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Interaction with the adaptor protein Shc prevents aberrant Erk activation in the absence of extracellular stimulus

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Abstract

Control mechanisms that prevent aberrant signaling are necessary to maintain cellular homeostasis. We describe a novel mechanism by which the adaptor protein Shc binds directly to the MAP-kinase Erk, preventing its activation in the absence of extracellular stimulus. The Shc–Erk complex restricts Erk nuclear translocation, restraining Erk-dependent transcription of genes, including those responsible for oncogenic growth. The complex is formed through unique binding sites on both the Shc PTB domain and N-terminal lobe of Erk. Upon receptor tyrosine kinase stimulation, a conformational change within Shc—induced through interaction with the phosphorylated receptor—releases Erk allowing it to fulfill its role in signaling. Thus, in addition to its established role in promoting MAP-kinase signaling in stimulated cells, Shc negatively regulates Erk activation in the absence of growth factors and thus could be considered as a tumor suppressor in human cells.

Despite having no intrinsic enzymatic activity, Shc is an integral part of several signal transduction pathways that are found to be disrupted in various cancers. For instance, clinical studies indicate that the activation of signaling pathways involving Shc is linked

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K.M.S., C.-C.L., R.G., F.A.M., E.R.B., Z.A. and S.T.A. carried out experiments and analyzed data, K.M.S., M.N.D. and S.A. performed the *C. elegans* experiments and analysed the data. K.M.S., S.A., S.T.A. and J.E.L. conceived the experiments and wrote the manuscript.

Competing financial interests

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with poor prognosis in breast cancer patients^{1–3}. Also, the receptor tyrosine kinase (RTK) Erb2, whose level of expression is inversely correlated with patient survival, must retain its Shc binding site in order to efficiently transform the mammary gland⁴. Shc similarly plays important roles in increasing the migratory and invasive properties of breast cancer cells^{5–10}, and is involved in the regulation of angiogenesis, cell proliferation, cell survival and stress signaling during normal development. The cooption of these Shc-dependent processes by transformed cells also suggests a potential role for Shc during cancer progression¹¹.

Of the three genes in the Shc family (*shcA*, *B* and *C*) only *shcA* is ubiquitously expressed. There are three isoforms of the ShcA protein (p46, p52 and p66). In this work we focus on p52^{ShcA} (henceforth referred to simply as Shc). Structurally, Shc consists of an N-terminal phosphotyrosine binding (PTB) domain, a collagen homology 1 (CH1) domain and a C-terminal Src homology 2 (SH2) domain which, like the PTB domain, binds to tyrosine phosphorylated proteins. The best understood role of Shc is in linking RTKs to the mitogen activated protein kinase (MAPK) pathway^{12–14}. The canonical view of this process is that upon receptor stimulation, Shc binds to the phosphorylated RTK (or other activated kinase) via a phosphotyrosine (pY) residue-containing site (bearing the consensus sequence NPXpY where X is any amino acid¹²) through its PTB domain. Recruitment to the activated receptor results in the phosphorylation of Shc at tyrosine residues within its CH1 domain that then provides a binding site for the adaptor protein Grb2. The phosphorylation of Shc also causes a conformational change, which exposes its SH2 domain to interactions with phosphorylated downstream signaling proteins^{15,16}. The Shc–Grb2 complex activates the MAPK pathway, through a sequence of events—involving the recruitment of Sos and up-regulation of Ras and Raf—that ultimately results in the phosphorylation of Erk by Mek^{17,18}.

Erk plays a fundamental role in RTK-mediated intracellular signals controlling cell growth, differentiation and survival. It is also up-regulated in many forms of cancer; including ovarian, prostate and Hodgkin's disease^{19–21}. Erk has two well-studied functionally and structurally similar isoforms: Erk1 and Erk2, (henceforth communally referred to as Erk) that act as the terminal kinase in the MAPK pathway. Phosphorylation of Erk by Mek is necessary and sufficient for the dissociation of the Erk–Mek complex²². Upon phosphorylation Erk acts as a serine-threonine kinase on numerous substrate proteins^{23–25} and/or translocates to the nucleus to stimulate the transcription of specific genes^{18,26}. Prolonged Erk phosphorylation results in cell proliferation^{27,28}. Erk can also regulate the MAPK pathway through negative feedback inhibition. For example, Erk2 can hyperphosphorylate Raf1 and Sos, inactivating them and resulting in the down-regulation of the signaling cascade²⁹.

Here we describe the mechanism by which direct interaction between Shc and Erk restrains Erk's activity in the absence of growth factor stimulus. This interaction functions to prevent the phosphorylation-dependent activation of Erk. The binding sites identified in this study—located on the PTB domain of Shc and the N-terminal lobe of Erk—have not previously been implicated in protein-protein interactions. Upon extracellular stimulation, recruitment of Shc–Erk to phosphorylated RTKs through Shc's PTB domain causes a conformational change within Shc that results in the release of Erk from the complex, allowing it to play its activating role in the MAPK signal transduction pathway. To confirm the role of Shc in

regulating Erk signaling, *C. elegans* was used to demonstrate that the absence of Shc has a clear up-regulatory effect on Erk activity *in vivo*. Thus, Shc is revealed as a key negative regulator of MAPK signaling in non-stimulated cells that becomes a positive regulator upon binding to activated RTKs, both through the release of Erk and recruitment of downstream effector proteins to the receptor complex. In the context of constitutively up-regulated RTKs, the loss of Shc regulation of Erk could be a contributing factor in exacerbating oncogenic phenotypes.

