Protein tyrosine phosphatase SHP2 suppresses podosome rosette formation in Src-transformed fibroblasts

Yi-Ru Pan[1,*], Ke-Huan Cho[1,*], Hsiao-Hui Lee[2], Zee-Fen Chang[3] and Hong-Chen Chen[1,4,‡]

[1]Department of Life Sciences, National Chung Hsing University, Taichung 402, Taiwan
[2]Department of Life Sciences and Institute of Genome Sciences, National Yang-Ming University, Taipei 112, Taiwan
[3]Institute of Biochemistry and Molecular Biology, National Yang-Ming University, Taipei 112, Taiwan
[4]Agricultural Biotechnology Center, National Chung Hsing University, Taichung 402, Taiwan

*These authors contributed equally to this work
‡Author for correspondence (hcchen@nchu.edu.tw)

Accepted 29 October 2012
Journal of Cell Science 126, 657–666

doi: 10.1242/jcs.116624

Summary

Podosomes are actin-enriched membrane protrusions that play important roles in extracellular matrix degradation and invasive cell motility. Podosomes undergo self-assembly into large rosette-like structures in Src-transformed fibroblasts, osteoclasts and certain highly invasive cancer cells. Several protein tyrosine kinases have been shown to be important for the formation of podosome rosettes, but little is known regarding the role of protein tyrosine phosphatases in this process. We found that knockdown of the Src homolog domain-containing phosphatase 2 (SHP2) significantly increased podosome rosette formation in Src-transformed fibroblasts. By contrast, SHP2 overexpression suppressed podosome rosette formation in these cells. The phosphatase activity of SHP2 was essential for the suppression of podosome rosette formation. SHP2 selectively suppressed the tyrosine phosphorylation of Tks5, a scaffolding protein required for podosome formation. The inhibitory effect of SHP2 on podosome rosette formation was associated with the increased activation of Rho-associated kinase (ROCK) and the enhanced polymerization of vimentin filaments. A higher content of polymerized vimentin filaments was correlated with a lower content of podosome rosettes. Taken together, our findings indicate that SHP2 serves as a negative regulator of podosome rosette formation through the dephosphorylation of Tks5 and the activation of ROCK-mediated polymerization of vimentin in Src-transformed fibroblasts.

Key words: Phosphatase, SHP2, Podosome, Src, ROCK, Vimentin

Introduction

The reciprocal regulation of protein tyrosine phosphorylation by protein tyrosine kinases and phosphatases is involved in the control of many important cellular structures and functions. The Src homolog domain-containing phosphatase 2 (SHP2), which is a cytoplasmic protein tyrosine phosphatase (PTP), has been found to play an important role in a variety of cellular functions, including cell migration (Yu et al., 1998), cell proliferation (Qu and Feng, 1998), and cell survival (Yang et al., 2006). However, the role of SHP2 in tumorigenesis remains controversial. SHP2 has been suggested to function as an oncogene by several studies (Aceto et al., 2012; Matozaki et al., 2009; Xu, 2007) and as a tumor suppressor by others (Bard-Chapeau et al., 2011). Several cellular proteins have been shown to be substrates of SHP2, including Src (Peng and Cartwright, 1995), focal adhesion kinase (FAK; Tsutsumi et al., 2006), paxillin (Ren et al., 2004), and Gab1 (Montagner et al., 2005). The dephosphorylation of Src at Y527 by SHP2 induces Src activation, which may lead to activation of the Ras/ERK signaling pathway (Zhang et al., 2004). The dephosphorylation of the focal adhesion proteins FAK and paxillin by SHP2 has been suggested to regulate focal adhesion dynamics and cell motility (Mañes et al., 1999). We have shown previously that the phosphorylation of Rho-associated kinase (ROCK) II at tyrosine 722 (Y722) by Src leads to its inhibition (Lee et al., 2010), whereas the dephosphorylation of this site by SHP2 potentiates its activation by RhoA (Lee and Chang, 2008). The reciprocal regulation of ROCK II by Src and SHP2 is important for cell contractility and motility (Lee et al., 2010).

Podosomes are F-actin-enriched structures that are important for extracellular matrix degradation and invasive cell motility (Linder, 2007). Podosomes often undergo self-assembly into large rosette-like structures in Src-transformed fibroblasts, osteoclasts, endothelial cells, and some highly invasive cancer cells (Linder and Aepfelbacher, 2003). Podosome rosettes are more potent than individual podosome dots in matrix degradation (Pan et al., 2011). Podosome rosettes are dynamic structures that have lifespans ranging from minutes to hours (Pan et al., 2011). Many scaffolding proteins, such as Tks5 (Seals et al., 2005; Stylli et al., 2009), cortactin (Oser et al., 2009), and paxillin (Badowski et al., 2008), are essential components of podosomes. The tyrosine phosphorylation of these proteins has been shown to be important for podosome formation. Several protein tyrosine kinases, such as Src and FAK, are important for the organization and function of podosome rosettes (Destaing et al., 2008; Pan et al., 2011). However, little is known regarding the identity and mechanisms of the PTPs that are involved in podosome rosette formation. The aim of the present study is to identify the PTPs

Journal of Cell Science

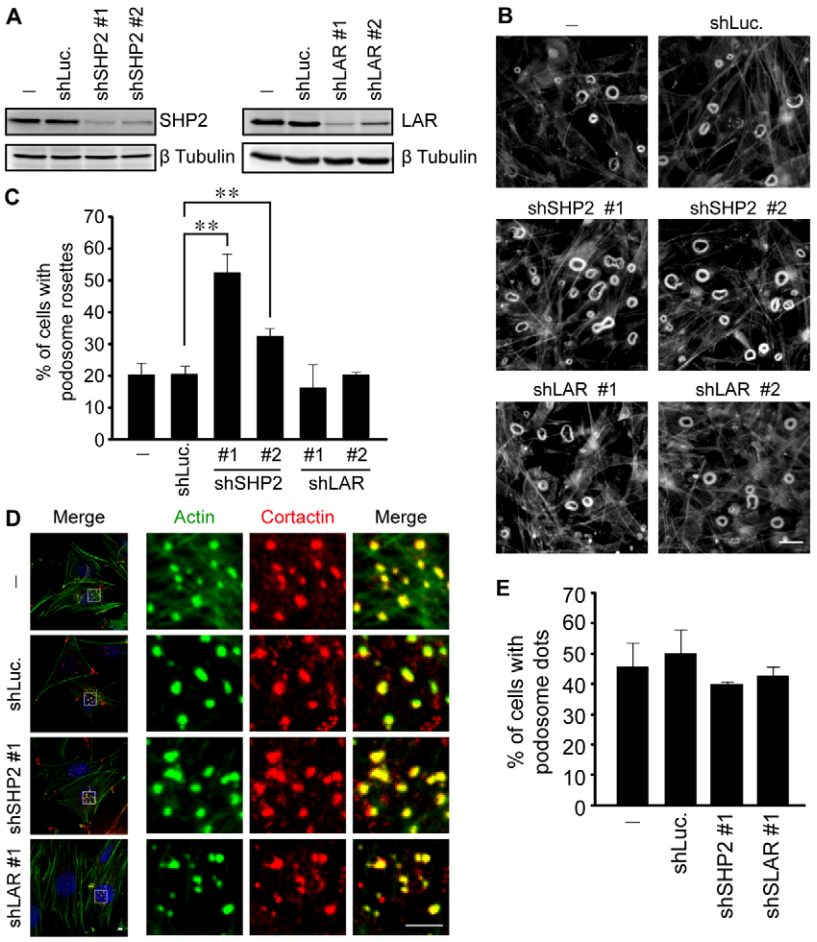

Fig. 1. Knockdown of SHP2 by shRNA increases the formation of podosome rosettes in Src-transformed MEFs. (**A**) v-Src-transformed MEFs were infected with recombinant lentiviruses encoding shRNAs specific to luciferase (shLuc), SHP2 (shSHP2; clones #1 and #2), or LAR (shLAR; clones #1 and #2) and then selected in the medium containing puromycin. Equal amounts of whole cell lysates were analyzed by immunoblotting with the indicated antibodies. (**B**) Cells (2×10^5) were grown on fibronectin-coated glass coverslips for 48 hours and then stained for F-actin. Scale bar: 20 μm. (**C**) The formation of podosome rosettes was quantified and expressed as the percentage of cells containing podosome rosettes relative to the total counted cells ($n\geq400$). (**D**) v-Src-transformed MEFs were infected with recombinant lentiviruses encoding shRNAs specific to luciferase (shLuc.), SHP2 (shSHP2; clone #1), or LAR (shLAR; clone #1). The cells were seeded on fibronectin-coated coverslips for 48 hours and then fixed. The fixed cells were stained for F-actin and cortactin as a marker for podosomes. Scale bar: 5 μm. (**E**) Podosome dots were quantified and expressed as the percentage of the cells containing podosome dots in total counted cells ($n\geq200$). Values (mean ± s.d.) are from three independent experiments; $^{**}P<0.005$.

