Cleaved Caspase 3 specific antibodies. The data depicts the average positively stained cells ± SEM and is representative of 9-10 tumors per group. $ShcA^{WT}$ vs. $ShcA^{S29A}$ or $ShcA^{\Delta 10}$: **P<0.05, **P<0.01*.

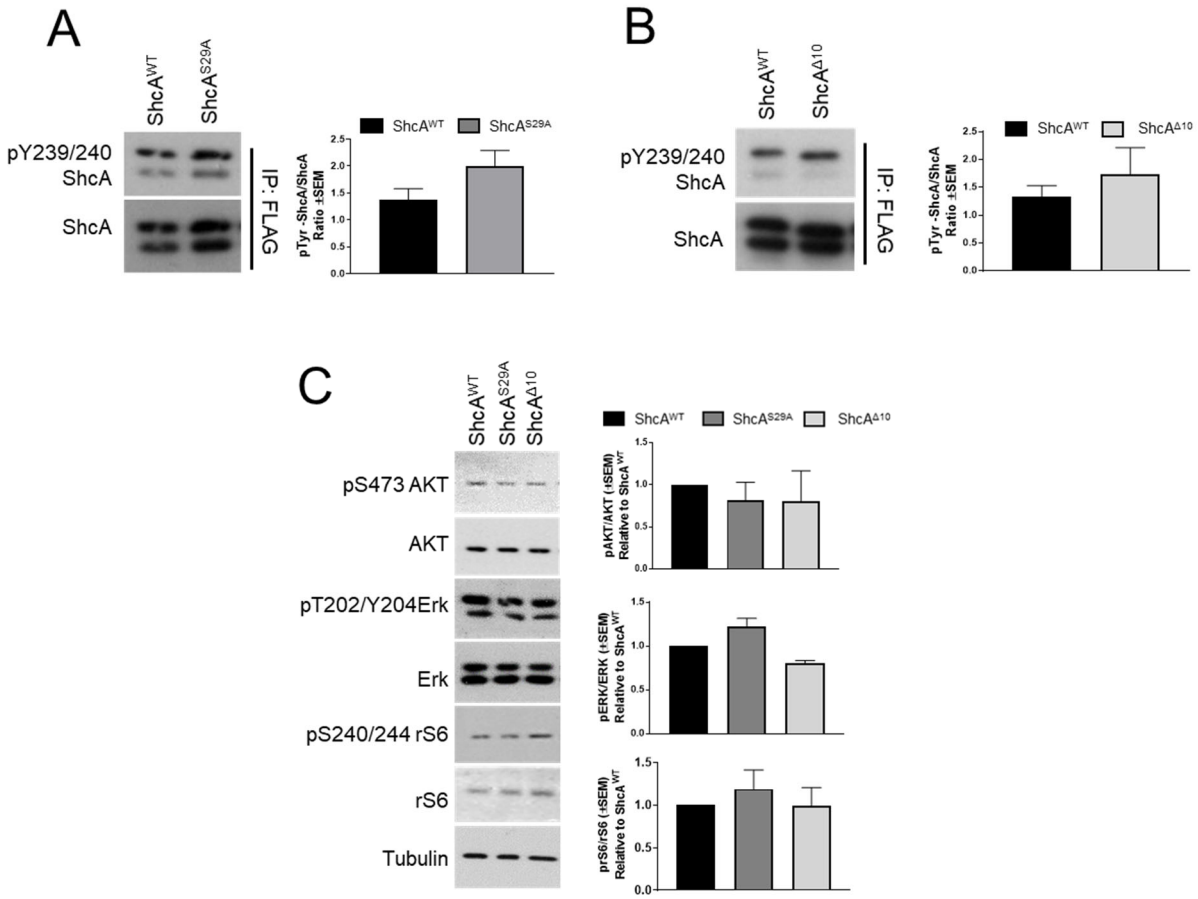

Figure 4: Increased tumor outgrowth mediated by the loss of Serine 29 phosphorylation and S29-W38 motif of PTB-dependent ShcA pools is independent of ShcA tyrosine phosphorylation, *in vitro.* (A, B) Baseline tyrosine phosphorylation levels of FLAG-tagged ShcA^{WT}, ShcA^{S29A}, and ShcA$^{\Delta 10}$ constructs expressed in NMuMG-NeuNT cell lines assessed by immunoprecipitation using Flag antibody and analyzed by immunoblot using pTyr239/240 ShcA antibody. The data is representative of 3 independent experiments (means ± SEM). Densitometric quantification was performed using Image J software. **(C)** Immunoblot analysis using indicated antibodies of ErbB2-driven breast cancer cells (NMuMG-NeuNT) expressing specified FLAG-tagged ShcA alleles. The data is representative of 3-5 independent experiments (means ± SEM). Densitometric quantification was performed using Image J software.

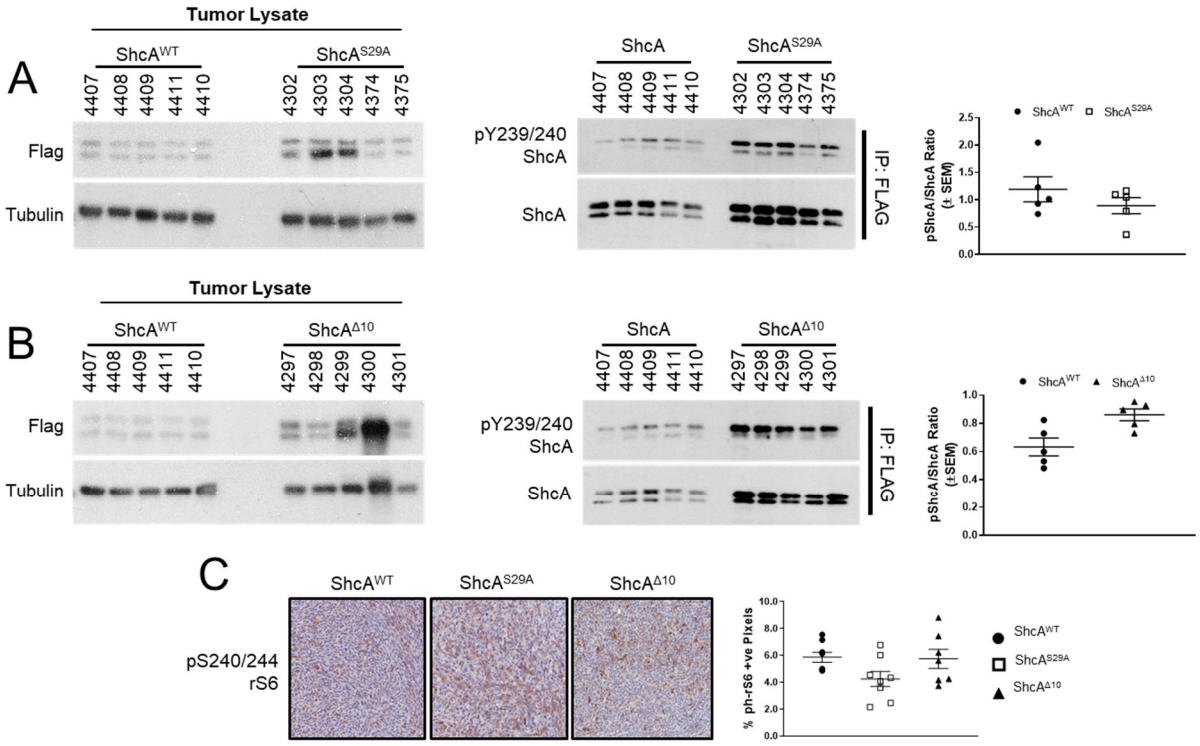

Figure 5: Increased tumor growth mediated by the loss of Serine 29 phosphorylation and S29-W38 motif of PTB-dependent ShcA pools is independent of ShcA tyrosine phosphorylation, *in vivo.* Tumor lysates and immunoprecipitates of **(A)** ShcA^{WT} and ShcA^{S29A}, **(B)** ShcA^{WT} and ShcA$^{\Delta 10}$ alleles expressed in NMuMG-NeuNT breast tumors harvested at endpoint depicted in Figure 2A. Five tumors per genotype were immunoprecipitated using Flag antibody and analyzed by immunoblot using pY239/240 ShcA and ShcA antibody. Densitometric quantification was performed using Image J software.

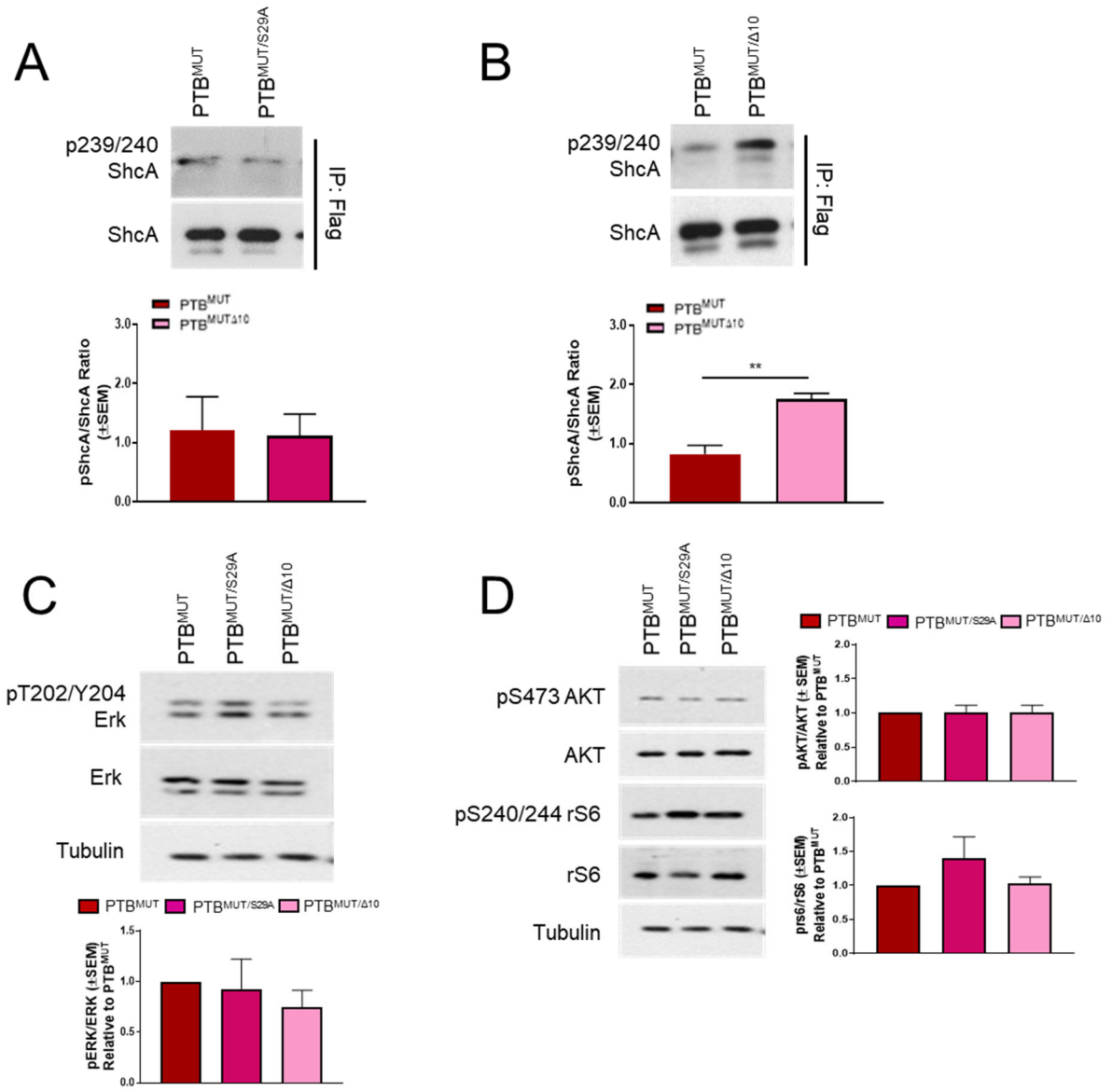

Figure 6: Breast tumors engaging PTB-independent ShcA pools do not rely on ShcA tyrosine phosphorylation for tumor growth. Baseline tyrosine phosphorylation of FLAG-tagged **(A)** PTB^{MUT} and $PTB^{MUT/S29A}$ and **(B)** PTB^{MUT} and $PTB^{MUT/\Delta10}$ expressed in NMuMG-NeuNT cell lines assessed by immunoprecipitation using Flag antibody and analyzed by immunoblot using pTyr239/240 ShcA antibody. The data is representative of 4 independent experiments (means ± SEM). Densitometric quantification was performed using Image J software. PTB^{MUT} vs $PTB^{MUT/\Delta10}$: $^{**}P<0.01$. **(C, D)** Immunoblot analysis using indicated antibodies of ErbB2-driven breast cancer cells expressing specified FLAG-tagged ShcA alleles. The data is representative of 3-6 independent experiments (means ± SEM). Densitometric quantification was performed using Image J software.