RESULTS

Shc directly binds to Erk

As part of a broader investigation into the multiple-protein binding capability of the adaptor protein, we investigated whether Shc and Erk interact in mammalian cells. We immunoprecipitated Shc in three different cell lines and immunoblotted for Erk. In serum starved NIH3T3, MKN28 and PC12 cells, we were able to observe an endogenous complex between Shc and Erk (Fig. 1). Stimulation of the epidermal growth factor receptor (EGFR) by EGF reduced the association of Shc with Erk in all three cell lines (Fig. 1). Since Shc is a known binding partner for EGFR upon growth factor stimulation, it appeared that the activated—phosphorylated—receptor might directly disrupt the interaction between Erk and Shc. In a reciprocal experiment, GST-tagged Erk immobilized on glutathione agarose beads was incubated with HEK293T lysates and immunoblotted for endogenous Shc. The formation of the Shc–Erk complex was again apparent, and it dissociated upon stimulation by EGFR (Fig. 1d). To characterize this interaction better, we quantified the binding affinity between Shc and Erk *in vitro* using isothermal titration calorimetry (ITC). Both proteins were expressed in *E. coli*, and Erk was titrated into Shc. Shc and Erk form a high affinity, 1:1 complex (equilibrium dissociation constant, $K_d = 0.9 \mu\text{M}$; Fig. 1f and Supplementary Table 1).

Shc–Erk interaction occurs through unique binding sites

We next sought to characterize the molecular basis for the interaction between Shc and Erk. To date, the CH1 domain has been shown to form complexes only once Shc is phosphorylated^{15,30}. As a result, we hypothesized that the constitutive binding site for Erk on Shc would be found in either the PTB domain (ShcPTB) or the SH2 domain (ShcSH2). To test this, GST-pulldowns from HEK293T or PC12 cell extracts were carried out using GST-ShcPTB and GST-ShcSH2 fusion proteins. The results of those experiments clearly indicated that the ShcPTB, but not the ShcSH2, interacts with Erk (Fig. 2a,b). The direct interaction between ShcPTB and Erk was further demonstrated using an *in vitro* fluorescence resonance energy transfer (FRET)-based method. ShcPTB and full length Erk were recombinantly expressed as N-terminal fusions with the blue and green fluorescent proteins respectively (BFP-Shc, GFP-Erk). The addition of increasing concentrations of GFP-Erk to BFP-ShcPTB resulted in increased FRET acceptor emission at 510nm and decreased FRET donor emission at 480nm due to excitation of the GFP acceptor fluorophore by the proximal BFP donor (Fig. 2c). This spectroscopic assay provides strong evidence for the interaction of ShcPTB with Erk. This interaction was confirmed by ITC ($K_d = 9.5 \mu\text{M}$; Fig. 2d and Supplementary Table 1). The affinity of the ShcPTB interaction with Erk was

about an order of magnitude weaker than the interaction with the full length Shc protein (Fig. 1f). The intact protein therefore appears to stabilize the interaction of the PTB domain with Erk.

Small angle X-ray scattering (SAXS), which allows determination of an *ab initio* low-resolution volume representation of proteins in solution, was used to provide a model for the molecular juxtaposition of ShcPTB and Erk in the complex. The SAXS models obtained had a volume and shape compatible with a compact 1:1 complex of Erk and ShcPTB (Fig. 3a and Supplementary Fig. 1) Best-scored SAXS docking models placed the atomic structure of ShcPTB (PDB 1SHC) onto the N-terminal lobe of Erk (PDB 1TVO). These docking models fitted well to the *ab initio* SAXS reconstruction (Fig. 3a and Supplementary Fig. 1). This structural analysis suggested that a long loop within ShcPTB (RRRKPCSRPLS, residues 97–107; henceforth referred to as Shc3R) contacts Erk. However the resolution obtained from SAXS was not sufficient to unambiguously determine the orientation of ShcPTB on Erk. We therefore used mutational analysis and binding assays with ShcPTB peptides to determine the orientation of ShcPTB toward its Erk binding site. Based on all our structural, biophysical and functional data, a most likely molecular model of the interaction between Erk and the Shc3R sequence was produced by placing an atomic model of the Shc3R peptide by hand in the experimentally determined binding site on Erk. This model was then energy minimized using Refmac⁵³¹ (Fig. 3b). According to this model, the interaction of Erk with Shc appeared to be mediated through a previously unidentified binding site, which is distal to both the canonical phosphotyrosine and phospholipid binding sites on ShcPTB^{13,32}.

To validate this model, a peptide based on the Shc3R sequence was synthesized and tested for binding to full length Erk. Using ITC the Shc3R binds to Erk with a K_d of 5.7 μ M (Fig. 3c and Supplementary Table 1). The model predicted that arginine Arg98 in ShcPTB would play a major role in the interaction with Erk (Fig. 3b). To test the involvement of Arg98 in Erk binding, this residue was mutated to glutamine (ShcPTB^{R98Q}). Two control Shc variants were also generated: a key residue in a potential consensus Erk substrate binding site, Trp24, was mutated to Ala (ShcPTB^{W24A}), while Arg175, a critical residue in the phosphotyrosine binding pocket recognized by RTKs, was mutated to Glu (ShcPTB^{R175Q}). ShcPTB^{R98Q} was unable to pull down endogenous Erk from HEK293T cell lysates (Fig. 3d), confirming the identity of the amino acid sequence on ShcPTB required for binding as including the sequence incorporated in Shc3R (Fig. 3b). The two other point-mutants, ShcPTB^{W24A} and ShcPTB^{R175Q}, retained the ability to interact with Erk, suggesting that the interaction site for Erk on Shc does not include sites bound by previously reported Shc-binding proteins.

The region of Erk involved in binding Shc also represents a novel site of interaction for this kinase³³. To confirm the location of the binding site on the N-terminal lobe of Erk, two aspartic acid residues which were predicted to bind to Shc3R in our model (Fig. 3b), Asp20 and Asp100, were simultaneously mutated to alanines (D20A, D100A; Erk^{D20A, D100A}). This Erk mutant was unable to pull down Shc from HEK293T cell lysate (Fig. 3d), confirming the binding site location.

Physiological role of the Shc–Erk complex

Having demonstrated the existence of a Shc–Erk complex in non-stimulated cells and identified the binding sites on the respective proteins, we next investigated the physiological relevance of this interaction. To test whether the Shc–Erk complex affects Erk phosphorylation, we established stable HEK293T cell lines that over-express wild type Shc (Shc^{WT}) or the mutant Shc^{R98Q} (in which Shc binding to Erk is compromised). The level of phosphorylated Erk (pErk) in the cell reports on the ability of the Shc–Erk complex to modulate kinase and/or phosphatase activity toward Erk. Under serum-starved conditions, the level of pErk was decreased by over-expression of Shc^{WT} but not Shc^{R98Q} (Fig. 4a).