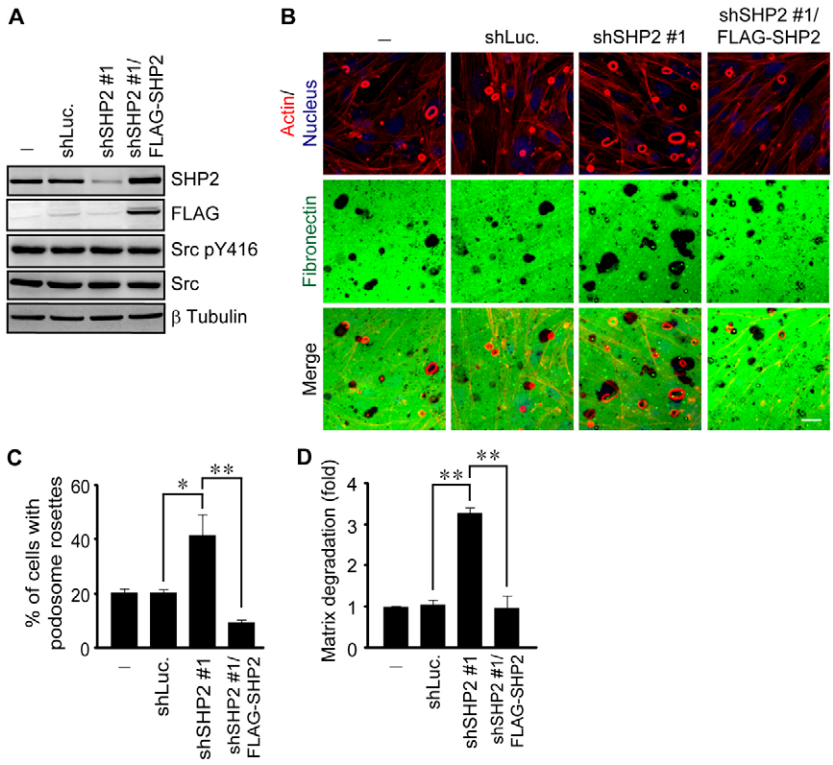

Fig. 2. Re-expression of FLAG-SHP2 reverses the effect of SHP2 knockdown on podosome rosette formation. (**A**) shRNAs specific to SHP2 (shSHP2; clone #1) or luciferase (shLuc) were stably expressed in v-Src-transformed MEFs. FLAG-tagged SHP2 was re-expressed in the cells expressing shSHP2#1 (shSHP2#1/FLAG-SHP2). Equal amounts of whole cell lysates were analyzed by immunoblotting with the indicated antibodies. (**B**) Cells were grown on Alexa-Fluor-488-conjugated fibronectin for 48 hours and then stained for F-actin and nuclei. The dark areas in the images (middle row) represent the areas in which the fibronectin was degraded. Scale bar: 20 μm. (**C**) Quantitative results for podosome rosette formation. Cells were grown on fibronectin-coated glass coverslips for 48 hours and stained for F-actin. The percentage of the cells containing podosome rosettes relative to the total counted cells ($n\geq400$) was determined. (**D**) Quantitative results from the matrix degradation assay. The data are expressed as fold relative to the level of the control v-Src-transformed MEFs. Values (mean ± s.d.) are from at least three independent experiments; $^{*}P<0.05$; $^{**}P<0.005$.

that are involved in podosome rosette formation using v-Src-transformed mouse embryo fibroblasts (MEFs) as a model.

Results

SHP2 plays an inhibitory role in the formation of podosome rosettes in Src-transformed MEFs

A short-hairpin RNA (shRNA) approach was used to identify the PTPs that are important for podosome rosette formation in Src-transformed MEFs. Among the examined shRNAs, those specific to SHP2 (sh-SHP2) appeared to increase the formation of podosome rosettes (supplementary material Fig. S1). We found that the shRNA-mediated knockdown of SHP2, but not LAR (a transmembrane PTP), significantly increased the formation of podosome rosettes in Src-transformed MEFs (Fig. 1). The stimulatory effect of SHP2 knockdown on the podosome rosette formation was also observed in another two independent clones of Src-transformed MEFs (supplementary material Fig. S2). This increase by SHP2 knockdown was suppressed by the re-expression of FLAG-tagged human SHP2 (FLAG-SHP2) (Fig. 2), indicating that the shRNA was specific to SHP2. In addition, the overexpression of FLAG-SHP2 apparently inhibited podosome rosette formation in Src-transformed MEFs (Fig. 3). The formation of podosome rosettes was correlated with the capability of the cells to degrade matrix proteins and to invade through Matrigel (Figs 2, 3), which was in keeping with our previous findings (Pan et al., 2011). Although SHP2 was previously reported to modulate Src activity (Zhang et al., 2004), we found that the Y416 phosphorylation of Src was not affected by SHP2 in Src-transformed MEFs (Figs 2, 3), suggesting that the inhibitory effect of SHP2 on podosome rosette formation is not due to its effect on Src. We also observed that the knockdown of SHP2 or LAR had no effect on individual podosome dots in Src-transformed MEFs (Fig. 1D,E). These findings together suggest that SHP2 negatively regulates the formation of podosome rosettes but not of podosome dots in Src-transformed fibroblasts.

Phosphatase activity of SHP2 is required for the suppression of podosome rosette formation by SHP2 but not for the localization of SHP2 in podosome rosettes

FLAG-SHP2 and its phosphatase-defective mutant (C459S mutant) were both found to be localized in podosome rosettes (Fig. 4C), indicating that the phosphatase activity of SHP2 is not required for its localization in podosome rosettes. However, the C459S mutant was deficient in the ability to suppress podosome rosette formation (Fig. 4E), indicating that the catalytic activity of SHP2 is important for the downregulation of podosome rosette formation in Src-transformed fibroblasts. A SHP2 mutant with deletion of both its Src homolog domain 2 (SH2) domains was found to be diffusively distributed in the cytoplasm and not localized in podosome rosettes (Fig. 4C). This finding suggests that the interaction of the SH2 domains with certain tyrosine phosphorylated proteins in podosome rosettes is important for the targeting of SHP2 to rosettes. Although the PTP domain per se did not specifically target podosome rosettes (Fig. 4C), it inhibited rosette formation (Fig. 4E). Because the PTP domain per se displayed strong catalytic activity (Fig. 4B), it is possible that the dephosphorylation of certain podosomal scaffolding proteins by the SHP2 PTP domain may prevent the initial assembly of podosome rosettes.

SHP2 selectively decreases tyrosine phosphorylation of the podosomal protein Tks5

The tyrosine phosphorylation of Tks5 has been shown to be important for the formation of podosomes and invadopodia (Seals et al., 2005; Stylli et al., 2009). We found that SHP2 selectively inhibited the tyrosine phosphorylation of Tks5 in Src-transformed MEFs (Fig. 5A). In contrast, the tyrosine phosphorylation of several other scaffolding proteins, including FAK, paxillin, cortactin, and p130Cas, was not affected by SHP2 (Fig. 5A; supplementary material Fig. S3A). Tks5 was apparently co-precipitated with the substrate-trapping mutant (D425A/C459S)