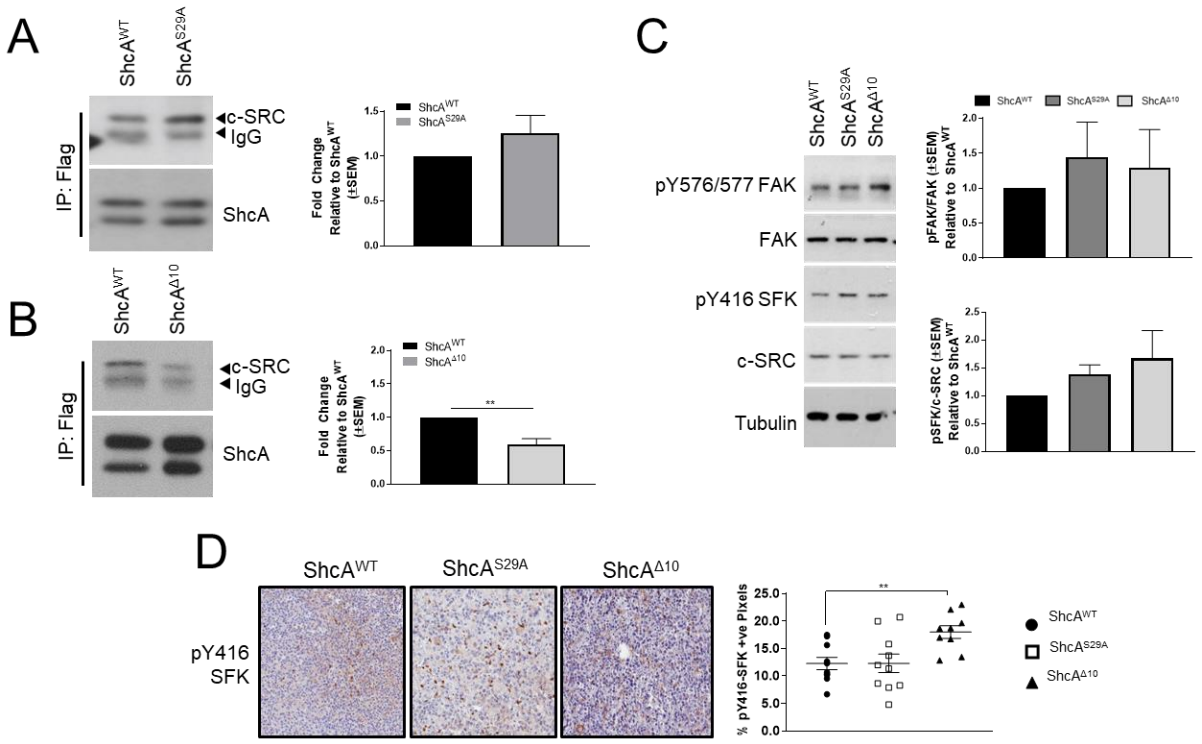

Figure 7: Loss of Serine 29 phosphorylation and S29-W38 motif in PTB-dependent ShcA pools increases tumor growth independent of Src recruitment and activity. Immunoprecipitation using Flag antibody of **(A)** ShcA^{WT} and ShcA^{S29A} and **(B)** ShcA^{WT} and ShcA$^{\Delta 10}$ alleles expressed in NMuMG-NeuNT cell line and characterized by immunoblot using Src and ShcA antibodies. The data is representative of 4 independent experiments (means ± SEM). Densitometric quantification was performed using Image J software. **(C)** Immunoblot analyses of steady state FAK and SFK activation in ErbB2-driven breast cancer cells expressing specified FLAG-tagged ShcA alleles. The data is representative of 3-5 independent experiments (means ± SEM). Densitometric quantification was performed using Image J software. **(D)** Immunohistochemical staining of mammary tumors using pY416 SFK antibody. The data depicts the average positively stained pixels ± SEM and is representative of 8-9 tumors per group.

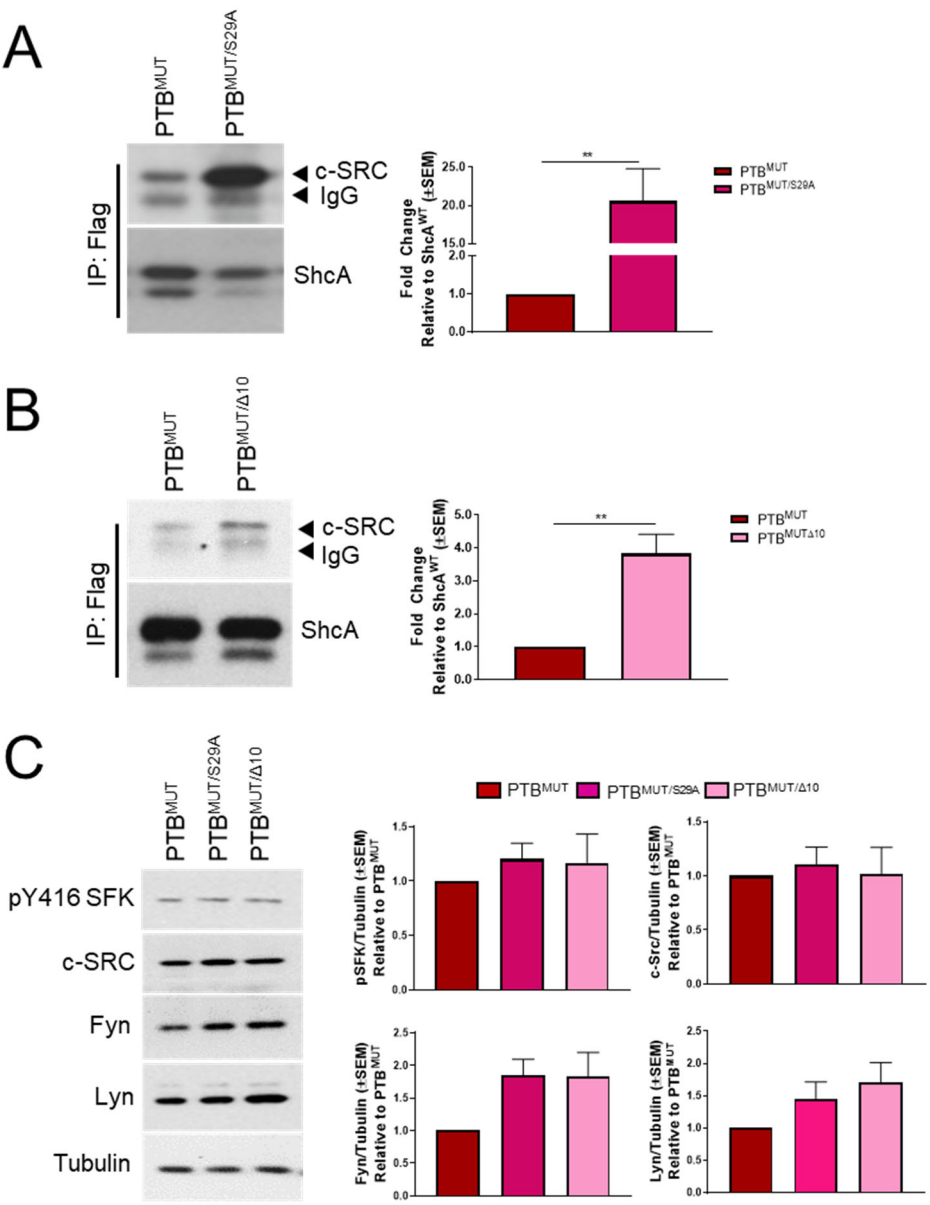

Figure 8: Src recruitment and activation are uncoupled in breast tumors engaging PTB-independent ShcA pools upon the loss of Serine 29 phosphorylation and S29-W38 motif. Immunoprecipitation using Flag antibody of **(A)** PTB^{MUT} and $PTB^{MUT/S29A}$ and **(B)** PTB^{MUT} and $PTB^{MUT/\Delta10}$ alleles expressed in NMuMG-NeuNT cell line and characterized by immunoblot using Src and ShcA antibodies. The data is representative of 4 independent experiments (means ± SEM). Densitometric quantification was performed using Image J software. PTB^{MUT} vs $PTB^{MUT/S29A}$ or $PTB^{MUT/\Delta10}$: ***P<0.01*. **(C)** Immunoblot analyses of steady state activation of SFKs and levels of SFK members, Src, Fyn and Lyn. Densitometric quantification was performed using Image J software. Barplots are representative of 3-6 independent experiments (means ± SEM).

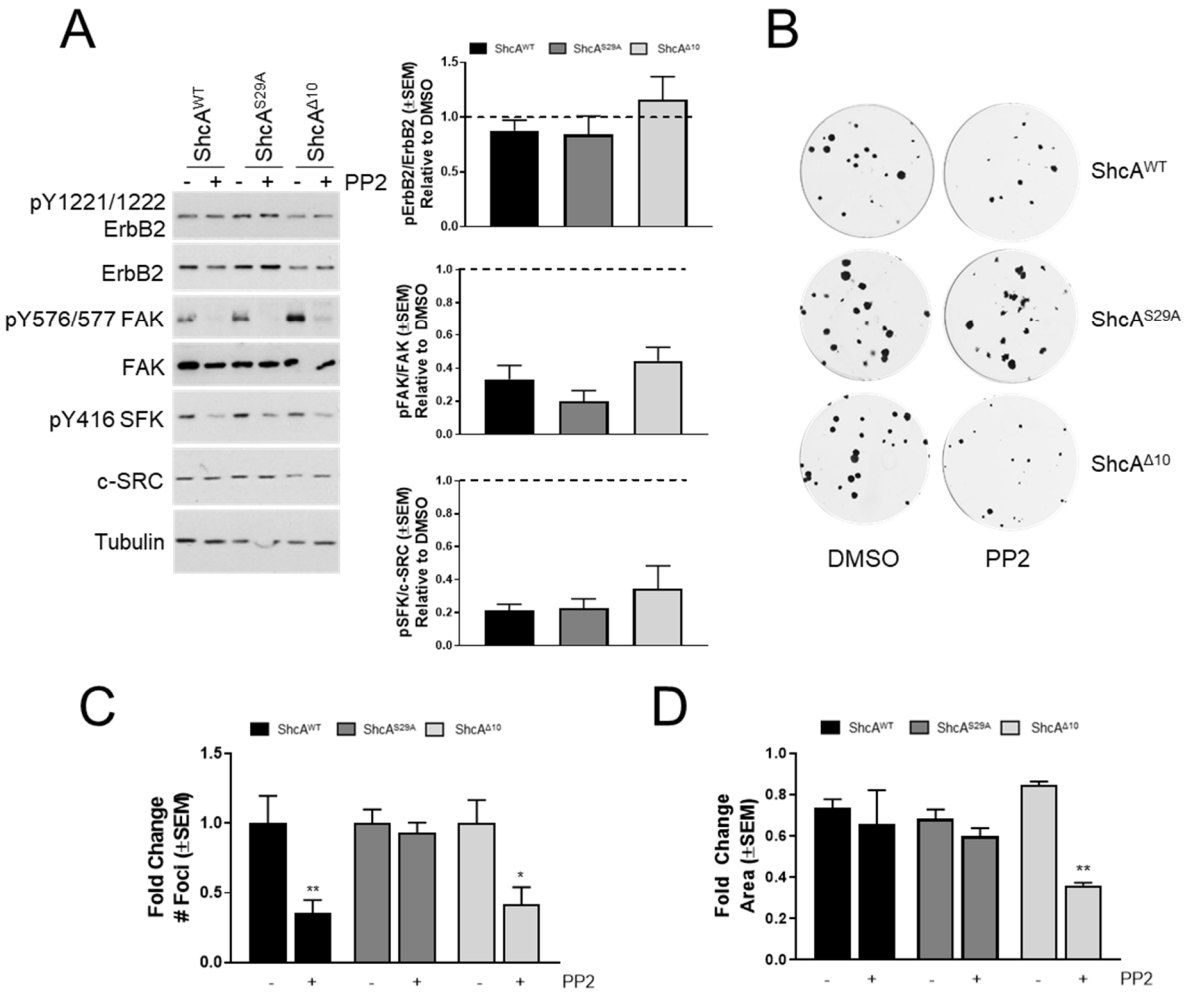

Figure 9: Loss of Serine 29 promotes resistance to SFK inhibition in ErbB2-transformed breast cancer cells. (A) Immunoblot analysis of specified cell lines upon treatment of vehicle alone (DMSO) or PP2 (2.0 μM). Densitometric quantification of immunoblots was performed using Image J software. The data is representative of 3-5 independent experiments (means ± SEM). **(B)** Clonogenic Assay—representative images of ErbB2 transformed breast cancer cell lines expressing specified FLAG-tagged PTB^{MUT} alleles upon treatment with DMSO or PP2 (2.0 μM) after 10 days. Clonogenic assay of specified cell lines treated with DMSO or PP2 (2.0 μM). The data is shown as fold change in **(C)** number of foci or **(D)** average area relative to DMSO control. The data is representative of 4 independent experiments (means ± SEM). *$P<0.05$, **$P<0.01$, ***$P<0.001$.

