The SHCA adapter protein cooperates with lipoma-preferred partner in the regulation of adhesion dynamics and invadopodia formation

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Abstract

SHC adaptor protein (SHCA) and lipoma-preferred partner (LPP) mediate transforming growth factor β (TGFβ)-induced breast cancer cell migration and invasion. Reduced expression of either protein diminishes breast cancer lung metastasis, but the reason for this effect is unclear. Here, using total internal reflection fluorescence (TIRF) microscopy, we found that TGFβ enhances the assembly and disassembly rates of paxillin-containing adhesions in an SHCA-dependent manner through the phosphorylation of the specific SHCA tyrosine residues Tyr-239, Tyr-240, and Tyr-313. Using a BioID proximity labeling approach, we show that SHCA exists in a complex with a variety of actin cytoskeletal proteins, including paxillin and LPP. Consistent with a functional interaction between SHCA and LPP, TGFβ-induced LPP localization to cellular adhesions depended on SHCA. Once localized to the adhesions, LPP was required for TGFβ-induced cell migration and adhesion dynamics. Mutations that impaired LPP localization to adhesions (mLIM1) or impeded interactions with the actin cytoskeleton via α-actinin (ΔABD) abrogated migratory responses to TGFβ. Live-cell TIRF microscopy revealed that SHCA clustering at the cell membrane precedes LPP recruitment. We therefore hypothesize that, in the presence of TGFβ, SHCA promotes the formation of small, dynamic adhesions by acting as a nucleator of focal complex formation. Finally, we defined a previously unknown function for SHCA in the formation of invadopodia, a process that also required LPP. Our results reveal that SHCA controls the formation and function of adhesions and invadopodia, two key cellular structures required for breast cancer metastasis.

Introduction

Cellular migration and invasion are fundamental processes that are required for metastasis. Some cancer cells employ a mesenchymal mode of cell migration, which is highly dependent on adhesions that link the actin cytoskeleton to the extracellular matrix (ECM). Cellular adhesions consist of a complex network of transmembrane integrin heterodimers and cytoplasmic proteins that form a plaque containing hundreds of components with a multitude of potential protein-protein interactions (1-3). Together, these protein interactions mediate cellular signaling and allow the cell to generate traction forces that are important for controlling cell migration. Equally important to the metastatic cascade are invadopodia, which are cellular structures that mediate cancer cell invasion through barriers otherwise impenetrable to migratory cells. Invadopodia are characterized as F-actin rich and proteolytically active cell protrusions capable of degrading ECM components and breaching epithelial and endothelial basement membranes (4). These structures share many protein components with adhesions, such as cortactin, but can be distinguished by the presence of Tks5 (4-6). Together, adhesions and invadopodia are two fundamental structures that enable cancer cells to escape from the primary tumor and establish distant metastases (7-12). Consequently, upregulation and/or increased signaling of proteins that enhance adhesion and invadopodia formation are often observed in invasive and metastatic cancer cells (13-17).
Previous work by our group and others showed that ErbB2 and transforming growth factor β (TGFβ) signaling pathways cooperate to enhance the metastatic ability of breast cancer cells (18-23). SHC adapter protein (SHCA) is a critical downstream integrator of these pathways and is essential for breast tumor growth, migration, invasion and metastasis (24-32). SHCA is part of a Src-homology/collagen (SHC) family of proteins that also includes SHCB, SHCC and SHCD. Alternative translational initiation and RNA splicing result in the expression of three different SHCA isoforms: p46, p52 and p66 (33). All breast cancer cells express p46/52 SHCA isoforms, while p66SHCA is more highly expressed in breast cancers with mesenchymal features (34). Recently, p66SHCA has been shown to be a context-dependent promoter of breast cancer metastasis (35). Loss of p66SHCA expression results in slower adhesion dynamics, reduced cell migration rates, and diminished lung metastasis (35). SHCA harbors an N-terminal phosphotyrosine binding domain (PTB), a central collagen homology domain (CH1) with three key tyrosine residues (Y239/Y240/Y313) and a C-terminal SH2 domain (33). Our previous studies revealed that the PTB domain, but not the SH2 domain, is required for TGFβ-induced migration and invasion of ErbB2+ breast cancer cells (32). Moreover, phosphorylation of tyrosine residues was required for migratory and invasive phenotypes in vitro and breast cancer lung metastasis in vivo (32).

More recently, we have characterized lipoma-preferred partner (LPP) as an important regulator of breast cancer cell migration, invasion and metastasis (36,37). LPP is a member of the zyxin family of LIM proteins and is known to promote mesenchymal migration (38). LPP contains three LIM domains and a proline-rich amino-terminal region (PRR), which allow it to localize to adhesions and interact with numerous proteins (39). While loss of SHCA negatively impacts breast tumor initiation and growth (24), LPP is dispensable for primary tumor growth (37). However, loss of LPP recapitulates the migratory and invasive defects seen in SHCA-depleted cells. Namely, breast cancer cells with diminished LPP expression do not exhibit increased migration in response to TGFβ stimulation (36). The ability of LPP to localize to adhesions via its LIM domains and interact with α-actinin are required for the pro-migratory and pro-invasive functions of LPP (36). Src-mediated phosphorylation of LPP, while dispensable for cell migration, is required for invadopodia formation and efficient breast cancer lung metastasis (37).

In the current study, we show for the first time that SHCA acts as a nucleator of focal complex formation by promoting the formation of small, dynamic adhesions in response to TGFβ. We suggest that SHCA serves as a molecular scaffold to facilitate the recruitment of actin cytoskeletal and adhesion proteins, including paxillin and LPP. Indeed, TGFβ enhances adhesion targeting of paxillin and LPP, which permits faster assembly and disassembly of these structures. TGFβ-induced migration and adhesion dynamics require LPP localization to adhesions and interaction with the actin cytoskeleton. Furthermore, we show that tyrosine phosphorylation on SHCA is required for TGFβ-induced adhesion dynamics. We also implicate SHCA as an
Important regulator of invadopodia formation, which requires phosphorylation of tyrosine residues within the CH1 domain. The requirement of SHCA for efficient invadopodia formation is reminiscent of the role of LPP in the formation of these structures (37). Taken together, we delineate critical roles for SHCA and LPP as critical mediators of adhesion fate and invadopodia formation.

Results

Cooperation between TGFβ and ErbB2 signaling pathways promotes single cell migration – NMuMG cells expressing activated ErbB2 spontaneously metastasize to the lung from the primary tumor (31). We have previously observed that cells with constitutively active ErbB2 (ErbB2-NT) exhibit increased movement through porous membranes in response to TGFβ (31,36). In contrast, NMuMG cells expressing a variant of ErbB2 that lacks five important tyrosine phosphorylation sites (ErbB2-NYPD) fail to exhibit this phenotype (31). While transwell assays are useful for defining gross cellular phenotypes at a population level, they fail to provide insights into the mechanisms of migration and invasion at the single-cell level. To gain a more in-depth understanding of TGFβ-induced migration of ErbB2+ breast cancer cells, we employed live-cell time-lapse microscopy. Using this approach, we could readily assess the behavior, mean net displacement and speed of individual breast cancer cells.

Rose plots of breast cancer cells treated with TGFβ demonstrated that ErbB2-NT cells stimulated with TGFβ migrated further and faster than untreated cells, a response that was not observed with ErbB2-NYPD breast cancer cells (Fig. 1, A-C; Movie 1). Population-based analysis of single cells showed that ErbB2-NT cells began to increase in speed after 9 hours of TGFβ treatment and achieved a maximal speed of ~33 μm h⁻¹ (Fig. 1C). In contrast, ErbB2-NYPD expressing cells continued to migrate at ~21 μm h⁻¹ even after 27 hours of TGFβ stimulation (Fig. 1C). These results were confirmed with an independent set of mammary tumor explants generated from mice injected with ErbB2-NT or ErbB2-NYPD expressing breast cancer cells (Supplemental Fig. S1).

TGFβ is known to induce an epithelial-to-mesenchymal transition (EMT) in ErbB2+ breast cancer cells (40). Cells engaging a mesenchymal mode of migration depend on integrin-mediated adhesion dynamics (41-43). Therefore, we sought to investigate the assembly and disassembly rates of adhesions using fluorescently-labelled paxillin, a bona fide marker of adhesions (44,45). TGFβ significantly increased the assembly and disassembly rates of mCherry-paxillin containing adhesions in protrusive cell regions of ErbB2-NT, but not ErbB2-NYPD, expressing breast cancer cells (Fig. 1D). In particular, a larger proportion of rapid events was observed in ErbB2-NT expressing cells (Movie 2). The observed rapid adhesion dynamics in ErbB2-NT cells following TGFβ stimulation correlated well with the TGFβ-induced increase in migration speeds exhibited by these cells.

Signaling through the SHCA adapter protein is required for TGFβ-induced migration – SHCA is recruited to tyrosine phosphorylated residues (Y1226/Y1227) in the cytoplasmic tail of the ErbB2 receptor and this association is required for the development of aggressively
growing mammary tumors (46). Indeed, SHCA has been shown to be important for primary tumor growth and metastasis of ErbB2+ breast cancer cells (26,29,32). Therefore, we applied time-lapse microscopy to assess the impact of SHCA loss on cell migration. NMuMG ErbB2-NT cells expressing shRNAs against the 3'UTR of mouse SHCA mRNA (SHCA low) or luciferase-targeting shRNAs (SHCA endo) were previously generated in our laboratory (32). Immunoblot analysis demonstrated that cells from ErbB2-NT/SHCA low explants expressed significantly reduced levels of SHCA (p46, 52 and 66 isoforms) when compared to parental controls (Fig. 2A). Rose plots revealed that ErbB2-NT/SHCA endo cells exhibited an increase in cell migration following TGFβ treatment while ErbB2-NT/SHCA low cells did not (Fig. 2B). Treatment with TGFβ also significantly increased the mean net displacement of cells expressing endogenous levels of SHCA (SHCA endo), which was not observed in cells with low SHCA expression (SHCA low) (Fig. 2C). This effect was due to an increase in migration speed, as ErbB2-NT breast cancer cells expressing wild-type levels of SHCA (SHCA endo) exhibited significantly faster speeds after 18 hours of TGFβ treatment (Fig. 2D) rather than a change in persistence (data not shown). Results from an independent set of ErbB2-NT/SHCA endo and ErbB2-NT/SHCA low mammary tumor explants supported these findings (Supplemental Fig. S2).

Next, we investigated adhesion dynamics in protrusive cell regions of SHCA endo and SHCA low expressing cells. We found that TGFβ enhanced the assembly and disassembly rates of mCherry-paxillin containing adhesions in cells expressing endogenous levels of SHCA, but not in ErbB2-NT/SHCA low cells (Fig. 2E). Moreover, TGFβ increased the formation of paxillin-bearing adhesions in ErbB2-NT/SHCA endo cells (Fig. 3, A-C). These newly formed adhesions appeared to be smaller in size (Fig. 3D), which is consistent with the idea that smaller adhesions are more dynamic (47). In contrast, TGFβ did not affect the number and size distribution of adhesions in ErbB2-NT/SHCA KD cells (Fig. 3, B-D). Taken together, our results suggest that SHCA is required for the formation of small, dynamic adhesions in response to TGFβ stimulation.