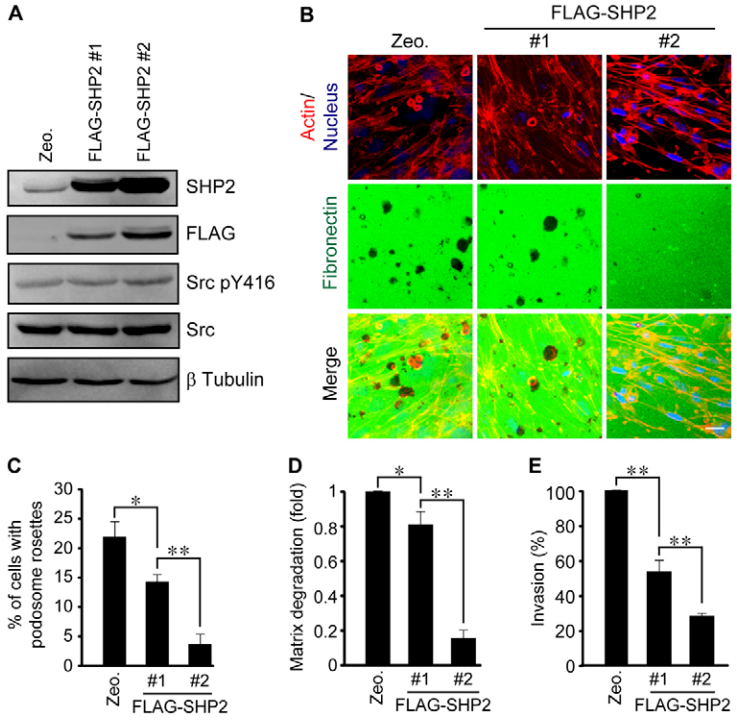

Fig. 3. Overexpression of FLAG-SHP2 suppresses podosome rosette formation. (**A**) Equal amounts of whole cell lysates from v-Src-transformed MEFs stably overexpressing FLAG-SHP2 (clones #1 and #2) and the Zeocin-resistant control clone (Zeo) were analyzed by immunoblotting with the indicated antibodies. (**B**) Cells were grown on Alexa-Fluor-488-conjugated fibronectin for 48 hours and then stained for F-actin and nuclei. The dark areas in the images (middle row) represent the areas in which the fibronectin was degraded. Scale bar: 20 μm. (**C**) Quantitative results for podosome rosette formation. Cells were grown on fibronectin-coated glass coverslips for 48 hours and stained for F-actin. The percentage of the cells containing podosome rosettes relative to the total counted cells ($n \geq 400$) was determined. (**D**) Quantitative results of the matrix degradation assay. The data are expressed as fold relative to the level of the control v-Src-transformed MEFs. (**E**) Quantitative results of the Matrigel invasion assay. The data are expressed as percentage relative to the level of the control v-Src-transformed MEFs, which defines as 100%. Values (mean ± s.d.) are from at least three independent experiments; $^{*}P<0.05$; $^{**}P<0.005$.

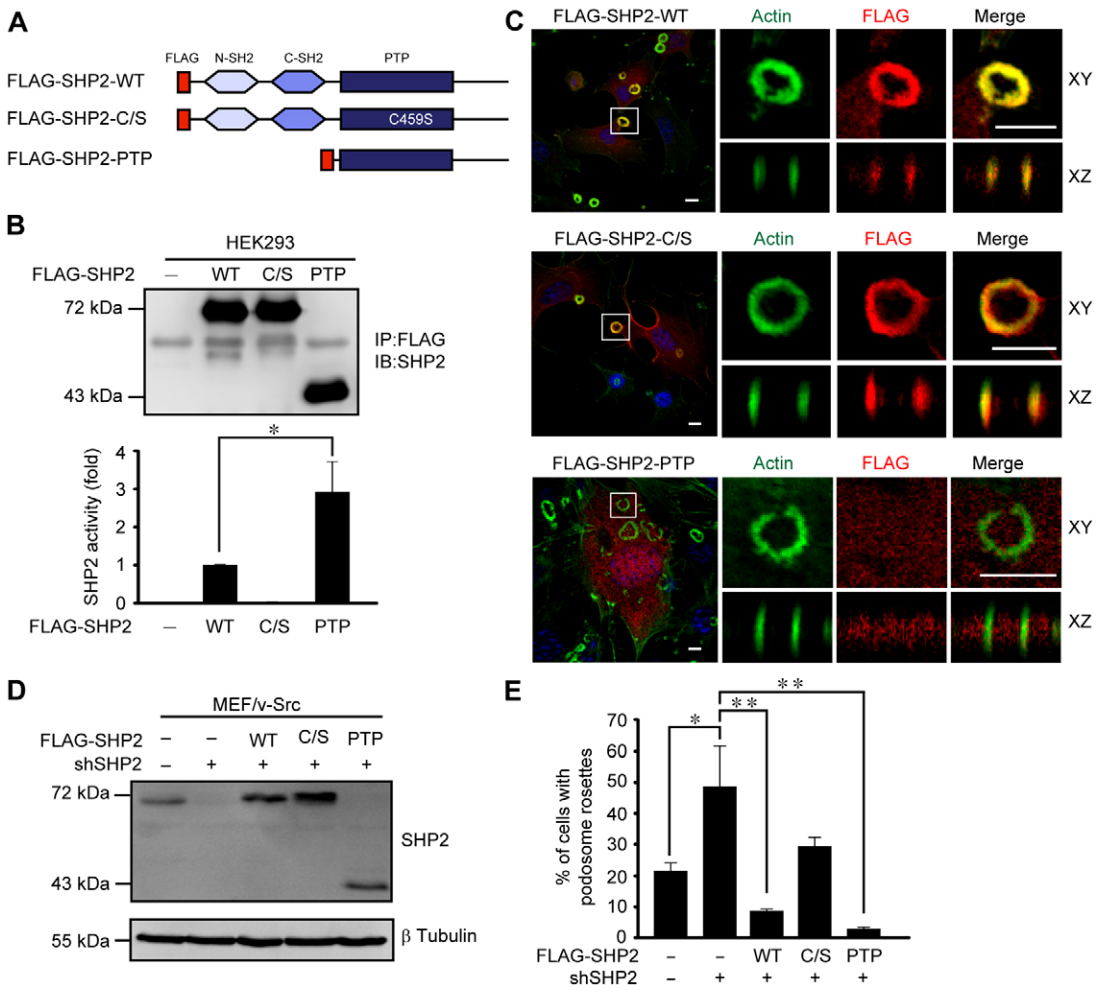

Fig. 4. Phosphatase activity of SHP2 is not required for the localization of SHP2 in podosome rosettes but is essential for the suppression of podosome rosette formation. (**A**) Schematic representation of wild-type FLAG-SHP2 (FLAG-SHP-WT) and its mutants. A single substitution of Cys549 by Ser (FLAG-SHP-C/S) abolishes the phosphatase activity of SHP2. A mutant in which both of the SH2 domains (FLAG-SHP2-PTP) are deleted contains the PTP domain alone (aa 268–593). (**B**) FLAG-SHP2 and its mutants were transiently expressed in HEK293 cells. Their phosphatase activities were analyzed using ρNPP as the substrate. The precipitated FLAG-SHP2 proteins were analyzed by immunoblotting (IB) with anti-SHP2. The phosphatase activity was quantified and expressed as fold relative to the level of FLAG-SHP2-WT. (**C**) FLAG-SHP2 and its mutants were transiently expressed in Src-transformed NIH3T3 cells. The cells were fixed and then stained for F-actin, FLAG epitope-tagged proteins and nuclei. The Z-stack images were obtained and reconstituted by confocal microscopy. The *XY* and *XZ* sections of the selected area containing a podosome rosette are shown. Scale bars: 10 μm. (**D**) FLAG-SHP2 and its mutants were stably expressed in v-Src-transformed MEFs in which endogenous SHP2 had been depleted by shRNA. Equal amounts of whole cell lysates were analyzed by immunoblotting with anti-SHP2. (**E**) The cells as described in D were fixed and stained for F-actin. The percentage of the cells containing podosome rosettes relative to the total counted cells ($n \geq 400$) was determined. Values (mean ± s.d.) are from three independent experiments; $^{*}P<0.05$; $^{**}P<0.005$.

of SHP2 (Fig. 5B) and directly dephosphorylated by SHP2 *in vitro* (Fig. 5C).

Activation of ROCK by SHP2 suppresses the formation of podosome rosettes

Rho and ROCK have been shown to be involved in the regulation of podosome rosette formation (Moreau et al., 2003; Pan et al., 2011; Varon et al., 2006). We reported recently that Src phosphorylates ROCK II at Y722, leading to the inhibition of ROCK II activity (Lee et al., 2010). Conversely, the dephosphorylation of ROCK II at Y722 by SHP2 potentiates its activation by RhoA (Lee and Chang, 2008). In the present study, we found that although the activity and expression of Rho, Rac, and Cdc42 were not affected by SHP2 in Src-transformed fibroblasts (supplementary material Fig. S3B), the Y722 phosphorylation of ROCK II was inversely correlated with SHP2 expression (Fig. 6A,B). The reduction in Y722 phosphorylation of ROCK II by SHP2 was correlated with an increase in ROCK II activity (Fig. 6C,D).

