Integration of Distinct ShcA Signaling Complexes Promotes Breast Tumor Growth and Tyrosine Kinase Inhibitor Resistance

Jacqueline R. Ha1,2, Ryuhi Jin Ahn1,2, Harvey W. Smith3,4, Valerie Sabourin1, Steven Hébert1, Eduardo Cepeda Cañedo1,2, Young Kyuen Im1,2, Claudia L. Kleinman1,5, William J. Muller3,5, and Josie Ursini-Siegel1,2,3,4,6

Abstract

The commonality between most phospho-tyrosine signaling networks is their shared use of adaptor proteins to transduce mitogenic signals. ShcA (SHC1) is one such adaptor protein that employs two phospho-tyrosine binding domains (PTB and SH2) and key phospho-tyrosine residues to promote mammary tumorigenesis. Receptor tyrosine kinases (RTK), such as ErbB2, bind the ShcA PTB domain to promote breast tumorigenesis by engaging Grb2 downstream of the ShcA tyrosine phosphorylation sites to activate AKT/mTOR signaling. However, breast tumors also rely on the ShcA PTB domain to bind numerous negative regulators that limit activation of secondary mitogenic signaling networks. This study examines the role of PTB-independent ShcA pools in controlling breast tumor growth and resistance to tyrosine kinase inhibitors. We demonstrate that PTB-independent ShcA complexes predominate on the ShcA SH2 domain to activate multiple Src family kinases (SFK), including Src and Fyn, in ErbB2-positive breast cancers. Using genetic and pharmacologic approaches, we show that PTB-independent ShcA complexes augment mammary tumorigenesis by increasing the activity of the Src and Fyn tyrosine kinases in an SH2-dependent manner. 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. This study mechanistically dissects how the interplay between diverse intracellular ShcA complexes impacts the tyrosine kinase to affect breast tumorigenesis.

Implications: The ShcA adaptor, within distinct signaling complexes, impacts tyrosine kinase signaling, breast tumor growth, and resistance to tyrosine kinase inhibitors. Mol Cancer Res; 16(5); 894–908. ©2018 AACR.

Introduction

Aberrant phospho-tyrosine signaling networks are essential drivers of breast cancer progression (1). With the knowledge that breast cancer is a heterogenous disease, intense research efforts have reinforced the understanding that tyrosine kinases contribute to the emergence of specific breast cancer subtypes. For example, HER2+ breast cancer is an aggressive disease driven by the dysregulated activation of the HER2/ErbB2 receptor tyrosine kinase (RTK; refs. 2, 3). Introduction of trastuzumab, a HER2-directed monoclonal antibody, significantly improved patient outcome. Unfortunately, numerous tyrosine kinases, including Met, EGFR, and Lyn (4–6), contribute to the emergence of basal-like breast cancer that has confounded the identification of effective targeted therapies against this poor outcome subtype. In this regard, HER2+ breast cancer cells reprogram their tyrosine kinase through the aberrant activation of numerous receptor and nonreceptor tyrosine kinases, including but not limited to ErbB3, Met, and Src, to confer trastuzumab resistance (7–10). Thus, tyrosine kinases serve an essential role in establishing breast cancer heterogeneity and therapeutic sensitivity.

Adaptor proteins serve an equally important role in phospho-tyrosine signaling. Although many adaptor proteins lack intrinsic catalytic activity, they are critical intermediates that transduce signals downstream of tyrosine kinases by integrating multimeric signaling complexes. The ShcA adaptor protein is an essential signaling node downstream of ErbB2 to promote breast cancer initiation (11). Specifically, ShcA possesses two phospho-tyrosine binding domains (PTB, SH2) and three tyrosine phosphorylation sites (Y239/Y240/Y313) to transduce phospho-tyrosine-dependent signals (12). Key RTKs that are activated in HER2+ breast tumors, including ErbB2, EGFR, and ErbB3, bind the ShcA PTB domain through a conserved NPxY motif (13, 14) and require a functional PTB domain to promote breast tumor initiation and progression (15). Once activated, RTKs bind the PTB domain of ShcA, leading to phosphorylation of tyrosine residues

Note: Supplementary data for this article are available at Molecular Cancer Research Online (http://mcr.aacrjournals.org/).

Corresponding Author: Josie Ursini-Siegel, Lady Davis Institute for Medical Research, 5755 Cote St. Catherine Road, Montréal, Quebec H3T 1E2, Canada. Phone: 514-340-8222, ext. 26557; Fax: 514-340-7502; E-mail: giuseppina.ursini-siegel@mcgill.ca.

doi: 10.1158/1541-7786.MCR-17-0623

©2018 American Association for Cancer Research.
in the CH1 domain (Y239/Y240/Y313). This creates docking sites for Grb2/SOS and Grb2/Gab1 complexes, which activate the Ras/ERK and PI3K/AKT pathways, respectively (16, 17). In addition to its protumorigenic properties, the ShcA PTB domain is also paradoxically important for signal termination. This property of ShcA ensures that the strength and duration of protumorigenic responses is tightly controlled. Indeed, the ShcA PTB domain binds numerous negative regulators, including SHIP2, PTp, and ERK. While some of these interactions require the phospho-tyrosine binding pocket of the ShcA PTB domain (PTP-PEST, SHIP2, PTp), others are phospho-tyrosine independent (PTp, ERK; refs. 18–20). Several studies have established the ShcA PTB domain as a temporal switch to control EGFR signaling networks by facilitating the delayed recruitment of PTP-PEST to terminate ShcA-coupled mitogenic responses (21–25). This corroborates in vivo studies, which demonstrated that the loss of PTB-driven ShcA signaling delayed breast tumor initiation but paradoxically potentiated subsequent tumor growth (15).

We previously demonstrated that PTB-dependent signaling complexes promote breast cancer growth by increasing AKT/mTOR signaling downstream of the ShcA tyrosine phosphorylation sites (24). In addition, we showed that the ShcA PTB domain dynamically controls signaling networks in breast cancer cells, not only to transduce tumorigenic signals downstream of RTKs including ErbB2 (PTB-dependent), but also to create a negative feedback loop that prevents secondary activation of ShcA-SH2–driven complexes (PTB-independent). 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. In this study, we show that PTB-independent complexes augment mammary tumorigenesis by increasing the activity of the Src and Fyn tyrosine kinases in an SH2-dependent manner. Surprisingly, the ShcA tyrosine phosphorylation sites are dispensable for the ability of these PTB-independent ShcA pools to amplify tumor growth. Finally, we establish that increased Src activation downstream of PTB-independent ShcA signaling complexes increases resistance to lapatinib, a dual EGFR/ErbB2 inhibitor. These results show that perturbation of discrete ShcA-dependent signaling complexes significantly impacts breast tumor growth and therapeutic responsiveness.