**Phosphorylation of SHCA is required for TGFβ-induced migration** – The CH1 domain of SHCA contains three phosphotyrosine residues (Y239/Y240/Y313) that are critical for breast cancer metastasis (32). Given the importance of SHCA signaling in breast cancer progression, we sought to investigate single cell migration of ErbB2-NT/SHCA low cells rescued with wildtype p46/52SHCA or various SHCA mutants that lacked specific tyrosine phosphorylation sites (Shc313F: Y313F; Shc2F: Y239F/Y240F; Shc3F: Y239F/Y240F/Y313F) (Fig. 4A). Immunoblot analysis revealed that wildtype and mutant SHCA alleles were expressed in ErbB2-NT/SHCA low breast cancer cells at comparable levels (Fig. 4B). As expected, TGFβ stimulated the migration of cells rescued with SHCA-WT in a manner similar to ErbB2-NT/SHCA endo cells (Fig. 4C). In particular, the mean net displacement and average speed of cells was increased 18 hours after TGFβ treatment (Fig. 4, D and E). Additionally, TGFβ promoted an increase in adhesion assembly and disassembly rates in SHCA-WT cells. (Fig. 4F). In contrast,
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SHCA-3F cells failed to respond to TGFβ. Indeed, the mean net displacement, migration speeds, and adhesion dynamics of SHCA-3F expressing ErbB2-NT/SHCA\textsuperscript{low} cells was unaffected by TGFβ stimulation (Fig. 4, C-F). Expression of SHCA-313F or SHCA-2F was sufficient to restore responsiveness to TGFβ, as measured by mean net displacement and cell speed (Supplemental Fig. S3). We confirmed these results by re-expressing SHCA-WT, SHCA-313F, SHCA-2F and SHCA-3F in an independent ErbB2-NT/SHCA\textsuperscript{low} explant (Supplemental Fig. S4). Altogether, these results revealed that SHCA controls TGFβ-induced migration of ErbB2-overexpressing breast cancer cells by regulating adhesion dynamics, which requires pY239/pY240 or pY313-dependent SHCA signaling.

**SHCA regulates LPP recruitment and retention in cellular adhesions** – Our data demonstrate a role for SHCA in regulating adhesion dynamics in rapidly migrating cells. To outline the molecular mechanisms by which SHCA impacts cellular adhesions, we sought to delineate its potential protein partners in signaling complexes using BioID proximity labeling. To achieve this, we generated cells expressing wildtype SHCA fused to a mutant BirA biotin ligase that covalently adds biotin to adjacent proteins (Myc-SHCA-WT-BirA). Biotinylated proteins were pulled-down from cell lysates and identified by mass spectrometry, using Myc-BirA as a control. A biological triplicate was performed and non-specific interactions were eliminated using the Significance Analysis of INTeractome (SAINT) algorithm (48,49). Strikingly, several adhesion and actin cytoskeletal proteins were identified as proximal to SHCA, including arpin, paxillin (Pxn), talin (Tln) and Lipoma Preferred Partner (LPP) (Table 1; Supplemental Table S1). LPP is a member of the zyxin family of LIM proteins (50). It is able to localize to adhesions and interact with LIM and SH3 protein (LASP), palladin, protein phosphatase 2A (PP2A), scrib, supervillin and vasodilator-stimulated phosphoprotein (VASP) (39). We have previously identified LPP as a promoter of efficient breast cancer lung metastasis (37). Therefore, we decided to investigate whether SHCA regulates the function of LPP in response to TGFβ.

We first validated that the Myc-SHCA-WT-BirA construct biotinylates LPP using an immunoblot-based method that is orthogonal to BioID (Supplemental Fig. 5). A BirA-only vector was included as a negative control. Next, we introduced mCherry-LPP into ErbB2-NT/SHCA\textsuperscript{endo} and ErbB2-NT/SHCA\textsuperscript{low} cells to assess LPP localization in the presence or absence of TGFβ (Fig. 5, A and B). TGFβ enhanced LPP targeting to adhesions in ErbB2-NT/SHCA\textsuperscript{endo} cells, which was not observed in ErbB2-NT/SHCA\textsuperscript{low} breast cancer cells (Fig. 5C). In addition to paxillin and LPP, we also investigated the number of vinculin-bearing adhesions found in ErbB2-NT/SHCA\textsuperscript{endo} and ErbB2-NT/SHCA\textsuperscript{low} cells treated with TGFβ. Vinculin stabilizes and strengthens adhesions and is therefore an important indicator of more mature adhesions (51,52). The number of vinculin-bearing mature adhesions did not change with TGFβ treatment (Fig. 5D) further supporting the notion that TGFβ promotes the formation of more nascent adhesions. Based on these observations, we performed time-lapse imaging to determine the assembly and disassembly rates of cellular adhesions containing LPP. ErbB2-NT/SHCA\textsuperscript{endo} cells treated with TGFβ
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exhibited increased dynamics of LPP containing adhesions in protrusive cell regions, while ErbB2-NT/SHCA\textsubscript{low} cells did not (Fig. 5E). Expression of SHCA-WT in SHCA\textsubscript{low} cells restored TGFβ-induced increased LPP dynamics in ErbB2-NT/SHCA\textsubscript{low}; however, expression of the SHCA-3F phosphomutant was not sufficient to rescue this phenotype (Fig. 5F). Altogether, our data suggest that SHCA controls paxillin and LPP localization to dynamic adhesions in response to TGFβ.

**LPP is an important component of adhesions that interacts with α-actinin to mediate TGFβ-enhanced migration** – Previous studies show that LPP is an important regulator of mesenchymal cell migration (53, 54). Given that SHCA regulates LPP recruitment to adhesions in response to TGFβ, we sought to determine whether LPP also regulates TGFβ-enhanced migration and adhesion dynamics. ErbB2-NT cells with endogenous levels of SHCA were infected with an shRNA control against LucA (LPP\textsubscript{endo}) or LPP knockdown (LPP\textsubscript{KD}) (Fig. 6A; Supplemental Fig. S6A). LPP knockdown was also confirmed by immunofluorescence staining and linescan analysis of paxillin-bearing adhesions (Supplemental Fig. S6, B-D). Cells with endogenous levels of SHCA and LPP (LPP\textsubscript{endo}) migrated further and faster in response to TGFβ treatment (Fig. 6, B-D). Cells with LPP knockdown, however, did not exhibit TGFβ-induced migration despite remaining responsive to TGFβ-induced signaling (pSmad/Smad) (Fig. 6B; Supplemental Fig. S6A). Furthermore, TGFβ did not affect assembly and disassembly rates of paxillin-bearing adhesions in LPP\textsubscript{KD} cells (Fig. 6E). Together, these data suggest that both SHCA and LPP are required for enhancing adhesion dynamics in TGFβ-stimulated cells.

LPP has three LIM domains, which are necessary for localization to adhesions. LPP also contains an α-actinin binding domain (ABD), which allows it to interact with the actin cytoskeleton. Previous studies by our group show that LPP must localize to adhesions and interact with the actin cytoskeleton to promote metastasis (37). Therefore, we rescued LPP\textsubscript{KD} cells with three fluorescently tagged LPP constructs: wildtype LPP (EGFP-LPP-WT), a mutant that cannot localize to adhesions (EGFP-LPP-mLIM1), or a mutant that cannot interact with α-actinin (EGFP-LPP-ΔABD) (Supplemental Fig. S7, A and B). Immunofluorescence staining and linescan analysis showed successful co-localization between EGFP-LPP-WT and paxillin in adhesions (Supplemental Fig. S7, C-E). LPP-ΔABD also co-localized with paxillin at adhesions (Supplemental Fig. S7, C-E). LPP-mLIM1, however, failed to localize to adhesions despite being expressed at levels comparable to endogenous LPP (Supplemental Fig. S7, C-E). Migration and adhesion dynamics of LPP-WT, LPP-mLIM1 and LPP-ΔABD cells were then analyzed using live-cell microscopy. Re-expression of wildtype LPP in LPP\textsubscript{KD} cells successfully rescued the migratory phenotype observed in LPP\textsubscript{endo} cells (Supplemental Fig. S8). Expression of either LPP-mLIM1 and LPP-ΔABD, however, was not able to rescue TGFβ-enhanced cell migration and adhesion dynamics (Supplemental Fig. S8). Thus, LPP must localize to adhesions and interact with the actin cytoskeleton to facilitate faster migration and adhesion dynamics in response to TGFβ treatment.
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SHCA localizes to adhesions to regulate focal complex formation – Given that SHCA interacts with several adhesion proteins, we explored the possibility that SHCA may also localize to adhesions. Using a far-red fluorescent protein (miRFP670) (55), we generated a wildtype SHCA construct (SHCA-WT-iRFP) and performed TIRF microscopy to limit fluorescent illumination to the first 80 nm of the cell. A far-red fluorescent protein was chosen to ensure that the emission/excitation spectra did not overlap with existing mCherry constructs (mCherry-paxillin and mCherry-LPP). ErbB2-NT/SHCAlow cells were infected with SHCA-WT-iRFP or SHCA-3F-iRFP (Fig. 7A; Supplemental Fig. S9A) and assessed for their responsiveness to TGFβ. The migratory phenotype was successfully rescued with SHCA-WT-iRFP but not the SHCA-3F-iRFP mutant (Supplemental Fig. S9, B-D). SHCA-WT-iRFP cells were then infected with mCherry-LPP and fixed to assess the cellular localization of these adapter proteins. TIRF microscopy revealed more SHCA clusters at the plasma membrane after TGFβ treatment (Fig. 7B). SHCA-positive signals co-localized with mCherry-LPP, suggesting that SHCA is targeted to adhesions upon stimulation (Fig. 7C). Image quantification revealed that the number of LPP-bearing adhesions increased upon TGFβ treatment, as well as the percentage of LPP-containing adhesions positive for SHCA (Fig. 7, D and E). Taken together, these results suggest that SHCA facilitates the recruitment of LPP into cellular adhesions in response to TGFβ treatment.

Adhesion assembly is a highly regulated process that begins with integrin binding to ECM ligands, such as fibronectin, vitronectin and laminin. Ligand binding induces a conformational change that unMASKS the short cytoplasmic tail of integrins, enabling the recruitment of scaffold and signaling proteins (56). TGFβ can regulate the expression of integrin subunits (57-59) and cause clustering of ErbB2 and integrins at the cell membrane (22). Considering that SHCA regulates the recruitment of LPP, we wondered whether SHCA directs adhesion formation. We performed semi-continuous time-lapse imaging of SHCA-WT-iRFP and EGFP-LPP to obtain high temporal resolution of adhesion dynamics (2 second intervals). SHCA appeared to assemble at the site of an adhesion prior to LPP recruitment (Fig. 8A; Movie 3). Once an adhesion formed and began to increase in size, the rate of SHCA recruitment decreased and eventually stabilized (Fig. 8B; Movie 4). Occasionally, a second nucleation point or “treadmilling” of SHCA was observed at the growing end of an adhesion (Fig. 8C). Interestingly, SHCA fluorescence intensity at some adhesions decreased prior to loss of LPP signal (Fig. 8D; Movie 5). Finally, there were instances where SHCA appeared to nucleate an adhesion but LPP was not recruited (Fig. 8E; Movie 6). In these cases, the adhesion often disassembled and was characterized by a short lifespan. These adhesions likely coincided with nascent adhesion formation and disassembly. Collectively, these four examples demonstrate that SHCA is an early component of adhesions and is required for the recruitment of LPP.

MAPK and PI3K activation are required for LPP localization to adhesions – In addition to the engagement of Smad proteins, TGFβ is known to modulate mitogen-activated protein kinase (MAPK) and phosphatidylinositol-3-kinase pathways.
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(PI3K)/AKT pathways (60-62). Tyrosine phosphorylation of SHCA by the TGFβ receptor complex causes the recruitment of a Grb2/son of sevenless (SOS)/Ras complex, which subsequently triggers activation of the Ras-MAPK pathway (63). Similarly, TGFβ stimulation causes the association of type I TGFβ receptor (TβRI) with p85, the regulatory subunit of PI3K, which mediates AKT activation and leads to the phosphorylation of S6 kinase 1 (64,65). MAPK and PI3K pathways are also known to regulate cell migration (66-70). Therefore, LPP recruitment to adhesions was analyzed in the absence or presence of trametinib (a MEK inhibitor) or pictilisib (a PI3K inhibitor) to determine if these signaling pathways play a role in regulating LPP recruitment in response to TGFβ stimulation.