The ROCK inhibitor Y27632 was found to reverse the inhibitory effect of SHP2 on podosome rosette formation (Fig. 7A), supporting the proposed role of ROCK in the SHP2-mediated suppression of podosome rosette formation. To test the idea that ROCK II is a negative regulator of podosome rosette formation, Myc-tagged ROCK II and its mutants were transiently expressed in Src-transformed 3T3 cells and their effects on podosome rosette formation were measured (Fig. 7B). The Y722F mutant (which is refractory to phosphorylation by Src)

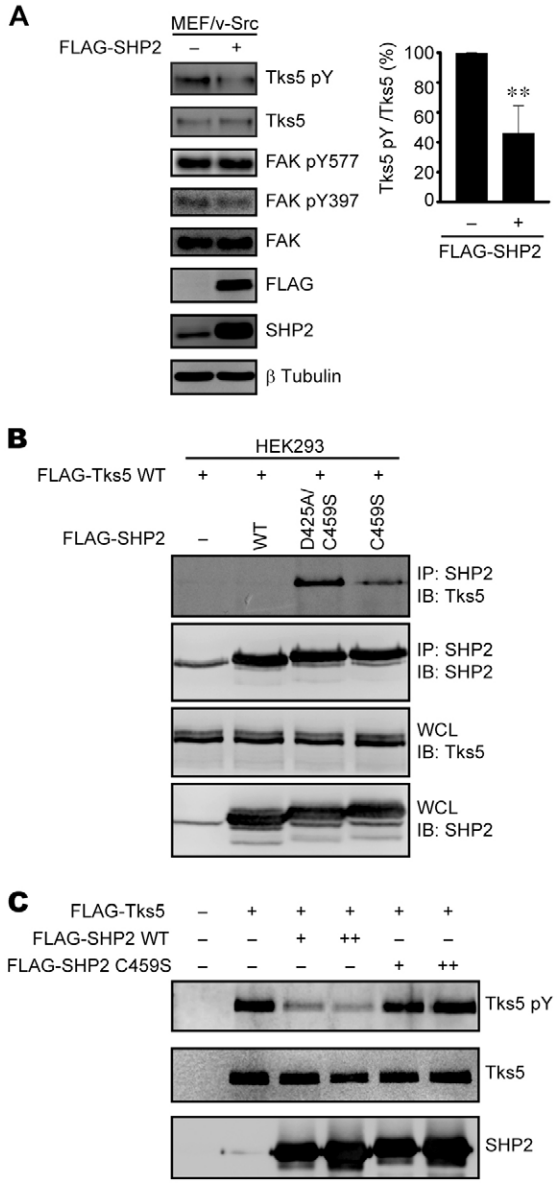

Fig. 5. SHP2 selectively dephosphorylates Tks5 in Src-transformed MEFs. (**A**) Equal amounts of cell lysates from v-Src-transformed MEFs with (+) or without (−) FLAG-SHP2 overexpression were analyzed for the tyrosine phosphorylation of Tks5 and FAK. The tyrosine phosphorylation of Tks5 was quantified and expressed as a percentage relative to the level in cells without FLAG-SHP2 overexpression, which was defined as 100%. Values (mean ± s.d.) are from three independent experiments; $^{**}P<0.005$. (**B**) Wild-type FLAG-Tks5 was transiently coexpressed with (+) or without (−) SHP2 or its mutants in HEK293 cells. SHP2 proteins were immunoprecipitated (IP) with anti-SHP2 and the immunocomplexes were analyzed by immunoblotting (IB) with anti-Tks5 or anti-SHP2. An equal amount of whole cell lysates (WCL) was analyzed by immunoblotting with anti-Tks5 or anti-SHP2. (**C**) HEK293 cells were transiently transfected with the plasmids encoding wild-type or mutant FLAG-SHP2 (+, 1 μg; ++, 2 μg). FLAG-SHP2 was immunoprecipitated by anti-FLAG and the immunocomplexes were subjected to an *in vitro* phosphatase assay in the presence (+) or absence (−) of purified FLAG-Tks5. The tyrosine phosphorylation of Tks5 was analyzed by immunoblotting with anti-phosphotyrosine. WT, wild-type; C459S, phosphatase-deficient mutant; D425A/C459S, substrate-trapping mutant.

displayed a stronger suppressing effect on podosome rosette formation than did the wild-type ROCK II. The constitutively active form of ROCK II completely inhibited the podosome rosette formation (Fig. 7B). In contrast, knockdown of endogenous ROCK II significantly increased the formation of podosome rosettes in Src-transformed MEFs (Fig. 7C).

SHP2 promotes the polymerization of vimentin intermediate filaments, which counteracts the formation of podosome rosettes

We demonstrated previously that the Rho-ROCK axis promotes the polymerization of vimentin intermediate filaments (VIFs) and thereby inhibits the formation of podosome rosettes (Pan et al., 2011). We found in the present study that the formation of VIFs was suppressed by the knockdown of SHP2 (Fig. 8A) but was promoted by the overexpression of SHP2 (Fig. 8B). A higher content of polymerized VIFs was correlated with a lower content of podosome rosettes (Figs 2, 3). The enhancement of VIF formation by SHP2 overexpression was suppressed by the ROCK inhibitor Y27632 (Fig. 8B), with a concomitant increase of podosome rosettes (Fig. 7A). These findings, taken together, suggest that the SHP2-mediated suppression of podosome rosette formation occurs, at least in part, through ROCK activation and VIF polymerization.

To directly measure the effect of ROCK on VIFs, ROCK II was suppressed or overexpressed in Src-transformed MEFs. The knockdown of endogenous ROCK II apparently decreased the content of polymerized VIFs in the cells (Fig. 8C). The transient expression of the constitutively active form of ROCK II enhanced the polymerization of VIFs (Fig. 8D). In contrast, the kinase-deficient mutant (K121G) of ROCK II had no such effect (Fig. 8D). These results suggest that the catalytic activity of ROCK II is required for promoting VIFs.

Discussion

In this study, we report that SHP2 functions as a negative regulator of podosome rosette formation in Src-transformed fibroblasts (Figs 1–3). Based on our findings, we propose that SHP2 suppresses podosome rosette formation through at least two routes: (i) the dephosphorylation of certain critical podosomal scaffolding proteins (e.g. Tks5); (ii) ROCK-mediated VIF polymerization (Fig. 9). The phosphatase activity of SHP2 is not required for the targeting of SHP2 to podosome rosettes but is essential for the suppression of podosome rosette formation by SHP2 (Fig. 4). Consistent with this finding, the tyrosine phosphorylation of Tks5 was selectively decreased by SHP2 (Fig. 5). Tks5 is a Src substrate and its tyrosine phosphorylation is important for podosome formation (Seals et al., 2005; Stylli et al., 2009). Tks5 has never been reported to be a substrate of SHP2 to the best of our knowledge. Although FAK is a known SHP2 substrate (Tsutsumi et al., 2006), its tyrosine phosphorylation was not affected by SHP2 in Src-transformed fibroblasts (Fig. 5).

We demonstrated previously that depletion of FAK leads to activation of Rho and ROCK and polymerization of VIFs and that these processes inhibit the assembly of podosome rosettes (Pan et al., 2011). In the present study, the SHP2 depletion reduced ROCK II activity, whereas SHP2 overexpression activated ROCK II (Fig. 6). The increased activation of ROCK by FAK depletion (Pan et al., 2011) or by SHP2 overexpression (present study) leads to enhanced VIF polymerization (Fig. 8).