The conclusion that complex formation is important for the ability of Shc to suppress Erk phosphorylation is further supported by similar experiments carried out in HEK293T cells that stably express the Erk^{D20A, D100A} mutant in which the Shc–Erk interaction is also abrogated. Accordingly, the level of phosphorylation was greater in the Erk^{D20A, D100A} mutant as compared to in wild type Erk-expressing cells (Fig. 4b). These data demonstrate that the ability of Shc to prevent Erk phosphorylation in unstimulated cells is mediated by direct interaction between the two proteins. Additional validation was obtained by depleting endogenous Shc from MCF7 cells by shRNA. In agreement with the above observations (Fig. 3d), the level of Erk phosphorylation was increased when the Shc–Erk complex was disrupted by knocking down endogenous Shc (Fig. 4c). Since activated Erk translocates into the nucleus, we performed nuclear extraction assays to assess the accumulation of Erk in the nucleus following Shc knock down. An increased amount of Erk was present in the nuclear fraction when endogenous Shc was depleted from MCF7 cells (Fig. 4d). This is again consistent with Erk becoming phosphorylated and translocating to the nucleus in the absence of interaction with Shc. We also demonstrated that a greater amount of pErk was localized to the nucleus in the context of the Shc–Erk interaction-disrupting mutant Erk^{D20A, D100A} compared to Erk^{WT} (Supplementary Fig. 2)

Activated Erk phosphorylates several proteins—including p90^{RSK} and Elk—which lead to the increased transcription of genes under the control of the promoter serum response element (SRE)³⁴. We therefore investigated if interacting with Shc affects the transcription stimulatory function of Erk in non-stimulated cells using a luciferase reporter assay system. HEK293T cells stably over-expressing RFP-tagged ShcPTB^{WT} or ShcPTB^{R98Q} were transfected with plasmids containing the firefly-luciferase gene under SRE control. Plasmids containing the Renilla-luciferase gene under the HSV-thymidine kinase promoter were also simultaneously transfected to serve as internal controls. In the non-stimulated state, cells over-expressing ShcPTB^{R98Q} exhibited a ~2-fold higher luciferase activity as compared with the ShcPTB^{WT} over-expressing cells (Fig. 4e). This suggests that in the absence of interaction with Shc, Erk translocates to the nucleus and stimulates transcription in response to background MAPK activity.

Shc regulates Erk activation in *Caenorhabditis elegans*

Having discovered a constitutive interaction between Shc and Erk and demonstrated its physiological relevance in mammalian cells, we wanted to confirm these observations in an *in vivo* context. Since there are multiple Shc isoforms in mammalian model systems, we

turned to the simple genetic model provided by *C. elegans* to assess the importance of the Shc and Erk interaction during development. *C. elegans* has single genes encoding for Ras (*let-60*), Raf (*lin-45*), Mek (*mek-2*) and Erk (*mpk-1*). This signaling pathway is conserved across evolution, and the amino acid sequence and structure of *C. elegans* Erk (CeErk) and human Erk2 (hErk2) are highly conserved (the amino acid sequence identity and similarity in the Shc binding region are 52% and 81%, respectively; Supplementary Fig. 3). The sequence of CeShc (*shc-1*) is conserved with hShc, particularly within the PTB domain (Supplementary Fig. 3). Using germ line development as a tissue context, we investigated the role of the Shc RQ mutation on Erk signaling and activation in *C. elegans*. The wild type *C. elegans* germ line exhibits a stereotypical biphasic mode of Erk activation (Fig. 5). Erk is first activated as visualized by an antibody that recognizes the active dually phosphorylated form of Erk (active Erk, red in the Fig. 5a). The two zones of activation are labeled Zone 1 and 2 (Fig. 5a). The down-regulation of Erk in the loop region is critical for normal meiotic progression of oogenic germ cells^{24,35}. Animals that lack *shc-1* (*shc-1(0)*) exhibit a continuous Erk activation pattern in the germ line (Fig. 5c), and lose the characteristic bimodal pattern of activation, and higher nuclear accumulation in oocytes (Fig. 5c, arrowheads). This higher ectopic and continuous activation of Erk is similar to that observed in a *ras* gain-of-function mutant—the *let-60* ga89(Ras^{V12G}) mutation (Fig. 5b). This mutation also results in the continuous activation of Erk and disrupts the bimodal pattern of Erk activation. Continuous activation of Erk causes the formation of multiple small oocytes in these germ lines as they age³⁶, the phenotype that causes sterility and growth defects in these differentiated meiotic cells (Supplementary Fig. 4). Because the *shc-1(0)* mutant has a similar Erk activation phenotype to a *ras* gain-of-function mutant, it suggests that—much like its vertebrate homolog—CeShc-1 can negatively regulate Erk activation and phosphorylation (Fig. 5a,b and Supplementary Fig. 3). To test the function of the Shc–Erk complex *in vivo*, we transformed WT human Shc (hShc^{WT}) into the *shc-1(0)* animals. The hShc constructs included a C-terminal GFP tag which was previously shown to have negligible effect on protein stability or function¹⁵. We found that hShc^{WT}-GFP suppressed ectopic nuclear accumulation of phosphorylated Erk in the *shc-1(0)* background, and restored the normal bimodal pattern of Erk activation typically observed in the *C. elegans* germ line^{24,35,36} (Fig. 5d). We generated five independent lines and characterized the most stable one (denoted vizIs18) in more detail. We found that the accumulation of pErk in all *shc-1(0);hShc^{WT}* lines was restored to wild type pattern (Fig. 5d). When we compared the levels of pErk accumulation between *shc-1(0);hShc^{WT}*, *shc-1(0)* and wild type animals, we find (after quantification using Image J) that the overall accumulation levels are ~40% lower in the transformed vizIs18 line compared to wild type (depicted by the dotted lines in Fig. 5d, and Supplementary Fig. 4). The difference likely resulted from a slightly higher affinity for Erk or greater cellular concentration of hShc^{WT} in vizIs18 as compared to *shc-1* animals. To confirm that hShc^{WT} is capable of binding to the *C. elegans* Erk-homolog MPK-1, we demonstrated that GST-tagged MPK-1 immobilized on agarose beads was able to pull down Shc overexpressed in HEK293T cells (Supplementary Fig. 4e).

