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Original Article

YES oncogenic activity is specified by its SH4 domain and regulates RAS/MAPK signaling in colon carcinoma cells

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Abstract: Members of the SRC family of tyrosine kinases (SFK) display important functions in human cancer, but their specific role in tumorigenesis remains unclear. We previously demonstrated that YES regulates a unique oncogenic signaling important for colorectal cancer (CRC) progression that is not shared with SRC. Here, we addressed the underlying mechanism involved in this process. We show that YES oncogenic signaling relies on palmitoylation of its SH4 domain that controls YES localization in cholesterol-enriched membrane micro-domains. Specifically, deletion of the palmitoylation site compromised YES transforming activity, while addition of a palmitoylation site in the SH4 domain of SRC was sufficient for SRC to restore the transforming properties of cells in which YES had been silenced. Subsequently, SILAC phosphoproteomic analysis revealed that micro-domain-associated cell adhesive components and receptor tyrosine kinases are major YES substrates. YES also phosphorylates upstream regulators of RAS/MAPK signaling, including EGFR, SHC and SHP2, which were not targeted by SRC due to the absence of palmitoylation. Accordingly, EGFR-induced MAPK activity was attenuated by YES down-regulation, while increased RAS activity significantly restored cell transformation that was lost upon YES silencing. Collectively, these results uncover a critical role for the SH4 domain in the specification of SFK oncogenic activity and a selective role for YES in the induction of RAS/MAPK signaling in CRC cells.

Keywords: YES, SRC, tyrosine kinase, colon cancer, oncogenic signaling, phosphoproteomics

Introduction

Colorectal cancer (CRC) is one of the leading causes of tumor-related death worldwide. Most CRCs are sporadic and under the control of genetic, epigenetic and environmental factors. Their current clinical management involves surgical removal of the primary tumor, often associated with chemotherapy. However, recurrences are detected in about 50% of patients with CRC and patients' survival after diagnosis is shorter than 5 years. Therapeutic failure is associated with high numbers of local or metastatic tumor recurrences [1]. While novel therapies targeting Receptor Tyrosine Kinases (RTK) have demonstrated some clinical benefit, they failed to significantly extend patients' survival [2]. Thus, there is an urgent need to identify new therapeutic targets in metastatic CRC and relevant biomarkers for the selection of patients that could benefit from such targeted therapies.

Cytoplasmic tyrosine kinases of the SRC family (SFK) play critical roles in signal transduction induced by growth and adhesive factors [3, 4] to control cell growth and adhesion. In mammals, the SFK family comprises eight cellular members, among which SRC, FYN and YES are widely expressed. SFKs share a common modular structure that includes a N-terminal SH4 domain with a myristilation site for membrane targeting, a unique domain with potential SRC regulatory function [5, 6], a SH3 and a SH2 domain involved in protein-protein interaction and a kinase domain bordered by two short

regulatory sequences [3]. SFKs also exhibit strong oncogenic activity when deregulated, a situation frequently observed in human cancer. Particularly, SFKs are deregulated in most colorectal cancers (CRC) and the extent of SRC and YES up-regulation has been associated with poor clinical prognosis and tumor recurrence [7, 8]. SRC deregulation primarily involves genetic- and epigenetic-dependent mechanisms, including gene overexpression or amplification in CRC [9, 10]. Oncogenic induction additionally involves inactivation of the SRC negative regulators Cbp/PAG [11, 12], which controls SFK catalytic activity, and SLAP, which controls SRC substrates stability [13]. As a result, SRC is highly active in tumour cells and has the capacity to promote tyrosine phosphorylation (pTyr) of several hundreds of proteins, including critical inducers of tumor cell growth, survival, angiogenesis and metastasis [9, 14]. The prominent role for SRC in CRC has been further supported by recent genetic evidence obtained in mice and flies that demonstrates SRC critical function in intestinal stem cell proliferation during tissue self-renewal, regeneration and tumorigenesis [15, 16]. Since then, SFKs have been considered as attractive therapeutic targets in CRC and several small inhibitors are currently tested in clinical trials [17].

SFK role in human cancer has focused on SRC because it is generally accepted that SFKs show significant redundant functions [3]. For instance, the analysis of mice in which *Src, Fyn* or *Yes* was genetically ablated and of the corresponding embryonic fibroblasts highlighted partial redundant functions during embryogenesis [18, 19] and in cell division [20]. More recently, similar gene knock-out experiments in the small intestine showed redundant functions in mouse intestinal homeostasis [15]. However, several reports are uncovering selective function of SFKs during tumorigenesis and malignant progression, such as in mouse breast [21] and intestinal cancer [15]. For instance, YES displays a specific role in cell migration [22] and in tumor cell resistance to EGFR-targeted therapy in human breast cancer [23]. In human CRC, several laboratories, including ours, reported that YES has a specific oncogenic role in the promotion of tumor cell survival, invasion, extravasation, metastasis formation and resistance to chemotherapy [24-26]. YES is also essential for the transformation and survival of β-catenin-dependent CRC cells [27]. Specifically, YES phosphorylates YAP1 to promote nuclear activation of the YAP1-beta-catenin-TBX5 complex and the induction of critical anti-apoptotic genes [27]. However, YAP1 may not be a highly specific target of YES because it can be phosphorylated by SRC as well [27]. Therefore, additional critical components of YES signaling may be expected in CRC.

SFK unique functions are thought to rely on their capacity to phosphorylate specific substrates, although experimental data supporting this hypothesis are still limited. Substrate recognition is primarily controlled by substrate access and/or interaction. For instance, the variable binding affinity of several substrates to SFK-SH3 allows the selection of specific substrates for efficient phosphorylation. However, in some SFKs, such as SRC and YES, SH2 and SH3 are very similar [3], implying that access to their substrates is the major mechanism of signaling specificity. Interestingly, a major structural difference between these two close SFK members is the presence of an additional palmitoylation site in YES, but not in SRC [3]. Palmitate modification specifies YES trafficking and localization in specific sub-cellular compartments [28], including cholesterol and sphingolipid-enriched membrane domains, also called membrane micro-domains [29, 30], that are present at tight and adherent junctions [31]. Due to the absence of such lipid attachment, SRC shows higher mobility at membranes and therefore is also localized at focal adhesions [32]. Such difference may significantly affect substrate access and SFK signaling specificity, as suggested in mouse embryonic fibroblasts during cell growth, migration and transformation [32-36]. Here, we investigated the mechanism underlying YES specific transforming activity in CRC cells and found that YES SH4 domain has a critical role in this process. Phosphoproteomic analysis revealed specific YES substrates localized in CRC micro-domains and an unsuspected function for YES in the regulation of RAS/MAPK signaling in these tumor cells.