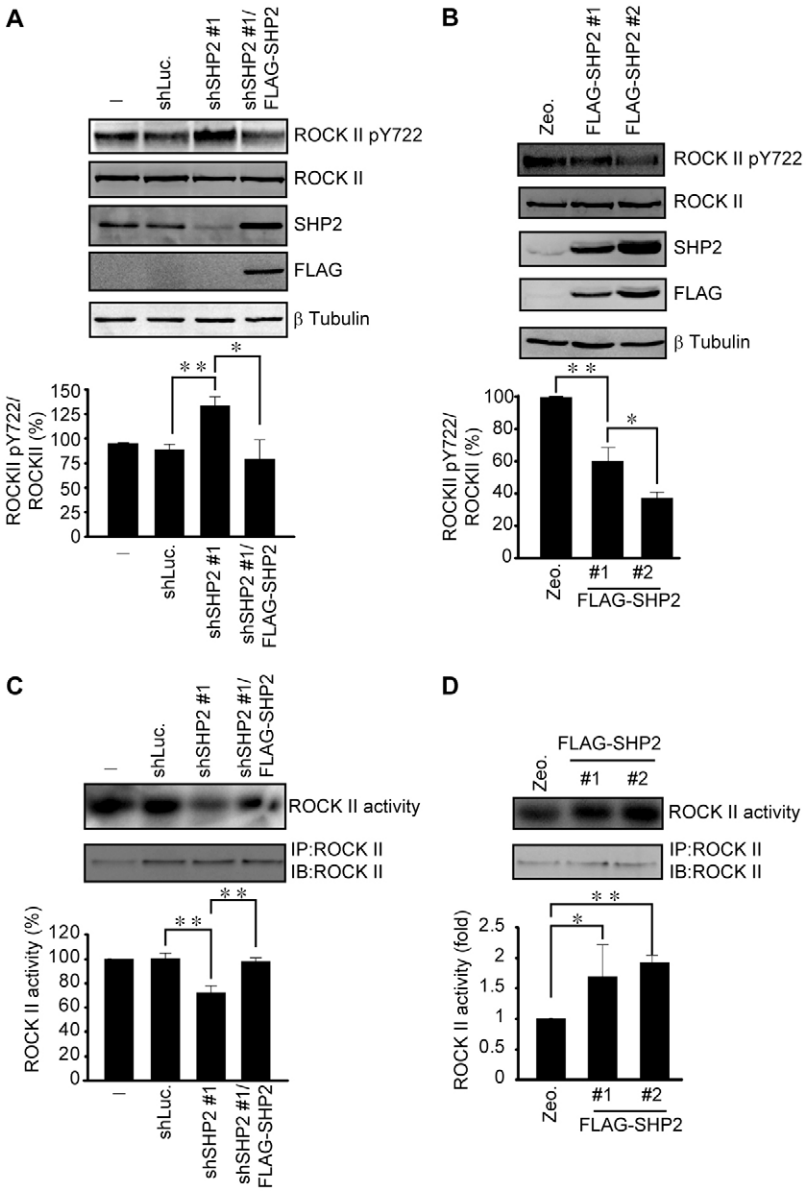

Fig. 6. SHP2 activates ROCK II through the dephosphorylation of Y722 of ROCK II in Src-transformed cells. (**A**) shRNAs specific to SHP2 (shSHP2; clone #1) or luciferase (shLuc) were stably expressed in v-Src-transformed MEFs. FLAG-SHP2 was re-expressed in the cells expressing shSHP2#1 (shSHP2#1/FLAG-SHP2). The phosphorylation of ROCKII at Y722 was analyzed and expressed as a percentage relative to the level in the control Src-transformed MEFs, which was defined as 100%. (**B**) The phosphorylation of ROCKII at Y722 in Src-transformed MEFs stably overexpressing FLAG-SHP2 (clones #1 and #2) and in a Zeocin-resistant control clone (Zeo) was analyzed. The data are expressed as percentages relative to the level in control Src-transformed MEFs, which was defined as 100%. (**C**) The kinase activity of ROCK II in the cells described in A was analyzed by an *in vitro* kinase assay using myelin basic proteins as the substrate. Data are expressed as a percentage relative to the level of the control Src-transformed MEFs, which was defined as 100%. (**D**) The kinase activity of ROCK II in the cells described in B was analyzed by an *in vitro* kinase assay using myelin basic proteins as the substrate. Data are expressed as fold relative to the level of the Zeocin-resistant control clone (Zeo). Values (mean ± s.d.) are from three independent experiments; $*P<0.05$; $**P<0.005$.

We provide direct evidence to support that ROCK II facilitates VIF polymerization. Our results indicate that a higher content of polymerized VIFs was correlated with a lower content of podosome rosettes, in keeping with our previous findings (Pan et al., 2011). The mechanism for the inhibitory effect of VIFs on podosome rosettes is currently under investigation.

To assess the possible role of SHP2 in the podosomal organization of other types of cells such as osteoclasts, we depleted SHP2 in mouse RAW264.7 cells, which can differentiate into osteoclast-like cells by receptor activator of NFκB ligand (RANKL; Boyle et al., 2003). However, SHP2 depletion did not appear to affect the podosomal organization of such osteoclast-like cells (supplementary material Fig. S4), indicating that SHP2 is not involved in the regulation of podosomal organization in osteoclasts. PTPε was shown to play a positive role in the podosomal organization and bone absorption function of osteoclasts (Chiusaroli et al., 2004). Taken together, these findings suggest that SHP2 may affect podosome rosette formation in certain types of cells but not in others. Experiments are in progress to examine the effect of SHP2 on podosome rosette formation in endothelial cells and carcinoma cells.

Podosomes and invadopodia are similar structures and are both important in extracellular matrix degradation and invasive cell motility (Linder, 2007). Very little is known regarding the role of PTPs in these structures. Only two PTPs have been reported to participate in the regulation of podosomes or invadopodia: PTPε in osteoclasts (Chiusaroli et al., 2004) and PTP1B in breast cancer cells (Cortesio et al., 2008). The stimulatory effects of PTPε and PTP1B on podosomes/invadopodia are through activating Src (Chiusaroli et al., 2004; Cortesio et al., 2008). In fact, many prior reports support a critical role of Src in podosomes/invadopodia in various types of cells. Src appears to be generally involved in podosome formation. We propose that when Src is highly activated, it phosphorylates and inhibits ROCK, which facilitates the formation of podosome rosettes. Under such circumstances, SHP2 becomes to counteract the

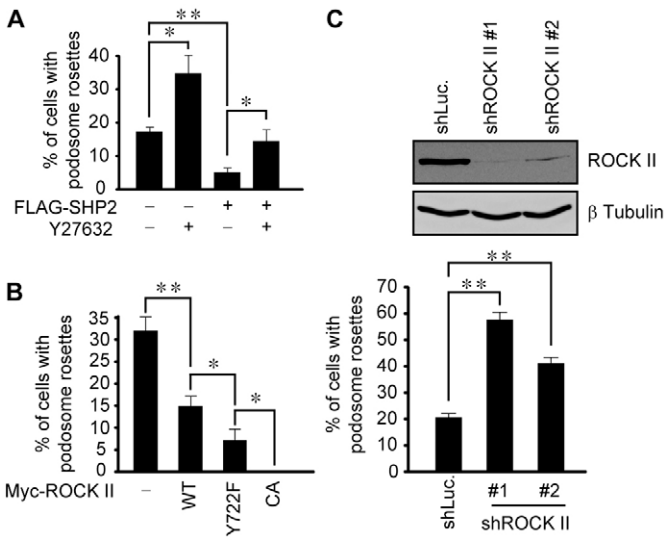

Fig. 7. ROCK plays a negative role in the formation of podosome rosettes. (**A**) v-Src-transformed MEFs stably expressing FLAG-SHP2 were grown on fibronectin-coated coverslips for 48 hours, treated with (+) or without (−) 10 μM Y27632 for 2 hours, fixed and stained for F-actin. The percentage of cells containing podosome rosettes relative to the total counted cells ($n \geq 400$) was determined. (**B**) Src-transformed NIH3T3 cells were grown on fibronectin-coated coverslips and transiently transfected with plasmids encoding Myc-tagged wild-type ROCK II or its mutants. The cells were fixed and stained for F-actin and Myc-tagged proteins. The percentage of the cells with podosome rosettes relative to the total counted cells ($n \geq 50$) expressing Myc-ROCK II was measured. WT, wild-type; Y722F, mutant refractory to phosphorylation by Src; CA, constitutively active. (**C**) v-Src-transformed MEFs were infected with recombinant lentiviruses encoding shRNAs specific to luciferase (shLuc.) or ROCK II (shROCK; clones #1 and #2). An equal amount of whole cell lysates was analyzed by immunoblotting with the indicated antibodies. The cells were grown on fibronectin-coated glass coverslips for 48 hours and then fixed. The fixed cells were stained for F-actin. The percentage of the cells containing podosome rosettes in total counted cells ($n \geq 500$) was determined. Values (mean ± s.d.) are from three independent experiments; $^{*}P<0.05$; $^{**}P<0.005$.

action of Src on ROCK in order to facilitate the dynamics of podosomal structures. However, it has been reported that SHP2 can also activate Src by dephosphorylating Src Y527 (Zhang et al., 2004). From this point of view, SHP2 may play a positive role in podosome formation by activating Src. Therefore, the role of SHP2 in podosomes may be cell-type and/or stimulus-dependent. We have demonstrated here that SHP2 plays an inhibitory role in podosome rosette formation in Src-transformed fibroblasts. Additional studies are needed to clarify the mechanisms whereby PTPs affect the formation of podosomes/invadopodia and the assembly of podosomal super structures in various types of cells.