NMuMG cells expressing constitutively active ErbB2 (NT118) exhibited increased ERK1/2 phosphorylation (T202/Y204) levels following 24 hours of TGFβ stimulation; however, S6 phosphorylation (S240/244) levels did not increase significantly (Supplemental Fig. 10A and B). As expected, TGFβ promoted the formation of small, dynamic adhesions in control cells (Supplemental Fig. 10C upper panels, D-F). Pre-treatment with trametinib effectively eliminated ERK1/2 phosphorylation in both TGFβ stimulated and unstimulated cells (Supplemental Fig. 10A and B). In line with previous observations (71), ERK inhibition resulted in the formation of large adhesions (Supplemental Fig. 10C middle panels, E and F). Cells pre-treated with trametinib also exhibited large stress fibers at the trailing edge and a robust lamellipodia (Supplemental Fig. 10C, middle panels). However, TGFβ stimulation did not affect the number or size of LPP adhesions compared to unstimulated trametinib-treated cells (Supplemental Fig. 10, D-F). On the other hand, pre-treatment with pictilisib reduced S6 phosphorylation (Supplementary Fig. 10, A and B). Cells pre-treated with pictilisib also exhibited large adhesions (Supplemental Fig. 10C lower panels, D-F). TGFβ stimulation did not affect the number of LPP adhesions; however, the size of LPP adhesions increased significantly (Supplemental Fig. 10, D-F). Together, these results suggest that both MAPK and PI3K signaling are required for LPP recruitment to small, dynamic adhesions in response to TGFβ. Additionally, the increased size of LPP containing adhesions in pictilisib-treated cells stimulated with TGFβ suggests that PI3K signaling may be important for the regulation of adhesion disassembly.

SHCA is a mediator of invadopodia formation – Invadopodia structures are unique mechanosensory structures that are central to the metastatic process (72). We have previously shown that LPP is required for TGFβ-induced invadopodia formation (37). Since SHCA regulates LPP recruitment to adhesions in response to TGFβ, we next investigated the effect of SHCA knockdown on invadopodia formation. ErbB2-NT/SHCAlow cells were plated onto fluorescently labeled gelatin for 24 hours in the presence or absence of TGFβ. Coverslips were then fixed and stained with phalloidin (F-actin) and assessed for gelatin degradation (as indicated by the loss of fluorescence signal). Coverslips without cells were imaged as a control to ensure uniform gelatin coating (Supplemental Fig. S11A). Our data revealed that shRNA-mediated knockdown of SHCA impaired the ability of cells to form invadopodia in response to TGFβ (Fig. 9, A and B). Of
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note, NMuMG ErbB2-NT cells did not degrade ECM in the absence of TGFβ stimulation (Supplemental Fig. S11B). Exogenous expression of wildtype SHCA restored TGFβ-induced gelatin degradation; however, ErbB2-NT/SHCA^low cells expressing the SHCA-3F mutant failed to respond to TGFβ (Fig. 9, A and B). These results indicate that phosphorylation of Y239/Y240/Y313 within SHCA is required for efficient invadopodia formation. To ensure that gelatin degradation was coincident with invadopodia formation, cells were stained with cortactin, a well-characterized marker of invadopodia (6,11) (Fig. 9C). Linescan analysis showed multiple sites of actin and cortactin co-localization at sites of reduced gelatin fluorescence (Fig. 9D). ErbB2-NT/SHCA^low cells expressing fluorescently tagged SHCA constructs (SHCA-WT-iRFP and SHCA-3F-iRFP) demonstrated a similar phenotype, with cells expressing SHCA-WT-iRFP exhibiting significantly greater TGFβ-induced gelatin degradation relative to SHCA-3F-iRFP expressing cells (Supplemental Fig. S11, C-F). Thus, our results indicate that tyrosine phosphorylation of SHCA is required for TGFβ-induced breast cancer cell migration and invadopodia formation.

Discussion

In this study, we establish SHCA (p46/52 isoforms) as a critical mediator of cell migration and invasion in ErbB2-overexpressing breast cancer cells. SHCA nucleates focal complex formation in the presence of TGFβ by enhancing the recruitment of key adhesion proteins, such as paxillin and LPP (Fig. 10). Beyond its function within cell adhesions, we further delineate a novel role for SHCA in TGFβ-induced invadopodia formation. In line with our observations that SHCA phosphorylation at Y239/Y240/Y313 mediates adhesion dynamics, SHCA phosphorylation is also required for these invasive structures to form.

Cells that undergo an epithelial-to-mesenchymal transition (EMT) adopt a mesenchymal mode of migration, which relies on adhesions to generate traction forces for movement (73,74). In order for cells to migrate, nascent adhesions must form, grow into focal contacts, couple to the actin cytoskeleton and then disassemble as the cell moves forward, a process that generally occurs on the order of minutes (75,76). Consequently, changes in adhesion dynamics dramatically affect the migration speed of cells (77). Our data demonstrate that TGFβ stimulates the formation of small, dynamic adhesions, which allow breast cancer cells to move at greater speeds. This is evidenced by a selective increase in the number of nascent paxillin-containing adhesions in response to TGFβ, with no change in more stable vinculin-containing mature adhesions. p46/52SHCA is required for this phenotype as shRNA-mediated knockdown impairs the ability of NMuMG ErbB2-NT cells to enhance assembly and disassembly rates in response to TGFβ. Mutating the cytoplasmic tail of ErbB2 such that p46/52SHCA can no longer bind (ErbB2-NYPD) also prevents TGFβ-induced migration. Accordingly, lung metastatic burden is significantly reduced when SHCA signaling is disrupted (31,32). This data is consistent with findings in triple negative breast cancer cells where p66SHCA is required for efficient cell migration and lung metastasis (35). Loss of p66SHCA expression results in the formation of large, elongated adhesions that exhibit
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slower assembly and disassembly rates whereas exogenous expression of p66SHCA induces an EMT in ErbB2+ luminal breast cancers (34,35).

TGFβ has been previously shown to induce the association of ErbB2 with the actin cytoskeleton (21,22). In addition, TGFβ stimulation induces the expression of various α and β integrin subunits, including α1, α2, α3, α5, αv, β1, and β3 (57,59,78-80). Of these integrin subunits, collagens are bound by α1β1 and α2β1; laminin is bound by α1β1, α2β1 and αvβ3; and vitronectin is bound by αvβ1 and αvβ3 (81). Fibronectin is also a ligand for many of these integrin receptors, including α5β1, which is widely regarded as the major fibronectin receptor (81). Immunoprecipitation experiments show that p52SHCA can bind α5β1, α1β1 and αvβ3, but not α2β1, α3β1 or α6β1 (82). Binding to integrin β3 is facilitated by the PTB domain (83). SHCA can also interact with the cytoplasmic domain of integrin β4 through its SH2 or PTB domains upon phosphorylation of Y1440 or Y1526, respectively (84). Here, we demonstrate that SHCA localizes to integrin-dependent adhesions using a fluorescently tagged construct and time-lapse TIRF microscopy. Our BioID results show that SHCA interacts with a variety of adhesion and actin cytoskeletal proteins such as arpin, crk, LPP, paxillin and talin. The SHCA-BirA construct also labelled known interacting partners, including Grb2, Shc SH2 domain-binding protein 1 (Shcbp1) and protein tyrosine phosphatase non-receptor type 12 (Ptpn12) (85-87). Cell migration experiments were performed on fibronectin as it has been implicated in the development of multiple cancers (88-90). In fact, human breast cancer cells that overexpress α5β1 integrins show a 3-fold increase in cell invasiveness compared to α5β1-depleted cells due to the generation of greater contractile forces (91). Given that SHCA can regulate cell migration, invasion and proliferation on collagen (92) laminin (93,94), vitronectin (92) and fibronectin (31), it would be interesting to further investigate adhesion dynamics and SHCA localization in response to different ECM components.

Our imaging results demonstrate that TGFβ induces clustering of SHCA in what appear to be nascent adhesions prior to LPP recruitment. In addition to nucleation, SHCA exhibits a treadmilling effect that may allow components to be recruited to the growing end of an adhesion. Three-dimensional super-resolution microscopy reveals that integrins and actin filaments are separated by a core region that spans ~40 nm (2). This region can be stratified into three spatial and function compartments: an integrin signaling layer, a force transduction layer and an actin regulatory layer. Paxillin and Focal Adhesion Kinase (FAK) appear to be key players in the membrane-proximal integrin signaling layer; vinculin is observed in the broader central zone responsible for force transduction; and zyxin localizes to the uppermost region that constitutes the actin regulatory layer (2). The ability of SHCA to engage with integrins and facilitate LPP localization to adhesions in the presence of TGFβ is necessary for enhanced cell migration. Moreover, LPP must interact with the actin cytoskeleton through an α-actinin binding domain (ABD) to mediate adhesion dynamics. Based on the results presented here, it is conceivable that SHCA is localized to the signaling layer whereas LPP is a constituent of the actin regulatory layer. Immunofluorescence analysis of motile cells shows that early
nascent adhesions are largely composed of integrins, talin and paxillin (95,96). Mass spectrometry of the adhesion machinery shows that paxillin is also one of the first components to dissociate during nocodazole-induced adhesion disassembly, with ~12% abundance remaining after 15 minutes (97). This is in contrast to vinculin, which is still an abundant constituent of adhesions (~73%) over this time frame (97). In comparison, LPP dynamics appear to fall between these two adhesion components (~43%) (97). It is interesting to note that p46/52SHCA does not appear in proteomics analyses of adhesions (97,98). However, human endothelial cells treated with fibronectin-bound paramagnetic microbeads show that all three splice isoforms of Shc (p46, p52 and p66) are found in adhesions (99). These results are in line with phosphoproteomic analyses that detect SHCA within isolated adhesion complexes and reveal SHCA phosphorylation on serine 139 (100). Importantly, our data also shows that p46/52SHCA isoforms localize to adhesions following TGFβ treatment, which occurs prior to LPP localization. Given the fact that SHCA directly interacts with FAK (99), and FAK is a component of early adhesions (101,102) which can localize prior to paxillin recruitment (103) and dissociate at roughly the same rate (97), it is conceivable that SHCA localizes to adhesions during the early stages of formation. It would be particularly interesting to generate a super-resolution map of SHCA and other adhesion components in ErbB2-overexpressing breast cancer cells and investigate whether SHCA forms multiple nucleation sites along the growing end of an adhesion. Such an approach would provide clarity on the precise relationship between SHCA and LPP within adhesions.

Our data suggest that both MAPK and PI3K signaling pathways are required for LPP recruitment to small, dynamic adhesions in response to TGFβ stimulation. However, the observation that trametinib-treated cells are phenotypically different than pictilisib-treated cells suggests that distinct signaling pathways engaged downstream of SHCA differentially impact adhesion dynamics. In particular, trametinib causes LPP localization to stress fibers with no further increases in adhesion size and number following TGFβ stimulation. In contrast, pictilisib does not impact the pattern of LPP localization or result in the formation of robust lamellipodia. The size increase of LPP containing adhesions following PI3K inhibition suggests it plays a role in regulating adhesion disassembly. This interpretation is consistent with previous observations that knockout of FAK, which is upstream of PI3K (104,105), specifically affects adhesion disassembly, but not adhesion assembly, rates (106).