Materials and Methods

Cell culture

NMuMG-NeuNT, an ErbB2-transformed mammary tumor cell line, was generated and cultured as described previously (11). NMuMG-NeuNT cell lines were stably transduced with cDNAs expressing: (i) wild-type ShcA (ShcAWT), (ii) a ShcA mutant harboring an arginine to glutamine substitution at amino acid 175 in the phospho-tyrosine binding pocket of the PTB domain of ShcA (PTBmut1), (iii) a PTBmut2 harboring tyrosine to phenylalanine substitution at amino acids 239, 240, and 313 (PTBmut2/3), or (iv) a PTBmut3 with an arginine to lysine substitution at amino acid 397 in the phospho-tyrosine binding pocket of the SH2 domain (PTB/H2mut). All ShcA constructs are C-terminally tagged with a 3×FLAG epitope. Four ShcA-expressing clones were pooled for each cell line, which were maintained in 0.5 mg/mL Hygromycin (Wisent Bio Products Cat: 450-141-WL). NIC/Src−/− cell lines were established from mammary tumor virus (MMTV)/Neu-internal ribosome entry site (IRES)-Cre ( Nic) mammary tumors, in which both c-Src alleles have been deleted by Cre-mediated excision (25–27). Culture conditions for these cells have also been described previously (25–27). NIC/Src−/− cells were stably transduced with a pQCXIB expression vector (Addgene catalog no. 22266) subcloned with Flag-tagged wild-type ShcA or the various ShcA mutants described above. Transfected cells were selected with 8 μg/mL blasticidin (Wisent Bio Products catalog no. 400-190-EM). Cell lines represent pools of six expressing clones. All cell lines were routinely screened for mycoplasma contamination using a Mycoprobe Mycoplasma Detection Kit (R&D Systems catalog no. CUL001B), at least once per month or one day prior to any mammary fat pad injection.

Cell line authentication

NMUUMG cells were purchased from ATCC and NMuMG-NeuNT cell lines (ErbB2 transformed) were generated from tumor explants and cultured as described previously (11). NIC/Src−/− and MT cell lines were generated and cultured as described previously (25, 27–29). Experiments performed on cell lines were passaged no more than 8 times per month.

Pharmacologic inhibitors

Cell lines were treated with media containing DMSO (BioShop catalog no. DMS666), PP2 pan-Src Family kinase inhibitor (2 μmol/L; Sigma-Aldrich catalog no. P0042), lapatinib ditosylate EGFR and HER2/ErbB2 inhibitor (500 nmol/L; Selleck Chemicals catalog no. S1028), or Torin1 mTOR inhibitor (50 nmol/L; Tocris catalog no. 4247).

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 catalog no. 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 Optrionx GelCount system. Quantification of the number of colonies and size of foci were analyzed with Aperio ImageScope software.

Soft agar assay

NMuMG-NeuNT. A total of 1.5 × 10^4 cells were plated into 1.5 mL of 0.4% Agar (BioShop catalog no. AGR001.500)/20% FBS (Wisent Bio Products catalog no. 080-150) DMEM (Wisent Bio Products catalog no. 319-005-CL) over a layer of 2 mL 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 μmol/L PP2 and 150 nmol/L Torin1 to obtain an approximate final concentration of 2 μmol/L and 50 nmol/L, respectively. For inhibitor studies, media containing inhibitors or DMSO was replenished every 3 days and monitored over a 10-day period.

NIC/Src−/−. A total of 1.5 × 10^4 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 catalog no. PHG0311), 0.5 mg/mL Hydrocortisone (Wisent Bio Products catalog no. 511-002-UG), 5 mg/mL insulin, 0.4% v/v bovine pituitary extract (Wisent Bio Products catalog no. 002-011-IL) over a layer of 2 mL 0.6% Agar/5 mg/mL insulin, 0.4% v/v bovine pituitary extract (Wisent Bio Products catalog no. 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.

**Statistical analyses**

Unless otherwise indicated, all in vitro studies were carried out with three biological replicates and with six technical replicates per experimental group. Data were normalized to the control groups as appropriate. For the in vitro replicates per experimental group. Data were normalized to the control groups as appropriate. For the in vivo studies, mammary fat pad injections were performed with 4\( \times 10^4 \) NMuMG-NeuNT or 2.5 \( \times 10^5 \) NIC/Sc3G-/- wild-type or mutant ShcA-expressing breast cancer cells were injected into the fourth mammary fat pad of 8- to 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 catalog no. 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.

**Mammary fat pad injection**

A total of 5 \( \times 10^4 \) NMuMG-NeuNT or 2.5 \( \times 10^5 \) NIC/Sc3G-/- wild-type or mutant ShcA-expressing breast cancer cells were injected into the fourth mammary fat pad of 8- to 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 catalog no. 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.

**Results**

**PTB-independent ShcA pools require an intact SH2 domain to potentiate mammary tumorigenesis**

To model the consequence of deregulated ShcA signaling from PTB-independent ShcA pools on breast tumorigenesis, we employed a loss-of-function mutant (R175Q) in the phospho-tyrosine binding pocket of the ShcA PTB domain (PTBMUT; refs. 15, 30). Ectopic expression of a ShcA-PTBMUT allele in breast cancer cells that endogenously express wild-type ShcA creates distinct intracellular ShcA pools that can independently transduce oncogenic signals downstream from RTKs, including ErbB2, (ShcA-PTB-dependent) and that also lack the ability to bind negative regulators, permitting amplification of pro-tumorigenic signals from PTB-independent ShcA complexes (PTBMUT; Fig. 1A). To determine how these PTB-independent ShcA pools transduce oncogenic signals, we either mutated all three ShcA tyrosine phosphorylation sites to phenylalanine residues (PTBMUT/3F) or introduced a loss of function mutation (R397K) in the phospho-tyrosine binding pocket of the SH2 domain (PTB/SH2MUT), in the context of PTB mutation (Fig. 1A). FLAG-tagged ShcA mutants were stably expressed in ErbB2-expressing breast cancer cells (Fig. 1B). FLAG-tagged ShcA alleles harboring a wild-type PTB domain (ShcAWT; PTB-dependent) or the loss-of-function PTB mutant (PTBMUT; PTB-independent) alone, were also stably expressed (Fig. 1B). We first confirmed that an intact PTB domain is required for ShcA to engage in PTB-dependent interactions, including ErbB2, PTP-PEST, and SHIP2 (refs. 18, 21–23, 31; Fig. 1C). Second, SH2-dependent interactions with ShcBP1 (32) were selectively abrogated in PTB/SH2MUT-expressing cells (Fig. 1C). Finally, mutation of the ShcA tyrosine phosphorylation sites ablated Grb2 binding (PTBMUT vs. PTBMUT/3F; Fig. 1D; ref. 17). These results confirm the specificity of the mutants generated.