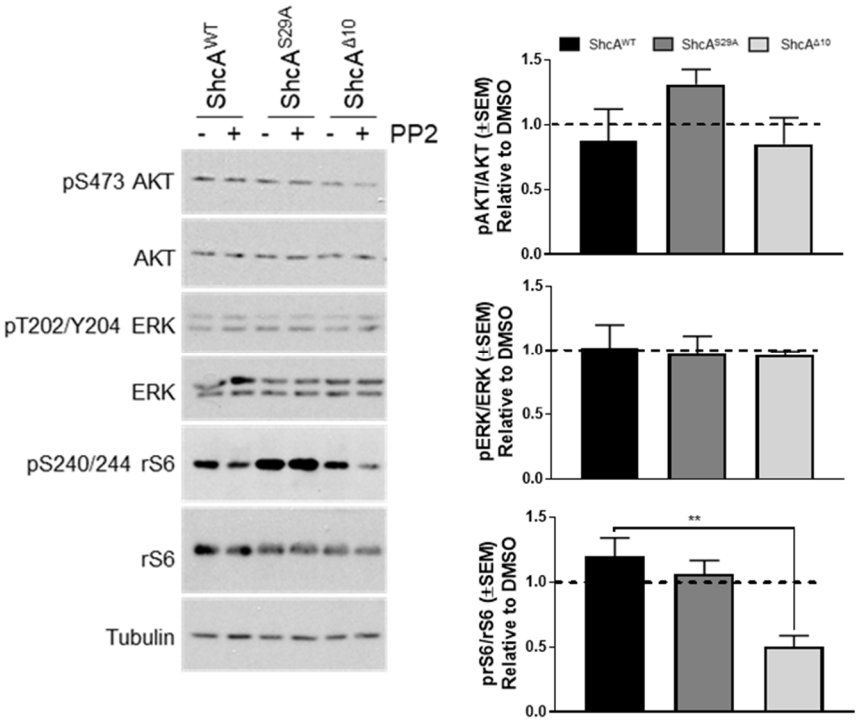

Figure 10: Immunoblot analysis using indicated antibodies of $ShcA^{WT}$, $ShcA^{S29A}$, and $ShcA^{\Delta 10}$, expressing NMuMG-NeuNT breast cancer cell lines upon treatment of vehicle alone (DMSO) or PP2 (2.0 μM) at 7 hours. Densitometric quantification of immunoblots was performed using Image J software. The data is representative of 3-5 independent experiments (means ± SEM). ***P<0.01.*

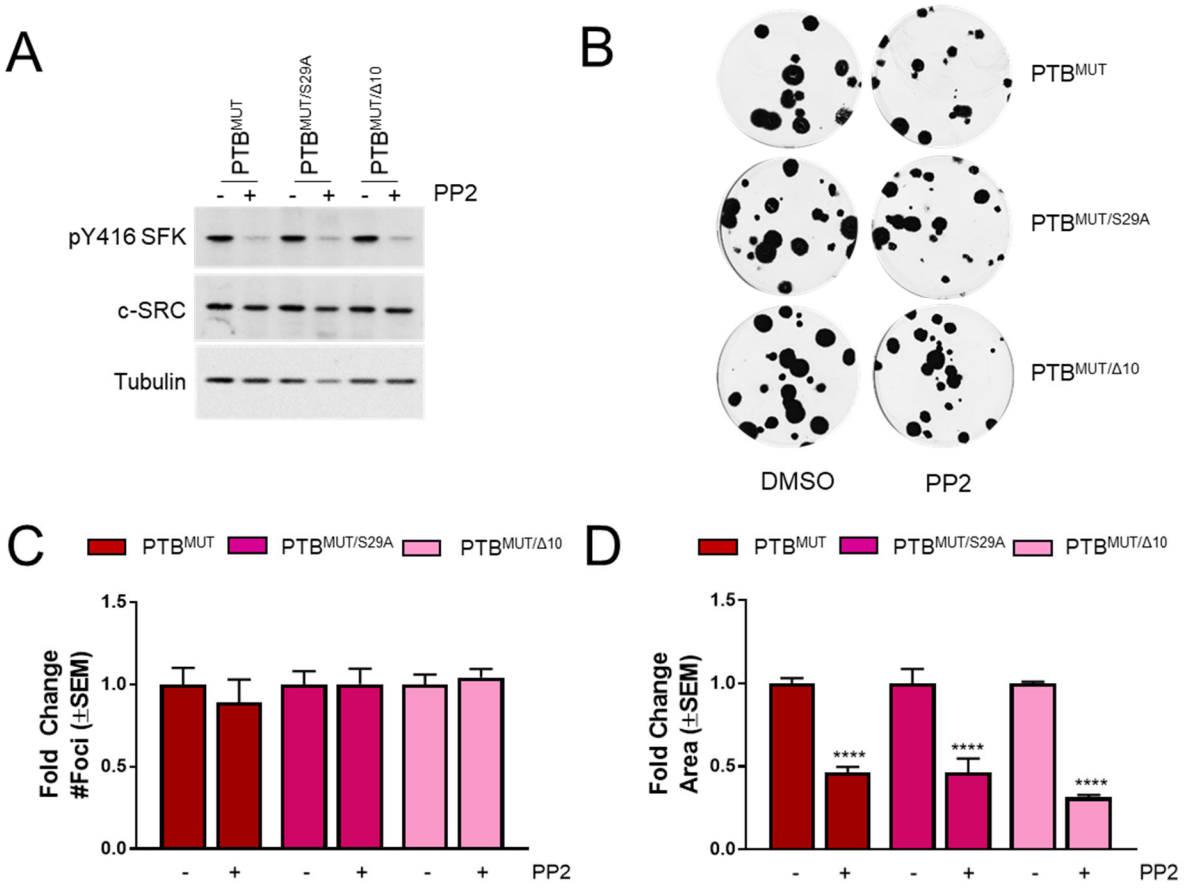

Figure 11: Src activation is necessary for PTB-independent breast tumors. (A) Immunoblot analysis of PTB^{MUT}, $PTB^{MUT/S29A}$ and $PTB^{MUT/\Delta10}$ expressing breast cancer cell lines (NMuMG-NeuNT) confirming Src inhibition using PP2 (2 μM) and vehicle alone (DMSO) at 7 hours. **(B)** Clonogenic Assay—representative images of ErbB2 transformed breast cancer cell lines expressing specified FLAG-tagged PTB^{MUT} alleles upon treatment with DMSO or PP2 (2 uM) after 10 days. Clonogenic assay of specified cell lines treated with DMSO or PP2 (2.0 μM). The data is shown as fold change in **(C)** number of foci or **(D)** average area relative to DMSO control. The data is representative of 3 independent experiments (means ± SEM). ****$P<0.0001$.

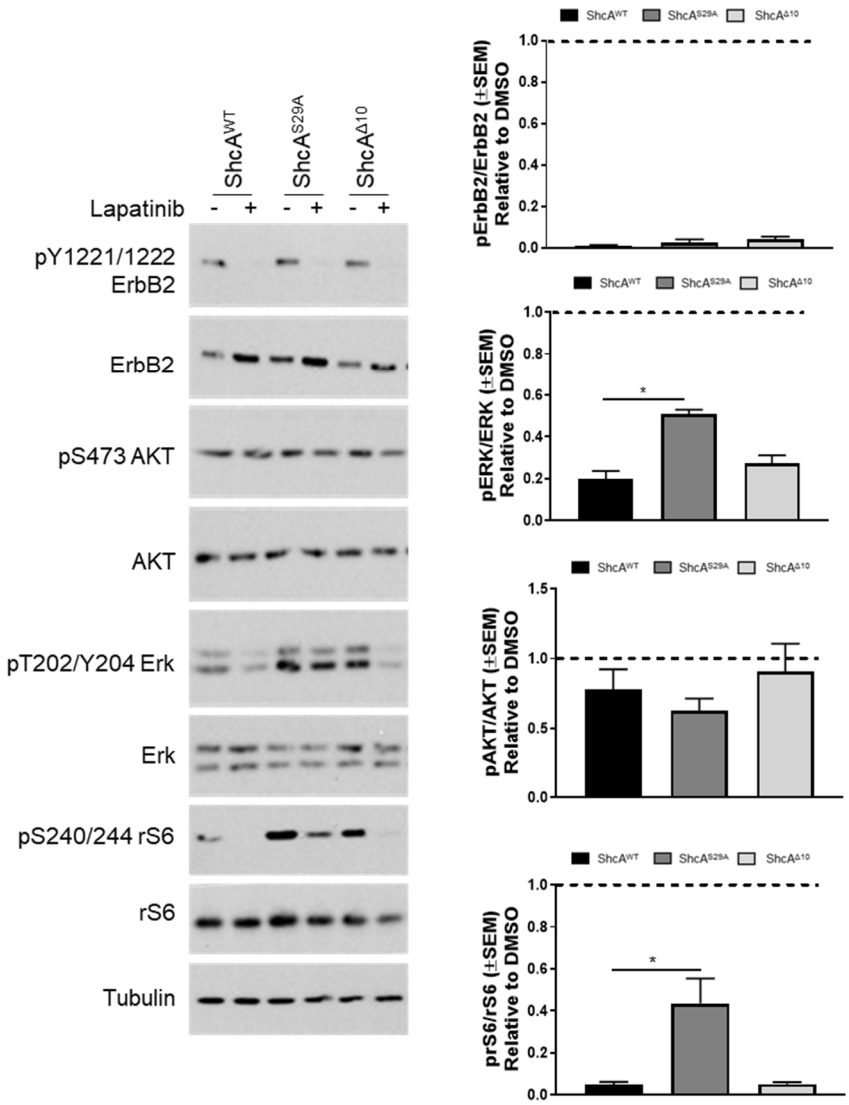

Figure 12: Serine 29 and S29-W38 motif differentially regulate mitogenic signaling response to Lapatinib in breast tumors engaging PTB-dependent ShcA pools. Immunoblot analysis of ShcA^{WT}, ShcA^{S29A}, and ShcA$^{\Delta 10}$ expressing NMuMG-NeuNT breast cancer cell lines upon treatment of vehicle alone (DMSO) or Lapatinib (0.5 μM) at 7 hours using indicated antibodies. Densitometric quantification of immunoblots was performed using Image J software. The data is representative of 3-6 independent experiments (means ± SEM). ShcA^{WT} vs. ShcA^{S29A}: *$P<0.05$.