LPP is a known regulator of migration in mesenchymal cells that has recently been characterized as a critical mediator of ErbB2+ breast cancer metastasis (37). LPP is also an important component of invadopodia (37). Invadopodia are mechanosensory structures (72,107) that allow tumor cells to penetrate the basement membrane and move through dense environments comprised of highly cross-linked ECM fibers (108). Increasing evidence suggests that invadopodia directly contribute to poor overall survival (13). Consequently, many studies have
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attempted to elucidate key regulators of invadopodia formation and target precursor formation. Current models propose three stages of invadopodia formation: precursor core initiation, precursor stabilization and maturation (109). Similar to adhesion assembly, SHCA may be involved in invadopodia formation. In the present study, we provide the first evidence that phosphotyrosine-dependent SHCA signaling is required for efficient invadopodia formation in breast cancer cells in response to TGFβ treatment. Interestingly, the results of our BioID screen show that SHCA is proximal to cortactin (Cttn), a core invadopodia component. Therefore, the finding that SHCA knockdown prevents efficient TGFβ-induced invadopodia formation may be due to a loss of LPP recruitment to these structures, a loss of interaction with cortactin, or both.

Several proteins (including LPP and Cttn (110)) have been identified in both adhesions and invadopodia, suggesting that these structures are intimately linked. Both cellular structures are connected to the actin cytoskeleton, albeit in a different fashion (5). The actin cytoskeleton couples to adhesions in a tangential orientation to generate traction forces while invadopodia require perpendicular alignment of filaments, with respect to the underlying ECM, to create protrusive forces (111,112). Whether invadopodia are discrete structures or derived from adhesions is still debated (113). Similar to adhesions, invadopodia are frequently surrounded by a multimeric protein complex consisting of integrins and integrin-associated proteins such as paxillin, talin and vinculin (114). These adhesion rings may help anchor invadopodia and promote their maturation. Given that SHCA regulates the recruitment of adhesion components in response to TGFβ, it is possible that SHCA may affect the formation of invadopodia anchors. Thus, the mechanism(s) through which SHCA regulates invadopodia formation awaits further investigation.

**Experimental procedures**

*Cell culture* − Normal murine mammary gland (NMuMG) cells were obtained from the American Type Culture Collection (ATCC; Cat. no.: CRL-1636) and grown in high glucose (4.5 g L⁻¹) Dulbecco’s Modified Eagle Medium (DMEM; Cat. no.: 319-005-CL, Wisent Bioproducts) supplemented with 5% fetal bovine serum (FBS; Cat. no.: 10082-147, Thermo Fisher Scientific), 10 µg mL⁻¹ insulin (Cat. no.: 511-016-CM, Wisent Bioproducts), 1 mM L-glutamine (Cat. no.: 609-065-CM, Wisent Bioproducts), 1% penicillin-streptomycin (Cat. no.: 450-201-EL, Wisent Bioproducts) and 0.2% amphotericin B (Cat. no.: 450-105-QL, Wisent Bioproducts).

NMuMG cells (NT 118/119 and NYPD 120/121) were previously infected with a pMSCV-puro viral vector to express the rat orthologue of ErbB2 with an activating transmembrane point mutation V664E (31). Cells were cultured as described above with the addition of 2 µg mL⁻¹ puromycin (Cat. no.: ant-pr-1, InvivoGen) antibiotic selection to maintain ErbB2 expression. NMuMG cells (SHCA<sub>endo</sub> 83/84 and SHCA<sub>low</sub> 87/92) were also previously infected with a pMSCV-hygro viral vector to express constitutively active ErbB2 (ErbB2-NT) and a pMSCV-puro viral vector containing an shRNA against endogenous SHCA (SHCA<sub>low</sub>) or an shRNA against luciferase (SHCA<sub>endo</sub>) (32). Cells were maintained in 0.8 mg mL⁻¹...
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1 hygromycin B (Cat. no: 450-141-XL, Wisent Bioproducts) to maintain ErbB2 expression and 2 μg mL⁻¹ puromycin to maintain SHCA knockdown.

To generate individual explants (NT 118/119, NYPD 120/121, SHCAendo 83/84, SHCAlow 87/92), NMuMG cells were injected into the mammary fat pad of athymic mice and subsequently derived from primary tumors (31,32). Explants 87 and 92 (SHCAlow) were infected with a pMSCV-blast viral vector harboring a variety of SHCA mutants, including wildtype SHCA (SHCA-WT), SHCA-313F, SHCA-2F and SHCA-3F. Finally, explant 87 (SHCAlow) was infected with wildtype SHCA fused to a far-red fluorescent protein (SHCA-WT-iRFP). Cells were maintained in 5 μg mL⁻¹ blasticidin (Cat. no: ant-bl-1, InvivoGen) to maintain SHCA re-expression.

In parallel, NMuMG cells were previously infected with an shRNA against endogenous LPP (LPPKD) or an shRNA against luciferase (LPPendo) (36). Cells were then infected with constitutively active ErbB2 (36). Cells were cultured in 2 μg mL⁻¹ puromycin to maintain LPP knockdown and 0.8 mg mL⁻¹ hygromycin B to maintain ErbB2 expression. Finally, NMuMG-ErbB2 cells with LPP knockdown were infected with a pMSCV-blast vector containing EGFP-LPP-WT, EGFP-LPP-mLIM1 or EGFP-LPP-ΔABD (37). Cells were cultured in 5 μg mL⁻¹ blasticidin.

MMTV/Polyoma Virus Middle T antigen expressing mammary tumor cells (MT864) were obtained from the laboratory of Dr. William J. Muller. Cells were cultured in high glucose DMEM supplemented with 5% FBS, mammary epithelial growth supplement (MEGS; 3 ng mL⁻¹ EGF [Cat. no.: PHG0311, Invitrogen], 0.5 μg mL⁻¹ hydrocortisone [Cat. no.: 511-002-UG, Wisent Bioproducts], 5 μg mL⁻¹ insulin, 0.4% v/v bovine pituitary extract [Cat. no.: 002-011-IL, Wisent Bioproducts]), 1% penicillin-streptomycin and 50 μg mL⁻¹ gentamycin (Cat. no.: 450-135-XL, Wisent Bioproducts). Wildtype SHCA tagged with BirA (Myc-SHCA-WT-BirA) and BirA-only vector were expressed in MMTV Middle T antigen mammary epithelial cells. Cells were maintained in 8 μg mL⁻¹ blasticidin (Cat. no: 400-190-EM, Wisent Bioproducts) antibiotic selection to maintain SHCA re-expression.

Retroviral production was performed using Retro-X Universal Packaging System (Cat. no.: 631530, Clontech) according to the manufacturer’s protocol. Cells were then incubated with polybrene (10 μg mL⁻¹) and virus containing media for 48 hours to allow for infection. Mycoplasma screening was routinely performed using MycoAlert mycoplasma detection kit (Cat. no.: LT07-318, Lonza).

DNA constructs – pMSCV-puro-ErbB2, pMSCV-hygro-ErbB2, pMSCV-puro-SHCA (shRNA), pMSCV-puro-LucA (shRNA), pMSCV-blast-SHCA, pMSCV-puro-LPP (shRNA) and pMSCV-EGFP-LPP constructs were previously generated in the laboratory of P.M.S. (31,32,37).

FLAG tagged SHCA was subcloned into pcDNA3.1 mycBioID (Cat. no.: 35700, Addgene) using the following primers and NotI and EcoRI restriction enzymes: 5’-CACGAGCGG CCGAAAAACAAAGCTGAGT-3’ and 5’- GCCGGATTCGAAATTTCACCTTGTCA CGTC-3’. BirA-SHCA-FLAG was then subcloned into the pQCXIB expression vector (Cat. no.: 22266, Addgene) using the following primers and AgeI and EcoRI
restriction enzymes: 5'–AGCTGGCAC CGGTAGCCACCATGGAACAAAAACT C-3' (a gift from Dr. Marc Fabian) and 5'-GCCGGAATTCGATTCGATTCGTCAT CGTC-3'.

To create SHCA-iRFP, SHCA was PCR-amplified from the previously generated constructs (pMSCV-SHCA-WT and pMSCV-SHCA-3F) with the following primers: 5'-CCCTTGAACCTCCTCGTTCGACC-3' and 5' -TAGGTAAGTGATCGTCATCCTAAGCGCGG-3' primers with EcoRI and BglII restriction sites were used to shuttle SHCA-iRFP into pMSCV-blast.

To create cells with stable expression of mCherry-paxillin, pSL301 (Invitrogen) and pmCherryPaxillin (Cat. no.: 50526, Addgene) were digested with Agel and XbaI restriction enzymes. The pSL301-mCherryPaxillin intermediate was then digested with HpaI and EcoRI restriction enzymes to shuttle mCherry-paxillin into pMSCV-blast.

To create cells with stable expression of mCherry-LPP, LPP was PCR-amplified from previously generated pMSCV-EGFP-LPP-WT construct with the following primers: 5'-ATTGGCAGCCGAGTCTCACCCCAT CTTGG-3' and 5'-GAGACGTGCTAC TTCCATTGTGTC-3'. NotI and EcoRI restriction enzymes were then used to replace paxillin with the amplified LPP product in pMSCV-blast-mCherryPaxillin.

**Cell migration assays** – NMuMG ErbB2 cells were seeded onto μ-slide 8-well plates (Cat. no.: 80821, ibidi) coated with 5 μg cm⁻² fibronectin (Cat. no.: F-0895, Sigma-Aldrich) diluted in 1x phosphate buffered saline (PBS). Cells were allowed to adhere and grow under exponential conditions for at least 12 hours prior to experimentation. Recombinant human TGFβ1 (2 ng mL⁻¹; Cat. no.: 100-21, PeproTech) was applied to the cells directly before imaging (t=0). Images were acquired on a Zeiss AxioObserver fully automated inverted microscope equipped with a Plan-Neofluar 10x/0.3NA Ph1 objective, AxioCam 506 camera (Carl Zeiss, Jena, Germany) and Chamlide TC-L-Z003 stage top environmental control incubator (Live-cell Instrument, Seoul, South Korea). Cells were imaged every 10 minutes in phase contrast for a total of 30 hours.

**Analysis of cell migration** – Cells were manually tracked in MetaXpress analysis software (Molecular Devices, Sunnyvale, CA). X,Y position data for each cell track was then exported to MATLAB (v. 8.6, Rel. R2015b; The MathWorks, Natick, MA). Rose plots of cell migration were created by superimposing the starting position of each track on the origin (0,0). Windrose plots were generated by determining the angle and mean net displacement of each track. The mean net displacement for each 45° segment was then plotted. The average speed was calculated by determining the mean distance travelled between each time point over the imaging interval. Speeds were then averaged into 3-hour segments. The data shown represents the mean ± standard error of the mean (s.e.m.) for all cells analyzed from at least three independent experiments.

**Imaging adhesion turnover** – NMuMG ErbB2 cells were seeded onto 35 mm cover-glass bottom cell culture dishes
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(Cat. no.: FD35-100, World Precision Instruments) coated with fibronectin and transfected with 1 µg of pmCherry paxillin using Effectene reagent (Cat. no.: 301425, Qiagen). Media was changed 18-24 h after transfection, and cells were allowed to recover for an additional 24 h in the absence or presence of TGFβ. NMuMG ErbB2 cells stably expressing mCherry-LPP were also seeded onto 35 mm dishes to analyze the dynamics of LPP. Cells with stable expression of mCherry-LPP were cultured in 5 µg mL⁻¹ blasticidin to maintain expression. Images were acquired every 30 seconds for 25 mins on a Total Internal Reflection Fluorescence (TIRF)-Spinning Disk Spectral DISkovey System (Spectral Applied Research, Richmond Hill, ON) coupled to a DMI6000B Leica microscope equipped with a Plan-Apochromat 63x/1.47NA oil DIC objective (Leica, Wetzler, Germany), ImagEM X2 EM-CCD camera (Hamamatsu Photonics K.K., Hamamatsu City, Japan), and Chamlide CU-501 top-stage incubator system (Live-cell Instrument, Seoul, South Korea). An EM-CCD camera was chosen at the cost of resolution (1 pixel = 0.155 µm) because of increased sensitivity. A 561 nm laser with an ET 620/60 nm filter cube was used to visualize mCherry-paxillin or mCherry-LPP (where indicated). The camera exposure time was set to 500 milliseconds with an EM gain of 255 and read speed of 22 MHz. A TIRF prism was used to limit fluorescence excitation to a depth of 100 nm.