Material and methods

Reagents

Epithelial Growth Factor, hexadimethrine bromide, G418 and puromycin were purchased from Sigma Aldrich (St Quentin, France). Anti-

SFK antibodies (cst1) were described in [37]. The anti-SRC specific antibody (2.17) was a generous gift of Dr S. Parsons (University of Virginia, VA, USA). Anti-pTyr 4G10, anti-ezrin, and anti-α tubulin antibodies were a gift from P. Mangeat and N. Morin respectively (CRBM, Montpellier). Additional antibodies used in this study include: anti-YES (1B7; Wako Chemicals), anti-ephrin 1, 2 and 3 (SC-18, Santa Cruz Biotech), anti-EPHA2 (C-20, Santa Cruz Biotech), anti-EPHB2 (1A6C9, Biosource), anti-EPHB3 (3F12, Abnova), anti-EPHB4 (3D7G8, Biosource), anti-RON (C-20, Santa Cruz Biotech), anti-MET (no. 3752, Cell Signaling Technology), anti-EGFR (SC-03, Santa Cruz Biotech), anti-SYK (sc-1077, Santa Cruz Biotech), anti-E-cadherin (H-108, Santa Cruz Biotech), anti-βcatenin (Clone 14, BD Transduction Laboratories), anti-p120 catenin (BD Biosciences), anti-flotillin 1 (Clone 18, BD Transduction Laboratories), anti-ZO-2 (H110, Santa Cruz), anti-SHC (C-20, Santa Cruz Biotech), anti-SHP2 (no. 3752, Cell Signaling Technology), anti-GAB1 (C-20, Santa Cruz), anti-MAPK1/2 (no. 4695, Cell signaling technology), anti-MAPK1/2 pT202/Y204 (no. 4370, Cell signaling technology) antibodies. Chicken SRC S3C/S4I (SRC palm+) and the human YES C3A (YES palm-), a YES mutant resistant to the YES shRNA [25], were generated with the QuickChange Site-Directed Mutagenesis Kit (Stratagene) using 5'-gtacgaccatggggtgcatcaagagcaagccca-3' and 5'-ccagtacccttcaccatgggcgccattaaaagtaaagaaaacaaa-3' respectively, and subcloned in pBABE. The human HRASV12 pBABE construct was a gift from A Eychene (Institute Curie, France) and the retroviral vectors pSIREN expressing control shRNA srb1 (Ambion), SRC and YES shRNA were described in [25].

Cell infection, growth and invasion

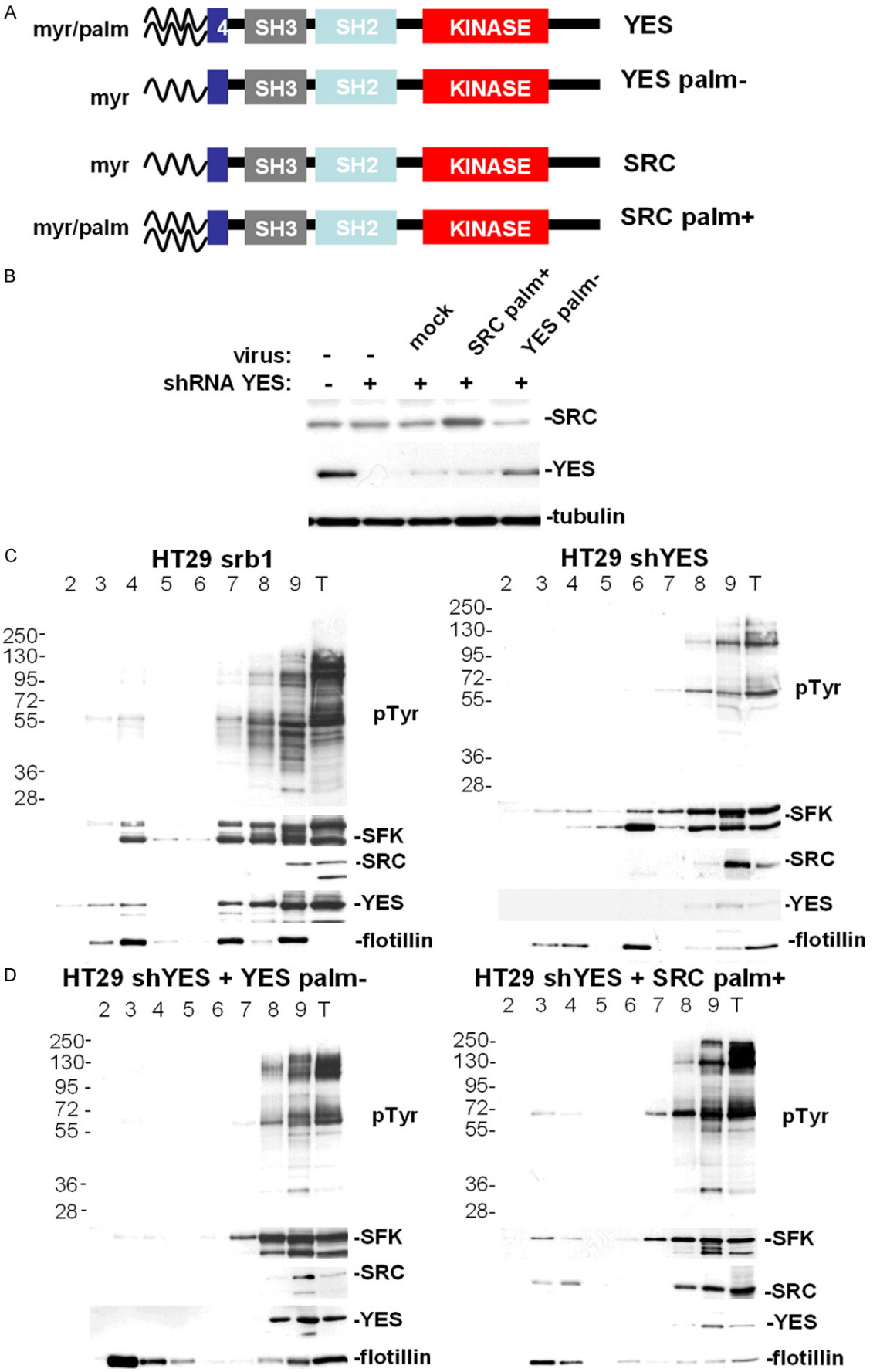
HT29 and HCT116 cells were described in [12]. HT29 srb1 (sh-control Ambion construct, srb1), HT29 shYES and HT29 shSRC cells were described in [25]. Cell lines were cultured at 37°C and 5% CO_2 in a humidified incubator in Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen) supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin and 100 µg/ml streptomycin. Retroviral infections were carried out as described in [12] and stable cell lines were obtained by selection with 1 µg/ml puromycin or 800 µg/ml geneticin. Soft-agar cell growth was performed as in [12]. After 18-21 days, colonies with > 50 cells were scored as positive. Cell invasion assays were performed in Boyden chambers (BD Bioscience, Le Pont de Claix, France) using 50,000 cells and in the presence of 100 µl Matrigel (2 mg/ml) (BD Bioscience) for 24 hrs. For EGF stimulation experiments, cells were cultured in 0.5% serum overnight before stimulation with 50 ng /ml EGF.