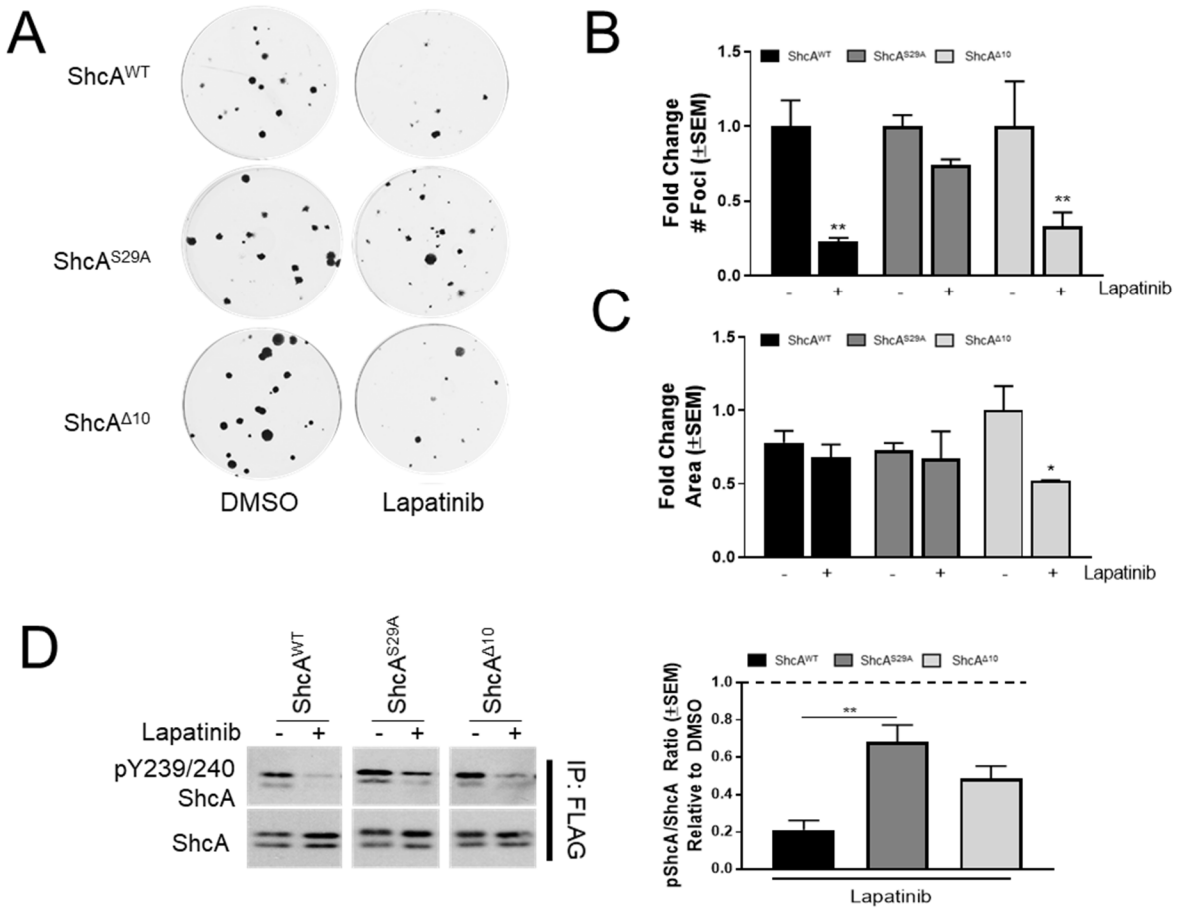

Figure 13: Loss of Serine 29 phosphorylation confers Lapatinib resistance in PTB-dependent ShcA expressing breast tumors and is associated with the activation of the ShcA phospho-tyrosine residues. (A) Clonogenic Assay—representative images of NMuMG-NeuNT cell lines expressing FLAG-tagged $ShcA^{WT}$, $ShcA^{S29A}$, $ShcA^{\Delta 10}$, and, alleles upon treatment with DMSO or Lapatinib (0.5 μM) every 2 days for 10 days. Clonogenic assay of specified cell lines treated with DMSO or Lapatinib (0.5 μM). The data is shown as fold change in **(B)** number of foci or **(C)** average area relative to DMSO control. *$P<0.05$, **$P<0.01$. **(D)** Level of tyrosine phosphorylation of ShcA relative to DMSO upon treatment of DMSO or Lapatinib (0.5 μM) assessed by immunoprecipitation of FLAG-tagged ShcA alleles using Flag and pY239/240 ShcA antibodies. The data is representative of 3 independent experiments (means ± SEM). Densitometric quantification was performed using Image J software. **$P<0.01$.

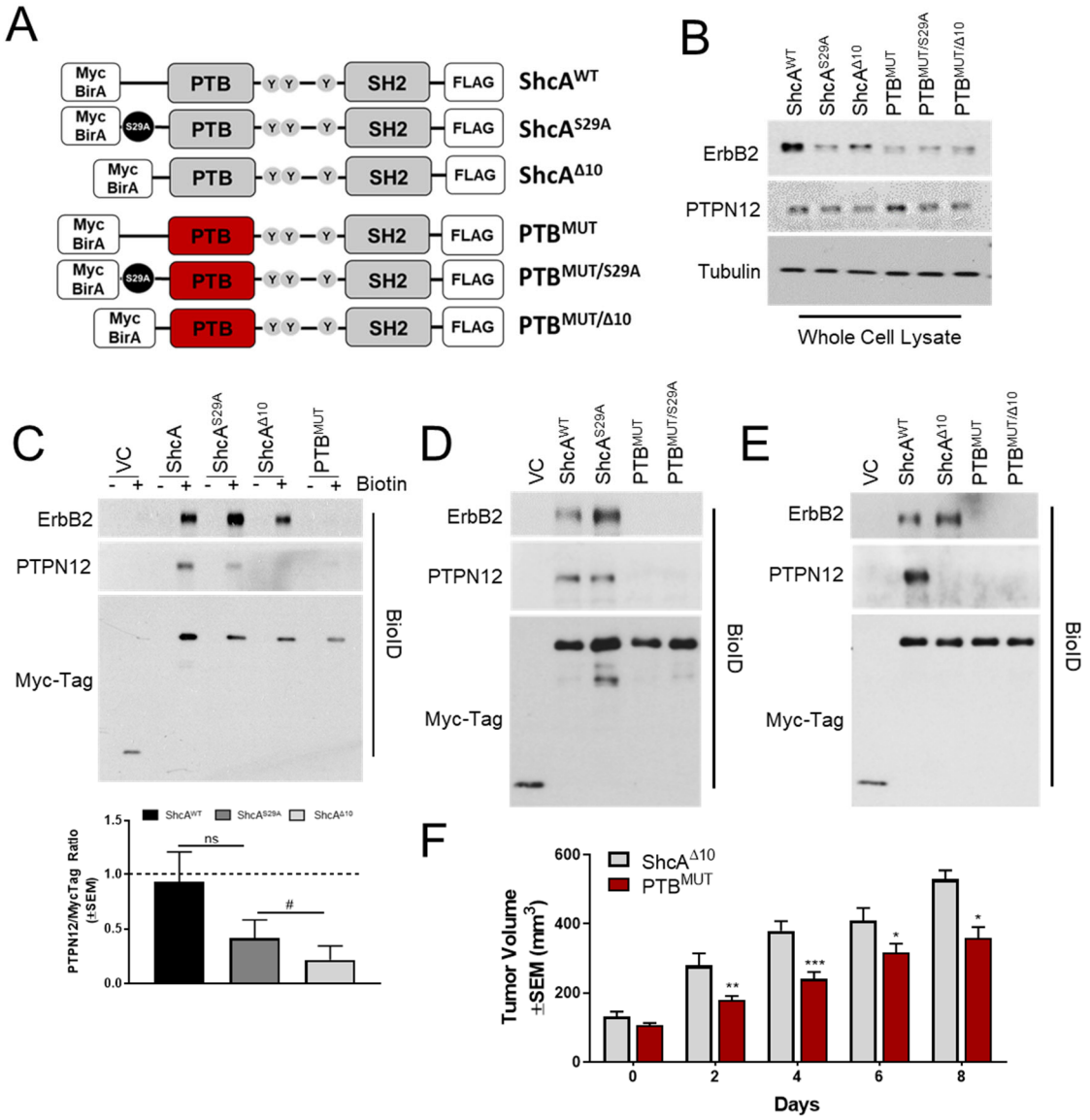

Figure 14: S29-W38 motif regulates PTPN12 engagement to PTB-dependent ShcA pools independent of the ShcA PTB domain. (A) Schematic diagram illustrating MycBirA fusion proteins expressed in NMuMG-NeuNT cell lines for BioID. **(B)** Immunoblot analysis confirming the expression of ErbB2 and PTPN12 in whole cell lysates of ShcA^{WT}, ShcA^{S29A}, ShcA$^{\Delta10}$, PTB^{MUT}, PTB$^{MUT/S29A}$, and PTB$^{MUT/\Delta10}$ expressing NMuMG-NeuNT breast cancer cell lines **(C)** BioID characterizing the interaction of ErbB2 and PTPN12 with ShcA^{WT}, ShcA^{S29A}, ShcA$^{\Delta10}$, PTB^{MUT} alleles. Densitometric analysis was performed using Image J software. Barplot is representative of 3-4 independent experiments (means ± SEM). ShcA^{S29A} vs. ShcA$^{\Delta10}$: $^{\#}P<0.05$. **(D, E)** BioID characterizing the interaction of ErbB2 and PTPN12 with PTB^{MUT}, PTB$^{MUT/S29A}$, and PTB$^{MUT/\Delta10}$ alleles **(F)** Tumor growth curves of ShcA$^{\Delta10}$ and PTB^{MUT} expressing breast tumors observed in Figure 2A and 2B, respectively to compare tumor growth potential. Tumor volumes were adjusted to 100 mm^3 on Day 0. ShcA$^{\Delta10}$ vs PTB^{MUT}: $*P<0.05$, $**P<0.01$, $***P<0.001$.

Chapter 4 Discussion

4.1 Discussion

In this study we focused on ShcA, a modular protein that is essential for breast cancer initiation and progression, as well as an integral convergence point downstream of multiple tyrosine kinases that potentiate breast tumor growth. Transgenic mouse models have reinforced the requirement of an intact ShcA signaling network during breast cancer development. Indeed, the complete loss of ShcA from ErbB2+ luminal mammary epithelial cells results in the dramatic impairment of breast tumor formation (9% penetrance) [9, 392]. Additionally, animals that are debilitated in ShcA tyrosine signaling are significantly delayed in mammary tumor onset and outgrowth [392, 395]. The engagement of the ShcA PTB domain to RTKs, including ErbB2, is considered a major initiating event that activates the pro-mitogenic signals necessary for breast tumorigenesis. Studies have shown that the deletion of the five main tyrosine phosphorylation residues within the cytoplasmic tail of ErbB2, including the ShcA binding site, compromises the ability of ErbB2 to induce mammary tumorigenesis [1]. Reconstitution of the ErbB2 ShcA binding site alone, is sufficient to restore breast tumor development [1]. On the other hand, the ShcA PTB domain is also important for signal termination. This property of ShcA ensures that the strength and duration of pro-tumorigenic responses is tightly controlled. For example, the ShcA PTB domain temporally controls EGFR signaling networks by facilitating the delayed recruitment of PTPN12 which terminates ShcA-dependent signaling downstream of its phospho-tyrosine residues [3, 7, 321]. In effect, through its ability to bind negative regulators, including protein tyrosine phosphatases (PTPN12, PTPε) and inositol phosphatases (SHIP2), the ShcA PTB domain can serve to limit the hyperactivation of ShcA-dependent mitogenic responses, independent of activated RTKs [2, 4, 5]. Given the absolute requirement for ShcA signaling in ErbB2+ breast cancer progression, unraveling how unique PTB-dependent and -independent signaling complexes integrate to promote mammary tumorigenesis deserves investigation.

Our previous work demonstrates that simultaneous expression of ShcA alleles that can ($ShcA^{WT}$) or cannot (PTB^{MUT}) partake in PTB-driven interactions, results in the paralleled activation of distinct ShcA pools that amplify breast tumor growth [2]. Using this approach, we aimed to dissect the molecular mechanisms by which PTB-dependent and -independent ShcA complexes potentiate mammary tumorigenesis. In effect, we identify a significant divergence of intracellular signaling pathways downstream of the ShcA PTB domain that positively and negatively regulate breast

tumor growth and response to tyrosine kinase inhibitors. Moreover, we establish that distinct regions in the N-terminal domain of ShcA (Serine 29 and S29-W38 region) utilize non-overlapping mechanisms to negatively regulate tumorigenic responses upstream of the ShcA PTB domain. The following discussion will address and evaluate the evidence of each study.

4.1.1 Distinct intracellular ShcA signaling complexes transduce diverse and non-redundant mitogenic signals.

Canonical ShcA signaling is primarily associated with the recruitment of the ShcA PTB domain to activated RTKs and the subsequent phosphorylation of its tyrosine residues to convey mitogenic and cell survival signals for breast cancer initiation and progression [1, 392, 393, 395]. It is also well established that PTB-coupled ShcA complexes require the phosphorylation of ShcA tyrosine residues to activate AKT/mTOR signaling [395, 406]. Interestingly, while ShcA tyrosine phosphorylation can activate AKT/mTOR signaling from PTB-independent ShcA complexes, these sites are surprisingly dispensable for mammary tumor growth *in vivo*. Instead, PTB-independent ShcA complexes primarily rely on the SH2 domain to activate SFKs and amplify tumor growth (Figure 1). Previous studies have identified a phosphorylation-dependent gating mechanism whereby tyrosine phosphorylation of ShcA induces a conformational change, which opens the SH2 domain to increase its ability to bind ligands [372]. This may explain why breast cancer cells expressing a mutant ShcA allele lacking both a functional PTB domain and the tyrosine phosphorylation sites (PTB^{MUT}/3F) are debilitated in their transforming potential *in vitro*. On the other hand, we show that ShcA-PTB^{MUT}/3F expressing cells can still robustly potentiate tumor growth *in vivo*. These data suggest that cross talk with the tumor microenvironment may transduce signals that permit SH2-driven signaling independently of ShcA tyrosine phosphorylation. As the aforementioned study was in the context of a wild-type ShcA allele [372], the impact of this gating mechanism on the ability of PTB-independent ShcA pools to activate the SH2 domain has yet to be determined.