To directly test the impact of Shc binding on Erk function, hShc^{R98Q} was expressed in the *shc-1(0)* background. We obtained 5 independent lines that had hShc^{R98Q}-GFP expression levels similar to those of the hShc^{WT}-GFP line. (Fig. 5d,e). In one of these lines, vizIs19,

we found that unlike hShc^{WT}, hShc^{R98Q} did not suppress the continuous pattern of Erk activation (Fig. 5e), and that it phenotypically resembled a *let-60 ras* gain-of-function animal (at the permissive temperature; Fig. 5b), consistent with the compromised binding of Shc^{R98Q} that results in the up-regulation of Erk activity in mammalian cells.

Morphologically, these animals are sluggish, cannot tolerate osmotic pressures, and often produce the small oocyte phenotype that results in sterility, all which are *ras* gain-of-function-like phenotypes. This suggests that Shc negatively regulates Erk function by sequestering Erk away from the canonical Ras-Raf-Mek MAPK pathway in those animals. In the absence of the Shc–Erk complex, the pathway is activated resulting in excessive Erk phosphorylation. The observations made in *C. elegans* are therefore consistent with the observations made in mammalian cells.

Phosphopeptide binding promotes Erk dissociation from Shc

The Shc–Erk complex, described above, forms in the absence of extracellular stimulation. Since the abundance of the endogenous Shc–Erk complex is reduced upon growth factor stimulation (Fig. 1), we investigated whether a ‘molecular switch’ existed that enables Erk to escape inhibitory interaction with Shc. Dissociation of the Shc–Erk complex does not appear to involve tyrosine phosphorylation of Shc by RTKs, since the ITC data showed that Erk can still bind to phosphorylated Shc ($K_D=0.8\ \mu\text{M}$. Supplementary Fig. 1 and Supplementary Table 1). Previously reported NMR spectroscopic structural data on ShcPTB indicated that the apo-form of the domain is partially disordered³⁷. Regions associated with the binding of a tyrosine phosphorylated peptide (pTrkA; derived from the TrkA nerve growth factor receptor, a physiological ligand) that are distal from the Erk binding site identified here, are particularly dynamic. The binding of pTrkA to Shc results in reorganization of the Erk binding site through local folding events which appear to trigger a conformational switch between the free and complex states^{32,37}. This conformational switch is exemplified by previously observed NMR chemical shift changes (δ) between apo- and peptide-bound ShcPTB³⁷. Considering the possible existence of an inducible conformational change between TrkA and Erk binding sites on ShcPTB, it is interesting to note that large δ values were not observed only in the $\beta 2$ - $\alpha 2$ loop region (residues 61–70; this region is in direct contact with pTrkA), but also in a sequence encompassing helix $\alpha 2$ (residues 72–87). Although not in direct contact with pTrkA, residues 75, 76, 78, 79 and 80 within ShcPTB helix $\alpha 2$ displayed the largest δ of all residues³⁷. Gln76, Arg79 and Glu80 point toward the $\alpha 2$ - $\beta 3$ loop that contains the Erk-binding Shc3R motif (Supplementary Fig. 1c). Residues from the $\alpha 2$ - $\beta 3$ loop region also experience significant δ , especially Ser103 which is facing Gln76, Arg79 and Glu80 of the $\alpha 2$ - $\beta 3$ loop³⁷. Based on these data we speculated that the observed disruption of the Shc–Erk complex by pTrkA results from an allosteric mechanism, in which pTrkA binding introduces structural changes onto the $\beta 2$ - $\alpha 2$ loop that are then propagated via $\alpha 2$ residues to the Erk-binding motif.

To confirm this allosteric model, we tested if the presence of a known phosphotyrosine peptide ligand could disrupt the Shc–Erk complex. In an ITC experiment, ShcPTB was titrated into Erk in the presence of pTrkA. No binding between Shc and Erk was observed under those conditions (Supplementary Fig. 1d), indicating that pTrkA prevents formation of the Shc–Erk complex, presumably by altering the conformation of Shc. Dissociation of the

Shc–Erk complex through phosphotyrosine ligand binding was confirmed with an *in vitro* FRET-based approach using either recombinant full-length phosphorylated cytoplasmic domain of EGFR (pEGFR_{cyto}; Fig. 6a) or pTrkA (Fig. 6b). The ShcPTB domain was C-terminally-tagged with BFP while Erk was N-terminally-tagged with GFP. The interaction of ShcPTB and Erk results in the fusion protein fluorophores being brought into proximity resulting in energy transfer between the BFP (donor) and GFP (acceptor). Addition of either the pTrkA peptide or pEGFR_{cyto} resulted in a reduction in the magnitude of the energy transfer. For example, Fig. 6a (lower panel) shows a gradual change in FRET (i.e. the donor emission fluorescence goes up whilst the acceptor emission goes down) as the concentration of pEGFR_{cyto} is increased. This confirmed that the binding of ligand to the known pY binding site of ShcPTB results in the dissociation of the Shc–Erk complex.

In addition we demonstrated that the Shc–Erk interaction was inhibited by tyrosine-phosphorylated ligand binding in a pull-down experiment in a cellular context (Fig. 6c). HEK293T cells over-expressing strep-tagged Shc^{WT} were starved overnight and the exogenous Shc precipitated by Strep-Tactin agarose beads then washed under non-stringent conditions before pTrkA or pEGFR peptides were incubated with the precipitated material. In the presence of these ligands the interaction between Erk and Shc was abrogated as evidenced by the absence of Erk from the eluted fractions. These data strongly support a mechanism for phosphotyrosine ligand-stimulated dissociation of Shc from Erk (Supplementary Fig. 1e).