Immunostaining

Immunostaining was performed as described in [25]. Confocal microscopy was performed using Zeiss LSM780 multi-photon microscopes. Images were acquired using Zeiss Zen 2010 (Zeiss) software and treated using the ImageJ software.

Biochemistry

Cell lysate, immunoprecipitation (ip) and western blotting were performed as described in [38]. Briefly, cells were lysed at 4°C with lysis buffer (20 mM Hepes pH7.5, 150 mM NaCl, 0.5% Triton X-100, 6 mM β-octylglucoside, 10 µg/ml aprotinin, 20 µM leupeptin, 1 mM NaF, 1 mM DTT and 100 µM sodium orthovanadate). Ip was performed with 500 µg proteins and 2 µg of the specific antibody. Immunoprecipitates or 20-50 µg of whole cell lysates were loaded on SDS-PAGE gels and transferred onto Immobilon membranes (Millipore). Detection was performed using the ECL System (Amersham Biosciences). Detergent-resistant membrane (DRM) purification was performed as described in [33]. Briefly, 10^8 cells were rinsed with PBS, scraped in ice-cold PBS-containing 1 mM vanadate and pelleted. Pellets were suspended in ice-cold 2x Lysis Buffer containing 1% Triton X-100, 10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 10 microg/ml aprotinin and 1 mM vanadate, for 20 min. Cell suspensions were homogenized in a tight fitting Dounce homogenizer with 10 strokes and centrifuged for 5 min at 1300 g to remove nuclei and large cellular debris. Supernatants were fractionated through a 5-42.5%, w/v sucrose gradient in 4 ml tubes. After centrifugation, nine fractions were collected from the top to the bottom of the gradient and protein expression was analyzed by western blotting in frac-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-flotillin

Figure 1. YES membrane distribution in HT29 cells is specified by its SH4 domain. A. Schematic representation of the SRC and YES mutants used in this study. The SH4 domain is shown in blue. B. Western blotting showing SRC and YES levels in lysates of HT29 cells in which *YES* was silenced or not and that were then infected with control virus (mock) or viruses expressing the indicated SRC and YES mutants. Tubulin was used as loading control. C. Western blot analysis of SFK, SRC and YES membrane distribution in sucrose density gradient fractions from 1% Triton X100 lysates of HT29 cells expressing the srb1 (control) or the anti-YES shRNAs. Detergent-resistant membranes (DRMs) correspond to fractions 2-4 and non-DRMs to fractions 5-9. 10% of total cell lysate (T) was included as loading control and flotillin expression was used as a marker of DRM fractions. D. YES membrane distribution in DRMs is specified by palmitoylation of the SH4 domain of YES. Sucrose density gradient fractions were obtained from lysates of *YES*-silenced HT29 cells that were infected with retroviruses expressing YES palm-, in which the palmitoylation site was mutated, or SRC palm+, in which a palmitoylation site was introduced.

tions 2-9. 10% of total cell lysate prior fractionation (T) was used as loading control.

SILAC phosphoproteomic analysis

Mass Spectrometry analysis SILAC ($^{13}C_6{}^{15}N_4$-Arg and $^{13}C_6{}^{15}N_2$-Lys as heavy labeling, Eurisotope), phosphotyrosine immuno-affinity purification (using a mixture of 4G10 and pY100 antibodies) and tryptic digests were done as described in [39]. Purified proteins were separated on SDS-PAGE gels. Trypsin-digested samples (1 µl) obtained from 44 cut gel slices were then analyzed online using nanoflow HPLC separation and nano-electrospray ionization on a quadrupole time-of-flight (Q-TOF) mass spectrometer (QSTAR Pulsar-*i,* Applied Biosystems, Foster City, CA) coupled to an Ultimate 3000 HPLC apparatus (Dionex, Amsterdam, Netherland). Sample desalting and pre-concentration were done on-line on a Pepmap® precolumn (0.3 mm × 10 mm). A gradient consisting of 0-40% solution B in 60 mn and 80% B in 15 min (A = 0.1% formic acid, 2% acetonitrile in water ; B = 0.1% formic acid in acetonitrile) at 300 nl/min was used to elute peptides from the capillary reverse-phase column (0.075 mm × 150 mm; Pepmap®, Dionex), fitted with an uncoated silica PicoTip Emitter (NewOjective, Woburn, USA) with an outlet diameter of 8 µm. Spectra were recorded using the Analyst QS 1.1 software (Applied Biosystems). Parameters were adjusted as follows: ion spray voltage (IS), 1800 V; curtain gas (CUR), 25; declustering potential 1 (DP1), 75 V; focusing potential (FP), 265 V; declustering potential 2 (DP2), 15 V. Spectra were acquired with the instrument operating in the information-dependent acquisition mode throughout the HPLC gradient. Every 7 s, the instrument cycled through acquisition of a full-scan spectrum (1 s) and two MS/MS spectra (3 s each). Peptide fragmentation was performed using nitrogen gas on the most abundant doubly or triply charged ions detected in the initial MS scan, with a collision energy profile optimized according to the peptide mass (using the manufacturer's parameters), and an active exclusion time of 0.60 min. All MS/MS spectra were analyzed against the *Homo sapiens* entries of the Swiss-Prot or Trembl database (release 53.0: http://www.expasy.ch), by using the Mascot v 2.1 algorithm (http://www.matrixscience.com). Search parameters were: mass accuracy, 0.1 Da for MS and MS/MS data; 1 miscleavage; variable modifications, oxidized methionine and phospho (Y); SILAC-labels, Lys-8 and Arg-10. All significant hits ($P < 0.05$) were manually inspected. Quantification was performed by using the MSQuant v1.4.1 software developed by Mann and colleagues (http://msquant.sourceforge.net). Data were manually inspected and corrected when necessary. All significant hits were manually inspected.