The activation of AKT/mTOR signaling downstream of RTKs, has also been demonstrated to involve the ShcA SH2 domain. Indeed, the ShcA SH2 domain can serve an accessory role to engage 14-3-3 adaptor proteins and PI3K to augment AKT/mTOR signaling [358]. However, we show that the SH2 domain is dispensable for AKT/mTOR signaling from PTB-independent ShcA

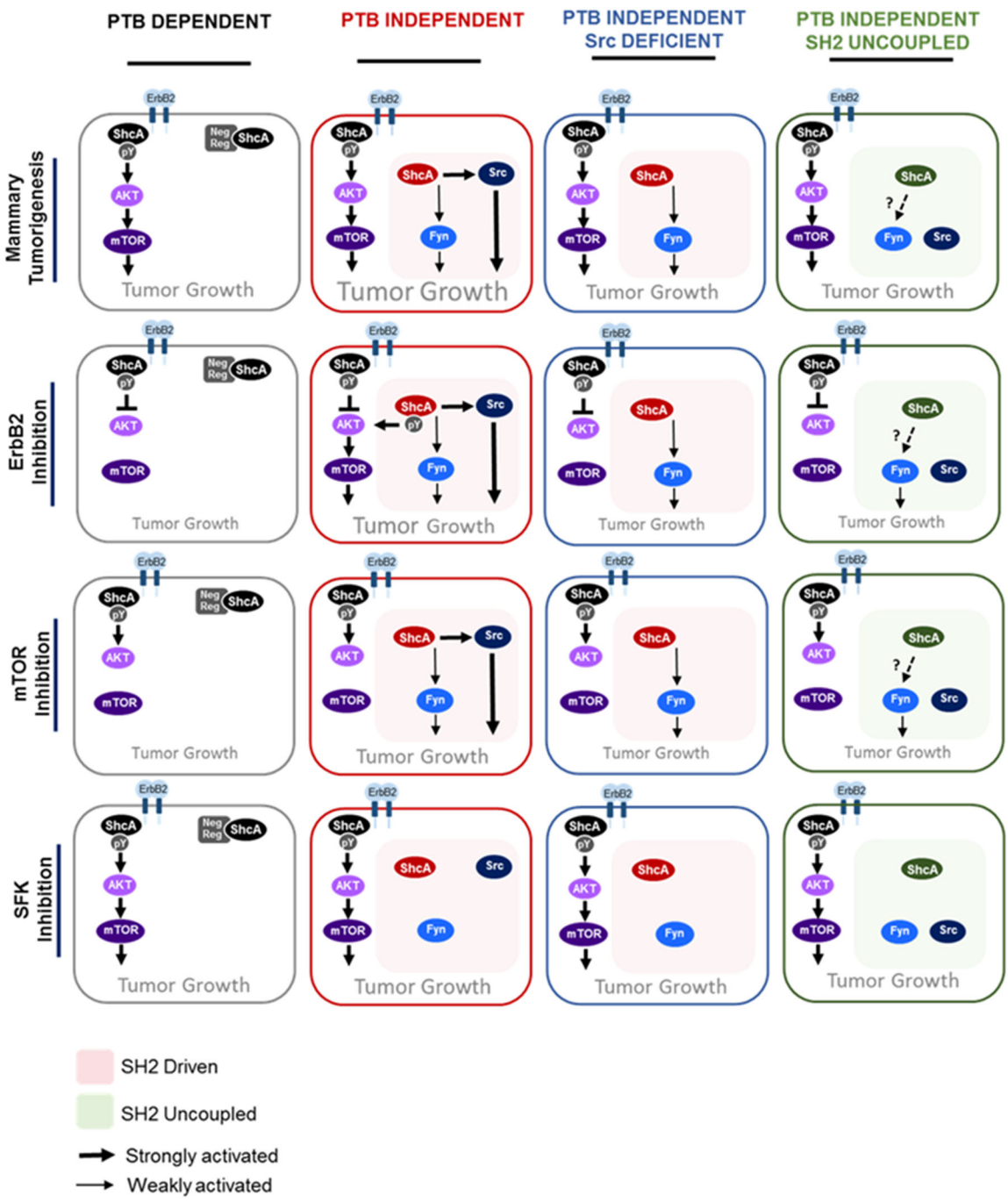

Figure 1: Distinct ShcA dependent pools influence breast tumor growth and therapeutic responsiveness downstream of RTKs during mammary tumorigenesis. Schematic diagram summarizing the biological impact of PTB-dependent and -independent ShcA signaling complexes on breast tumor growth along with sensitivity to pharmacological and/or genetic inhibition of the ErbB2, mTOR and Src family kinase pathways.

pools which highlights the inherent plasticity of these breast cancers to adapt to the loss of extracellular stimuli required for tumor initiation. Yet, this does not suggest that the hyperactivation of PTB-independent ShcA signaling complexes renders breast tumors insensitive to the mTOR pathway. We demonstrate that the transforming potential of breast tumors are sensitive to pharmacological mTOR inhibitors regardless of whether they are exclusively reliant on PTB-coupled or PTB-uncoupled ShcA pools (Figure 1). Rather, these data established that the activation of the AKT/mTOR pathway in breast tumors was primarily through PTB-dependent ShcA signaling complexes. Corroborating these results, studies have shown that increasing eIF4E activity is sufficient to restore the tumorigenic potential of breast cancer cells that are deficient in phospho-tyrosine ShcA signaling from PTB-coupled complexes [395, 415]. Despite this fact, loss of SH2-driven ShcA signaling from PTB-independent complexes, attenuates Src activation and consequently reduces tumor growth. Altogether, these data corroborate the notion that distinct intracellular ShcA signaling complexes transduce diverse and non-redundant mitogenic signals, that activate the AKT/mTOR (PTB-dependent) and Src (PTB-independent) pathways to cooperatively promote mammary tumorigenesis.

4.1.2 Aberrant activation of distinct ShcA signaling complexes results in therapeutic resistance to tyrosine kinase inhibitors.

Intense research efforts continue to focus on the inhibition of RTKs in the treatment of specific breast cancer subtypes. For example, in ErbB2-driven breast cancers, targeted therapies inhibiting ErbB2 and/or ErbB3 signaling are either the standard of care or are actively being pursued in clinical trials. Although numerous studies show that mutational activation, amplification or overexpression of multiple components in the tyrosine kinome promote therapeutic resistance to ErbB2-targeted therapies in breast cancer [9], our study also suggests that the loss of intracellular negative feedback loops through the aberrant activation of distinct ShcA signaling complexes can result in therapeutic resistance to tyrosine kinase inhibitors. Indeed, we show that the hyperactivation of PTB-independent ShcA signaling complexes confers Lapatinib resistance by increasing SH2-driven Src signaling. Moreover, debilitating the ShcA SH2 domain or inactivating Src overcomes Lapatinib resistance conferred by PTB-independent ShcA signaling complexes (Figure 1). This is consistent with previous studies demonstrating that Src is hyperactivated in

Trastuzumab resistant, HER2+ breast cancers and that Src inhibition is able to re-sensitize them to ErbB2-targeted therapies [235].

Our data reinforces the importance of SFKs in enhancing tumorigenic signals from PTB-independent ShcA pools (PTB^{MUT}) through multiple experimental approaches. First, Src or Fyn deletion by CRISPR/Cas9 genomic editing and/or Cre-mediated excision selectively attenuated transformation from PTB-independent ShcA pools. In contrast, both Src and Fyn were dispensable for tumor growth transduced exclusively by PTB-coupled ShcA complexes ($ShcA^{WT}$). On the other hand, Lyn was dispensable in its transforming ability downstream of both PTB-dependent and -independent ShcA signaling pools. This highlighted that potential differences may exist for SFK dependency across breast cancer subtypes. Indeed, studies have described Lyn as an important effector of tumorigenicity in basal breast cancers [139]. We also show that sub-optimal doses of pharmacological Src inhibitor, PP2, selectively sensitized breast cancer cells that engaged PTB-independent ShcA complexes (Figure 1). Finally, while the loss of SH2-driven ShcA signaling from PTB-independent complexes reduced Src activity and impaired mammary tumorigenesis, emerging breast tumors re-activated Src at the experimental end-point. This demonstrated that there are significant selective pressures exerted upon these cells to restore Src signaling. Corroborating this observation, we found that Src deficiency in concert with impaired SH2-driven signaling from PTB-independent ShcA pools in ErbB2+ breast cancer cells resulted in increased Fyn expression. Taken together, we propose that the ability of HER2+ breast cancers to augment auxiliary ShcA signaling pathways independently of PTB-driven interactions with oncogenes such as ErbB2 or ErbB3, may represent one mechanism by which these tumors increase Src activity and develop Trastuzumab resistance.

4.1.3 SFK activity but not its recruitment is required to enhance tumor growth from PTB-independent ShcA complexes.

While PTB-independent ShcA pools can no longer bind ErbB2, this does not preclude the fact that ShcA is competent to participate in SH2 driven interactions, including Met [416], PDGFR [417], FGFR [418], and, the SFKs, Src and Lyn [349, 356], to increase ShcA tyrosine phosphorylation. Indeed, we previously demonstrated that mammary tumors expressing PTB-independent pools of ShcA establish an autocrine loop to activate Src. Src, in turn, laterally activates Met, FGFR, and

PDGFR [2]. Several studies have revealed a complex interplay between ShcA and SFKs. For example, the ShcA SH2 domain has been shown to bind both Src and Lyn [349, 356]. On the other hand, SFK recruitment to ShcA is not exclusively dependent on the SH2 domain and can also involve alternative phospho-tyrosine independent interactions. Fyn associates with a proline-rich region in the CH1 domain of ShcA through its SH3 domain [368]. In effect, the ShcA phospho-tyrosine residues, Y239/240 and Y313 can be phosphorylated by either Src and Fyn, respectively, leading to Grb2 recruitment and the consequent transduction of mitogenic signals [368, 373]. Therefore, it is likely that ShcA and Src influence signal transduction pathways in a reciprocal manner. In support of these findings, while tyrosine phosphorylation of ErbB2-coupled (PTB-dependent) ShcA pools is independent of SFKs, PTB-independent ShcA complexes require SFK expression and activity to phosphorylate these ShcA tyrosine residues. Despite this observation, we see an inverse correlation between Src, Fyn and Lyn recruitment to PTB-independent ShcA pools with Src activity and the transforming potential of these ShcA complexes. In fact, PTB-independent ShcA complexes with a non-functional SH2 domain most strongly bound Src, Fyn and Lyn even though total Src activity is largely debilitated in these cells. With the knowledge that both SFK activity and an intact SH2 domain is required to enhance tumor growth from PTB-independent ShcA complexes, these data suggest that the ability of ShcA to interact with SFKs was not functionally significant for increased tumorigenesis in ErbB2+ breast cancers. In support of this, ShcA tyrosine phosphorylation, which requires Src, is only dispensable for increased tumorigenesis from PTB-independent ShcA pools. Rather, these data imply that the ShcA SH2 domain indirectly activates Src from PTB-independent ShcA complexes by recruiting a yet to be identified protein, whose signaling properties are also likely to be restrained by one or more negative regulators that normally bind the PTB domain of ShcA.

4.1.4 PTB-dependent ShcA pools utilize the S29-W38 motif to restrict the activation of Src while retaining the ability to transduce signals through ErbB2/ShcA complexes.

Given that PTB-independent breast tumors (PTB^{MUT}) are highly resistant to Lapatinib by increasing SH2-driven Src signaling, we hypothesized that this response was a consequence of losing a negative regulator of Src activation. We have since observed that Src can also associate with the amino-terminal of ShcA, exposing another mode of Src regulation in our model system. Previous reports have established that a 10-amino acid region upstream of the ShcA PTB domain

is necessary to engage Src, leading to its activation [8, 408]. Corroborating these previous reports, we have shown that loss of the S29-W38 motif from PTB-dependent complexes (ShcA$^{\Delta 10}$) did not affect the ability of ShcA to engage ErbB2 but did reduce Src recruitment ~2-fold. Paradoxically, despite having lost the ability to fully interact with Src, breast tumors that engaged ShcA$^{\Delta 10}$ had increased Src activity at end-stage with no apparent alterations in ShcA tyrosine phosphorylation levels. These data, combined with the observed increase in tumor growth (2.5-fold), mirrored those breast tumors that had engaged PTB-independent ShcA complexes. However, unlike ShcA$^{\Delta 10}$ expressing breast tumors, for PTB-independent breast tumors, having hyperactivated Src signaling, the loss of the S29-W38 motif had no consequence on breast tumor growth. This suggested that elevating Src activity due to S29-W38 deletion did not further increase the transforming potential of breast tumors engaging PTB-independent ShcA pools. These data reinforced that PTB-dependent ShcA pools utilize the S29-W38 region to restrict the activation of Src while still retaining the ability to transduce signals through ErbB2/ShcA complexes (Figure 2). Indeed, PTB-dependent breast tumors that have lost the S29-W38 motif are particularly responsive to Lapatinib and Src inhibition, underscoring the dependence of these breast tumors on signaling emanating from both ErbB2 and Src, respectively (Figure 2). Thus, we propose that in ErbB2+ breast cancers, PTB-coupled ShcA signaling responses work in concert with the S29-W38 motif to recruit a negative regulator of the PTB domain to restrain Src activation and prevent the acquisition of a PTB-independent phenotype.