Our previous studies demonstrated that loss of PTB-dependent inhibitory signals augments ErbB2-driven mammary tumorigenesis (15). To mechanistically define how these PTB-independent ShcA pools (PTBMUT) 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. PTBMUT-expressing breast cancer cells had a reduced capacity to form colonies in soft agar relative to ShcAWT controls (Fig. 1E). This corroborated previous observations, which defined an important role for the ShcA PTB domain in ErbB2-driven tumor growth (15, 22, 33). However, once formed, PTBMUT-expressing foci were significantly larger than ShcAWT controls (Fig. 1F). This indicated that both phospho-tyrosine and SH2-dependent ShcA signals are important for breast cancer cell growth in vitro.

We next investigated whether PTB-uncoupled ShcA pools (PTBMUT) relied on the tyrosine phosphorylation sites and/or SH2 domain to amplify ErbB2-driven mammary tumor growth in vivo. As expected, unrestrained ShcA, signaling from PTB-independent complexes (PTBMUT) significantly accelerated tumor growth relative to ShcAWT controls (Fig. 1G). Surprisingly, the ShcA tyrosine phosphorylation sites were dispensable for the enhanced tumor growth observed from PTB-independent ShcA complexes (PTBMUT vs. PTBMUT/3F). Rather, loss of SH2-dependent signaling from these ShcA pools (PTB/SH2MUT) profoundly delayed breast tumor onset (Fig. 1G). To better understand the mechanism underlying this phenotype, we performed Ki67 and cleaved caspase-3 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 ShcAWT, PTBMUT, and PTBMUT/3F–expressing tumors. However, we did observe that PTB/SH2MUT–expressing tumors had the greatest proliferative potential, which was counteracted by a significantly increased apoptotic rate (Fig. 1H and I). This suggests that loss of SH2-dependent signaling from PTB-uncoupled ShcA complexes initiates an apoptotic response, leading to a compensatory increase in mitogenic responses to permit tumor growth.

Published OnlineFirst February 16, 2018; DOI: 10.1158/1541-7786.MCR-17-0623
PTB-independent ShcA pools simultaneously increase mTOR signaling and Src activity

We previously demonstrated that PTB-uncoupled ShcA signaling complexes (PTB\textsuperscript{MUT}) activate Src (15). We confirmed this observation and further show that these PTB-independent ShcA pools required an intact SH2 domain, and not the tyrosine phosphorylation sites, to activate Src (Fig. 2A). Moreover, PTB\textsuperscript{MUT}-expressing cells upregulated mTOR signaling, as evidenced by elevated pS6\textsuperscript{240/244}-4EBP1 and pT\textsuperscript{389}-S6K levels, compared with ShcA\textsuperscript{WT} controls (Fig. 2A). This increase in mTOR signaling required the ShcA tyrosine phosphorylation sites. Interestingly, AKT activation was relatively unchanged, irrespective of the mutational status of these PTB-uncoupled ShcA signaling complexes (Fig. 2A). 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\textsuperscript{MUT}/3F tumors compared with PTB\textsuperscript{MUT} controls (Fig. 2B), corroborating an important role for ShcA tyrosine phosphorylation in promoting mTOR signaling. Given that PTB\textsuperscript{MUT} and PTB\textsuperscript{MUT}/3F-expressing tumors display comparable growth rates in vivo (Fig. 1G), these data suggest that mTOR activation is not primarily responsible for the increased
tumorigenic potential of PTB-uncoupled ShcA pools (Fig. 2B). On the other hand, pY416-Src levels were profoundly elevated in PTBMUT and PTBMUT/3F–expressing tumors relative to ShcAWT controls (Fig. 2C). This is consistent with our in vitro studies showing that increased tumor growth was associated with an SH2-dependent increase in Src activity (Fig. 2A). At endpoint, Src signaling was restored in PTB/SH2MUT tumors (Fig. 2C) even though their growth potential was severely impaired in vivo (Fig. 2C). 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, there were significant selective pressures to reacquire Src activity to permit eventual tumor growth.

Once activated, ErbB2 recruits and phosphorylates ShcA in a PTB-dependent manner (34, 35). We now demonstrate that PTB-independent ShcA pools (PTBMUT) are also tyrosine phosphorylated, albeit to a significantly lower extent (3 fold) relative to PTB-dependent ShcA complexes (ShcAWT; Fig. 2D). Given that numerous Src family kinases (SFK) bind ShcA, both in a constitutive and phospho-tyrosine inducible manner (36–39), we employed a pan SFK inhibitor (PP2) at lower doses (2 μmol/L) to test whether PTB-independent ShcA pools are more reliant on SFKs to phosphorylate ShcA (Supplementary Fig. S1A). Pharmacologic SFK inhibition (2 μmol/L PP2) did not impact ShcA tyrosine phosphorylation from PTB-coupled complexes (ShcAWT) but attenuated pY-ShcA levels from PTB-uncoupled ShcA pools (PTBMUT) relative to their respective DMSO controls (Fig. 2D).
addition, PP2 inhibition of SFKs at a higher dose (20 μmol/L) completely abrogated ShcA tyrosine phosphorylation, specifically from PTB-independent ShcA complexes (Supplementary Fig. S1B). As expected, a ShcA mutant lacking the tyrosine phosphorylation sites (PTBMUT/3F) was not phosphorylated in control or PP2-treated cells. Remarkably, ShcA pools that cannot engage in PTB- or SH2-driven interactions (PTB/SH2MUT) were residually tyrosine phosphorylated in an SFK-dependent manner (Fig. 2D; Supplementary Fig. S1B). This highlights an accessory role for constitutive ShcA/SFK interactions in transducing phosphotyrosine driven ShcA signals. These results suggest that PTB-uncoupled ShcA pools are more reliant on SFKs to phosphorylate 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 (PTBMTF vs. PTB/SH2MTF; Fig. 2E). Paradoxically, independent ShcA pools with an intact SH2 domain (PTBMTF) exhibited the lowest level of SFK binding, relative to ShcAMTF controls (Fig. 2E), despite displaying a 2-fold increase in Src activation (Fig. 2A). Collectively, these results suggest that the ability of PTB-uncoupled ShcA pools to activate Src required a functional SH2 domain but was uncoupled from direct SFK recruitment to ShcA signaling complexes.