Materials and Methods

Materials

Polyclonal anti-Cdc42, anti-cortactin (H-191), anti-Tks5 (M300), and anti-ROCK II (H-85 for immunoblotting) antibodies and monoclonal anti-SHP2 (B-1), anti-Myc (9E10), anti-RhoA (26C4), and anti-β tubulin (D-10) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal anti-Myc antibody was purchased from Cell Signaling Technology (Beverly, MA). Monoclonal anti-p130Cas, anti-LAR, anti-FAK, anti-phosphotyrosine (4G10), anti-paxillin, and anti-Rac1 antibodies, and Matrigel were from BD Transduction Laboratories (San Jose, CA). Monoclonal anti-FLAG, and anti-vimentin (clone VIM 13.2 for immunofluorescence staining) antibodies, myelin basic protein (MBP), gelatin, ρ-NPP (para-nitrophenylphosphate), and protein A-Sepharose beads were from Sigma-Aldrich (St Louis, MO). Polyclonal anti-FAK pY577, anti-FAK pY397, anti-paxillin pY118, and anti-Src pY416 antibodies, Dulbecco's modified Eagle's medium (DMEM), Zeocin, and Lipofectamine were from Life Technologies-Invitrogen (Carlsbad, CA). Polyclonal anti-ROCK II (for immunoprecipitation) antibody, fibronectin, puromycin, and Y27632 were from Millipore (Billerica, MA). Polyclonal anti-ROCK II pY722 antibody was prepared as described previously (Lee and Chang, 2008). Mouse ascites containing monoclonal anti-Src (peptide 2-17) antibody produced by hybridoma (CRL-2651) was prepared in our laboratory.

Plasmids

The plasmids pFLAG-CMV2-human SHP2 WT and C459S were kindly provided by D.-L. Wang (Tzu Chi University, Hualien, Taiwan). The plasmids pLKO-AS2.zeo-FLAG-SHP2 WT, C459S, and PTP [amino acids (aa) 268–593] were constructed in our laboratory. The plasmids pEF-myc-ROCKII WT and Y722F were described previously (Lee and Chang, 2008). pEF-Bos-myc-ROCK C.A. (constitutively active; aa 85–355) was described previously (Chen et al., 2002). The plasmids pEF-myc-ROCK II KD (aa 6–553; K121G) and pFLAG-CMV2-human SHP2 D425A/C459S (substrate-trapping mutant) were kindly provided by H.-H. Lee (National Yang-Ming University, Taipei, Taiwan). The plasmid pCMV-Tag-2B-Tks5 was kindly provided by T. Oikawa (Keio University, Tokyo, Japan) and described previously (Oikawa et al., 2012).

Cell culture and transfection

v-Src-transformed MEFs and SrcY527F-transformed NIH3T3 cells were described previously (Pan et al., 2011). For transient expression, cells were transfected with plasmids encoding FLAG-SHP2, FLAG-Tks5, or Myc-ROCK II using Lipofectamine.

shRNA and lentivirus production

The lentiviral expression system was provided by the National RNAi Core Facility, Academia Sinica, Taiwan. For FLAG-SHP2 expression, human FLAG-SHP2 cDNA was amplified by polymerase chain reaction and subcloned in frame to the *Nhe*I and *Eco*RI site of the pLKO-AS2-zeo vector. The plasmids pLKO-AS1-puro, which encodes shRNAs, were obtained from the National RNAi Core Facility, Academia Sinica. The target sequences for mouse SHP2 are 5′-CCTGATGAGT-ATGCGCTCAAA-3′ (#1) and 5′-GACATCCTTATTGACATCATT-3′ (#2). The target sequences for mouse LAR are 5′- GCCGTATGTGAAATGGATGAT-3′ (#1) and 5′- CCACCAGTGTTACTCTGACAT-3′ (#2). The target sequences for mouse ROCKII are 5′-GCATCTCTTGAAGAAACAAAT-3′ (#1) and 5′-CCCATG-GATCAGAGATAATTA-3′ (#2). To produce lentiviruses, HEK293T cells were co-transfected with pCMV-ΔR8.91 (2.25 μg), pMD.G (0.25 μg), and pLKO-AS1-puro-shRNA (or pLKO-AS2-zeo-FLAG-SHP2; 2.5 μg) using Lipofectamine. After 3 days, medium containing lentivirus particles was collected and stored at −80℃. Cells were infected with lentiviruses encoding shRNAs or FLAG-SHP2 for 24 hours and subsequently selected in the growth medium containing 1.8–2.5 μg/ml puromycin.

Immunoprecipitation and immunoblotting

Immunoblotting and immunoprecipitation were performed as described previously (Chen and Chen, 2006). Chemiluminescent signals were detected and quantified using a luminescence image system (LAS-3000, Fujifilm).

Phosphatase activity assay

FLAG-SHP2 WT and its mutants were transiently expressed in HEK293 cells and FLAG-SHP2 proteins were immunoprecipitated by anti-FLAG antibody. The immune complexes were washed three times with 1% NP-40 lysis buffer (1% Nonidet P-40, 20 mM Tris-HCl, pH 8.0, 137 mM NaCl, 10% glycerol) and twice with washing buffer (50 mM Tris pH 7.4, 137 mM NaCl, 2 mM EDTA) and subjected to a phosphatase activity assay using ρNPP as the substrate. This assay was performed in phosphatase buffer (50 mM Tris pH 7.4, 137 mM NaCl, 2 mM EDTA, 5 mM dithiothreitol, 20 mM ρNPP) at 37℃ for 1.5 hours and the reaction was terminated by the addition of 0.2 N NaOH. Upon release of the phosphate group from ρNPP, a ρ-nitrophenolate anion was produced and determined by absorbance at 405 nm. To ensure equal amounts of FLAG-SHP2 proteins, immune complexes were analyzed by immunoblotting with anti-SHP2.

To assay whether Tks5 is a substrate of SHP2, FLAG-Tks5 proteins were transiently co-expressed with v-Src in 293 cells and purified by anti-FLAG affinity chromatography. The purified Tks5 proteins (100 ng) were incubated with immobilized FLAG-SHP2 or its phosphatase mutants in 50 μl of the buffer (25 mM HEPES pH 7.4, 150 mM NaCl, 5 mM EDTA, and 2 mM dithiothreitol) at 37℃ for 60 minutes. The reaction was terminated by SDS sample buffer and the proteins were fractionated by SDS-PAGE. The tyrosine phosphorylation of Tks5 was analyzed by immunoblotting with anti-phosphotyrosine.