DISCUSSION

Significant basal kinase activity prevails in cells in the absence of extracellular stimulation³⁸. This requires that control mechanisms be in place to prevent improper cellular responses to this activity. This work focused on the role of the adaptor protein Shc in preventing aberrant signal transduction by Erk in non-stimulated cells. We uncovered a control mechanism in which Shc binds to, and inhibits the recruitment of Erk to the MAPK pathway. This interaction occurs between a non-canonical binding interface on the ShcPTB and the N-terminal lobe of Erk. As a consequence, Shc suppresses Erk phosphorylation and, potentially, undesirable cellular outcomes such as oncogenic cell proliferation. The function of Shc as a negative regulator of Erk phosphorylation was confirmed *in vivo* in an animal model. Deletion of *shc-1* in *C. elegans* resulted in the elevation of pErk in the germ line which could be rescued by the expression of hShc. Upon growth factor stimulation, Shc binds to an appropriate RTK which induces a conformational change in the ShcPTB and results in the release of Erk from Shc. Both Shc and Erk have numerous known intracellular binding partners, and the distinct domains of Shc provide docking sites for a diverse array of signaling molecules^{39–41}. Erk also has several cognate sites for binding partners^{33,42}. However, the ShcPTB–Erk interaction maps to sites unique on the surfaces of both Shc and Erk, suggesting a specialized function. The previously reported site closest to the one we identified for the ShcPTB–Erk interaction involves the yeast scaffold protein Ste5 binding to a site on the N-terminal lobe of the Fus3—an Erk homologue⁴³. However the corresponding site on the N-terminal lobe of human Erk is occluded by Erk's own N-terminus and hence prevents ligand binding. Alignment of Shc sequences from vertebrates and invertebrates indicate that the binding site for Erk is conserved⁴⁰. The fact that there are—to date—no

reported competing interactions for this site suggest that the maintenance of the Shc–Erk complex is important for cellular homeostasis and likely disrupted only in the context of signaling. Thus control of Erk by Shc is preserved in the presence of other potential functional interactions of these proteins.

The role of Shc as an adaptor protein in stimulated cells by forming a docking platform for enzymes involved in receptor-mediated signaling is well established. The involvement of Shc in signal transduction in response to growth factor stimulation is contrasted by its function in the absence of cellular stimulation. One notable interaction in this context is the binding of Shc to protein phosphatase 2 (PP2) under basal conditions⁴⁴. Similarly to Erk, PP2 is released upon stimulation by EGF or insulin-like growth factor 1 (IGF-1). Furthermore, the Shc–PP2 complex is believed to negatively regulate growth factor signaling. This is because the presence of PP2 prevents phosphorylation of Shc and the subsequent recruitment of Grb2. Thus, although the result of the dissociation of the Shc–PP2 complex is similar to that of the Shc–Erk complex, the mechanism of regulating MAPK signaling is entirely different.

Erk activity has also been shown to be regulated by cellular proteins other than Shc. For example, the phosphoprotein enriched in astrocytes 15 (Pea-15) protein has been demonstrated to regulate the subcellular localization of Erk and control the outcomes of MAPK signaling^{45,46}. Pea-15 binds to Erk regardless of its phosphorylation status and as a result cells depleted of Pea-15 show increased levels of Erk in the nucleus. In addition, the similar expression to EGF (Sef) protein also regulates Erk cellular localization. This is achieved through Sef binding to the activated forms of Mek and preventing the dissociation of Erk from Mek⁴⁷. Again, knock down of Sef results in nuclear accumulation of Erk in stimulated cells. Importantly, although Pea-15 and Sef exert control over the spatial distribution of Erk (the latter in stimulated cells only), neither are involved in induction of MAPK signaling as shown here for Shc. Thus Shc is unique in exerting a dual influence on Erk activation and its downstream response.

ShcPTB binds to a distinct set of activated growth factor receptors including TrkA, EGFR, ErbB2, and ErbB3. The resulting tyrosine phosphorylation of Shc promotes signal transduction through the MAPK pathway¹⁵. However, this work highlights that the recruitment to an appropriate RTK also results in the release of Erk from Shc, increasing the cellular concentrations of free Erk able to increase MAPK signaling and downstream gene transcription. Shc therefore plays a central role in modulating MAPK signaling through two distinct mechanisms, and could be fundamental in controlling Erk activation-sensitive cellular outcomes of MAPK-mediated signal transduction²⁵. In the context of oncogenic activating mutations of receptors, there will be increased recruitment of Shc to pY-containing binding sites and hence elevated levels of free Erk. This will have an effect on amplification of MAPK signal transduction and hence increased proliferative potential.

Erk plays multiple roles in the acquisition of complex malignant phenotypes, hence down-regulation of Erk activity is expected to produce anti-proliferative, anti-metastatic and/or anti-angiogenic effects in tumor cells. It has already been demonstrated that reducing the cellular concentration of Erk by RNA interference leads to the suppression of tumor cell

proliferation in ovarian cancer cells¹⁹. Small molecule inhibition of Shc–RTK interaction that preserve Shc–Erk binding could have a similar effect by maintaining the engagement of the Shc–Erk complex.

Online Methods

Reagents

Antibodies for western blotting were as follows: Cell Signalling - Erk1/2 (4695), phospho-Erk1/2 (4377), EGFR (2646), tubulin (2125), laminA/C (4777), Hsp90 (4874) and GST (2625); Santa Cruz – Shc (sc-967). Antibodies for immunoprecipitations were obtained from BD Biosciences (anti-Shc, 610879); Abnova (anti-strep, PAB-16601); Santa Cruz (rabbit-IgG, sc-2027); ShRNA for Shc was obtained from Santa Cruz (sc-29480-v); Lysis Buffer (LB): 50mM HEPES pH 7.4, 50 mM NaCl, 1mM Na₃VO₄, 10mM NaF, 0.1% NP-40, 10% glycerol, supplemented with 1x protease inhibitors (Roche). pTrkA peptide (HIIENPQpYFSDA) and pEGFR peptide (TAVGNPEpYLNTVQ) were obtained from GenScript. HRP-conjugated secondary antibodies were obtained from Santa Cruz. Fluorescent secondary antibodies were obtained from LI-COR.