PTB-independent ShcA signaling complexes cooperatively activate SFKs in ErbB2-positive breast cancer cells. Thus, we assessed whether their hyperactivation altered sensitivity of breast cancer cells to pharmacologic inhibition of ErbB2 and EGFR (lapatinib), both of which bind the ShcA PTB domain. Recall that PTBMTF-expressing cells retain the ability to engage both PTB-independent ShcA complexes alongside PTB-coupled interactions from endogenous ShcA alleles. We demonstrate that increased PTB-independent ShcA signaling promoted lapatinib resistance in ErbB2-transformed breast cancer cells (Fig. 2F). Indeed, the ERK and AKT pathways remained elevated in lapatinib-treated PTBMUT-expressing cells compared with ShcA WT controls (Supplementary Fig. S1C). Loss of either phosphotyrosine (PTBMUT/3F)- or SH2 (PTB/SH2MUT)-driven signaling from these PTB-independent ShcA pools reversed lapatinib resistance (Fig. 2F) and impaired ERK and AKT/mTOR signaling (Supplementary Fig. S1C). Significant inhibition of ErbB2 signaling was confirmed upon treatment with lapatinib (500 nmol/L) (Supplementary Fig. S1C). Moreover, ErbB2 inhibition had no impact on Src activation in any cell line tested (Supplementary Fig. S1C). Combined, these data suggest that increased signaling from PTB-independent ShcA pools can induce lapatinib resistance by increasing ERK and AKT activation both through the SH2 domain and tyrosine phosphorylation sites.

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

Although these studies demonstrate an essential role for the ShcA-SH2 domain in promoting mammary tumorigenesis from PTB-independent complexes, they do not identify a causal role for Src in this phenotype. To test this, we ectopically expressed ShcAMTF and PTBMTF alleles in ErbB2 transformed...
mammary tumors established from transgenic mice lacking Src (NIC/Src−/−) in the mammary epithelial compartment (Fig. 3A; ref. 27). Consistent with our previous studies (Fig. 2D), PTB-independent ShcA pools were significantly hypophosphorylated relative to ShcAWT controls in Src-deficient cells (Fig. 3B). In the absence of Src, mammary tumor growth was comparable between ShcAWT- and PTBMUT-expressing cells, both in vitro and in vivo (Fig. 3C; Supplementary Fig. S2A). IHC staining further revealed that both Src-deficient ShcAWT and PTBMUT breast tumors displayed comparable rates of proliferation and apoptosis (Fig. 3D and E; Supplementary Fig. S2B). Moreover, pY416-SFK levels were comparable between ShcAWT- and PTBMUT-expressing mammary tumors (Fig. 3F; Supplementary Fig. S2B), suggesting that the ability of PTB-independent ShcA pools to promote mammary tumorigenesis required Src. In addition, reduced pS240/244-rS6 levels in Shc-PTBMUT-expressing mammary tumors (Fig. 3G) did not correlate with their tumorigenic potential (Fig. 3C). This confirms our previous observation that mTOR signaling is likely not 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 these tumors significantly rewired their kinome in response to Src loss. To confirm that 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 ShcAWT- and PTBMUT-expressing, ErbB2-transformed (ErbB2/Src+/+) breast cancer cells (Fig. 4A). While Src was dispensable for the growth of ShcAWT mammary tumors (Fig. 4B), Src deletion impaired the ability of PTB-independent ShcA pools to promote mammary tumorigenesis (Fig. 4C). At the experimental endpoint, proliferation and apoptosis were comparable in all groups, irrespective of the presence or absence of Src (Supplementary Fig. S3A–S3D). IHC analysis confirmed that both ShcAWT/Src-CR and PTBMUT/Src-CR tumors were debilitated in SFK activation relative to their respective vector controls (Fig. 4D; Supplementary Fig. S3E). However,
pS240/244-rS6 levels were selectively reduced in Src-deficient tumors compared with PTB\textsuperscript{MUT} controls (Fig. 4E; Supplementary Fig. S3F). Thus, using two independent model systems, we show that loss of Src signaling from PTB-independent ShcA pools debilitates mTOR signaling (Figs. 3G and 4E). While SFK activity was required to phosphorylate ShcA residing within PTB-independent ShcA pools (Fig. 2D), Src deletion severely reduced, but did not ablate, ShcA tyrosine phosphorylation from these complexes (PTBMUT \textsuperscript{vs} PTBMUT/Src-CR; Fig. 4F). In contrast, tyrosine phosphorylation of PTB-dependent ShcA signaling complexes remained unaffected by Src deletion (ShcA\textsuperscript{WT} vs. ShcA\textsuperscript{WT/Src-CR}; Fig. 4F). These data further confirm our observation that Src, and potentially other SFKs, selectively promote ShcA tyrosine phosphorylation in PTB-independent signaling complexes.

We previously established that increased SH2 signaling from PTB-independent ShcA complexes contributes to lapatinib resistance, presumably through increased Src activation (Fig. 2F). We next examined whether the loss of Src impacted lapatinib sensitivity in mammary tumors that can (PTB\textsuperscript{MUT}) or cannot (ShcA\textsuperscript{WT}) activate ShcA from PTB-independent complexes. As expected, Src deficiency had no impact on the sensitivity of ShcA\textsuperscript{WT} tumors to lapatinib but profoundly sensitized PTB\textsuperscript{MUT} expressing tumors to this ErbB2 inhibitor (Fig. 4G). This suggests that in the absence of Src, tumors lose their ability to engage PTB-independent ShcA complexes and instead are more reliant on ErbB2/ShcA signaling. On the other hand, Src deficiency minimally impacted sensitivity to PP2, in both ShcA\textsuperscript{WT}, and PTB\textsuperscript{MUT}-expressing cells (Fig. 4G). This indicated that Src is likely the primary SFK to transduce mitogenic signals in ErbB2-transformed breast cancer cells in vitro. On the other hand, while the loss of Src resulted in a 3-fold increase in the sensitivity of PTB\textsuperscript{MUT}-expressing cells to lapatinib treatment, coincubation with lapatinib and PP2 resulted in a 22-fold decrease in cell viability. This was contrasted by ShcA\textsuperscript{WT}, expressing breast cancer cells in which cell viability upon combined lapatinib/PP2 treatment was comparable, irrespective of Src status (Fig. 4G). These results suggest that in the absence of Src, breast tumors that engage PTB-independent signaling complexes become codependent on mitogenic signals emanating from both ErbB2 and other SFKs.