Several negative regulators have been shown to bind the PTB domain of ShcA. Thus far, only PTPN12 and SHIP2 require the phospho-tyrosine binding pocket, which was specifically mutated in our study. The recruitment of PTPN12 and SHIP2 to multiple RTKs that bind the ShcA PTB domain, including ErbB2, EGFR and InsR, have been shown to attenuate downstream mitogenic signals [7, 193, 196, 419, 420]. This would suggest that the loss of either PTPN12 or SHIP2 expression would amplify both PTB-dependent and independent ShcA signaling complexes. Using genomics approaches, we have identified an inverse correlation between a PTB-independent ShcA gene signature and PTPN12, but not SHIP2 copy number levels in human breast cancers. Thus, PTPN12 was a candidate negative regulator for ShcA. To our surprise, we observed that the loss of S29-W38 motif was sufficient to abrogate the interaction of PTPN12 from PTB-dependent ShcA complexes (ShcA$^{\Delta 10}$), which paralleled the loss of PTPN12 engagement upon debilitating the PTB domain phosho-tyrosine binding pocket (PTB^{MUT}). Given that both PTB^{MUT} and ShcA$^{\Delta 10}$

breast tumors are relatively similar in tumor growth potential, these results provide further evidence that PTPN12 is a major negative regulator of PTB-coupled ShcA signaling. Moreover, these data suggested that the S29-W38 motif can independently regulate PTPN12 interactions with the ShcA PTB domain. However, these data do not preclude other novel negative regulators that may attenuate PTB domain dependent tumorigenesis. It must be noted here, that the PTPN12/ShcA interaction possesses another unique property involving Serine 29 phosphorylation (encompassed by the S29-W38 motif) which stabilizes but is not absolutely necessary for this protein-protein interaction. Thus, the loss of Serine 29 within the 10-amino acid region may affect the ability of ShcA to stabilize the interaction itself. Therefore, we can hypothesize that the S29-W38 motif is necessary to initiate the binding of PTPN12 to the ShcA PTB domain, while the phosphorylation of Serine 29 is required to stabilize this interaction. However, to address this possibility, a mutational analysis that deletes 9-amino acids (F30-W38), while retaining a functional Serine 29 phosphorylation site ($ShcA^{\Delta 9}$) would be required to assess the dynamics between these two regulatory motifs.

4.1.5 Serine 29 exhibits non-overlapping growth inhibitory properties contingent on the engagement of either PTB-dependent or -independent ShcA complexes.

The regulation of Serine 29 is dependent on feedback mechanisms downstream of activated RTKs which permit the delayed recruitment of PTPN12 and the subsequent termination of PTB-dependent ShcA signaling [7]. Indeed, we observe that the loss of Serine 29 phosphorylation reduces the ability of ShcA to engage PTPN12 in PTB-dependent ShcA complexes ($ShcA^{S29A}$), resulting in accelerated tumor growth, *in vivo*. Yet, the very same loss of Serine 29 phosphorylation in PTB-independent ShcA breast tumors ($PTB^{MUT/S29A}$), which already cannot bind PTPN12, was able to further increase breast tumor growth relative to PTB^{MUT} controls. Thus, the increased growth kinetics of PTB-independent breast tumors that can no longer engage Serine 29, cannot be explained by the loss of PTPN12 interaction alone. These data suggest that Serine 29 phosphorylation regulates the recruitment of another, yet undefined negative regulator that controls mitogenic signals from Serine 29 dependent complexes (Figure 2). This is the first *in vivo* report associating the Serine 29 phosphorylation site with additional tumor suppressive properties that are not contingent upon a functional PTB domain (Figure 2).

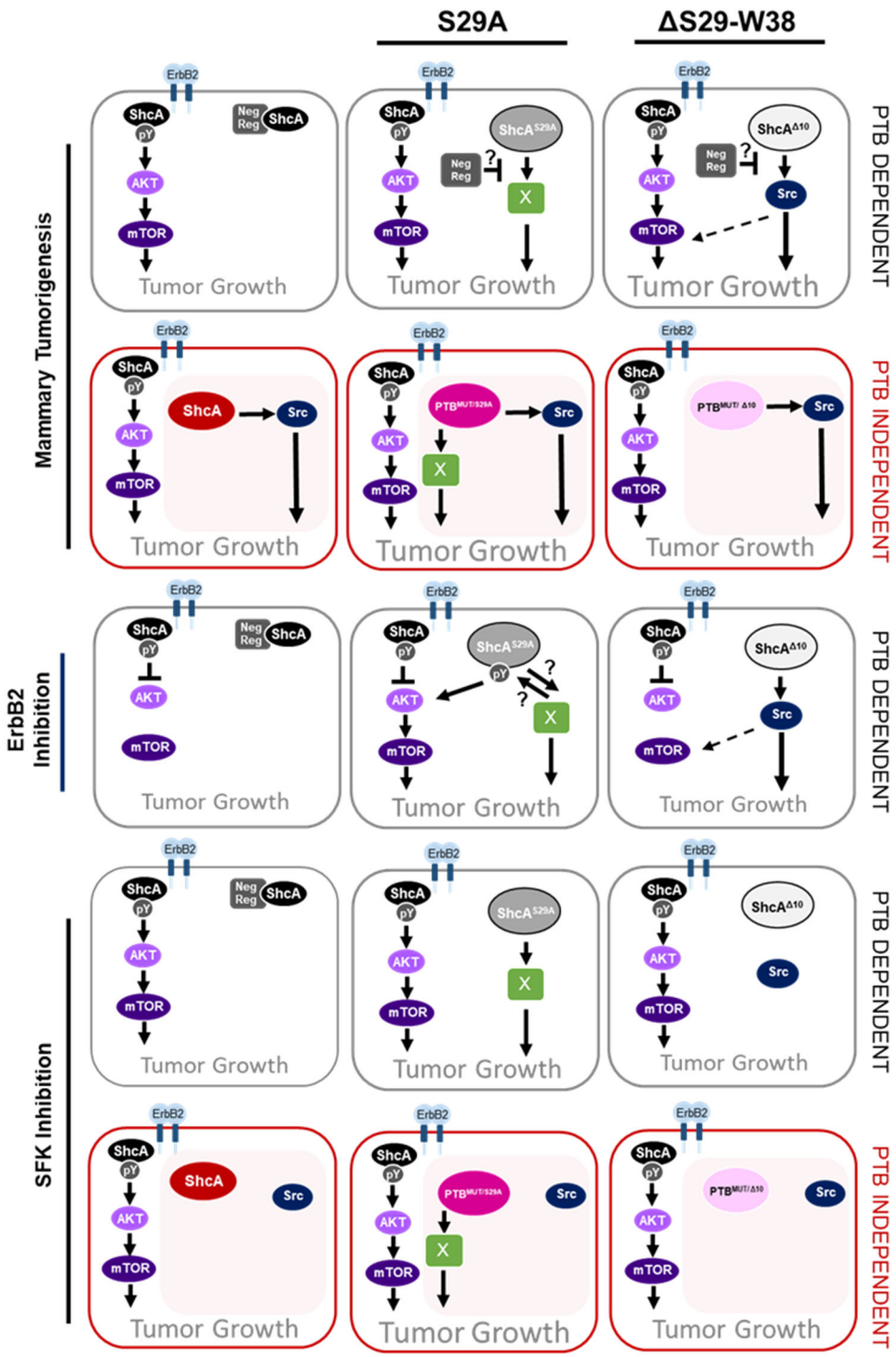

Figure 2: Independent regions of the ShcA amino terminus negatively regulate breast tumor growth and sensitivity to tyrosine kinase inhibitors. Schematic diagram summarizing the biological impact of N-terminal regulatory elements of PTB-dependent and -independent ShcA signaling complexes on breast tumor growth and response to tyrosine kinase inhibitors.

Given that Serine 29 phosphorylation of both PTB-coupled ($ShcA^{WT}$) or -uncoupled (PTB^{MUT}) ShcA complexes is necessary to negatively regulate tumor growth suggests that this phosphorylation site controls a mitogenic signaling network(s) that is independent of the ShcA PTB domain. Indeed, PTB-dependent signaling complexes lacking the Serine 29 phosphorylation site render breast tumors resistant to Src inhibition with no apparent alterations in ERK and AKT/mTOR activity. This suggested that PTB-coupled ShcA pools in response to the loss of Serine 29, can increase mitogenic signaling that was independent of Src. In fact, we see that Serine 29 does not regulate the recruitment or the activity of Src in PTB-dependent ShcA complexes. Alternatively, the loss of Serine 29 from PTB-independent pools increases Src recruitment 20-fold compared to PTB^{MUT} alone. However, this increase in Src recruitment did not correlate with the activation of SFKs, nor involve the upregulation of alternative SFKs, including Fyn and Lyn. Moreover, the loss of Serine 29 phosphorylation did not further sensitize $PTB^{MUT/S29A}$ tumors to SFK inhibition relative to PTB^{MUT} controls, which are characteristically hyperactivated in Src. These data warrant that there is an unidentified growth inhibitory mechanism that bifurcates from Serine 29 within either PTB-coupled or -uncoupled ShcA complexes that is independent of Src.

Similarly, we also observe that the loss of Serine 29 phosphorylation increases the resistance of PTB-dependent breast tumors ($ShcA^{S29A}$) to Lapatinib. PTB-dependent ShcA complexes still retain a functional PTB domain. Thus, it was surprising to observe that the loss of Serine 29 was able to protect these breast tumors from ErbB2/EGFR inhibition. The resistance mechanism mirrored that of PTB-independent breast tumors, as we also observed an increase in both ShcA phosho-tyrosine activation and the re-engagement of ERK and mTOR signaling. However, unlike PTB-independent ShcA complexes, the phosphorylation of the ShcA tyrosine sites was not dependent on Src activation. As such, we must consider that Lapatinib resistance may be caused by other RTKs or non-RTKs that independently increase ERK and mTOR signaling. For example, inherent to the AKT signaling network is the ability to cross talk with Ras/ERK and PLCγ/PKC pathways [421]. Although PI3K/AKT and Ras/ERK pathways are considered cross-inhibitory, they can also act in a cooperative manner to robustly regulate many of the same downstream effectors, including mTOR activity through the TSC complex or the phosphorylation of p90 ribosomal S6 kinase [152]. Interestingly, PKC activation is sufficient to phosphorylate Serine 29 [385] and increase the interaction of PTPN12 to ShcA, 8-10 fold [384]. Indeed, PKCε was found to be an interactor of ShcA upon EGF stimulation [7]. PKCε expression and activation has also

been shown to engage MAPK and AKT signaling cascades [422, 423]. Moreover, PKCε can indirectly regulate AKT through the engagement of integrins [423, 424]. Therefore, this does not exclude the fact that there may be compensation by PKC driven pathways in our model system upon the loss of Serine 29 phosphorylation. Collectively, these data suggest that Serine 29 exhibits non-overlapping growth inhibitory properties that regulate an unidentified signaling network in breast tumorigenesis depending on the engagement of PTB-dependent or -independent ShcA complexes. Thus, further evaluation is required to specifically characterize the PTB-dependent and -independent interactome upon the loss of Serine 29 phosphorylation and identify these novel regulatory networks.

4.1.6 ShcA is required for the transforming potential of PTPN12 in breast cancer.

There is no evidence for any point mutations within ShcA across cancers. Thus, our data would suggest that the loss or inactivation of genes that negatively regulate ShcA, would amplify both PTB-dependent and independent ShcA signaling complexes. Several studies have interrogated the impact of PTPN12 loss in breast cancer. For instance, PTPN12 is deleted or mutated in a subset of human primary breast cancers [192]. Moreover, the ability of PTPN12 to transform mammary epithelial cells requires ShcA [193]. Intriguingly, like our model system, several independent studies have demonstrated that PTPN12 loss only marginally impacts AKT signaling but profoundly impairs mTOR activation, particularly S6K and rS6 phosphorylation. These data suggest that PTPN12 and/or other negative regulators may augment mTOR signaling through a distinct mechanism that is independent of AKT [193, 196, 197]. Moreover, corroborating our data, germline PTPN12 deletion did not alter Src phosphorylation levels, even though downstream effectors of integrin signaling (Cas, Pyk2) were hyper-phosphorylated [196]. Three-dimensional structural modeling of the PTB domain, including the amino-terminal end of the ShcA, placed Serine 29 in a fragment protruding away from the PTB domain [385]. This suggests that Serine 29 dependent PTPN12 interaction to the ShcA PTB domain is not through steric hindrance by the phosphate group [385]. Thus, authors of this study speculate that Serine 29 may also play a regulatory role in enhancing ShcA interactions with additional targets where the preferred ShcA PTB domain binding sequence is not available [385]. For example, as PTPN12 is a scaffold protein itself, it can engage other proteins including paxillin, Csk, and Cas [195]. Thus, PTPN12/ShcA complexes may induce signals not only through PI3K/AKT and Ras/ERK signaling pathways, but

also through other pathways independent of ShcA. Indeed, it is known that PTPN12 plays an important role in actin remodeling and phosphatidylinositol signaling to increase ERK and AKT activation, independently of ShcA [196, 425]. This suggests that ShcA-independent effects of PTPN12 deficiency on actin remodeling may confound the ability to observe any ShcA-regulated effects on Src activation. In order to address this question, the introduction of a PTPN12 mutant that contains an intact tyrosine phosphatase domain but lacks the NPXH ShcA PTB domain binding motif, would be required [3]. Alternatively, we must also consider that one or more alternative negative regulators that bind the ShcA PTB domain may be contributing to the biological phenotypes observed.