Cell culture

MKN28 cells and MCF7 cells were kind gifts from Dr. Peek (Vanderbilt University Medical Center) and Dr. Brown (MD Anderson Cancer Center), respectively. HEK293T, NIH3T3, MKN28 and MCF7 cells were maintained in DMEM supplemented with 10% FBS. PC12 cells were maintained in DMEM supplemented with 10% HS and 5% FBS. All cell lines used in this study were grown at 37 °C with 5% CO₂. For experimental procedures cells were starved at 80% confluency for a period of 16–24 h before stimulation with EGF. For transfections and stable cell selection cells were seeded the day before for 80% confluency on the day of transfection. Transfection was performed with Metafectene Pro according to manufacturer's instructions. Stable cell lines were maintained by 100µg/ml blasticidine (HEK293T Erk-WT/D20/100A), 160µg/ml hygromycin (HEK293T Shc WT/R98Q), 4 µg/mL puromycin (MCF7 Shc knockdowns)

Immunoprecipitation

Cells were lysed in lysis buffer and cleared by centrifugation. 1mg of lysate was used per IP experiment. Santa Cruz Optima C kit was used according to the manufacturers' protocol. Briefly, cleared lysate was incubated with pre-clearing mix for 1 h before 10 µl of anti-Shc or Strep-tactin beads was incubated overnight at 4 °C. Protein A/G beads were added for 2h. Immunoprecipitants were washed 3 times and boiled with 2X sample buffer (Bio-rad) for 5 min. Samples were then analyzed by western blotting.

Protein expression and purification

Erk2 was sub-cloned into a pET28a vector to yield His-tagged Erk2; Full-length Shc and ShcPTB (residues 1–207 of Shc) were sub-cloned into a pET28a vector as described previously¹⁵. For mammalian expression: myc-tagged Erk2 and strep-tagged Shc were cloned into pcDNA6 myc/His and pcDNA3.1 vectors, respectively. Mutants were generated by site-directed mutagenesis. Recombinant proteins were expressed in Rosetta 2 under

appropriate antibiotic selection and were carried out as follows: GST-tagged Erk2, His-tagged full-length Shc and His-tagged Erk2 were induced at $OD_{600}=0.5$ with 100 μ M IPTG at 20 °C overnight; His-tagged BFP-Shc-PTB, His-tagged GFP-Erk2 and His-tagged EGFR cytoplasmic domain were induced at $OD_{600}=0.5$ with 0.5mM IPTG at 20 °C overnight; GST-tagged SH2, GST-tagged PTB and His-tagged PTB were induced at $OD_{600} = 0.8$ with 1mM IPTG at 30 °C for 4 h. All proteins were first purified by affinity chromatography and finally by size exclusion chromatography. Purification of full-length Shc requires an additional step using ion-exchange chromatography. The EGFR cytoplasmic domain was autophosphorylated in the presence of ATP and $MgCl_2$.

Isothermal titration calorimetry (ITC)

Experiments were performed using either a VP or iTC200 instrument (GE, Northampton, MA). All experiments were done at 15 °C in 20 mM HEPES, pH 7.4, 50 mM NaCl, 3 mM EDTA and 5 mM β ME. ITC binding data were corrected for heats of dilution. ORIGIN7 software was used for data analysis using the single site model.

Fluorescence spectroscopy

All experiments were performed using the QuantaMaster-4-CW (PTI). Steady-state fluorescence was measured in triplicate at 15 °C. Spectra were measured from 400–550 nm with excitation at 383 nm. Excitation and emission bandwidths were set at 0.5 nm.

Small angle X-ray scattering

For SAXS analysis, purified recombinant protein samples of Erk and ShcPTB were dialyzed into 50 mM Hepes pH 7.4, 125 mM NaCl, 5 mM β -mercapto ethanol. For data collection, Erk was used at 3.5 and 7.0 mg/ml, and ShcPTB at 3 and 1.5 mg/ml. The 1:1 complex of Erk2-ShcPTB was prepared at 4.0 mg/ml. SAXS data were recorded at the Advanced Light Source synchrotron, beamline bl12.3.1, at 10 °C using a wavelength of 1 Å. Data were recorded between resolutions of $q = (4\pi\sin\theta)/\lambda = 0.01 - 0.32 \text{ Å}^{-1}$. The same sample was sequentially exposed to X-rays for 0.5 s, 1 s and 5 s. Samples containing buffer only were measured before and after protein samples. The buffer contributions were subtracted from protein scattering data using the oGreNew program available at SIBYLS. Data were controlled for radiation damage which would result in a higher apparent R_g of the Guinier region for subsequent sample exposures. A combined SAXS pattern was obtained through scaling and merging selected regions of buffer-subtracted scattering pattern for the 0.5s, 1s and 5s exposures. Data were analyzed using PRIMUS, GNOM, SASREF, DAMMIF, DAMMIN, CRY SOL and DAMAVER of the ATSAS program package⁴⁸. The radii of gyration R_g determined by Guinier analysis or by GNOM for Erk2-ShcPTB were very similar (30.8 ± 0.5 and $32.2 \pm 0.7 \text{ Å}$, respectively). The maximum particle diameter D_m , as determined by GNOM, was $120 \pm 5 \text{ Å}$. The average (hydrated) particle volume from 30 individual DAMMIF *ab initio* shape calculations was $99,900 \pm 8,000 \text{ Å}^3$. The R_g ($32 \pm 1 \text{ Å}$), D_m ($120 \pm 15 \text{ Å}$) and hydrated volumes ($102,000 \pm 6,000 \text{ Å}^3$) calculated from the final ShcPTB-Erk complex model agreed well with the values derived directly from the SAXS data (the variance of the calculated values comes mainly from the positioning of the flexible 37 N-terminal residues of ShcPTB). Protein regions not included in the atomic structures

were modeled by short flexibly linked protein fragments. The final structural model was energy minimized using Refmac5³¹

Fluorescence lifetime imaging microscopy of nuclear localization of pErk

The EKAR_{nuclear} reporter was transfected into cells and localizes primarily to the nucleus. It contains an N-terminal GFP, followed by a phospho-binding WW domain, a substrate peptide, an Erk-docking domain and a C-terminal RFP fusion⁴⁹. Activated Erk binds to this reporter through the Erk docking-domain and then phosphorylates the substrate peptide, which subsequently serves as a docking site for the phospho-binding domain. This binding event causes a conformational change in the reporter such that the GFP is brought into close proximity to the RFP and allows fluorescence resonance energy transfer between the two fluorophores. This results in a shortening of the lifetime of the GFP emission. A shortening of the lifetime of the GFP emission therefore indicates the presence of pErk in the nucleus. The FLIM measurements were performed on Leica TCP SP5 confocal microscope system with internal PMT FLIM detector. Samples were excited with titanium-sapphire pumped laser (Mai Tai BB, Spectral Physics) and SPC830 data and image acquisition card for time-correlated single photon counting. Data processing and analysis were carried out using B & H SPC FLIM analysis software as previously reported⁵⁰.