\textbf{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 (Fig. 5A and B; Supplementary Fig. S4A). We did not observe a compensatory increase in Src levels when either Fyn or Lyn were deleted. By the same token, Src deletion did not appreciably alter Fyn or Lyn expression levels (Fig. 5A and B; Supplementary Fig. S4A). Moreover, while pY416-SFK and pY576/7-FAK levels (Src specific phosphorylation site) were significantly reduced in ShcA\textsuperscript{WT/Src-CR} and PTB\textsuperscript{MUT/Src-CR}–expressing cells, SFK activation was unaltered by Fyn or Lyn loss in either cell line (Fig. 5A and B; Supplementary Fig. S4A). 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 transformation potential of Src-, Fyn-, or Lyn-deficient breast cancer cells in a soft agar assay. In support of our in vivo study, Src deletion impaired tumor initiation (number of foci) in breast cancer cells that retained a functional PTB domain (ShcAWT) but was dispensable for tumor growth (average area of foci; Fig. 5C and D). In contrast, neither Fyn nor Lyn deletion appreciably altered the transforming potential of ShcAWT breast cancer cells (Fig. 5C and D). In contrast, Src loss in breast cancer cells that hyperactivate PTB-independent ShcA complexes (PTBMUT) significantly reduced both the number and size of foci formed (Fig. 5E and F). This validated our in vivo observations where Src was necessary for the ability of these PTB-independent ShcA pools to augment mammary tumor growth (Fig. 4C). Fyn deletion in PTBMUT-expressing breast cancer cells did not impact foci formation but reduced their growth potential ($/C24^2$ fold) relative to vector controls (Fig. 5E and F). Lyn, on the other hand, was dispensable for the transforming potential of ErbB2-driven breast cancer cells, irrespective of whether they had engaged PTB-independent ShcA complexes (Fig. 5E and F). These data suggest that Src cooperates with Fyn

Figure 6.
PTB-independent ShcA complexes hyperactivate mTOR signaling to maintain mammary tumorigenesis in the absence of Src. A, Mammary fat pad injection of NIC/Src$^{-/-}$ PTB/H2MT-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. B, Vector control and Src-CR of NMuMG-NeuNT PTB/H2MT-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 per group. *, $P < 0.05$; **, ***, $P < 0.01$; ****, $P < 0.001$. C, 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. D, Immunoblot blot analysis of whole-cell lysates of NMuMG-NeuNT PTB/H2MT mammary tumors using pY416-SFK, K67, cleaved Casp3 and pS240/244-rS6 specific antibodies. The data depict the average positively stained cells or pixels ± SEM and is representative of 7-9 tumors per group. *, $P < 0.05$; **, ***, $P < 0.01$; ****, $P < 0.0001$. E, Immunoblot analysis of the indicated NIC/Src$^{-/-}$ PTB/H2MT-expressing cell lines using Src, Fyn, Lyn with specified antibodies. IHC staining of NIC/Src$^{-/-}$ PTB/H2MT (E) and vector control (F) and Src-CR NMuMG-NeuNT PTB/H2MT mammary tumors using pY416-SFK, Ki67, cleaved Casp3 and pS240/244-rS6 specific antibodies. The data depict the average positively stained cells or pixels ± SEM and is representative of 7-9 tumors per group. *, $P < 0.05$; **, ***, $P < 0.01$; ****, $P < 0.0001$. F, Clonogenic assays of specified cell lines treated with DMSO, lapatinib (0.5 mol/L), PP2 (2 mol/L) 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). *, $P < 0.05$. G, FLAG immunoprecipitates from NMuMG-NeuNT PTB/H2MT cell line probed with pY239/240-ShcA or ShcA-specific antibodies by immunoblot analysis. The barplot represents densitometric quantification of three independent experiments using ImageJ software. H, Immunoblot analysis of the indicated NIC/Src$^{-/-}$ PTB/H2MT-expressing cell lines using ShcA-specific antibodies. I, Number ($I$) and average area ($J$) of foci formed in a soft agar assay from the specified cell lines. The data are representative of three independent experiments (means ± SEM), *, $P < 0.05$. J, Number ($K$) and average area ($L$) of foci formed in soft agar of indicated cell lines relative to DMSO controls in the absence or presence of PP2 (2 mol/L) or Torin1 (50 mmol/L). The data is representative of three independent experiments (means ± SEM). DMSO versus PP2 or Torin1 treatment: *, $P < 0.05$; **, $P < 0.01$.
downstream of PTB-independent Shc pools to increase the tumorigenic potential of ErbB2+ breast cancers. To test this, we first assessed how Src deletion, in combination with, SFK inhibition (2 μmol/L PP2) impacted the tumorigenic potential of Shc\textsuperscript{WT}- and PTB\textsuperscript{Mut}-expressing cells. As expected, pharmacologic SFK inhibition did not appreciably impact the transforming potential of Shc\textsuperscript{WT}-expressing cells (Fig. 5G and H; Supplementary Fig. S4B). In addition, genetic loss of Src in Shc\textsuperscript{WT}-expressing cells impacted the number but not the size of foci formed in a soft agar assay. Similarly, even in the absence of Src, pharmacologic SFK inhibition moderately reduced the number of foci but did not impact the size of the foci (Fig. 5G and H). These data further support the observation that Shc\textsuperscript{WT} breast tumors 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 (24). Consistent with this observation, Shc\textsuperscript{WT}-expressing breast cancer cells were exquisitely sensitive to suboptimal doses of mTORI inhibitor, Torin1 (50 nmol/L) in a soft agar assay, showing a significant reduction in the number and size of foci formed (Fig. 5G and H; Supplementary Fig. S4B). Moreover, the ability of Torin1 to impair focus formation (# foci) was independent of Src in Shc\textsuperscript{WT}-expressing cells. Alternatively, the tumorigenic potential of breast cancer cells engaging PTB-independent ShcA signaling complexes (PTB\textsuperscript{Mut}) were similarly sensitive to Torin1 (Fig. 5I and J; Supplementary Fig. S4B). These results highlighted an important role for mTOR signaling in ErbB2+ breast cancers, irrespective of whether they transduce signals from PTB-dependent ShcA pools. Suboptimal doses of SFK inhibitor, PP2, had no impact on the tumor-forming potential of Src proficient PTB\textsuperscript{Mut}-expressing cells but did attenuate the growth of these foci (Fig. 5I and J; Supplementary Fig. S4B). In contrast, Src deletion was sufficient to decrease both the number and size of foci formed by PTB\textsuperscript{Mut} expressing cells (Fig. 5I and J). The average area of PTB\textsuperscript{Mut} expressing foci was further and significantly diminished upon SFK inhibition by PP2 (Fig. 5I and J). These data suggest that Src is principally responsible for the increased tumorigenic potential of PTB-independent ShcA complexes but that other SFKs, including Fyn play a cooperative role in this process.