Statistical analysis

All analyses were performed using GraphPad Prism and ImageJ. Data are presented as the mean ± SEM. When distribution was normal (assessed with the Shapiro Wilk test), the two-tailed t test was used for between-group comparisons. Statistical significance level was illustrated with *p* values: *$P \leq 0.5$, **$P \leq 0.01$, ***$P \leq 0.001$.

Results

YES oncogenic activity is specified by its SH4 domain

We previously showed, using an RNA interference approach, that in HT29 CRC cells YES specifically drives the oncogenic signaling required for cell-cell adhesion, anchorage-independent cell growth and invasion [25]. The specificity of this YES-mediated effect was then confirmed by the incapacity of SRC to restore this trans-

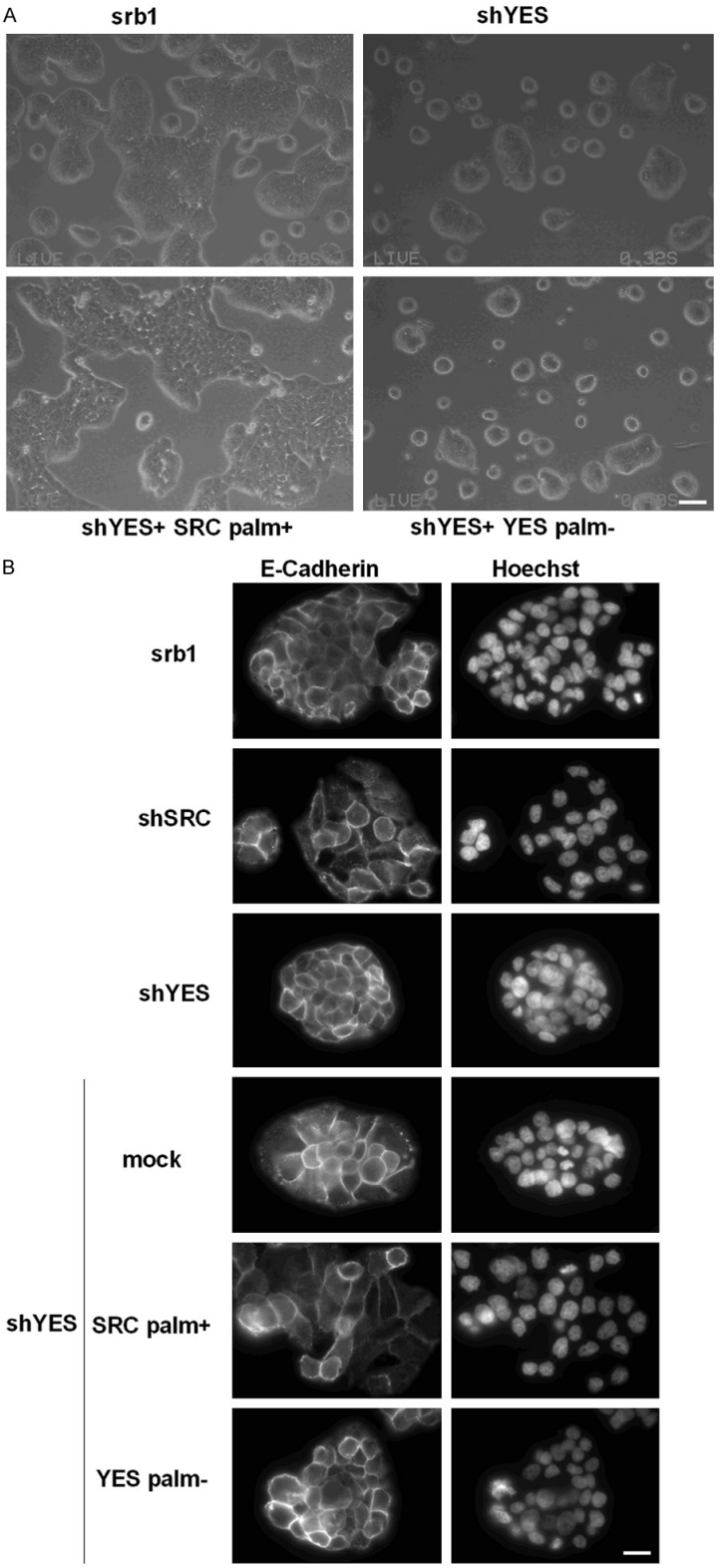


Figure 2. YES cell adhesive activity is specified by the SH4 domain. SRC palm+ but not YES palm- expression restores cell adhesive properties of *YES*-silenced HT29 cells. A. Cell morphology of HT29 cells that express the indicated shRNAs and were infected with indicated retroviruses. Scale bar, 100 µm. B. Confocal microscopy of E-Cadherin immunostaining of HT29 cells that express the indicated shRNAs and were infected with indicated retroviruses. Nuclear staining (Hoechst) is also shown. Scale bar, 20 µm.