Nematode strains and culture

The following mutations were used: LGI: shc-1(tm1729). Standard procedures for culture and genetic manipulation of *C. elegans* strains were followed with growth at 20°C unless otherwise noted⁵¹. Descriptions of genes, alleles and phenotypes related to this study are reported elsewhere^{52–55}.

C. elegans antibody staining, image capture and processing

For antibody staining, dissected gonads (as described⁵⁶) of the indicated animals were prepared, fixed with 3% formaldehyde with 0.1 M K₂HPO₄ (pH 7.2) for 10 min. at room temperature. Dissected gonad arms were then post-fixed with 100% methanol (–20 °C) for 5 min⁵⁷. Fixed germ lines were blocked with 30% Normal goat serum (NGS) in 1X PBS with 0.1% Tween-20 (termed blocking buffer) for 1 hour at room temperature before being incubated with the desired primary antibody. anti-MAPKYT antibody (Sigma, MO) was used at 1:400, anti-GFP from Molecular Probes, Clone 3E6. Secondary antibodies were donkey anti-mouse alexa 594-goat anti-rabbit alexa 488-goat anti-rabbit alexa 594, obtained from Molecular Probes (Invitrogen, CA). In all cases the primary and secondary antibody incubations were at indicated dilutions in blocking buffer followed by washes with 1X PBS with 0.1% Tween-20 as previously described⁵⁸. For MAPKYT staining and dissections were performed as previously described⁵⁹. For most experiments, where two different genotypes were compared for antibody accumulation patterns, the gonad arms were dissected, blocked, stained, mounted and processed identically to avoid any internal biases. Fluorescent images were captured with a Zeiss Apotome microscope. All images were taken as a montage at 63X and processed identically with Adobe Photoshop v7. All images for a given antibody staining were taken with identical exposures, unless otherwise indicated. For the Measurement of dpMPK-1 staining intensity, Shc-1(tm1729) animals transformed with wild

type or p52R98Q Shc constructs termed vizIs18 and vizIs19 respectively, were synchronized at L4 stage, grown at 20 °C for 24 h, then dissected, and stained with GFP antibody and MAPKYT antibody. To measure the dpMPK-1 staining intensity, the dpMPK-1 images were imported into NIH Image J, and a line was drawn from start of pachytene stage to the end of pachytene and measured the pixel intensity represented as a graph for pachytene signal.

Luciferase assays

Stable HEK293T cells over-expressing RFP-alone, RFP-tagged WT ShcPTB domain or RFP-tagged R98Q ShcPTB domain were seeded on 24-well plates ~24 h prior to transfection. pGL4.33 and pRL-TK were co-transfected into cells in a 5:1 ratio. Cells were starved the next day for 14–16 h and luciferase activity was measured using the Dual-luciferase reporter assay (Promega) according to manufacturer's instructions.

Additional methods can be found in a supplementary note online.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Shc forms a complex with Erk in the absence of extracellular stimulation

(a–c) Western blots on Shc immunoprecipitates from (a) NIH3T3, (b) MKN28, (c) PC12 cells. The cells were serum starved overnight then stimulated with 20ng/ml EGF (NIH3T3 and MKN28 cells), or 100 ng/ml (++) and 50 ng/ml (+) EGF (PC12 cells) before lysis and precipitation with Shc antibodies, followed by immunoblotting for both Shc and Erk. (d) Purified and immobilized GST-Erk was incubated with HEK293T cell lysate and the presence of bound Shc detected by western blotting. (e) Quantitation of Erk co-precipitated with Shc (n=3, error bars represent s.d.) normalized against the loading control in non-stimulated cells. Dark grey – basal; light grey – EGF treated. (f) ITC measurement of the Shc and Erk interaction. Thirteen 3 μl injections of Erk (60 μM) were titrated into Shc (6 μM) at 15 $^{\circ}\text{C}$. Top panel: baseline-corrected power versus time plot for the titration (black) and the heat of dilution of Erk titrated into buffer (red; offset by $-0.04 \mu\text{cal/s}$). Bottom panel: the integrated heats and the molar ratio of Erk binding to Shc. The data were corrected for the heats of dilution of Erk and fit to a one-site binding model.

Figure 2. ShcPTB–Erk interaction

Western blot detection of Erk bound to Shc. Purified and immobilized GST-ShcSH2 or GST-ShcPTB was incubated with either (a) HEK293T or (b) PC12 cell lysates with or without EGF stimulation the amount of bound Erk measured by western blotting. (c) FRET analysis of the interaction between BFP-ShcPTB and GFP-Erk. Fluorescence (arbitrary units) plotted against wavelength. A FRET-induced decrease in donor (440 nm) and increase in acceptor emissions (510 nm) are observed as BFP-ShcPTB binds to GFP-Erk. Signal from the control addition of GFP alone to BFP-ShcPTB have been subtracted from the spectra. (d) ITC measurement of the ShcPTB and Erk interaction. Twenty-nine 10 μ l injections of ShcPTB (203 μ M) were titrated into Erk (20 μ M) at 15 $^{\circ}$ C. Top panel: baseline-corrected power versus time plot for the titration (black) and the heat of dilution of buffer titrated into Erk (red; offset by -0.04 μ cal/s). Bottom panel: the integrated heats and the molar ratio of ShcPTB binding to Erk.