Loss of c-Src signaling downstream of PTB-independent ShcA complexes increases the dependency of ErbB2-driven breast tumors on mTOR signaling

Our data suggest that breast tumors require a functional ShcA SH2 domain to activate Src downstream from PTB-independent ShcA complexes. Given that Src deficiency reduced the tumorigenic potential of PTB-uncoupled ShcA pools (Figs. 3C and 4C), we interrogated the impact of simultaneous Src loss and impaired SH2-driven signaling from tumor-amplifying ShcA complexes (PTB/SH2\textsuperscript{Mut}). Surprisingly, upon ectopic expression of PTB/SH2\textsuperscript{Mut} alleles in NIC/\textsuperscript{Src\textsuperscript{-/-}} breast cancer cells, loss of SH2-driven ShcA signaling did not ablate breast tumor formation in vivo (Fig. 6A). In addition, Src deficiency (by Crisp/Cas9) paradoxically increased the growth potential of tumors that lack the ability to transduce SH2-driven signals from PTB-independent ShcA complexes (Fig. 6B). This indicated that, in the absence of Src and SH2 signaling from these PTB-uncoupled ShcA pools, secondary adaptive responses likely facilitate breast tumor growth. We already showed that Fyn cooperates with Src to amplify the tumorigenic potential of PTB-independent signaling complexes (Fig. 5). Moreover, Src deficiency in two independent models (NIC/\textsuperscript{Src\textsuperscript{-/-}} and ErbB2/Src-CR) significantly upregulated Fyn levels when PTB-independent ShcA pools could no longer signal through the SH2 domain (Fig. 6C and D, Supplementary Fig. S5A and S5B). Lyn expression levels, on the other hand, were minimally impacted in these cells (Fig. 6C and D, Supplementary Fig. S5A and S5B). Moreover, we did not observe increased pY416-SFK levels in Src-deficient PTB/SH2\textsuperscript{Mut} cancer cells, both in vitro and in vivo (Fig. 6C–F; Supplementary Fig. S5A and S5B). This is consistent with the observation that the growth potential of these cells was insensitive to SFK inhibition (PP2) in a clonogenic assay (Fig. 6G; Supplementary Fig. S5C). Rather, Src deficiency coupled with the lack of SH2-driven signaling from PTB-independent ShcA pools (PTB/SH2\textsuperscript{Mut}), significantly upregulated pS240/244-rs6 levels in mammary tumors (Fig. 6E and F). This was coincident with an increased proliferative index compared with SH2-proficient controls (Fig. 6E and F; Supplementary Fig. S56A and S6B). Thus, in the absence of Src and a functional SH2 domain, PTB-independent ShcA pools elicit a compensatory increase in mTOR signaling to facilitate eventual tumor growth. However, the hyperactivation of mTOR signaling in these cells was not associated with increased pY-ShcA levels (Fig. 6H), suggesting that increased mTOR signaling was independent of the ShcA tyrosine phosphorylation sites. These observations reinforce the importance of mTOR signaling in ErbB2-driven breast cancer, particularly in response to Src inhibition, and demonstrate the ability of mammary tumors to reprogram their kinomes.

We next tested whether Src-null breast tumors that engage PTB-independent ShcA signaling, become more reliant on ErbB2. As expected, Src deletion sensitized PTB/SH2\textsuperscript{Mut}-expressing cells to lapatinib, demonstrating an increased reliance on ErbB2/ShcA signaling complexes (Fig. 6G). Moreover, simultaneous ErbB2 and SFK inhibition (lapatinib and PP2) completely ablated the viability of PTB/SH2\textsuperscript{Mut} breast cancer cells, suggesting a potential accessory role for Fyn or Lyn in the absence of Src. To test this, Fyn and Lyn were also stably deleted from PTB/SH2\textsuperscript{Mut}-expressing cells using Crisp/Cas9 genomic editing (Fig. 6D). Fyn deletion reciprocally increased Src expression levels in PTB/SH2\textsuperscript{Mut}-expressing cells, again highlighting the adaptive responses triggered in ErbB2-driven breast tumors to retain SFK function (Fig. 6D; Supplementary Fig. S5B). To test whether these perturbations in SFK levels were biologically relevant, 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 (Fig. 6B), deletion of Src in PTB/SH2\textsuperscript{Mut}-expressing cells increased the average size but not number of foci formed, relative to Src-proficient controls (Fig. 6I and J). These data suggest that compensatory activation of mTOR signaling likely sustained their growth in soft agar. To test this, we examined the sensitivity of both Src-proficient and Src-deficient PTB/SH2\textsuperscript{Mut}-expressing cells to mTORI inhibition (Torin1) in a soft agar assay. As expected, the transforming potential of these cells was insensitive to pharmacologic (PP2) or genetic (Src-CR) Src inhibition (Fig. 6K and L). Rather, the loss of a functional ShcA-SH2 domain from PTB-independent ShcA complexes was sufficient to render these cancer cells exquisitely sensitive to Torin1, independently of Src (Fig. 6K and L). These data support the notion that PTB-independent ShcA signaling complexes rely on the ShcA SH2 domain to activate Src signaling to augment mammary tumor growth; however, loss of the ShcA/Src signaling axis from these
PTB-independent pools enable the reprogramming of signal networks to increase their reliance on mTOR signaling. **Increased Src and mTOR signaling correlates with a PTB\textsuperscript{MUT} gene signature in human breast cancers**