Chapter 5 Future Directions

5.1 Prospective Studies

Collectively, our work demonstrates that the PTB domain of ShcA is essential to transduce both pro- and anti-mitogenic signals in breast cancer cells. We mechanistically dissected how breast tumors control PTB-driven signaling and how the loss of negative regulators of the PTB domain perturbs mitogenic signaling to promote the development of more aggressive breast tumors. We have identified unique regulatory elements within the ShcA amino terminus, including Serine 29 and a S29-W38 motif, to have non-overlapping mechanisms that control ShcA-dependent tumorigenic responses. Given the new developments of our study, further investigation is warranted to address the mechanisms underlying the biological phenotypes observed. Potential avenues of investigation have been outlined below.

5.1.1 To identify novel interacting proteins that attenuate the tumorigenic phenotype through the ShcA PTB domain.

We have established that the ShcA PTB domain is important for signal termination through its ability to engage one or more negative regulators that attenuate ShcA signaling complexes that are uncoupled from the transforming oncogene, including ErbB2. Indeed, we have shown that the loss of a functional PTB domain (PTB^{MUT}) creates a ShcA pool that cannot bind its known negative regulators yet retains the ability to activate ShcA signaling through the SH2 domain and/or tyrosine phosphorylation sites. As there are no point mutations observed in ShcA, this suggests that breast cancers have the propensity to lose a negative regulator that dampens PTB-dependent ShcA signaling. The recruitment of these negative regulators can be mediated through phospho-tyrosine-dependent and -independent interactions. Of interest to our model system, is Arginine 175 of the PTB domain phospho-tyrosine binding pocket, the loss of which is sufficient to uncouple negative regulators from ShcA, including PTPN12 [426] and SHIP2 [4]. Corroborating recent literature, we have confirmed that Serine 29 phosphorylation is necessary to stabilize the interaction of PTPN12 to ShcA. Interestingly, the loss of Serine 29 phosphorylation alone can accelerate tumor growth in breast tumors that have engaged either PTB-dependent or -independent ShcA complexes. Provided that PTB-independent ShcA pools (PTB^{MUT}) have already lost the ability to bind PTPN12, the observed acceleration in tumor growth suggests that Serine 29 possess additional negative regulatory mechanisms to dampen ShcA signaling complexes that are uncoupled from the PTB

domain. Given these unique growth inhibitory properties of Serine 29, we propose that a systematic assessment on the protein interactome should be addressed in our model system, namely through a high throughput genome wide mapping of the ShcA interactome. To do so, we propose that proximity-dependent biotin identification (BioID) proteomic or affinity-purificated/mass spectrometry (AP/MS) approaches are necessary to biochemically screen and identify novel binding partners of PTB-dependent and -independent complexes in the absence or presence of Serine 29 phosphorylation in ErbB2 transformed breast cancer cells. Following the identification of novel interactors of ShcA signaling, such candidates would be functionally validated for their ability to attenuate tumorigenic responses, using CRISPR/Cas9 or RNAi approaches in both *in vitro* and *in vivo* assays.

5.1.2 To determine the impact of Serine 29 phosphorylation and S29-W38 motif on PTPN12-dependent regulation of ShcA.

Through our studies, we discovered that the S29-W38 motif can independently determine the engagement of PTPN12 to the ShcA PTB domain. The loss of this region alone was sufficient to disengage PTPN12 from ShcA, suggesting that the S29-W38 region is necessary for the formation of ShcA PTB domain/PTPN12 interactions. We hypothesize that the S29-W38 motif is necessary to initiate the interaction of PTPN12 to the ShcA PTB domain, while the phosphorylation of Serine 29 is required to stabilize this interaction. Therefore, a mutational analysis that deletes the 9 amino acids (F30-W38) proximal to the Serine 29 phosphorylation site ($ShcA^{\Delta 9}$) would be required to assess the dynamics between these two regulatory motifs. Additionally, we also observe that the S29-W38 region of ShcA can engage negative regulators of Src activation in ErbB2+ breast tumors. The mechanism underlying this interplay between Src activation and PTPN12 engagement is currently unknown. While a high throughput analyses would be warranted to establish any novel interactors of the S29-W38 region, it must be noted that PTPN12 itself is comprised of scaffolding domains that interact with various proteins involved in numerous cellular processes including, cellular morphology, migration, adhesion, and programmed cell death. Thus, ShcA-independent effects of PTPN12 deficiency may confound the ability to observe any ShcA-dependent responses on breast tumorigenesis. To establish the direct effects of ShcA/PTPN12 interactions, we recommend the expression of PTB-dependent and -independent ShcA alleles in the context of wild-type ShcA alleles or the loss of Serine 29 phosphorylation or S29-W38 motif, together with

the reconstitution of a PTPN12 mutant that contains an intact tyrosine phosphatase domain but lacks the ShcA binding site in two model systems. This includes utilizing (1) CRISPR/Cas9 gene editing technology to target PTPN12 from ErbB2 transformed mammary epithelial cells proficient in PTPN12, or (2) breast tumors arising from ErbB2-driven transgenic mammary tumors that either retain or have deleted both PTPN12 alleles [196]. These tools will define how Serine 29 phosphorylation and S29-W38 motif specifically regulate PTPN12 dependent mammary tumorigenesis within distinct pools of ShcA.

5.1.3 To determine the interplay between N-terminal regulatory motifs and SH2-driven Src activation in ErbB2+ breast tumorigenesis.

We have established that PTB-independent ShcA pools require the SH2 domain to hyperactivate Src to sustain and amplify tumor growth. In addition, we demonstrate that S29-W38 motif is also able to negatively regulate Src activation upstream of the ShcA PTB domain. Indeed, the loss of S29-W38 motif from PTB-dependent ShcA complexes was sufficient to accelerate tumor growth and hyperactivate Src, *in vivo.* This suggest that the S29-W38 region is either able to cooperate with the SH2 domain for Src activation or is itself, independently regulating Src activity. Previous studies have identified a phosphorylation-dependent gating mechanism whereby tyrosine phosphorylation of ShcA induces a conformational change, which opens the SH2 domain to increase its ability to bind ligands [372]. However, we do not see any correlation between the loss of S29-W38 motif and ShcA tyrosine phosphorylation. Although these data suggest that there is no association between the activation of ShcA phospho-tyrosine residues, it does not ascertain whether the ShcA tyrosine sites are dispensable to the accelerated growth phenotype. To further distinguish the interplay between the S29-W38 motif downstream of the ShcA phospho-tyrosine residues, mutational studies that assess the impact of the loss of the S29-W38 motif alongside debilitating the SH2 domain in ErbB2+ breast cancer cells would highlight the spatial and temporal regulation of ShcA, both upstream and downstream of the ShcA PTB domain. In parallel, the expression of PTB-dependent and -independent ShcA alleles with debilitated ShcA phospho-tyrosine residues in the context of S29-W38 loss would provide an interesting assessment of alternative signaling networks that may contribute to mammary tumorigenesis independent of ShcA phospho-tyrosine sites.

Chapter 6 Overall Summary and Implications of the Study

6.1 Overall Summary

The objectives of our studies were as follows:

1. To determine whether PTB-independent ShcA tumorigeneisis is dependent on the hyperactivation of ShcA signaling.

2. To identify ShcA interactors that contribute to the amplified tumor growth phenotype of breast tumors engaging in PTB-independent ShcA complexes.

3. To understand how signaling complexes upstream of the ShcA PTB domain differentially regulate mammary tumorigenesis.

The objectives of our studies were accomplished as follows:

1. We provide the first evidence that the ShcA adaptor protein is a critical convergence point downstream of numerous tyrosine kinases and that perturbation of discrete ShcA-dependent signaling complexes significantly impacts breast tumor growth and therapeutic responsiveness. Specifically, we demonstrate that PTB-independent ShcA complexes serve to augment mammary tumorigenesis by increasing the activity of the Src and Fyn tyrosine kinases through the SH2 domain, while ShcA tyrosine phosphorylation sites are dispensable for these PTB-independent ShcA pools to amplify tumor growth. Finally, we establish that increased Src activation downstream from ShcA PTB-independent signaling complexes increases resistance to Lapatinib.

2. Through genetic and pharmacologic approaches, we establish a ShcA-dependent resistance mechanism underlying PTB-independent breast tumorigenesis. PTB-independent ShcA signaling complexes rely on the ShcA SH2 domain to activate Src signaling to augment mammary tumor growth. However, the loss of the ShcA/Src signaling axis from these PTB-independent pools enables the reprogramming of signal networks to increase their reliance on mTOR signaling.

3. Using a previously generated dataset identifying 100 differentially expressed genes that distinguished tumors that did or did not augment mitogenic signals from PTB-independent ShcA complexes, we stratify 1218 human breast cancers using publicly available TCGA

RNA-seq platform. Primary human breast tumors display characteristic features observed in our model system, including reduced pY317-ShcA levels, elevated pY416-Src, pS235/6-rS6 levels, and increased Src, Fyn, and Lyn mRNA expression. Lastly, the genomic loss of PTPN12 is a characteristic of breast tumors that have engaged PTB-independent ShcA complexes, establishing a possible relationship between this phosphatase and its role in restricting ShcA-dependent Src activation in human breast tumorigenesis.

4. We provide the first *in vivo* evidence evaluating the biological significance of unique regulatory elements within the ShcA N-terminal domain, including Serine 29 and a S29-W38 motif. We show that these two regulatory elements have non-overlapping mechanisms that control ShcA regulated tumorigenic responses.

In summary, we establish that ShcA intracellular complexes (PTB-dependent and PTB-independent) rely on ShcA's modular domains to transduce non-redundant signals to cooperatively accelerate breast cancer development. We provide significant insight into a novel mechanism by which breast tumors can direct the ShcA adaptor into distinct intracellular signaling complexes to increase breast cancer heterogeneity and influence responsiveness to tyrosine kinase inhibitors.

6.2 Implications of the study

During the transition from good to poor outcome breast cancer subtypes, the tyrosine kinome is extremely fluid, with the capacity to re-adjust itself across a wide spectrum of selection pressures. This plasticity in the tyrosine kinome provides tumor cells with the competitive edge required for cell proliferation, growth, and survival. As such, intense research efforts have been reinforced to fully understand the mechanisms by which cancer cells inherently utilize the tyrosine kinome to circumvent tyrosine kinase inhibition and to better increase the efficacy of existing tyrosine kinase-based therapies.

Due to the inherent ability of breast tumors to overcome tyrosine kinase inhibition, novel and alternative approaches are being investigated to specifically target the phospho-tyrosine interactome. This includes antagonistic peptide hybrids that inhibit SH2 and PTB domain interactions. For example, a Grb2 SH2 domain phospho-tyrosine-clamp targeted toward tyrosine phosphorylated Y239/240 of ShcA has been successfully developed [427]. Alternatively,

significant efforts have focused on the development of small-molecule phospho-peptide mimetic inhibitors of the SH2 domain, such as Src kinase, p85 regulatory subunit of PI3K, and Grb2 [428]. This also includes an inhibitory ShcA SH2 domain peptide hybrids developed to restrain SH2 domain interactions with Met receptor [428]. These innovative technologies have significant implications for the development of SH2 domain targeted therapy and presents a useful biochemical tool to functionally assess specific protein interaction networks that mediate unique biological processes. However, the biological significance of this technology in the context of ShcA has yet to be determined.

Given the transformative properties of ShcA in the various stages of breast tumorigenesis, we believe that inhibiting the ShcA interactome may only provide alternative mechanisms for therapeutic resistance. Indeed, we have demonstrated that therapeutic strategies that uncouple ShcA from ErbB2 may only delay the eventual acquisition of *de novo* resistance mechanisms, including the hyperactivation of SFKs and AKT/mTOR pathways. Our pre-clinical studies would advise against the development of inhibitors that target the ShcA PTB domain and warrant further investigation as to whether targeting the ShcA SH2 domain, or one or more of its tyrosine phosphorylation sites would have clinical impact [9]. Moreover, the additional regulation of phospho-tyrosine networks by ShcA N-terminal regulatory motifs including Serine 29 and S29-W38 motif, suggests that the disruption of negative feedback loops targeting upstream the PTB domain would also lead to the eventual acquisition of resistance to tyrosine kinase inhibition. Importantly, we establish that ShcA plays an important role in regulating the balance of distinct intracellular signaling complexes. The ability of ShcA to redistribute signaling complexes is a unique mechanism that enables the differential regulation of tyrosine kinases and eventual resistance to ErbB2 inhibitors. As such, due to the capacity of ShcA to integrate multiple compensatory signaling nodes downstream of RTKs, it is evident that we must consider ShcA as a critical intermediate that contributes to the pleiotropic mechanisms underlying therapeutic resistance in breast cancer. Considering the diverse means of resistance that coexist in these tumors, our data would suggest that a multi-level strategy will be necessary to restore sensitivity to tyrosine kinase inhibitors.