formed phenotype in HT29 cells in which *YES* was silenced [25]. Here, we investigated the role of the YES-SH4 domain in these transforming properties by a similar approach. As the SH4 domain of YES, but not that of SRC, can be palmitoylated on the Cys 3 residue [3], we analyzed whether this post-translational modification was required for YES signaling and conversely, whether palmtoylation of SRC-SH4 was sufficient to bestow similar transforming properties to SRC. The palmitate-defective YES-C3A mutant (YES palm-) and the palmitoylated SRC-S3C/S4I mutant (SRC palm+) (**Figure 1A**) were expressed in *YES*-silenced HT29 cells by retroviral transduction to induce a moderate level of ectopic protein expression (**Figure 1B**). The role of this modification on SFK microdomain distribution was then investigated using purified detergent-resistant membrane (DRM) fractions, isolated from the light fractions of Triton X-100 cell lysates by sucrose gradient centrifugation [33]. Proper DRM purification was confirmed by the finding that the bulk of flotillins, a major structural component of these domains [29, 30], was localized in the light fractions (**Figure 1C**). In control HT29 srb1 cells, 20% of YES and 15% of SFKs were recovered in DRM fractions (**Figure 1C**). YES down-regulation strongly reduced SFK localization in DRM fractions (**Figure 1C**), suggesting that YES is one of the main SFKs expressed in CRC microdomains. Moreover, SRC was hardly detected in DRM fractions of both control and silenced HT29 cells (**Figure 1C**), probably due to the absence of palmitate modification. In support to this idea,

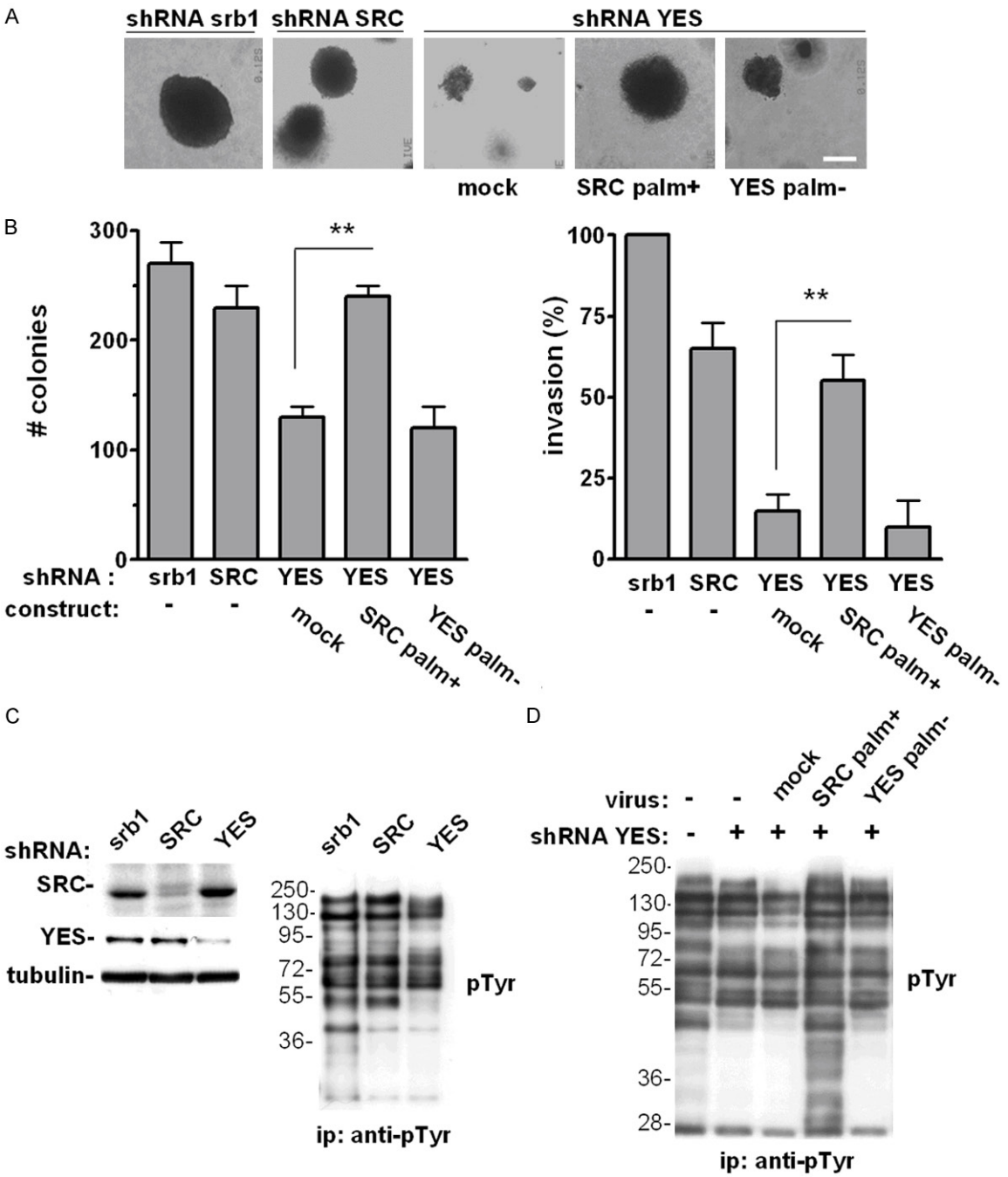
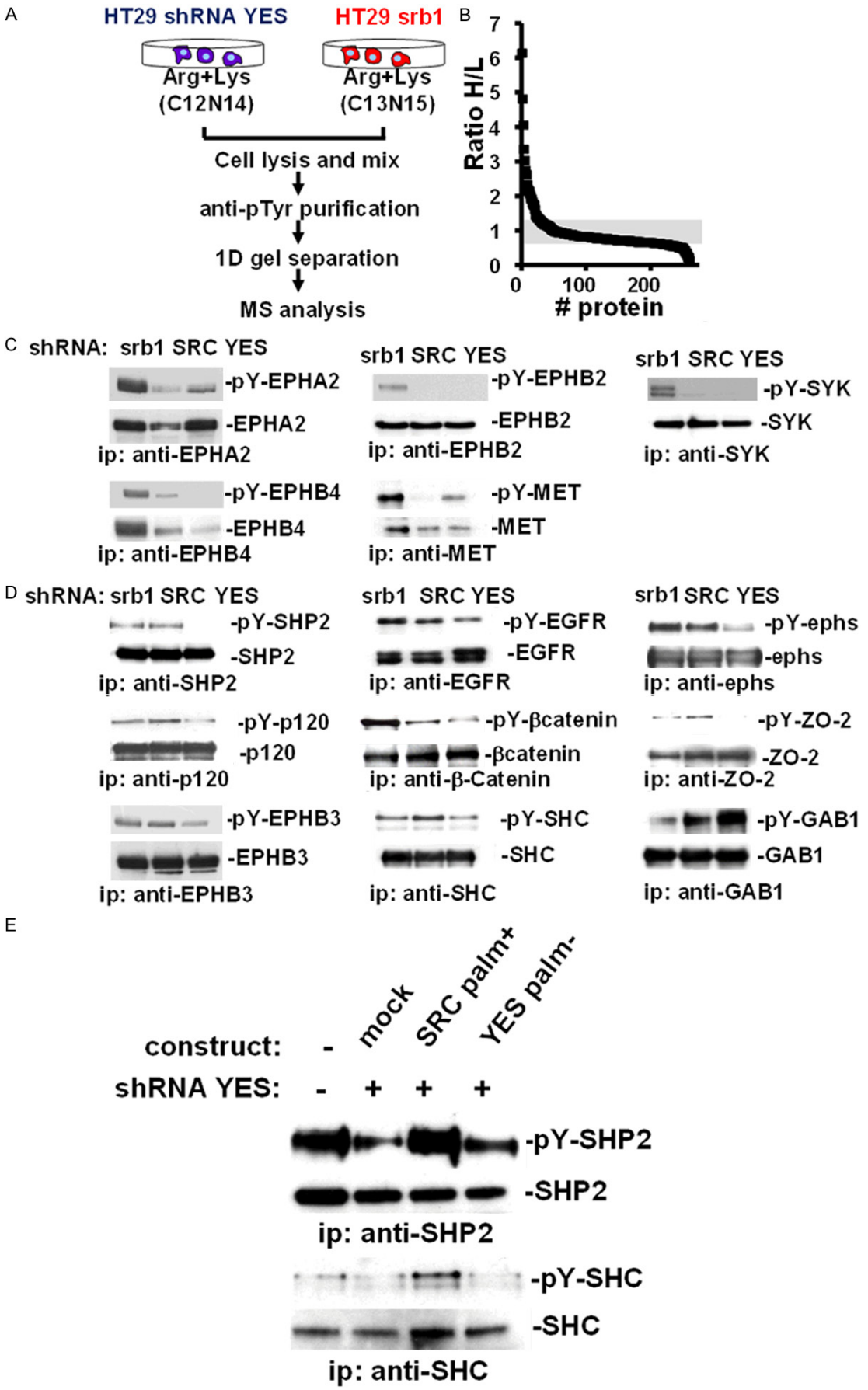