We previously generated a gene signature that distinguished tumors that did (PTB\textsuperscript{MUT}) or did not (ShcA\textsuperscript{WT}) augment mitogenic signals from PTB-independent ShcA complexes (15). We used this gene signature (>2.5-fold differentially expressed; Supplementary Table S1); to stratified 1,218 human breast cancers using the publicly available TCGA RNA-seq dataset. 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 (40). Breast cancer patients were stratified into four quartiles based on the degree to which they most resemble a PTB\textsuperscript{MUT} gene signature (quartile 1: most ShcA\textsuperscript{WT}/like/PTB-dependent vs. quartile 4: most PTB\textsuperscript{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 (Supplementary Fig. S7A). We show that the acquisition of a PTB\textsuperscript{MUT}/like gene signature is associated with a modest but significant increase in Src mRNA levels (1.14-fold increase between 1st and 4th quartiles). In contrast Fyn and Lyn mRNA levels are much more significantly increased in PTB\textsuperscript{MUT}/like human breast tumors compared with those that are most like ShcA\textsuperscript{WT}/like breast tumors (1.37-fold and 1.47-fold increase respectively between 1st and 4th quartiles). We also took advantage of the fact that the TCGA database contains RPPA data for 747 of these patients (n = 171:1st quartile; n = 164: 2nd quartile; n = 196: 3rd quartile; n = 216: 4th quartile). We demonstrate that PTB\textsuperscript{MUT}/like breast tumors display significantly reduced pY317-ShcA levels coincident with elevated pY416-Src and pS235/6-rS6 levels compared with ShcA\textsuperscript{WT}/like breast tumors (Supplementary Fig. S7B). These results correlate with our preclinical studies, demonstrating that PTB-independent ShcA complexes hyperactivate Src and mTOR signaling, independently of the ShcA tyrosine phosphorylation sites. Finally, as described previously, several negative regulators, particularly PTP-PEST and SHIP2, have been shown to bind the PTB domain of ShcA to dampen ShcA-driven mitogenic signaling (18, 21–23, 31). Copy number analyses recently showed that the SHC1 (ShcA) gene, residing within the genomic region 1q21–1q23, is amplified in a subset of human breast cancers (41). Thus, breast cancers could potentially 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. We asked whether copy number variations of SHC1, PTPN12 (PTP-PEST), INPP11 (SHIP2) were correlated with tumors that most closely resemble a ShcA\textsuperscript{WT}/like or PTB\textsuperscript{MUT}/like gene signature. Interestingly, ShcA amplification was selectively enriched in ShcA\textsuperscript{WT}/like breast tumors (Supplementary Fig. S7C). In contrast, PTP-PEST, and not SHIP2, genomic loss was specifically enriched in PTB\textsuperscript{MUT}/like breast tumors (Supplementary Fig. S7C). Although correlatively, these data suggest a possible relationship between PTP-PEST, ShcA signaling, and Src activation in human breast cancers.

**Discussion**

We previously demonstrated that simultaneous expression of ShcA alleles that can (ShcA\textsuperscript{WT}) or cannot (PTB\textsuperscript{MUT}) partake in PTB-driven interactions result in parallel activation of distinct ShcA pools that amplify breast tumor growth (15). This approach enabled us to specifically dissect the molecular mechanisms by which PTB-independent ShcA complexes augment mammmary tumorigenesis without altering the strength or duration of signaling responses from PTB-coupled ShcA pools. In this study, we identified a significant divergence in the signaling properties of PTB-dependent versus PTB-independent signaling complexes. One of the most striking differences between these two ShcA pools lies in their use of the ShcA tyrosine phosphorylation sites to promote tumor growth. PTB-coupled ShcA complexes require phosphorylation of the tyrosine residues in the CH1 domain to activate AKT/mTOR signaling (16, 24). Interestingly, while ShcA tyrosine phosphorylation activates 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 relied on the SH2 domain to activate SFKs and amplify tumor growth (Fig. 7). 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 (42). This may explain why ShcA\textsuperscript{PTB\textsuperscript{MUT}/3F}–expressing cells are debilitated in their transforming potential in vivo. On the other hand, we show that a mutant ShcA allele lacking both a functional PTB domain and the tyrosine phosphorylation sites (PTB\textsuperscript{MUT}/3F) can still engage ShcB\textsubscript{P1}, an SH2-specific ShcA interactor, and 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 previous study was in the context of a wild-type ShcA allele (42), the impact of this gating mechanism on the ability of PTB-independent ShcA pools to activate the SH2 domain has yet to be determined.

Although the ShcA SH2 domain can serve as an accessory role to augment AKT/mTOR signaling downstream of activated RTKs (43), the very fact that the SH2 domain is dispensable for AKT/mTOR signaling from PTB-independent ShcA pools, highlights the inherent plasticity of these breast cancers to adapt to the loss of extracellular stimuli required for tumor initiation. However, this does not suggest that the hyperactivation of PTB-independent ShcA signaling complexes renders breast tumors insensitive to the mTOR pathway. Indeed, we show that breast tumors are exquisitely sensitive to pharmacologic mTOR inhibitors regardless of whether they are reliant on PTB-coupled or PTB-independent ShcA pools (Fig. 7). Rather, these data establish that activation of the AKT/mTOR pathway in breast tumors is primarily through PTB-dependent ShcA signaling complexes. Despite this fact, loss of SH2-driven ShcA signaling from PTB-independent complexes attenuated Src activation and consequently reduced tumor growth. Altogether, these data corroborate the notion that distinct intracellular ShcA signaling complexes transduce diverse and nonredundant mitogenic signals, that activate the AKT/mTOR (PTB-dependent) and Src (PTB-independent) pathways to cooperatively promote mammary tumorigenesis.

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. However, mutational activation, amplification, or overexpression of multiple...
components of the tyrosine kinome inevitably promote therapeutic resistance to ErbB2-targeted therapies (1). Our study 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 demonstrate 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 (Fig. 7). This is consistent with previous studies demonstrating that Src is hyperactivated in trastuzumab-resistant HER2+ breast cancers and that Src inhibition is able to resensitize them to ErbB2-targeted therapies (10). Our observations also reinforce the importance of SFKs in enhancing tumorigenic signals from PTB-independent ShcA pools 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. This highlighted that potential differences may exist for SFK dependency across breast cancer subtypes. Indeed, recent studies describe Lyn as an important effector of tumorigenicity in basal breast cancers (4). Moreover, reduced Src expression or diminished SH2-driven Src activation reduced tumor growth, which imposes selective pressures on breast tumors to restore Src signaling (Fig. 7). We propose that the ability of HER2+ breast cancers to augment auxiliary ShcA signaling pathways, independently of the PTB domain may represent one mechanism by which these tumors increase Src activity and acquire resistance to ErbB2 inhibitors.