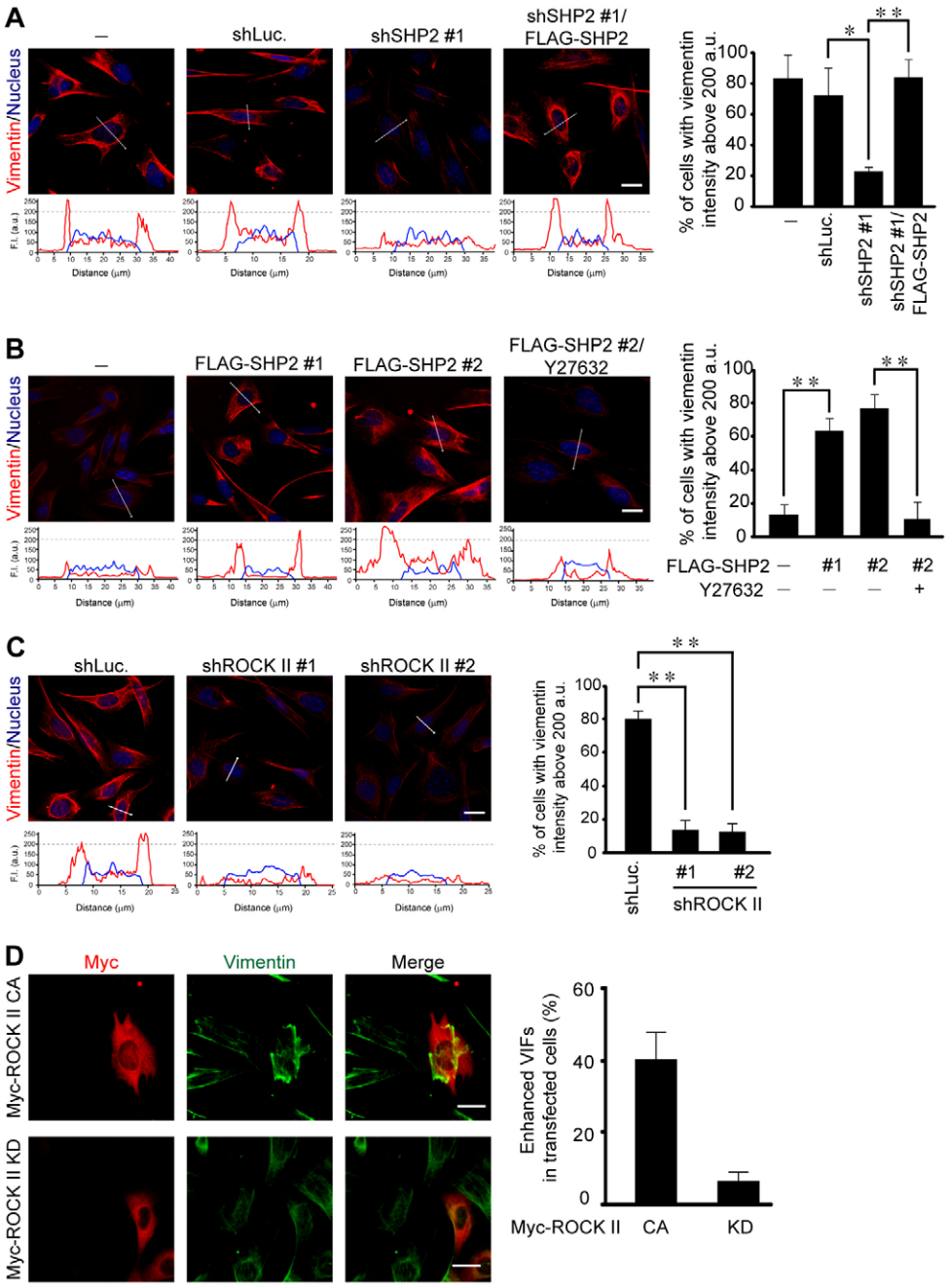

Fig. 8. Enhanced polymerization of vimentin intermediate filaments by SHP2 or ROCK II inhibits podosome rosette formation. (**A**) shRNAs specific to SHP2 (shSHP2; clone #1) or luciferase (shLuc) were stably expressed in v-Src-transformed MEFs. FLAG-SHP2 was re-expressed in the cells expressing shSHP2#1 (shSHP2#1/FLAG-SHP2). The cells were grown on fibronectin-coated coverslips for 48 hours and stained for vimentin and nuclei. The white lines in the images indicate the regions in which the fluorescence intensity of vimentin and nuclei was detected. The percentage of cells with vimentin fluorescence intensity greater than 200 arbitrary units (a.u.) relative to the total counted cells ($n \geq 50$) was measured. (**B**) v-Src-transformed MEFs and derived cells stably expressing FLAG-SHP2 were grown on fibronectin-coated coverslips for 48 hours and then treated with (+) or without (−) 10 μM Y27632 for 2 hours. The cells were fixed, stained for vimentin and nuclei and the fluorescence intensity of vimentin and nuclei was measured. The percentage of cells with vimentin fluorescence intensity greater than 200 arbitrary units (a.u.) relative to the total counted cells ($n \geq 50$) was measured. (**C**) shRNAs specific to ROCK II (shROCK II; clone #1 and #2) or luciferase (shLuc) were stably expressed in v-Src-transformed MEFs. The cells were grown on fibronectin-coated coverslips for 48 hours and stained for vimentin and nuclei. The white lines in the images indicate the regions where the fluorescence intensity of vimentin and nucleus was detected. The percentage of the cells with vimentin fluorescence intensity above 200 arbitrary units (a.u.) in the total counted cells ($n \geq 50$) was measured. (**D**) v-Src-transformed MEFs were grown on fibronectin-coated coverslips and transiently transfected with the plasmids encoding Myc-tagged ROCK II CA (constitutively active) or ROCK II KD (kinase deficient). The cells were fixed and then stained for vimentin and Myc-ROCK II. The percentage of enhanced vimentin intermediate filaments (VIFs) in Myc-ROCK II-expressed cells ($n \geq 100$) was measured. Values (mean ± s.d.) are from three independent experiments; $^{*}P<0.05$; $^{**}P<0.005$. Scale bars: 10 μm (A,B); 20 μm (D,E).

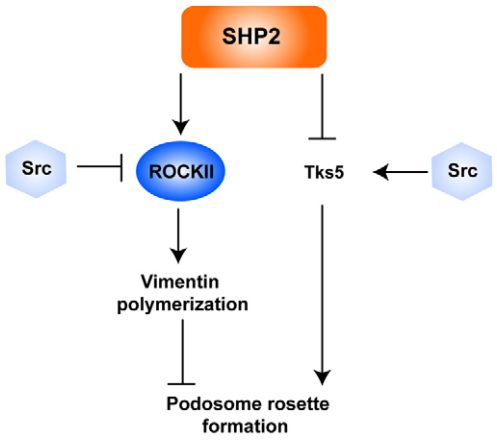

Fig. 9. How SHP2 suppresses the formation of podosome rosettes. Schematic representation of two proposed routes through which SHP2 might suppress podosome rosette formation. Right: dephosphorylation of certain critical podosomal scaffolding proteins (e.g. Tks5). Left: ROCK-mediated polymerization of vimentin filaments.

In vitro kinase assay

Cell lysates were incubated with 2 µg polyclonal anti-ROCK II antibody for 1.5 hours at 4℃. Immunocomplexes were collected on protein A-Sepharose beads and washed three times with 1% Nonidet P-40 lysis buffer and twice with 20 mM Tris-HCl, pH 7.4. Kinase reactions were performed in 40 µl of kinase buffer (20 mM Tris pH 7.4, 10 mM $MgCl_2$, 3 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA) containing 10 µCi of [γ-^{32}P]ATP and 2.5 µg of MBP for 30 minutes at 25℃. The reaction was terminated by the addition of SDS sample buffer and the proteins were fractionated by SDS-PAGE.

Matrix degradation assay

This assay was performed as described previously (Pan et al., 2011). v-Src-transformed MEFs were seeded on coverslips coated with Alexa-Fluor-488-conjugated fibronectin for 48 hours. The matrix degraded areas were determined using the Image-Pro Plus® software version 5.1. Ten random fields equivalent to 2 mm^2 were measured.

Matrigel invasion assay

This assay was performed in a 24-well transwell chamber (pore size 8 µm, Costar) coated with 100 µl Matrigel. Cells (5×10^3) were added to the upper compartment of the chamber. After 48 hours, the cells that had migrated through the Matrigel were fixed by methanol, stained by Giemsa Stain, and counted.

Immunofluorescence staining and laser-scanning confocal microscopy

Immunofluorescence staining was performed as described previously (Pan et al., 2011). The primary antibodies used were monoclonal anti-Myc (1:100), anti-FLAG (1:500), and anti-vimentin (1:100) and polyclonal anti-cortactin (1:200). Rhodamine-conjugated phalloidin and Alexa-Fluor-488-conjugated phalloidin (Molecular Probes, Invitrogen) were used to stain actin filaments. Coverslips were mounted in anti-Fade Dapi-Fluoromount-G™ (Southern Biotechnology Associates). Images were detected using a Zeiss LSM510 laser-scanning confocal microscopic imaging system (LSM 510; Carl Zeiss) with a Zeiss 63 Plan-Apochromat (NA 1.2 W Korr).

Statistical analysis

The data were analyzed by Student's *t*-test. Differences were considered to be statistically significant at $P<0.05$.

Acknowledgements

We are grateful to D.-L. Wang (Tzu Chi University, Hualien, Taiwan) for SHP2 cDNA and T. Oikawa (Keio University, Tokyo, Japan) for Tks5 cDNA. All authors declare no potential conflict of interest.

Funding

This work was supported by grants from the National Science Council, Taiwan [grant numbers NSC99-2628-B-005-010-MY3 and NSC100-2320-B-005-004-MY3]; by the National Health Research Institutes, Taiwan [grant number NHRI-EX101-10103BI]; and by the ATU plan from the Ministry of Education, Taiwan.