Figure 3. YES transforming activity is specified by the SH4 domain. A. SRC palm+ but not YES palm- expression restores growth in soft agar of *YES*-silenced HT29 cells. Size (top panel) and number (bottom panel) of colonies obtained using HT29 cells that express the indicated shRNAs and were infected with retroviruses expressing YES palm-, in which the palmitoylation site was mutated, or SRC palm+, in which a palmitoylation site was introduced. B. SRC palm+ but not YES palm- expression restores the cell invasion capacity of *YES*-silenced HT29 cells in Boyden chambers. The graph shows the percentage (mean ± SD; n > 3) of cells that invaded the Matrigel matrix relative to control cells (parental HT29 cells); **P < 0.01. D. SRC palm+ but not YES palm- expression restores the tyrosine phosphorylation level of *YES*-silenced HT29 cells. C. YES but not SRC depletion induces a strong decrease of tyrosine phosphorylation level in HT29 cells. Western blotting showing SRC, YES and tyrosine phosphorylation levels in lysates of HT29 cells expressing or not YES shRNA as shown and infected with control virus (mock) or viruses expressing indicated SFK constructs. Tubulin was used as loading control. Scale bar, 200 μm.

ectopic expression of SRC palm+, but not of YES palm-, significantly restored SFK localization in DRMs (**Figure 1D**). Therefore, SH4 palmitoylation defines a major mechanism for the


A
HT29 shRNA YES
HT29 srb1
Arg+Lys (C12N14)
Arg+Lys (C13N15)
Cell lysis and mix
anti-pTyr purification
1D gel separation
MS analysis
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Ratio H/L
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-SHC
ip: anti-SHC

Figure 4. SILAC-based phosphoproteomic analysis of YES substrates in HT29 cells. A. Outline of the SILAC-based phosphoproteomic analysis in HT29 cells in which *YES* was silenced (shRNA YES) or not (srb1; control). B. Quantification of the phosphorylation changes by SILAC. A Heavy/Light (H/L) ratio > 1.3 indicates that the tyrosine phosphorylation content of that specific protein was increased and a H/L ratio < 0.7 that the tyrosine phosphorylation content was decreased in *YES*-silenced cells compared to controls. The grey zone highlights proteins in which tyrosine phosphorylation was not significantly modified following *YES* silencing. See also Table S1 for a summary of the results. C, D. Biochemical validation of YES substrates revealed by the SILAC analysis. Tyrosine phosphorylation content and cell expression of selected YES targets identified by phosphoproteomics. Proteins were immunoprecipitated from HT29 cells infected with retroviruses that express the indicated shRNAs using the indicated antibodies. C. Common SRC and YES targets. D. Specific YES targets. E. Phosphorylation of selected YES substrates is controlled by palmitoylation of the SH4 domain. Western blot analysis of tyrosine phosphorylation content and SHP2 and SHC protein expression in HT29 cells expressing the indicated constructs.