Calculating adhesion dynamics – Images collected with the TIRF microscope were processed in Imaris (v. 8.3.1; Bitplane AG, Zurich, CH) using the Surfaces function. A protruding edge of each cell was manually selected using the region of interest (ROI) tool. Surfaces detail was smoothed and set to 0.300 µm with a local background subtraction of 0.300 µm. Adhesions were then masked by a manual refinement of the autothreshold feature. Finally, adhesions were tracked over time using an autoregressive algorithm with a maximum distance of 2 µm and maximum gap size of three time points. Surfaces smaller than 5 pixels were removed by filtering.

Mean intensity data for each adhesion tracked in Imaris was exported to Excel for further analysis. Curves were visually inspected for assembly and disassembly events. A string of five or more points upwards was interpreted as assembly, while five of more points downwards was interpreted as disassembly. A log-linear fitting method was then used to determine the rate for each event. Fits with an $R^2$ value greater than 0.7 were considered acceptable. Finally, assembly and disassembly rates were pooled together into a box plot each condition. The mean for each condition is denoted by a red line. The data shown represents the results from at least three independent experiments.

To verify the results of our semi-automatic analysis, a custom algorithm was created in MATLAB (35,115). Here, a spline curve was first fitted to each intensity trace to identify segments of assembly and disassembly. The difference in intensity between each time point was calculated and changes greater than 15% were considered significant. A log-linear fitting method was then used to determine the rate for each event, as described above. Our automatic algorithm generated the same results as the semi-automatic analysis.

Immunoblotting – Cells were cultured to 80% confluency and lysed in ice cold
TNE lysis buffer, as previously described (31). Where indicated, cells were cultured in the presence of TGFβ for 48 hours. Total cell lysates (20 μg) were resolved by 6-12% SDS-polyacrylamide gel electrophoresis. Proteins were transferred onto polyvinylidene difluoride membranes (Cat. no.: IPVH00010, Millipore) and membranes were blocked in 5% fat-free milk for 1 h. Membranes were incubated with the following antibodies overnight at 4°C: ErbB2 (1:4000; Cat. no.: sc-284, Santa Cruz Biotechnology), SHCA (1:4000; Cat. no.: 610082, BD Transduction Laboratories), FLAG (1:4000; Cat. no.: F1804, Sigma-Aldrich), LPP (1:4000; Cat. no.: sc-104343, Santa Cruz Biotechnology), paxillin (1:4000, Cat. no.: ab23510, Abcam), Smad2/3 (1:1000, Cat. no.: 3102, Cell Signaling), pSmad2 (S465/467) (1:1000, Cat. no.: 3101, Cell Signaling), Myc (1:3000; Cat. no.: TAG003, BioShop), α-Tubulin (1:20,000; Cat. no.: T9026, Sigma-Aldrich) and GAPDH (1:10,000; Cat. no.: MAB374, Millipore). The appropriate HRP-conjugated secondary antibodies (1:10,000; Jackson Immuno Research Laboratories) were added to the membranes for 1 h. Finally, the membranes were visualized using SuperSignal West Pico PLUS Chemiluminescent Substrate (Cat. no.: 34578, Thermo Fisher Scientific).

For MAPK and PI3K inhibitor experiments, cells were pre-treated with 500 nM trametinib (Cat. no.: T-8123, LC Laboratories) or 500 nM pictilisib (Cat. no.: 11600-10, Cayman Chemical) for 12 h. TGFβ was then added to the media and cells were cultured for an additional 24 h. Proteins were transferred onto polyvinylidene difluoride membranes (Cat. no.: IPFL00010, Millipore) and membranes were blocked with Intercept blocking buffer (Cat. no.: 927-60001, Licor) for 1 h. Membranes were incubated with the following antibodies overnight at 4°C: ERK1/2 (1:1000; Cat. no.: 9107S, Cell Signaling), pErk1/2 (T202/Y204) (1:1000; Cat. no.: 9101S, Cell Signaling), S6 (1:500; Cat. no.: 2317S, Cell Signaling) and pS6 (S240/244) (1:500; Cat. no.: 5364S, Cell Signaling). IR dyes 680RD donkey anti-mouse (1:10,000; Cat. no.: 926-68072, Licor) and 800CW donkey anti-rabbit (1:10,000; Cat. no.: 926-32213, Licor) were added to the membranes for 1 h. Finally, the membranes were visualized with the Odyssey Imager (Licor, Lincoln, NE). Quantification was performed with ImageJ (NIH, Bethesda, MD). Phosphorylated protein levels were normalized by their respective total protein levels. Fold change for each experiment was determined relative to unstimulated cells. The data shown represents the mean ± s.e.m. for three independent experiments.

Immunofluorescence – Cells were plated onto 35 mm cover-glass bottom dishes coated with fibronectin and allowed to settle overnight. Cells were then cultured in the absence or presence of TGFβ for 24 h before being fixed with 4% paraformaldehyde (PFA) for 10 mins at 37°C. Cells were permeabilized with 0.3% Triton X-100 for 10 mins, rinsed with 100 mM glycine in PBS, and blocked with 10% FBS in PBS for 1 h. Antibodies against LPP (1:500; Cat. no.: sc-27312, Sigma-Aldrich) and vinculin (1:1500; Cat. no.: V9131, Sigma-Aldrich) were applied overnight at 4°C (where indicated). Alexa Fluor 488 (1:1000; Cat. no.: A11034, Invitrogen) and Alexa Fluor 546 (1:1000; Cat. no.: A10036, Invitrogen) dye conjugated secondary antibodies were then applied for 1 hr at room temperature to visualize LPP and vinculin,
respectively. Alexa Fluor 488 Phalloidin (1:500; Cat. no.: A12379, Invitrogen) was used to visualize F-actin, and 4',6-diamidino-2-phenylindole (DAPI; 1:20,000; Cat. no.: D1306, Thermo Fisher Scientific) was used to visualize nuclei (where indicated). Cells were kept in 1x PBS with 0.05% sodium azide (Cat. no.: SAZ001, BioShop).

For MAPK and PI3K inhibitor experiments, cells were seeded onto cover-glass bottom dishes and pretreated with 500 nM trametinib or 500 nM pictilisib for 12 h. TGFβ was then added to the media and cells were cultured for an additional 24 h. Antibodies against LPP (1:1000) and paxillin (1:1000) were applied overnight at 4°C. Alexa Fluor 546 (1:1000) and Alexa Fluor 647 (1:1000; Cat. no.: A21447, Invitrogen) dye conjugated secondary antibodies were then applied for 1 h at room temperature to visualize paxillin and LPP, respectively. Alexa Fluor 488 Phalloidin (1:1000) was used to visualize F-actin. Cells were kept in 1x PBS.

Images were acquired on a Zeiss LSM 710 confocal microscope with a Plan Apochromat 63x/1.4 NA objective. For Figs. 3 and 5, the following parameters were used: 1 airy unit, 900 master gain, 1 digital gain, 3.15 μs pixel dwell time, and 4 line averaging. An Ar Ion laser tuned to 488 nm (1% power; 488/594 nm beam splitter; 493-556 nm detection) was used to visualize actin, a HeNe Green 543 nm laser (8% power; 458/543/633 nm beam splitter; 548-643 nm detection) was used to visualize paxillin, and a HeNe Red 633 nm laser (5% power; 488/543/633 nm beam splitter; 638-755 nm detection) was used to visualize mCherry-paxillin. Images were saved as 8-bit with 1024x1024 pixels. For MAPK and PI3K inhibitor experiments (Supplemental Fig. S10), the following parameters were used: 1 airy unit, 850 master gain, 1 digital gain, 6.3 μs pixel dwell time, and 4 line averaging. The 488 nm laser (3.5% power; 488/594 nm beam splitter; 493-556 nm detection) was used to visualize actin, the 543 nm laser (8% power; 458/543 nm beam splitter; 548-643 nm detection) was used to visualize paxillin, and a HeNe Red 633 nm laser (5% power; 488/543/633 nm beam splitter; 638-755 nm detection) was used to visualize LPP. Images were saved as 8-bit with 512x512 pixels.

Images were also acquired on a Zeiss LSM 780 confocal microscope with a Plan Apochromat 63x/1.4 NA objective. For Supplemental Fig. S6, the following parameters were used: 1 airy unit, 800 master gain, 1 digital gain, 6.3 μs pixel dwell time, and 4 line averaging. A 405 nm blue diode laser (1% power; 405 nm beam splitter; 410-514 nm detection) was used to visualize DAPI, an Ar Ion laser tuned to 488 nm (2% power; 488 nm beam splitter; 490-606 nm detection) was used to visualize LPP, and a 561 nm DPSS laser (2% power; 458/561 nm beam splitter; 578-696 nm detection) was used to visualize mCherry-paxillin.
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Images were saved as 8-bit with 1024x1024 pixels.

Determining the number and size of adhesions per cell area – Immunofluorescence images were imported into Imaris for analysis using the Surfaces function. Surfaces detail was smoothed and set to 0.200 µm with a local background subtraction of 0.200 µm. All cellular adhesions (vinculin, paxillin and/or LPP signals) were masked by a manual refinement of the autothreshold feature and splitting touching objects with a seed points diameter of 0.500 µm. Surfaces smaller than 10 pixels were removed by filtering. A contour was then manually drawn around the cell to determine the total surface area in contact with the dish. Finally, the number of adhesions for each cell was divided by the total cell area. An intensity threshold was used to classify small adhesions. Adhesions with a mean intensity less than 35 (A.U.) were considered to be small. The number of small adhesions in each cell was then divided by the total number of adhesions identified. The data shown represents the mean ± s.e.m. for all cells analyzed from three independent experiments.

For MAPK and PI3K inhibitor experiments, surfaces detail and local background subtraction were uniformly changed to 0.300 µm to accommodate for the larger adhesions present in some conditions. Surfaces smaller than 2 pixels were removed by filtering. Measurements of adhesion size were obtained directly from masked adhesions. The data shown represents the mean ± s.e.m. for all cells analyzed from two independent experiments.

BioID assay and streptavidin pulldown – MMTV/MT cells were starved overnight and subsequently treated with 50 µM biotin supplemented 10% FBS/DMEM growth media for 24 h. Two 15 cm plates of cells at 70% confluency were used per pull-down sample. The next day, cells were trypsinized, washed with PBS, and centrifuged at 250 x g for 5 mins. Cells collected from each 15 cm plate were lysed with 700 µL RIPA lysis 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 protease inhibitor cocktail, 50 U benzonase, 0.5% sodium deoxycholate for 1 h on ice. Cells were vortexed every 15 mins for 10 s. Lysates were sonicated at 50% amplitude 2 times for 10 s on ice. Lysates were centrifuged at maximum speed for 30 mins at 4°C. Pierce Avidin agarose beads (30 µL bead volume per pull-down; Cat. no.: 20219, Thermo Fisher Scientific) were used on supernatants from the centrifuged lysates (6% by volume of the supernatant was used as input control). Samples were rotated end-over-end for 3 h at 4°C. Afterwards, beads were centrifuged at 3000 rpm for 1 min at 4°C and washed with RIPA buffer three times. For immunoblots: both input and pull-down samples were incubated with protein loading dye for 10 mins at 95°C. Elution for MS: following the wash with RIPA buffer, samples were washed twice with LIGHT buffer (50 mM Tris-HCl pH 7.5 using HPLC grade water) and eluted with 150 µL 50 mM H$_3$PO$_4$ (pH 1.5-2) on ice for 10 mins. Supernatants were collected. This was repeated two more times and all three elutions were pooled and kept in -80°C until mass spectrometry analysis. Three technical replicates per condition were used for mass spectrometry analysis.