Supplementary material available online at
http://jcs.biologists.org/lookup/suppl/doi:10.1242/jcs.116624/-/DC1

References

Aceto, N., Sausgruber, N., Brinkhaus, H., Gaidatzis, D., Martiny-Baron, G., Mazzarol, G., Confalonieri, S., Quarto, M., Hu, G., Balwierz, P. J. et al. (2012). Tyrosine phosphatase SHP2 promotes breast cancer progression and maintains tumor-initiating cells via activation of key transcription factors and a positive feedback signaling loop. *Nat. Med.* **18**, 529-537.

Badowski, C., Pawlak, G., Grichine, A., Chabadel, A., Oddou, C., Jurdic, P., Pfaff, M., Albigès-Rizo, C. and Block, M. R. (2008). Paxillin phosphorylation controls invadopodia/podosomes spatiotemporal organization. *Mol. Biol. Cell* **19**, 633-645.

Bard-Chapeau, E. A., Li, S., Ding, J., Zhang, S. S., Zhu, H. H., Princen, F., Fang, D. D., Han, T., Bailly-Maitre, B., Poli, V. et al. (2011). Ptpn11/Shp2 acts as a tumor suppressor in hepatocellular carcinogenesis. *Cancer Cell* **19**, 629-639.

Boyle, W. J., Simonet, W. S. and Lacey, D. L. (2003). Osteoclast differentiation and activation. *Nature* **423**, 337-342.

Chen, S. Y. and Chen, H. C. (2006). Direct interaction of focal adhesion kinase (FAK) with Met is required for FAK to promote hepatocyte growth factor-induced cell invasion. *Mol. Cell. Biol.* **26**, 5155-5167.

Chen, B. H., Tzen, J. T. C., Bresnick, A. R. and Chen, H. C. (2002). Roles of Rho-associated kinase and myosin light chain kinase in morphological and migratory defects of focal adhesion kinase-null cells. *J. Biol. Chem.* **277**, 33857-33863.

Chiusaroli, R., Knobler, H., Luxenburg, C., Sanjay, A., Granot-Attas, S., Tiran, Z., Miyazaki, T., Harmelin, A., Baron, R. and Elson, A. (2004). Tyrosine phosphatase epsilon is a positive regulator of osteoclast function in vitro and in vivo. *Mol. Biol. Cell* **15**, 234-244.

Cortesio, C. L., Chan, K. T., Perrin, B. J., Burton, N. O., Zhang, S., Zhang, Z. Y. and Huttenlocher, A. (2008). Calpain 2 and PTP1B function in a novel pathway with Src to regulate invadopodia dynamics and breast cancer cell invasion. *J. Cell Biol.* **180**, 957-971.

Destaing, O., Sanjay, A., Itzstein, C., Horne, W. C., Toomre, D., De Camilli, P. and Baron, R. (2008). The tyrosine kinase activity of c-Src regulates actin dynamics and organization of podosomes in osteoclasts. *Mol. Biol. Cell* **19**, 394-404.

Lee, H. H. and Chang, Z. F. (2008). Regulation of RhoA-dependent ROCKII activation by Shp2. *J. Cell Biol.* **181**, 999-1012.

Lee, H. H., Tien, S. C., Jou, T. S., Chang, Y. C., Jhong, J. G. and Chang, Z. F. (2010). Src-dependent phosphorylation of ROCK participates in regulation of focal adhesion dynamics. *J. Cell Sci.* **123**, 3368-3377.

Linder, S. (2007). The matrix corroded: podosomes and invadopodia in extracellular matrix degradation. *Trends Cell Biol.* **17**, 107-117.

Linder, S. and Aepfelbacher, M. (2003). Podosomes: adhesion hot-spots of invasive cells. *Trends Cell Biol.* **13**, 376-385.

Mañes, S., Mira, E., Gómez-Mouton, C., Zhao, Z. J., Lacalle, R. A. and Martínez-A, C. (1999). Concerted activity of tyrosine phosphatase SHP-2 and focal adhesion kinase in regulation of cell motility. *Mol. Cell. Biol.* **19**, 3125-3135.

Matozaki, T., Murata, Y., Saito, Y., Okazawa, H. and Ohnishi, H. (2009). Protein tyrosine phosphatase SHP-2: a proto-oncogene product that promotes Ras activation. *Cancer Sci.* **100**, 1786-1793.

Montagner, A., Yart, A., Dance, M., Perret, B., Salles, J. P. and Raynal, P. (2005). A novel role for Gab1 and SHP2 in epidermal growth factor-induced Ras activation. *J. Biol. Chem.* **280**, 5350-5360.

Moreau, V., Tatin, F., Varon, C. and Génot, E. (2003). Actin can reorganize into podosomes in aortic endothelial cells, a process controlled by Cdc42 and RhoA. *Mol. Cell. Biol.* **23**, 6809-6822.

Oikawa, T., Oyama, M., Kozuka-Hata, H., Uehara, S., Udagawa, N., Saya, H. and Matsuo, K. (2012). Tks5-dependent formation of circumferential podosomes/invadopodia mediates cell-cell fusion. *J. Cell Biol.* **197**, 553-568.

Oser, M., Yamaguchi, H., Mader, C. C., Bravo-Cordero, J. J., Arias, M., Chen, X., Desmarais, V., van Rheenen, J., Koleske, A. J. and Condeelis, J. (2009). Cortactin regulates cofilin and N-WASp activities to control the stages of invadopodium assembly and maturation. *J. Cell Biol.* **186**, 571-587.

Pan, Y. R., Chen, C. L. and Chen, H. C. (2011). FAK is required for the assembly of podosome rosettes. *J. Cell Biol.* **195**, 113-129.

Peng, Z. Y. and Cartwright, C. A. (1995). Regulation of the Src tyrosine kinase and Syp tyrosine phosphatase by their cellular association. *Oncogene* **11**, 1955-1962.

Qu, C. K. and Feng, G. S. (1998). Shp-2 has a positive regulatory role in ES cell differentiation and proliferation. *Oncogene* **17**, 433-439.

Ren, Y., Meng, S., Mei, L., Zhao, Z. J., Jove, R. and Wu, J. (2004). Roles of Gab1 and SHP2 in paxillin tyrosine dephosphorylation and Src activation in response to epidermal growth factor. *J. Biol. Chem.* **279**, 8497-8505.

Seals, D. F., Azucena, E. F., Jr, Pass, I., Jr, Tesfay, L., Gordon, R., Woodrow, M., Resau, J. H. and Courtneidge, S. A. (2005). The adaptor protein Tks5/Fish is required for podosome formation and function, and for the protease-driven invasion of cancer cells. *Cancer Cell* **7**, 155-165.

Stylli, S. S., Stacey, T. T., Verhagen, A. M., Xu, S. S., Pass, I., Courtneidge, S. A. and Lock, P. (2009). Nck adaptor proteins link Tks5 to invadopodia actin regulation and ECM degradation. *J. Cell Sci.* **122**, 2727-2740.

Tsutsumi, R., Takahashi, A., Azuma, T., Higashi, H. and Hatakeyama, M. (2006). Focal adhesion kinase is a substrate and downstream effector of SHP-2 complexed with Helicobacter pylori CagA. *Mol. Cell. Biol.* **26**, 261-276.

Varon, C., Tatin, F., Moreau, V., Van Obberghen-Schilling, E., Fernandez-Sauze, S., Reuzeau, E., Kramer, I. and Génot, E. (2006). Transforming growth factor beta induces rosettes of podosomes in primary aortic endothelial cells. *Mol. Cell. Biol.* **26**, 3582-3594.

Xu, R. (2007). Shp2, a novel oncogenic tyrosine phosphatase and potential therapeutic target for human leukemia. *Cell Res.* **17**, 295-297.

Yang, W., Klaman, L. D., Chen, B., Araki, T., Harada, H., Thomas, S. M., George, E. L. and Neel, B. G. (2006). An Shp2/SFK/Ras/Erk signaling pathway controls trophoblast stem cell survival. *Dev. Cell* **10**, 317-327.

Yu, D. H., Qu, C. K., Henegariu, O., Lu, X. and Feng, G. S. (1998). Protein-tyrosine phosphatase Shp-2 regulates cell spreading, migration, and focal adhesion. *J. Biol. Chem.* **273**, 21125-21131.

Zhang, S. Q., Yang, W., Kontaridis, M. I., Bivona, T. G., Wen, G., Araki, T., Luo, J., Thompson, J. A., Schraven, B. L., Philips, M. R. et al. (2004). Shp2 regulates SRC family kinase activity and Ras/Erk activation by controlling Csk recruitment. *Mol. Cell* **13**, 341-355.