Table 1. Potential YES substrates identified by SILAC phosphoproteomics

H/L ratio	*Gene name*	*Description*
6.133	EPHB2	Ephrin type-B receptor 2
4.816	EFNB1	Ephrin-B1
4.047	EFNB2	Ephrin-B2
3.359	ZO2	Tight junction protein ZO-2
3.031	BI2L1	BAI1-associated protein 2-like protein 1
2.747	CTND1	Catenin delta-1
2.626	EPS8	Epidermal growth factor receptor kinase substrate 8
2.61	EZRI	Ezrin
2.322	EPHA2	Ephrin type-A receptor 2
2.19	SHIP2	Phosphatidylinositol-3,4,5-trisphosphate 5-phosphatase 2
2.138	EPHB4	Ephrin type-B receptor 4
2.137	LSR	Lipolysis-stimulated lipoprotein receptor
2.061	MET	Hepatocyte growth factor receptor
1.987	RON	Macrophage-stimulating protein receptor
1.908	EGFR	Epidermal growth factor receptor
1.904	DCBD2	Discoidin, CUB and LCCL domain-containing protein 2
1.825	PLAK	Junction plakoglobin
1.773	EPHB3	Ephrin type-B receptor 3
1.739	ERBB2	Receptor tyrosine-protein kinase erbB-2
1.727	PTN11	Tyrosine-protein phosphatase non-receptor type 11
1.608	CADH1	Epithelial cadherin
1.449	SHC1	SHC-transforming
1.44	SYK	Tyrosine-protein kinase SYK
1.398	PDC6I	Programmed cell death 6-interacting protein
1.359	CTNB1	Catenin beta-1
1.339	CTNA1	Catenin alpha-1
1.305	PYGB	Glycogen phosphorylase, brain form

control of SFK localization in CRC microdomains.

We next investigated the functional role of this lipid modification in YES signaling. YES knockdown induced formation of cell clusters due to modulation of adherent junctions, inhibition of anchorage independent-growth and invasion (**Figures 2**, **3A** and **3B**) [25]. In contrast, SRC knock-down resulted in a mild effect on these tumoral cell activities (**Figures 2**, **3A** and **3B**) [25]. Ectopic expression of YES palmdid not restore cell-cell adhesion, anchorage-independent growth and invasion in *YES*-silenced cells, while the SRC palm+ mutant significantly rescued these YES-mediated transforming properties (**Figures 2**, **3A** and **3B**). A comparison of SRC and YES mRNA levels indicated that these cells contain 3.8-times higher SRC levels [25]; nevertheless, YES depletion induced a strong reduction in cellular pTyr content (**Figure 3C**) indicating that YES plays a major role also in pTyr-dependent signaling, despite high level of SRC expression in CRC cells. Accordingly, cellular pTyr was largely restored by SRC palm+, but not by YES palm- (**Figure 3D**). We thus conclude that palmitoylation of the SH4 domain specifies YES oncogenic signaling in CRC cells.

SILAC phosphoproteomic analysis of YES signaling in CRC cells

To identify YES substrates involved in its oncogenic signaling, we used a SILAC-based phosphoproteomic approach [39] in HT29 cells in which *YES* was silenced or not (**Figure 4A**). This analysis identified 259 proteins, 28 of which exhibited reduced pTyr (H/L > 1.3), and 93

Table 2. Selected proteins in which pTyr level is increased upon *YES* silencing

H/L ratio	*Gene name*	*Description*
0.66	CAP1	Adenylyl cyclase-associated protein 1
0.65	PIK2R2	Phosphatidylinositol 3-kinase regulatory subunit beta
0.62	MYO1C	Myosin-Ic
0.61	MYH9	Myosin-9
0.58	RASA1	Ras GTPase-activating protein
0.55	GAB1	GRB2-associated-binding protein 1
0.55	GELS	Gelsolin
0.55	DC1I2	Cytoplasmic dynein 1 intermediate chain 2
0.49	CUL5	Cullin-5
0.35	BCAR1	Breast cancer anti-estrogen resistance protein 1

increased pTyr (H/L < 0.7) upon YES down-regulation (**Figure 4B**; **Tables 1**, **2** and S1). This analysis also revealed that most YES substrates have a membrane micro-domain distribution [29, 30, 40] and function as cell-cell adhesion (14/28), cell surface receptor (13/28) or signaling factors (19/28). We also noticed that YES induced phosphorylation of eight Tyrosine Kinases (TK). This suggests that, like SRC [9, 39], YES could orchestrate a TK signaling network to promote CRC cell transforming activity. We confirmed the SILAC results biochemically for 13/17 identified substrates (**Figure 4C-E**). We found that phosphorylation of several YES targets was also affected by *SRC* silencing, indicating that SRC and YES regulate the tyrosine phosphorylation of common targets (**Figure 4C**). However, *SRC* silencing also affected EPHA2, EPHB2 and MET receptor levels, suggesting that SRC-dependent pTyr of these substrates involves an indirect mechanism, such as gene expression and/or protein stabilization (**Figure 4C**). On the other hand, we confirmed that ephrin 1 and 2, EPHB3, ZO-2, p120 and β-catenin and, unexpectedly, upstream regulators of RAS/MAPK signaling (such as EGFR, SHP2 and SHC) are specific YES substrates (**Figure 4D**). The link between YES and RAS signaling was further supported by the YES-dependent increased pTyr level of GAB1, a well-established mediator of EGFR-RAS signaling and a substrate of the tyrosine phosphatase SHP2 (**Figure 4D**) [41]. Finally, we showed that the unique capacity of YES to phosphorylate these substrates, such as the RAS/MAPK signaling regulators, originated from the SH4 domain. Indeed, SRC palm+, but not YES palm-, restored substrate phosphorylation in YES-depleted cells (**Figure 4E**). Collectively, these results suggest that YES is an important regulator of cadherin- and ephrin-dependent cell adhesion and also, unexpectedly, of RAS/MAPK signaling in CRC cells.