Mass spectrometry and analysis of BioID data – Each BioID experiment was performed in triplicate, on different days and using cells from successive
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passages. In order to distinguish background from 
_bona fide_ protein associations, Myc-BirA (no biotin) and 
Myc-SHCA-WT-BirA (no biotin) were used as controls to perform SAINT analyses (48,116).

Peptide identification was carried out on an Orbitrap Fusion Tribrid mass spectrometer (Thermo Fisher Scientific) equipped with a nanoelectrospray ion source (Thermo Fisher Scientific) and coupled to an UltiMate 3000 nanoRSLC (Thermo Fisher Scientific). Data dependent acquisition of mass spectra was performed using Xcalibur (v. 3.0.63; Thermo Fisher Scientific). Full scan mass spectra (350 to 1800 m/z) were acquired in the orbitrap at a resolution of 120,000 using a maximum injection time of 50 ms and automatic gain control target of 4e5. The quadrupole analyzer allowed for the isolation of selected ions in a window of 1.6 m/z and fragmentation by higher energy collision-induced dissociation (HCD) with 35% energy. The resulting fragments were detected by the linear ion trap at a rapid scan rate. Dynamic exclusion of previously fragmented peptides was set for a period of 20 seconds and a tolerance of 10 ppm. All MS/MS peak lists were generated using Thermo Proteome Discoverer (v. 1.4.0.288; Thermo Fisher Scientific) with default parameters. MGF sample files were analyzed using Mascot (v. 2.5.1; Matrix Science, London, UK). The software was programmed to search against Uniprot _Mus musculus_ database (November 2014, 84646 entries) assuming trypsin digestion. Searches were carried out with a fragment ion mass tolerance of 0.60 Da and a parent ion tolerance of 10 ppm. Carbamidomethyl of cysteine was specified as a fixed modification. Variable modifications were deamidation of asparagine and glutamine, oxidation of methionine and phosphorylation of serine, threonine, and tyrosine. Two mis-cleavages were allowed.

Validation of the MS/MS-based peptide and protein identifications was carried out using Scaffold software (v. 4.7.5; Proteome Software Inc., Portland, OR). Criteria for protein identifications were fixed at greater than 99% probability to achieve an FDR less than 1% and required at least 1 peptide to be identified. The Protein Prophet algorithm was used to assign peptide and protein probabilities (117,118). For proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone, the principle of parsimony was applied. After being exported from Scaffold, spectral counts were formatted according to SAINT algorithm guidelines (48,49). For each prey, the SAINT express statistical analyses were carried out by using the maximal spectral count value from Myc-BirA (no biotin) or Myc-SHCA-WT-BirA (no biotin) as controls. Proteins with a SAINT score ≥0.9 were deemed genuine interactors.

**Co-localization of SHCA and LPP** – NMuMG ErbB2 cells expressing SHCA-WT-iRFP and mCherry-LPP were plated onto 35 mm cover-glass bottom dishes coated with fibronectin and allowed to settle overnight. Cells were cultured in the presence or absence of TGFβ for 24 h before being fixed with 4% PFA for 10 mins at 37°C. Images of SHCA and LPP were acquired sequentially on the TIRF microscope described above. A 561 nm laser with an ET 620/60 nm filter cube was used to visualize mCherry-LPP while a 642 nm laser with an ET 700/75 nm filter cube was used to visualize SHCA-WT-iRFP. The camera exposure time was set to 1 s with an EM gain of 255 and
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read speed of 22 MHz. The TIRF prism was set to limit fluorescence excitation to a depth of 80 nm.

Images were imported into Imaris for analysis using the Surfaces function. Surfaces detail was smoothed and set to 0.200 µm with a local background subtraction of 0.200 µm. The LPP signal was used to mask cellular adhesions; manual refinement of the autothreshold feature and splitting touching objects with a seed points diameter of 0.500 µm was used. A contour was then manually drawn around the cell to determine the total surface area in contact with the dish. The percentage of LPP adhesions positive for SHCA was determined by analyzing the mean intensity of SHCA within each mask. Adhesions with a mean intensity greater than diffuse cytoplasmic signal were considered positive for SHCA. The data shown represents the mean ± s.e.m. for all cells analyzed from three independent experiments. Linescan analysis was performed in ImageJ to show overlap of SHCA and LPP signals in fixed cells.

**Rapid imaging of adhesion dynamics** – NMuMG ErbB2 cells expressing SHCA-WT-iRFP and EGFP-LPP were plated onto 35 mm cover-glass bottom dishes coated with fibronectin and allowed to settle overnight. Cells were cultured in the presence of TGFβ for 24 h prior to imaging. Images of SHCA and LPP were acquired sequentially on the TIRF microscope described above. A 488 nm laser with an ET 525/50 nm filter cube was used to visualize EGFP-LPP while a 642 nm laser with an ET 700/75 nm filter cube was used to visualize SHCA-WT-iRFP. The camera exposure time was set to 350 ms with an EM gain of 255 and read speed of 22 MHz. Images were acquired semi-continuously with the imaging interval set to 2 s. This time delay was chosen to give the filter wheel sufficient time to switch between filters. The exact time of acquisition for each image was recorded. The TIRF prism was set to limit fluorescence excitation to a depth of 80 nm.

Image stacks were analyzed in Imaris, as described in the previous section. Given that SHCA appears at sites of adhesion before LPP, the SHCA signal was used to mask adhesions in these sets of experiments. The mean intensities of SHCA and LPP within an adhesion were then plotted against time after adjusting for the delay between the channels. Image stacks were also analyzed in ImageJ. Linescan analysis (ImageJ) was used to show treadmilling of SHCA, or the formation of a second nucleation point, in mature adhesions. Note that EGFP-LPP was chosen for these sets of experiments because EGFP is brighter than mCherry and there is no spectral overlap with iRFP.

**Gelatin degradation assays** – Degradation assays were performed on fluorescently conjugated gelatin-coated coverslips. Briefly, 35 mm glass-bottom dishes were coated with a mix of 0.1 mg mL⁻¹ poly-D-lysine (Cat. no.: P6407, Sigma-Aldrich) and 5 μg cm⁻² fibronectin in 1x PBS for 20 mins, followed by incubation with 0.4% glutaraldehyde for 10 mins. Oregon Green 488 conjugated gelatin (Cat. no.: G13186, Invitrogen) was diluted 1:20 with 0.1% unconjugated gelatin (Cat. no.: 07903, Stem Cell Technologies) and used to coat dishes for 10 mins at 37°C. Coverslips were then incubated with 10 mg mL⁻¹ sodium borohydride for 2 mins, followed by 70% ethanol for 20 mins. Three washes with 1x PBS were performed between each
step. DMEM media (37°C) was added to the coverslips 1 h before cell plating.

Cells were pre-treated with TGFβ for 24 h in a cell culture dish and counted before plating. Cells seeded onto gelatin dishes (32,000 cells) were allowed to invade for 24 h before fixing with 4% PFA. Cells were then permeabilized with 0.2% Triton X-100, rinsed with 100 mM glycine in PBS, and blocked with 10% FBS in PBS. Antibodies against cortactin (1:1000; Cat. no.: 05-180, EMD Millipore) and Atto 647N Phalloidin for F-actin (1:2000; Cat. no.: 65906, Sigma-Aldrich) were used. Alexa Fluor 546 dye conjugated secondary antibody (1:1000) was used to visualize cortactin. Cells were kept in 1x PBS with 0.05% sodium azide.

Images were acquired on a Zeiss LSM 710 confocal microscope with a Plan Apochromat 63x/1.4 NA objective with the following parameters: 1 airy unit, 800-900 master gain, 1 digital gain, 3.15 μs pixel dwell time, 4 line averaging. An Ar Ion laser tuned to 488 nm (0.5-2.5% power; 488/594 nm beam splitter; 493-549 nm detection) was used to visualize gelatin, a HeNe Green 543 nm laser (4-5% power; 458/543 nm beam splitter; 566-599 nm detection) was used to visualize cortactin, and a HeNe Red 633 nm laser (1-4% power; 488/543/633 nm beam splitter; 638-759 nm detection) was used to visualize actin. Z-stacks were acquired at 0.26-0.30 μm steps over 2.1-2.4 μm. Images of gelatin alone were captured with a 20x/0.8NA Plan Apochromat objective. Images were saved as 12-bit with 1024x1024 pixels.

Quantification of gelatin degradation was performed in Imaris using the Surfaces function. The lookup table was inverted to make areas of degradation appear bright. Surfaces detail was smoothed and set to 0.500 μm with a local background subtraction of 0.250 μm. Areas of degradation were then masked by a manual refinement of the autothreshold feature. Finally, orthogonal views were generated with the section viewer. Linescan analysis was performed in ImageJ. The data shown represents the mean ± s.e.m. for all cells analyzed from three independent experiments.

**Statistical analyses** – Statistical significance values (P-values) were obtained by performing an unpaired two-tailed Student’s t-test or Mann-Whitney U test. Data are presented as mean ± s.e.m (where indicated). For adhesion dynamics, top and bottom lines of box plots indicate the 3rd and 1st quartile, respectively, while the bold central lines indicate mean. The whiskers extend up to 1.5 times the interquartile range. Black dots represent outliers.

**Data Availability**

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (119) partner repository with the dataset identifier PXD018265 and 10.6019/PXD018265.
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Conflict of interest

The authors declare that they have no conflicts of interest with the contents of this article.

Author Contributions

A.K. generated several constructs, performed immunoblotting and microscopy experiments, analyzed data, and created MATLAB codes. E.V. performed gelatin degradation assays and helped with immunoblotting experiments. J.S. helped with microscopy, analysis and immunoblotting experiments. R.A. performed streptavidin pulldown assays. M.G.A. generated SHCA-iRFP constructs. K.J. and N.B. performed mass spectrometry analyses. G.T. provided preliminary results for cell migration. J.U.-S. contributed to the design and analysis of the SHCA BioID data. P.M.S. and C.M.B. conceptualized and supervised the project. A.K., E.V., P.M.S. and C.M.B. wrote and edited the manuscript.