Figure 3. Determination of the binding interface between ShcPTB and Erk

Depiction of the Erk-ShcPTB complex. **(a)** Two 90° rotated views of the atomic model of the Erk-ShcPTB complex (Erk, green, PDB 1SHC; ShcPTB, magenta, PDB 1TVO). The model was fitted in the bead volume (grey) determined independently by SAXS using *ab initio* shape reconstruction methods. See also Supplementary Fig. 1. **(b)** Two 90° rotated close-up views of a theoretical structural model of the interaction between the ShcPTB peptide (magenta stick model) and the Erk N-terminal lobe (surface representation: blue, positively charged atoms; red, negatively charged atoms; green, hydrophobic atoms; salmon, polar oxygens; marine, polar nitrogens; yellow, sulphur). The model was constructed based on all available experimental and structural data (see Methods online). **(c)** ITC measurement of the binding between Erk and ShcPTB peptide. Thirty-nine 7 μ l injections of Shc3R peptide (250 μ M) were titrated into Erk (10 μ M) at 15 °C. Top panel: baseline-corrected power versus time plot for the titration (black) and the heat of dilution of buffer titrated into Erk (22 injections shown in red; offset by $-0.04 \mu\text{cal}\cdot\text{sec}^{-1}$). Bottom panel: the integrated heats and the molar ratio of Shc3R peptide binding to Erk. **(d)** Top panel: GST-fusion of ShcPTB containing 3 different mutations (R98Q, W24A or R175Q) immobilized on agarose beads and incubated with unstimulated HEK293T lysates. The presence of bound Erk was detected by western blotting. Bottom panel: GST-Erk^{WT} or GST-Erk^{D20, 100A} were immobilized on agarose beads and incubated with unstimulated NIH3T3 cell lysates.

*Indicates non-specific protein bands.

Figure 4. Shc binding has an inhibitory effect on Erk phosphorylation

(a) HEK293T cells stably over-expressing either strep-tagged Shc^{WT}, Shc^{R98Q} or strep-tag alone (control) were starved overnight. The phosphorylation level of endogenous Erk was analyzed by western blotting. Erk is more phosphorylated in cells over-expressing Shc^{R98Q} than Shc^{WT} under non-stimulatory conditions. This blot was reproduced using fluorescent secondary antibodies (lower panels). (b) HEK293T cells stably over-expressing Myc-tagged Erk^{WT} or Erk^{D20, 100A} were starved overnight. Erk^{D20, 100A} is more phosphorylated than Erk^{WT} under non-stimulatory conditions. The blot was reproduced using fluorescent secondary antibodies (lower panels). (c) MCF7 cells were transfected with either scrambled shRNA (control) or Shc shRNA and starved overnight. 50 µg of total cell lysates were analyzed by western blotting for endogenous pErk. Shc-depleted cells exhibit a high level of pErk compared to those with the control shRNA. (d) Nuclear extraction was performed on Shc-depleted MCF7 cells. More total Erk was found in the nucleus when the endogenous Shc level is reduced. (e) HEK293T cells over-expressing WT or R98Q RFP-tagged ShcPTB or RFP-alone (control) were transiently co-transfected with SRE-firefly and Renilla luciferases. Cells were starved overnight, lysed and the luciferase activity was measured. SRE-firefly activity was normalized by Renilla-luciferase activity. The over-expression of ShcPTB^{R98Q} domain results in a higher transcription activity under the control of SRE (n = 3; error bars = standard deviation; p = 0.0027).

Figure 5. Shc controls pErk levels in *C. elegans*

Visualization of phosphorylated Erk in the *C. elegans* gonad by immunofluorescence. **(a)** Dissected adult *C. elegans* germ lines, with indicated genotypes, were stained with DAPI (blue, DNA), pErk (red) and GFP-hShc (green). All animals are in the mitotic-oocyte orientation from left to right. pErk is dynamic and in wild type germ lines. It is first activated in pachytene (Zone 1), down-regulated in loop and reactivated in oocytes (Zone 2). **(b)** *Let-60 ras* gain-of-function animals at the permissive temperature lose the bimodal pattern of activation and have ectopic and higher accumulation of pErk resulting in continuous activation of Erk. **(c)** In *shc-1(0)* animals, pErk is continuously phosphorylated from pachytene (much like *ras* gain-of-function animals), a pattern that is restored to normal by WT hShc transformation **(d)** and not rescued by the RQ mutant Shc transformation **(e)**. Arrowheads point to nuclear accumulation of Erk. Upon quantitation (Supplementary Figure 4), levels of pErk in hShc transformed animals in a *shc-1(0)* background are much lower than in *shc-1(0)* (dotted lines in **(d)** for Zone 1 and Zone 2 represent lower activation of Erk).

Figure 6. Binding of the ShcPTB domain to tyrosine phosphorylated protein inhibits Shc-Erk interaction

FRET measurements of the interaction between Shc and Erk. **(a)** Upper panel: FRET analysis of BFP-ShcPTB and GFP-Erk binding in the presence of the phosphorylated EGFR cytoplasmic domain. GFP-Erk (25 nM) was added to BFP-ShcPTB (25 nM) at 15 °C. The addition of GFP-Erk results in increasing FRET signals at 510 nm as BFP-ShcPTB binds to GFP-Erk, as shown by the decrease in donor and increase in acceptor emissions (fluorescence in arbitrary units). Insets: magnified versions of the spectral peaks (Left: BFP-ShcPTB emission, Right: GFP-Erk emission) Lower panel: Plot of the change in donor (red) and acceptor (blue) emission (measured at 440 nm and 510 nm respectively). **(b)** FRET analysis of BFP-ShcPTB and GFP-Erk binding in the presence or absence of pTrkA. GFP-Erk (87.5 nM) was added to BFP-ShcPTB (100 nM) at 15 °C. The addition of GFP-Erk results in increasing FRET signals at 510 nm as BFP-ShcPTB binds to GFP-Erk, as shown by the decrease in donor and increase in acceptor emissions. This increase in FRET signal is reduced in the presence of 2.66 μ M TrkA peptide. **(c)** Erk is displaced from immobilized Shc^{WT} precipitated from

serum starved HEK293T cells upon incubation with pTrkA or pEGFR peptides. The western blots were re-probed using a fluorescent secondary antibody (lower panels).