While PTB-independent ShcA pools can no longer bind ErbB2, this does not preclude the fact that it is competent to participate in SH2-driven interactions to increase ShcA tyrosine phosphorylation, including Met (44), PDGFR (45), FGFR (46), and, the SFKs, Src, and Lyn (36, 38). 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 (15). On the other hand, SFK

**Figure 7.**
Distinct ShcA-dependent pools influence breast tumor growth, heterogeneity, and therapeutic responsiveness downstream of RTKs during mammary tumorigenesis. Schematic diagram summarizing the biological impact of PTB-dependent versus -independent ShcA signaling complexes on breast tumor growth along with sensitivity to pharmacologic and/or genetic inhibition of the ErbB2, mTOR, and Src family kinase pathways.

![Diagram of Distinct ShcA Pools Cooperatively Increase Tumorigenesis](image-url)
recruitment to ShcA is not exclusively dependent on the SH2 domain and can also involve alternative phospho-tyrosine-independent interactions. For example, Src can constitutively associate with an amino-terminal region in ShcA (37), leading to increased Src activation (47). Moreover, Lyn interacts with a proline-rich region in the CH1 domain of ShcA through its SH3 domain (39). In effect, the ShcA phospho-tyrosine residues, Y239/240 and Y313, can be phosphorylated by either Src and Lyn, respectively, leading to Grb2 recruitment and the consequent transduction of mitogenic signals (39, 48). Therefore, PTB-independent ShcA complexes require SFKs to ShcA. It is likely then, that ShcA and Src signaling is reciprocally regulated. Indeed, we found an inverse correlation between Src, Lyn, and Lyn recruitment to PTB-independent ShcA pools with Src activity and the transforming potential of these ShcA complexes. 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. Correspondingly, ShcA tyrosine phosphorylation, which requires Src, is dispensable for increased tumorigenesis from PTB-independent ShcA pools. These data imply that the ShcA SH2 domain indirectly activates Src from PTB-independent ShcA pools 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.

Recruitment of negative regulators to the PTB domain of ShcA is dependent, in part, on serine phosphorylation of ShcA, which results in the termination of ShcA-dependent signaling responses and the subsequent shift in the ShcA interaction to favor cytoskeletal reorganization (23). Specifically, the recruitment of PTP-PEST depends on Serine 29 phosphorylation to stabilize ShcA/PTP-PEST interactions. Moreover, Threonine 214 phosphorylation of ShcA was recently shown to increase ERK and AKT activation (49). Thus, serine/threonine phosphorylation of ShcA dynamically regulates the signaling potential of the adaptor protein, yet it is still poorly understood. How serine/threonine phosphorylation of ShcA from PTB-independent complexes may impact breast tumorigenesis is yet to be studied and poses an interesting avenue for further investigation.

Although there is no evidence for any point mutations in ShcA, 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. Indeed, several studies have interrogated the impact of PTP-PEST loss in breast cancers. For instance, PTP-PEST is deleted or mutated in a subset of human primary breast cancers (50). Moreover, the ability of PTP-PEST loss to transform mammary epithelial cells requires ShcA (51). We demonstrate that signaling from PTB-independent pools increases mTOR, but not AKT signaling. In accordance with these observations, PTP-PEST loss only marginally impacted AKT signaling but profoundly increased mTOR activation, particularly S6K and rS6 phosphorylation. As such, PTP-PEST and/or other negative regulators may augment mTOR signaling through a distinct mechanism that is independent of AKT (51–53). Interestingly, mammary epithelial PTP-PEST deletion did not alter Src phosphorylation levels, even though downstream effectors of integrin signaling (Cas, Pyk2) were hyperphosphorylated (53). However, it is unlikely that genomic loss of PTP-PEST would phenocopy the observations found in our study as PTP-PEST is closely involved in actin remodeling independent of ShcA (53, 54). The effects of PTP-PEST loss on actin remodeling may confound the ability to observe any ShcA-regulated effects on Src activation. To address this question, the introduction of a PTP-PEST mutant that contains an intact phosphatase domain but lacks the ShcA binding motif, would be required (21). Alternatively, it is possible that one or more alternative negative regulators that bind the PTB domain of ShcA may contribute to increased Src activation from PTB-independent ShcA signaling complexes.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: J.R. Ha, J. Ursini-Siegel
Development of methodology: J.R. Ha
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J.R. Ha, R. Ahn, H.W. Smith, V. Sabourin, E.C. Cañedo, Y.K. Im, W.J. Muller
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J.R. Ha, S. Hebert, C.L. Kleinman, J. Ursini-Siegel
Writing, review, and/or revision of the manuscript: J.R. Ha, S. Hebert, C.L. Kleinman, J. Ursini-Siegel
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): J.R. Ha
Study supervision: J. Ursini-Siegel

Acknowledgments
This work was supported by CIHR (MOP-133670; MOP-111443) grants (to J. Ursini-Siegel). J.R. Ha is supported by a CIHR Doctoral Award. J. Ursini-Siegel is the recipient of a Senior FRQ-S salary support award. C.L. Kleinman is supported by a Junior I FRQ-S salary support award. W.J. Muller acknowledges a Canada Research Chair in Molecular Oncology. The authors thank Dr. Peter Siegel for critical reading of the manuscript. We also thank Drs. Marc Fabian and Michel Tremblay for providing reagents for BioID and PTP-PEST antibody, respectively. We further acknowledge support from the small-animal research and pathology cores at the Lady Davis Institute for Medical Research and Goodman Cancer Research Centre.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received October 25, 2017; revised December 20, 2017; accepted January 26, 2018; published first February 16, 2018.

References


43. Distinct ShcA Pools Cooperatively Increase Tumorigenesis. Mol Cancer Res; 16(5) May 2018. Published OnlineFirst February 16, 2018; DOI: 10.1158/1541-7786.MCR-17-0623. © 2018 American Association for Cancer Research.