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Abbreviations

ABD: α-actinin binding domain
CH: Collagen homology domain
Ctn: Cortactin
EMT: Epithelial to Mesenchymal Transition
ErB2: Avian erythroblastosis oncogene B
ERK: Extracellular signal-related kinase
FAK: Focal Adhesion Kinase
iRFP670: Far Red Fluorescent Protein
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LPP: Lipoma Preferred Partner
LPP\textsuperscript{endo}: Cells Expressing Luciferase targeting shRNA
LPP\textsuperscript{KD}: Cells Expressing LPP Targeting shRNA
LPP-mLIM1: LPP construct with mutation in the first LIM domain
LPP-\Delta\text{ABD}: LPP construct with a deleting in the \(\alpha\)-actinin binding domain
MAPK: Mitogen-Activated Protein Kinase
NMuMG: Normal Murine Mammary Gland
NT: Constitutively active ErbB2 receptor (V664E point mutation)
NYPD: Constitutively active ErbB2 receptor with five autophosphorylation sites (Y1028, Y1144, Y1201, Y1226/Y1227 and Y1253) mutated to phenylalanine
PI3K: Phosphoinositide 3-kinase
PTB: Phosphotyrosine-Binding Domain
SAINT: Significance Analysis of INTeractome
SH2: Src Homology 2 Domain
SHCA: Src Homology and Collagen
SHCA\textsuperscript{endo}: Cells Expressing Luciferase targeting shRNA
SHCA\textsuperscript{low}: Cells Expressing SHCA Targeting shRNA
Src: Avian Arcoma (Schmidt-Ruppin A-2) Viral Oncogene
TGF\(\beta\): Transforming Growth Factor-\(\beta\)
T\(\beta\)RI: Type I Transforming Growth Factor-\(\beta\) Receptor
TIRF: Total Internal Reflection Fluorescence
Tk5: Tyrosine Kinase Substrate with 5 SH3 domains
WT: Wildtype
313F: SHCA construct with tyrosine 313 mutated to phenylalanine
2F: SHCA construct with tyrosine residues 239 and 240 mutated to phenylalanine
3F: SHCA construct with tyrosine residues 239, 240 and 313 mutated to phenylalanine
### Table 1: BioID analysis to identify novel SHCA-interacting proteins

| Prey          | Spectral Counts* | Saint Score** | Fold Change*** | Function                                                                 |
|---------------|------------------|---------------|----------------|--------------------------------------------------------------------------|
| **Cellular Adhesion Proteins (SHCA Interactors)** |                  |               |                |                                                                         |
| Actr2         | 11.3 ± 2.3       | 0.96          | 4.3            | ATP-binding component of Arp2/3 complex                                  |
| Arhgef10l     | 9.3 ± 2.5        | 1             | 93.3           | RhoGEF                                                                  |
| Arhgef5       | 5.3 ± 3.5        | 0.98          | 53.3           | RhoGEF                                                                  |
| Arhgef7       | 3.3 ± 1.2        | 0.98          | 33.3           | RhoGEF                                                                  |
| Arpin         | 7.3 ± 2.9        | 1             | 73.3           | Regulates actin polymerization                                           |
| Cd2ap         | 24 ± 4           | 1             | 10.3           | Scaffold protein, binds actin                                            |
| Coro1b        | 37.3 ± 11.7      | 1             | 12.4           | Actin binding protein                                                    |
| Crk           | 5.7 ± 2.9        | 1             | 56.7           | Adaptor protein, cell adhesion, migration                               |
| Ctn           | 25 ± 4.6         | 1             | 18.8           | Focal adhesion protein, cytoskeletal organization                       |
| Eps8l2        | 25.3 ± 7.5       | 1             | 253.3          | Enhances SOS activity, cytoskeletal organization                        |
| Lims1         | 4.7 ± 0.6        | 1             | 46.7           | Integrin signaling, focal adhesion protein                              |
| Lpp           | 9.7 ± 5.5        | 0.98          | 9.7            | Focal adhesion protein, adhesion dynamics                               |
| Pak2          | 8.3 ± 2.3        | 1             | 25             | Ser/thr kinase, cytoskeletal organization                               |
| Parva         | 3.3 ± 0.6        | 0.96          | 10             | Focal adhesion protein, binds actin                                     |
| Pdlim5        | 20.3 ± 7         | 1             | 15.3           | Cytoskeletal associated protein, migration                               |
| Pxn           | 2.7 ± 1.2        | 0.96          | 26.7           | Focal adhesion protein, adhesion dynamics                               |
| Sept10        | 8.3 ± 2.5        | 1             | 12.5           | GTP binding protein, cytoskeletal organization                         |
| Sept11        | 27.7 ± 0.6       | 1             | 4.6            | GTP binding protein, cytoskeletal organization                         |
| Sept2         | 33 ± 3.6         | 0.97          | 3.1            | GTP binding protein, cytoskeletal organization                         |
| Sept6         | 11 ± 2.6         | 1             | 16.5           | GTP binding protein, cytoskeletal organization                         |
| Sept7         | 36 ± 3           | 1             | 6.8            | GTP binding protein, cytoskeletal organization                         |
| Tln1          | 37 ± 19.5        | 1             | 37             | Focal adhesion protein, binds actin                                     |
| **Known SHCA Interacting Proteins** |                  |               |                |                                                                         |
| Ptpn12        | 10.7 ± 2.9       | 1             | 32             | Protein tyrosine phosphatase                                            |
| Grb2          | 6.3 ± 3.2        | 1             | 63.3           | Adaptor protein; activates ERK and AKT pathways                         |
| Shcbp1        | 8.3 ± 1.5        | 1             | 83.3           | SHCA binding protein                                                    |

*Average of three biological replicates

**Cutoff: Saint Score >0.9 (From a total of 113 interacting proteins)

***Fold enrichment relative to Myc-BirA alone
Figure Legends

Fig 1. TGFβ stimulates the migration of ErbB2-NT expressing breast cancer cells. (A) Live-cell migration tracks for each condition and cell line are shown 18 h after treatment with or without TGFβ (2 ng mL$^{-1}$). Each line represents the migration path of a single cell over 6 h. The starting point of each cell was superimposed on the origin (0,0). Bracketed numbers refer to explant cell lines. (B) Cell displacements from (A) were pooled into 45° segments based on their angle of trajectory and averaged to determine mean net displacement. (C) Average migration speed over time was calculated by determining the mean distance travelled between each imaging time point by all cells in (A). The data for each population was then averaged into 3-hour segments. Data represent mean ± s.e.m. from five (NT) or six (NYPD) independent experiments. (*, $P < 0.05$, two-tailed Student’s $t$-test). (D) Adhesions in protrusive cell regions were tracked over time using total internal reflection fluorescence (TIRF) microscopy. Cells were transfected with mCherry-paxillin, a marker of cellular adhesions, 48 h prior to imaging and left untreated or treated with TGFβ for 24 h. Average assembly (green) or disassembly (red) rates were determined from changes in mean fluorescence intensity. Data represent individual assembly and disassembly events from three independent experiments. Top and bottom lines of the box indicate 3rd and 1st quartile, respectively, while the bold central line indicates mean. The whiskers extend up to 1.5 times the interquartile range. Black dots represent outliers. ($^* P < 0.0001$ calculated from the cell averages for assembly and disassembly rates; two-tailed Student’s $t$-test).

Fig 2. The SHCA adapter protein is required for TGFβ-induced migration. (A) Total cell lysates showing ErbB2 and SHCA levels in ErbB2-NT (explant 118), ErbB2-NYPD (explant 120), ErbB2-NT/SHCA$^{\text{endo}}$ (explant 83) and ErbB2-NT/SHCA$^{\text{low}}$ (explant 87) populations. $\alpha$-Tubulin was used as a loading control. (B) ErbB2-NT/SHCA$^{\text{endo}}$ (83) and ErbB2-NT/SHCA$^{\text{low}}$ (87) cells were seeded onto fibronectin-coated glass bottom dishes and allowed to migrate in the absence or presence of TGFβ (2 ng mL$^{-1}$). Live-cell migration tracks for each condition are shown 18 h after treatment. Each line represents the migration path of a single cell over 6 h. The starting point of each cell was superimposed on the origin (0,0). (C) Cell displacements from (B) were pooled into 45° segments based on their angle of trajectory and averaged to determine mean net displacement. (D) Average migration speed over time was calculated by determining the mean distance travelled between each imaging time point by all cells in (B). The data for each population was then averaged into 3-hour segments. Data represent mean ± s.e.m. from three (ErbB2-NT/SHCA$^{\text{endo}}$) or four (ErbB2-NT/SHCA$^{\text{low}}$) independent experiments. (*, $P < 0.05$, two-tailed Student’s $t$-test). (E) Adhesions in protrusive cell regions were tracked over time using TIRF microscopy. Cells were transfected with mCherry-paxillin 48 h prior to imaging and left untreated or treated with TGFβ for 24 h. Average assembly (green) or disassembly (red) rates were determined from changes in mean fluorescence intensity. Data represent individual assembly and disassembly events from three (ErbB2-NT/SHCA$^{\text{endo}}$) or four (ErbB2-NT/SHCA$^{\text{low}}$) independent experiments. Top and bottom lines of the box indicate 3rd and 1st quartile, respectively, while the bold central line indicates mean. The whiskers extend up to 1.5 times the interquartile range. Black dots represent outliers. ($^* P < 0.0001$ calculated from the cell averages for assembly and disassembly rates; two-tailed Student’s $t$-test).
Fig 3. SHCA is required for the formation of small, dynamic adhesions in breast cancer cells exposed to TGFβ. (A,B) ErbB2-NT/SHCA<sup>endo</sup> (83) and ErbB2-NT/SHCA<sup>low</sup> (87) cells were infected with mCherry-paxillin, seeded onto fibronectin-coated glass bottom dishes, and cultured in the absence or presence of TGFβ (2 ng mL<sup>-1</sup>) for 24 h. Cells were then fixed with 4% paraformaldehyde (PFA) and stained with phalloidin (F-actin). Images were taken on a Zeiss Confocal Laser Scanning Confocal Microscope (CLSM) using a Plan-Apochromat 63x/1.4 NA oil DIC objective lens (1 pixel = 0.132 μm). Arrowheads highlight examples of small adhesions formed after treatment with TGFβ. Scale bar is 10 μm. (C) Images were imported into Imaris to determine the average number of cellular adhesions for the indicated conditions. Data was normalized by dividing the number of adhesions in each cell by its total area. Cell area was determined by drawing a contour around each cell. (D) Cellular adhesions from (C) were subsequently analyzed for size. An intensity threshold was used to classify small adhesions. The number of small adhesions in each cell was then divided by total adhesions identified. Data represent mean ± s.e.m. from three independent experiments. (*, P < 0.003; Mann-Whitney U test).

Fig 4. Loss of SHCA phosphorylation abrogates TGFβ-induced increase in migration speed of ErbB2-expressing breast cancer cells. (A) Schematic diagram of SHCA showing the domain structure and location of three tyrosine residues (Y) that were mutated to phenylalanine (F). (B) Immunoblot analysis of whole cell lysates showing SHCA levels (anti-SHCA or anti-FLAG antibodies) in the indicated cell populations. α-Tubulin was used as a loading control. (C) ErbB2-NT/SHCA-WT (87) and ErbB2-NT/SHCA-3F (87) cells were seeded onto fibronectin-coated glass bottom dishes and allowed to migrate in the presence or absence of TGFβ (2 ng mL<sup>-1</sup>). Live-cell migration tracks for each condition are shown 18 h after treatment. Each line represents the migration path of a single cell over 6 hours. The starting point of each cell was superimposed on the origin (0,0). (D) Cell displacements from (C) were pooled into 45° segments based on their angle of trajectory and averaged to determine mean final displacement. (E) Average migration speed over time was calculated by determining the mean distance travelled between each imaging time point by all cells in (C). The data for each population was then averaged into 3-hour segments. Data represent mean ± s.e.m. from four independent experiments. (*, P < 0.05, two-tailed Student’s t-test). (F) Adhesions in protrusive cell regions were tracked over time using TIRF microscopy. Cells were transfected with mCherry-paxillin 48 h prior to imaging and left untreated or treated with TGFβ for 24 h. Average assembly (green) or disassembly (red) rates were determined from changes in mean fluorescence intensity. Data represent individual assembly and disassembly events from three independent experiments. Top and bottom lines of the box indicate 3<sup>rd</sup> and 1<sup>st</sup> quartile, respectively, while the bold central line indicates mean. The whiskers extend up to 1.5 times the interquartile range. Black dots represent outliers. (*, P < 0.0001 calculated from the cell averages for assembly and disassembly rates; two-tailed Student’s t-test).