Chapter 7 Comprehensive Bibliography

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Chapter 8 Appendices of Permissions and Waivers

RE: Request for Permissions: JCB-16-0144

Wiley Global Permissions <permissions@wiley.com>

Fri 2018-05-25 11:00 AM

To:Jacqueline Ha <jacqueline.ha2@mail.mcgill.ca>;

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To Whom It May Concern,

My name is Jacqueline Ha and I am the first author of the manuscript titled "The Tyrosine Kinome Dictates Breast Cancer Heterogeneity and Therapeutic Responsiveness" (doi: 10.1002/jcb.25561) which was accepted to the Journal of Cellular Biochemistry in September 2016. I am currently a senior PhD candidate and would like to kindly ask for permissions/waiver from the Journal of Cellular Biochemistry to use this work in full and/or in part for my doctoral dissertation.

Thank you for your time and consideration. Should you require any further information, please feel free to contact me.

I look forward to hearing from you,

Sincerely,

Jacqueline Ha

PhD Candidate

Lady Davis Institute for Medical Research

Division of Experimental Medicine, Faculty of Medicine, McGill University
3755 Cote Ste. Catherine Road, Room# F528
Montreal, Quebec, Canada, H3T 1E2
Phone: (514) 340-8222 ext. 22365
Fax: (514) 340-7502
E-mail: jacqueline.ha2@mail.mcgill.ca

RE: Permissions: MCR-17-0623

Rac, Karola <karola.rac@aacr.org>

Mon 2018-05-07 2:18 PM

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From: Jacqueline Ha [mailto:jacqueline.ha2@mail.mcgill.ca]
Sent: Sunday, April 29, 2018 10:53 AM

To: Permissions <Permissions@aacr.org>
Subject: Permissions: MCR-17-0623

To Whom It May Concern,

My name is Jacqueline Ha and I am the first author of the manuscript titled "Integration of Distinct ShcA Signaling Complexes Promotes Breast Tumor Growth and Tyrosine Kinase Inhibitor Resistance" (doi: 10.1158/1541-7786.MCR-17-0623) which was accepted to Molecular Cancer Research in February 2018. I am currently a senior PhD candidate and would like to kindly ask for permissions/waiver from Molecular Cancer Research to use this work in full and/or in part for my doctoral dissertation.

Thank you for your time and consideration. Should you require any further information, please feel free to contact me.

I look forward to hearing from you.

Sincerely,

Jacqueline Ha

PhD Candidate

Lady Davis Institute for Medical Research

Division of Experimental Medicine, Faculty of Medicine, McGill University
3755 Cote Ste. Catherine Road, Room# F528
Montreal, Quebec, Canada, H3T 1E2
Phone: (514) 340-8222 ext. 22365
Fax: (514) 340-7502
E-mail: jacqueline.ha2@mail.mcgill.ca

Re: PhD Thesis: Permission/waivers to use figures

Claudia Kleinman <claudia.kleinman@mcgill.ca>

Mon 2018-10-15 12:03 PM

To: Jacqueline Ha <jacqueline.ha2@mail.mcgill.ca>;

Cc: Giuseppina Ursini-Siegel, Dr <giuseppina.ursini-siegel@mcgill.ca>;

Dear Jackie,

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Best,
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On Mon, Oct 15, 2018 at 12:00 PM Jacqueline Ha <jacqueline.ha2@mail.mcgill.ca> wrote:

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> Jacqueline Ha
>
> PhD Candidate
>
> Ursini-Siegel Laboratory
>
> Lady Davis Institute for Medical Research
>
> Division of Experimental Medicine, Faculty of Medicine, McGill University
>
> 3755 Cote Ste. Catherine Road, Room# F528
>
> Montreal, Quebec, Canada, H3T 1E2
>
> Phone: (514) 586 6209

E-mail: jacqueline.ha2@mail.mcgill.ca

--

Claudia L. Kleinman

Assistant Professor
Department of Human Genetics, McGill University

http://functionalgenomics.ca
Lady Davis Institute for Medical Research
3999 Côte Ste-Catherine Road
Montreal, QC H3T 1E2
Canada
514-340-8222 ext 26129

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Eduardo Cepeda Cañedo

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To: Jacqueline Ha <jacqueline.ha2@mail.mcgill.ca>;

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Eduardo

De: Jacqueline Ha
Enviado: lunes, 15 de octubre de 2018 11:00:11 a. m.
Para: Claudia Kleinman, Ms; Harvey Wilmore Smith, Dr; Steven Hébert; William Muller, Dr.; Ryuhjin Ahn; Young Im; Valerie Sabourin; Eduardo Cepeda Cañedo
CC: Giuseppina Ursini-Siegel, Dr
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Thank you for your time and support. I look forward to hearing from you.

Sincerely,

Jacqueline Ha
PhD Candidate
Ursini-Siegel Laboratory
Lady Davis Institute for Medical Research
Division of Experimental Medicine, Faculty of Medicine, McGill University
3755 Cote Ste. Catherine Road, Room# F528
Montreal, Quebec, Canada, H3T 1E2
Phone: (514) 586 6209
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Re: PhD Thesis: Permission/waivers to use figures

Harvey Wilmore Smith, Dr

Mon 2018-10-15 12:09 PM

To: Jacqueline Ha <jacqueline.ha2@mail.mcgill.ca>;

Cc: Giuseppina Ursini-Siegel, Dr <giuseppina.ursini-siegel@mcgill.ca>;

Hi Jacqueline,

Yes, you certainly have my permission. Best of luck with preparing your dissertation!

All the best,

Harvey

Dr. Harvey W. Smith
Goodman Cancer Research Centre
McGill University
Tel: (01) 514 398 4147
Fax: (01) 514 398 6769

From: Jacqueline Ha
Sent: Monday, October 15, 2018 12:00:11 PM
To: Claudia Kleinman, Ms; Harvey Wilmore Smith, Dr; Steven Hébert; William Muller, Dr.; Ryuhjin Ahn; Young Im; Valerie Sabourin; Eduardo Cepeda Cañedo
Cc: Giuseppina Ursini-Siegel, Dr
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Sincerely,

Jacqueline Ha
PhD Candidate
Ursini-Siegel Laboratory
Lady Davis Institute for Medical Research
Division of Experimental Medicine, Faculty of Medicine, McGill University
3755 Cote Ste. Catherine Road, Room# F528
Montreal, Quebec, Canada, H3T 1E2
Phone: (514) 586 6209
E-mail: jacqueline.ha2@mail.mcgill.ca

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Peter Siegel, Dr.

Fri 2018-11-30 7:25 AM

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Best,

Peter

Sent from my iPhone

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> Division of Experimental Medicine, Faculty of Medicine, McGill University

3755 Cote Ste. Catherine Road, Room# F528

Montreal, Quebec, Canada, H3T 1E2

Phone: (514) 586 6209

E-mail: jacqueline.ha2@mail.mcgill.ca

Re: PhD Thesis: Permission/waivers to use figures

Ryuhjin Ahn

Mon 2018-10-15 12:04 PM

To:Jacqueline Ha <jacqueline.ha2@mail.mcgill.ca>;

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From: Jacqueline Ha
Sent: Monday, October 15, 2018 12:00:11 PM
To: Claudia Kleinman, Ms; Harvey Wilmore Smith, Dr; Steven Hébert; William Muller, Dr.; Ryuhjin Ahn; Young Im; Valerie Sabourin; Eduardo Cepeda Cañedo
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Jacqueline Ha
PhD Candidate
Ursini-Siegel Laboratory
Lady Davis Institute for Medical Research
Division of Experimental Medicine, Faculty of Medicine, McGill University
3755 Cote Ste. Catherine Road, Room# F528
Montreal, Quebec, Canada, H3T 1E2
Phone: (514) 586 6209
E-mail: jacqueline.ha2@mail.mcgill.ca

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Steven Hébert <steven.hebert06@gmail.com>

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Steven

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>
> Lady Davis Institute for Medical Research
>
> Division of Experimental Medicine, Faculty of Medicine, McGill University
>
> 3755 Cote Ste. Catherine Road, Room# F528
>
> Montreal, Quebec, Canada, H3T 1E2
>
> Phone: (514) 586 6209
>
> E-mail: jacqueline.ha2@mail.mcgill.ca

--

Steven Hébert M.Sc.

Research Assistant in bioinformatics
Lady Davis Institute for Medical Research at the Jewish General Hospital
3999 Côte Ste-Catherine Road, room E-542.1
Montréal, QC H3T 1E2
T: 514-340-8222 x6075
E: steven.hebert06@gmail.com

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Valérie Sabourin <valeriesabourin@yahoo.ca>

Mon 2018-10-15 2:02 PM

To:Jacqueline Ha <jacqueline.ha2@mail.mcgill.ca>;

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> Sincerely,
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> Jacqueline Ha
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> PhD Candidate
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> Ursini-Siegel Laboratory
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> Lady Davis Institute for Medical Research
>
> Division of Experimental Medicine, Faculty of Medicine, McGill University
>
> 3755 Cote Ste. Catherine Road, Room# F528
>
> Montreal, Quebec, Canada, H3T 1E2
>
> Phone: (514) 586 6209
>
> E-mail: jacqueline.ha2@mail.mcgill.ca

Re: PhD Thesis: Permission/waivers to use figures

William Muller, Dr.

Mon 2018-10-15 1:36 PM

To: Jacqueline Ha <jacqueline.ha2@mail.mcgill.ca>; Claudia Kleinman, Ms <claudia.kleinman@mcgill.ca>; Harvey Wilmore Smith, Dr <harvey.smith2@mcgill.ca>; Steven Hébert <steven.hebert06@gmail.com>; Ryuhjin Ahn <ryuhjin.ahn@mail.mcgill.ca>; Young Im <young.im@mail.mcgill.ca>; Valerie Sabourin <valeriesabourin@yahoo.ca>; Eduardo Cepeda Cañedo <eduardo.cepedacanedo@mail.mcgill.ca>;

Cc: Giuseppina Ursini-Siegel, Dr <giuseppina.ursini-siegel@mcgill.ca>;

I am fine with that

From: Jacqueline Ha
Sent: Monday, October 15, 2018 12:00:11 PM
To: Claudia Kleinman, Ms; Harvey Wilmore Smith, Dr; Steven Hébert; William Muller, Dr.; Ryuhjin Ahn; Young Im; Valerie Sabourin; Eduardo Cepeda Cañedo
Cc: Giuseppina Ursini-Siegel, Dr
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Thank you for your time and support. I look forward to hearing from you.

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Lady Davis Institute for Medical Research
Division of Experimental Medicine, Faculty of Medicine, McGill University
3755 Cote Ste. Catherine Road, Room# F528
Montreal, Quebec, Canada, H3T 1E2
Phone: (514) 586 6209
E-mail: jacqueline.ha2@mail.mcgill.ca

Re: PhD Thesis: Permission/waivers to use figures

Young Im

Mon 2018-10-15 1:08 PM

To: Jacqueline Ha <jacqueline.ha2@mail.mcgill.ca>;

I confirm of course,
Good luck with your writing!
Young Im

From: Jacqueline Ha
Sent: Monday, October 15, 2018 12:00 PM
To: Claudia Kleinman, Ms; Harvey Wilmore Smith, Dr; Steven Hébert; William Muller, Dr.; Ryuhjin Ahn; Young Im; Valerie Sabourin; Eduardo Cepeda Cañedo
Cc: Giuseppina Ursini-Siegel, Dr
Subject: PhD Thesis: Permission/waivers to use figures

Dear Collaborators,

I am writing to kindly ask you for permission to use all figures and data in-part or in-full from our manuscript titled "Integration of Distinct ShcA Signaling Complexes Promotes Breast Tumor Growth and Tyrosine Kinase Inhibitor Resistance" published in Molecular Cancer Research for my PhD dissertation.

Please confirm by responding to this email at your earliest convenience.

Thank you for your time and support. I look forward to hearing from you.

Sincerely,

Jacqueline Ha
PhD Candidate
Ursini-Siegel Laboratory
Lady Davis Institute for Medical Research
Division of Experimental Medicine, Faculty of Medicine, McGill University
3755 Cote Ste. Catherine Road, Room# F528
Montreal, Quebec, Canada, H3T 1E2
Phone: (514) 586 6209
E-mail: jacqueline.ha2@mail.mcgill.ca