Fig 5. LPP targeting to adhesions in response to TGFβ requires SHCA. (A,B) ErbB2-NT/SHCA<sup>endo</sup> (83) and ErbB2-NT/SHCA<sup>low</sup> (87) cells were infected with mCherry-LPP,
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Seeded onto fibronectin-coated glass bottom dishes, and cultured in the absence or presence of TGFβ (2 ng mL⁻¹) for 24 h. Cells were then fixed with 4% PFA and stained with antibodies against vinculin and phalloidin (F-actin). Images were taken on a Zeiss CLSM using a Plan-Apochromat 63x/1.4 NA oil DIC objective lens (1 pixel = 0.132 μm). Arrowheads highlight examples of small adhesions formed after treatment with TGFβ. Scale bar is 10 μm. (C,D) Images were imported into Imaris to determine the average number of LPP and vinculin-bearing adhesions over the whole cell for each condition. Data was normalized by dividing the number of adhesions in each cell by its total area. Cell area was determined by drawing a contour around each cell. Data represent mean ± s.e.m. from three independent experiments. (*, P = 0.001, Mann-Whitney U test). (E,F) Adhesions in protrusive cell regions were tracked over time using TIRF microscopy. Average assembly (green) or disassembly (red) rates were determined from changes in mean fluorescence intensity after 24 h with or without TGFβ treatment. Data represent individual assembly and disassembly events from three independent experiments. Top and bottom lines of the box indicate 3rd and 1st quartile, respectively, while the bold central line indicates mean. The whiskers extend up to 1.5 times the interquartile range. Black dots represent outliers. (*, P < 0.001 calculated from the cell averages for assembly and disassembly rates, two-tailed Student’s t-test).

Fig 6. LPP is required TGFβ-induced migration and adhesion dynamics. (A) Immunoblot analysis of whole cell lysates showing LPP levels in the indicated cell populations. α-Tubulin was used as a loading control. (B) ErbB2-NT/LPPendo and ErbB2-NT/LPPKD cells were seeded onto fibronectin-coated glass bottom dishes and allowed to migrate in the presence or absence of TGFβ (2 ng mL⁻¹). Live-cell migration tracks for each condition are shown 18 h after treatment. Each line represents the migration path of a single cell over 6 h. The starting point of each cell was superimposed on the origin (0,0). (C) Cell displacements from (B) were pooled into 45° segments based on their angle of trajectory and averaged to determine mean final displacement. (D) Average migration speed over time was calculated by determining the mean distance travelled between each imaging time point by all cells in (B). The data for each population was then averaged into 3-hour segments. Data represent mean ± s.e.m. from four independent experiments. (*, P < 0.05, two-tailed Student’s t-test). (E) Adhesions in protrusive cell regions were tracked over time using TIRF microscopy. Cells were transfected with mCherry-paxillin 48 h prior to imaging and left untreated or treated with TGFβ for 24 h. Average assembly (green) or disassembly (red) rates were determined from changes in mean fluorescence intensity. Data represent individual assembly and disassembly events from three independent experiments. Top and bottom lines of the box indicate 3rd and 1st quartile, respectively, while the bold central line indicates mean. The whiskers extend up to 1.5 times the interquartile range. Black dots represent outliers. (*, P < 0.0001 calculated from the cell averages for assembly and disassembly rates; two-tailed Student’s t-test).

Fig 7. SHCA is found in adhesions and co-localizes with LPP upon TGFβ treatment. (A) Immunoblot analyses of whole cell lysates showing SHCA (anti-SHCA or anti-FLAG antibodies) and LPP levels in ErbB2-NT/SHCAendo (83), ErbB2-NT/SHCALow (87) and ErbB2-NT/SHCALow; SHCA-WT-iRFP (87) cells. α-Tubulin was used as a loading control. (B) NMuMG ErbB2 cells expressing SHCA-WT-iRFP were infected with mCherry-LPP,
SHCA mediates adhesion and invadopodia formation

seeded onto fibronectin-coated glass bottom dishes, and cultured in the absence or presence of TGFβ (2 ng mL\(^{-1}\)) for 24 h. Cells were then fixed with 4% PFA and imaged on a TIRF Spectral Diskovery System coupled to a Leica microscope equipped with a Plan-Apochromat 63x/1.47NA oil DIC objective lens and EM-CCD camera (1 pixel = 0.155 μm). Scale bar is 10 μm. (C) Linescan analysis of SHCA and LPP from boxed region in (B). (D,E) Images were imported into Imaris to determine the average number of LPP-bearing adhesions and percent co-localization with SHCA over the whole cell. Data was normalized by dividing the number of adhesions in each cell by its total area. Cell area was determined by drawing a contour around each cell. Adhesions were considered positive for SHCA if the mean intensity of the SHCA channel within the adhesion exceeded the diffuse cytoplasmic signal. Data represent mean ± s.e.m. from three independent experiments. (*, \(P < 0.0012\); Mann-Whitney U test).

Fig 8. SHCA localization to adhesions precedes LPP recruitment. (A) Time-lapse montage of a newly forming adhesion (see Movie 3). White arrowheads indicate the site of adhesion nucleation; green arrowheads indicate the appearance of SHCA; and magenta arrowheads indicate the appearance of LPP. Intensity traces for SHCA and LPP are depicted in the adjacent graph. (B) Time-lapse montage of a maturing adhesion (see Movie 4). White arrowheads indicate the adhesion of interest. Intensity traces for SHCA and LPP are depicted in the adjacent graph. (C) Pseudo-colored montage (16-color) of SHCA and LPP in a mature adhesion. Black arrowhead points to a second nucleation point in the SHCA channel. Linescan analysis was performed every 20 s to show the formation of a second SHCA nucleation point at the growing end of the adhesion. Fluorescence intensity of SHCA and LPP are plotted as a function of distance and time. (D) Time-lapse montage of a disassembling adhesion (see Movie 5). White arrowheads indicate the adhesion of interest; green arrowheads indicate the start of SHCA disassembly; and magenta arrowheads indicate the start of LPP disassembly. Intensity traces for SHCA and LPP are depicted in the adjacent graph. (E) Time-lapse montage of a short-lived adhesion (see Movie 6). White arrowheads indicate the site of adhesion nucleation and green arrowheads indicate the appearance of SHCA. Intensity traces for SHCA and LPP are depicted in the adjacent graph. All scale bars are 2 μm.

Fig 9. SHCA promotes breast cancer cell-mediated gelatin degradation. (A) Representative images of ErbB2-NT/SHCA\(^\text{low}\), ErbB2-NT/SHCA\(^\text{low}\)/SHCA-WT and ErbB2-NT/SHCA\(^\text{low}\)/SHCA-3F cells taken on a Zeiss CLSM using a Plan-Apochromat 63x/1.4 NA oil DIC objective lens. Scale bar is 10 μm. (B) Images were imported into Imaris to determine the total surface area degraded per field of view (FOV). Data represent mean ± s.e.m. from three independent experiments. Individual data points are depicted with symbols. (*, \(P < 0.0001\); Mann-Whitney U test). (C) Z-stack acquisition was performed over a depth of 2.1 μm at 0.3 μm intervals. An orthogonal view (x-z plane, dotted black box) is presented to highlight an area of gelatin degradation where cortactin and actin are co-localized (black arrow). Scale bar is 10 μm. (D) Linescan analysis of zoomed region from (C) showing a slice view (x-y plane, solid white line) of cortactin and actin co-localization at sites of gelatin degradation. Intensity traces for cortactin, actin and gelatin are shown in the adjacent graph. Scale bar is 5 μm.
Fig 10. SHCA promotes the formation of small, dynamic adhesions in the presence of TGFβ by acting as a nucleator of focal complex formation. Schematic diagram illustrating the proposed role of SHCA during the different stages of adhesion formation. **Initiation**: TGFβ causes clustering of ErbB2 and integrins at the cell membrane. **Nascent adhesions**: SHCA associates with the cytoplasmic regions of several integrins both directly and indirectly. FAK and paxillin are also recruited. **Focal adhesions**: SHCA interacts with a variety of intracellular adapter/signaling molecules (including LPP) and enhances their recruitment to adhesions. **Mature adhesions**: SHCA exhibits treadmilling as more integrins are engaged. This promotes the recruitment of additional components to the growing end of an adhesion. **Force-bearing adhesion**: adhesion binding to actin cables establishes a link between the ECM and the cellular cytoskeleton for the generation of traction forces. **Disassembly**: SHCA begins to leave before other components.
Figure 1

A

ErbB2-NT (118) 
Constitutively active

ErbB2-NYPD (120) 
Phosphorylation deficient

B

Mean net displacement (μm)

C

Average speed (μm/h)

D

Paxillin dynamics (min\(^{-1}\))
Figure 2

(A) Western blot analysis showing ErbB2-NT, SHCA, and α-Tubulin proteins. The blot shows bands at 250 kDa, 150 kDa, 75 kDa, 50 kDa, and 25 kDa.

(B) Scatter plots showing the X and Y positions of SHCAendo (83) and SHCALow (87) in the absence (− TGFβ) and presence (+ TGFβ) of TGFβ. The blue line represents − TGFβ, and the red line represents + TGFβ.

(C) Polar plots showing the mean net displacement of SHCAendo (83) and SHCALow (87) in the absence and presence of TGFβ. The plots indicate a significant difference in displacement with p = 0.012 for SHCAendo and p = 0.59 for SHCALow.

(D) Bar graphs comparing the average speed of SHCAendo (83) and SHCALow (87) in the absence and presence of TGFβ. The graphs show a significant difference with p = 0.012 for SHCAendo and p = 0.59 for SHCALow.

(E) Violin plots showing the Paxillin dynamics (min⁻¹) for SHCAendo (83) and SHCALow (87) in the absence and presence of TGFβ. The plots indicate a significant difference with p = 0.012 for SHCAendo and p = 0.59 for SHCALow.

Legend:
- △: − TGFβ
- ▲: + TGFβ

Figure 2
Figure 3

(A) Paxillin and Paxillin / Actin stained cells. SHCA<sub>endo</sub> (83) and SHCA<sub>low</sub> (87) are shown with and without β treatment.

(B) The same cells as in (A) with an inset to highlight the region of interest.

(C) Graph showing the number of paxillin adhesions per unit area (µm²) for SHCA<sub>endo</sub> (83) and SHCA<sub>low</sub> (87) with and without β treatment. The graph includes error bars and statistical significance indicated by an asterisk (*).

(D) Graph showing the percentage of small adhesions for SHCA<sub>endo</sub> (83) and SHCA<sub>low</sub> (87) with and without β treatment. The graph includes error bars and statistical significance indicated by an asterisk (*).
**A**

**PTB** | **CH1** | **SH2**

![](attachment:ptb_ch1_sh2.png)

ErbB2 binding

**B**

| NMuMG-ErbB2 |
|--------------|
| **SHCA**<sup>low</sup> (87) |

![](attachment:nmugerb2.png)

**C**

**ErbB2-NT/SHCA<sup>low</sup>** (87)

**SHCA-WT**

![](attachment:erbb2nt_shca_low_shca_wt.png)

**SHCA-3F**

![](attachment:erbb2nt_shca_low_shca_3f.png)

**D**

Mean net displacement (μm)

![](attachment:mean_net_displacement.png)

**E**

Average speed (μm/h)

![](attachment:average_speed.png)

**F**

Paxillin dynamics (min<sup>-1</sup>)

![](attachment:paxillin_dynamics.png)

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**Figure 4**

SHCA-WT: all phosphorylation sites intact

SHCA-313F: Y313F

SHCA-2F: Y239F/Y240F

SHCA-3F: Y239F/Y240F/313F
Figure 5
Figure 6
Figure 8
Figure 9

A

+ TGFβ

SHCA+ 

SHCA-WT (87)

SHCA-3F (87)

Gelatin

Actin

Merge

B

Total Surface Area Degraded per FOV (µm²)

C

Cortactin / Actin / Gelatin

D

Cortactin / Actin / Gelatin

Normalized Intensity (A.U.) vs. Distance (µm)

SHCA+ (87) +β

SHCA-WT (87) +β

SHCA-3F (87) +β

(n=30) (n=39) (n=39)

ShcA low (87) + β

ShcA-WT (87) + β

ShcA-3F (87) + β
The SHCA adapter protein cooperates with lipoma-preferred partner in the regulation of adhesion dynamics and invadopodia formation
Alex Kiepas, Elena Voorand, Julien Senecal, Ryuhjin Ahn, Matthew G. Annis, Kevin Jacquet, George Tali, Nicolas Bisson, Josie Ursini-Siegel, Peter M. Siegel and Claire M Brown

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