A novel role for YES in the regulation of RAS/MAPK signaling in CRC cells

We then analyzed the effect of YES inhibition on EGF-induced MAPK activation in HT29 cells. Although these CRC cells harbor the oncogenic BRAF mutation, EGF still induced a dramatic increase in MAPK activity over time (**Figure 5A**). YES inactivation reduced this EGF response by 50%, while *SRC* silencing did not have any significant effect (**Figure 5A**). We next addressed the functional role of RAS/MAPK signaling in YES transforming activity by restoring MAPK activity in *YES*-silenced HT29 cells. Retroviral transduction of HRASV12 induced a 3-fold increase in MAPK activity in *YES*-silenced cells, thus restoring completely MAPK activity (**Figure 5B**). HRASV12 expression also recapitulated the specific contribution of SRC and YES to anchorage-independent growth (**Figure 5C**). Thus, these findings support a model where SRC and YES oncogenic signaling converge towards the activation of RAS-dependent components to promote anchorage-independent cell growth. Conversely, HRASV12 transduction could partially, but significantly, restore cell invasion in shYES cells, but not in shSRC cells (**Figure 5C**). We thus conclude that YES selectively regulates a RAS-dependent signaling cascade to promote cell invasion. Finally, we confirmed the oncogenic role of YES also in HCT116 cells that harbors one allelic RAS oncogenic mutation. EGF still induced MAPK activation, probably because of the activation of the wild-type RAS isoforms expressed in these cells. Interestingly, both MAPK activation and anchorage-independent cellular growth were impaired in YES-depleted HCT116 cells (**Figure 6**).

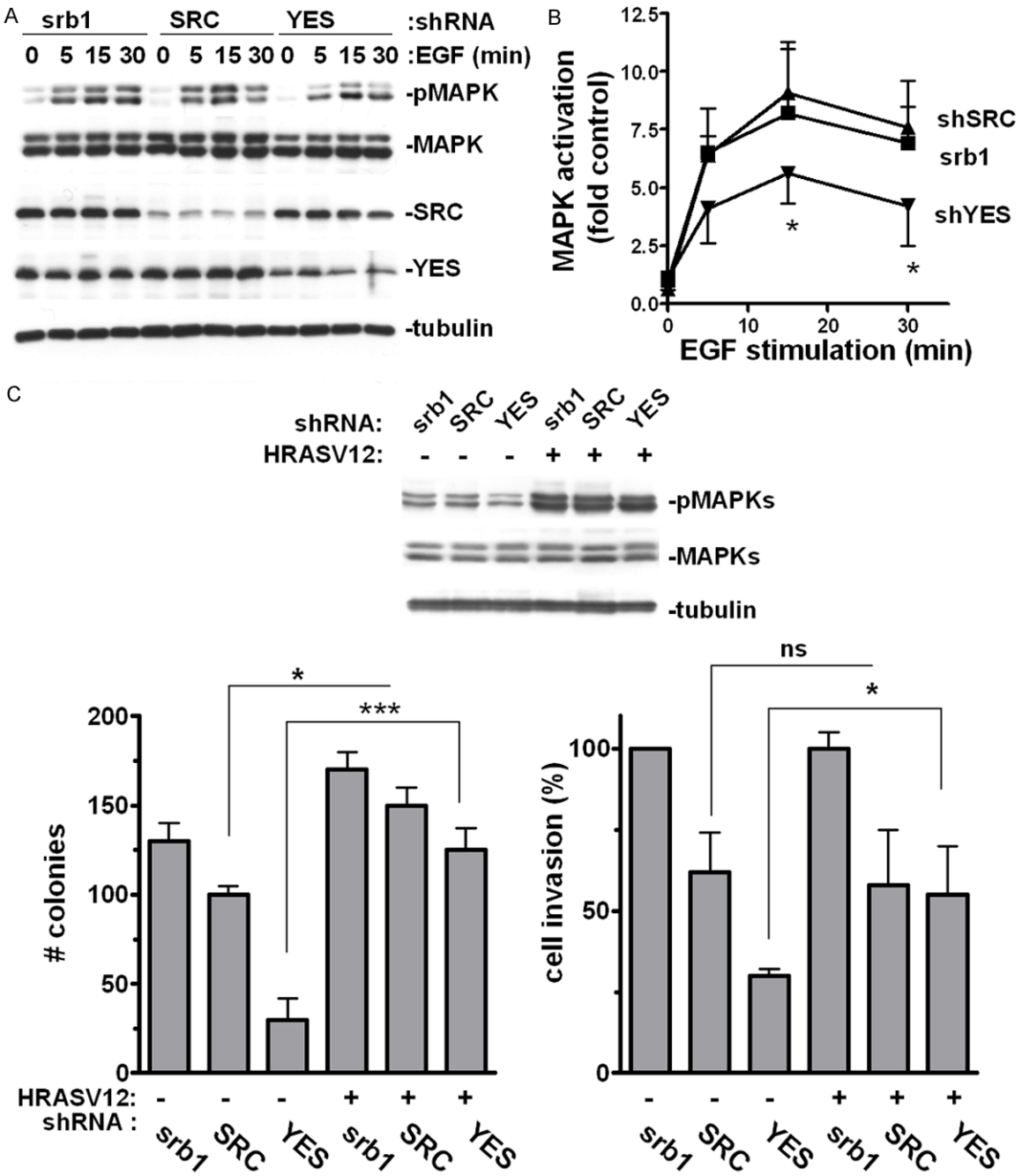

Figure 5. YES regulates RAS/MAPK signaling in HT29 cells. A. YES regulates MAPK activity in EGF-stimulated HT29 cells. A representative example (left panel) and quantification (right panel) of pMAPK, MAPK, SRC and YES levels in HT29 cells that express the indicated shRNAs (srb1, negative control) following stimulation with 50 ng/ml EGF for the indicated times. Tubulin was used as loading control. The mean ± SD (n = 4) is shown. *P < 0.05 compared to HT29 cells that express srb1. B. HRASV12 restores the MAPK activity in *YES*-silenced cells. Western blotting showing pMAPK and MAPK levels in lysates of HT29 cells that express the indicated shRNAs and infected or not with retroviruses expressing HRASV12. Tubulin was used as loading control. C. HRASV12 restores anchorage-independent growth and invasion in *YES*-silenced cells. Number of colonies in soft agar (left panel) and relative cell invasion in Boyden chambers (right panel) of HT29 cells expressing the indicated shRNAs and infected or not with HRAV12 retroviruses. The mean ± SD (n > 3) is shown. *P < 0.05; ***P ≤ 0.001; ns, not significant.

Collectively, these results indicate that RAS/MAPK signaling plays an important role in YES-dependent transforming properties of CRC cells.

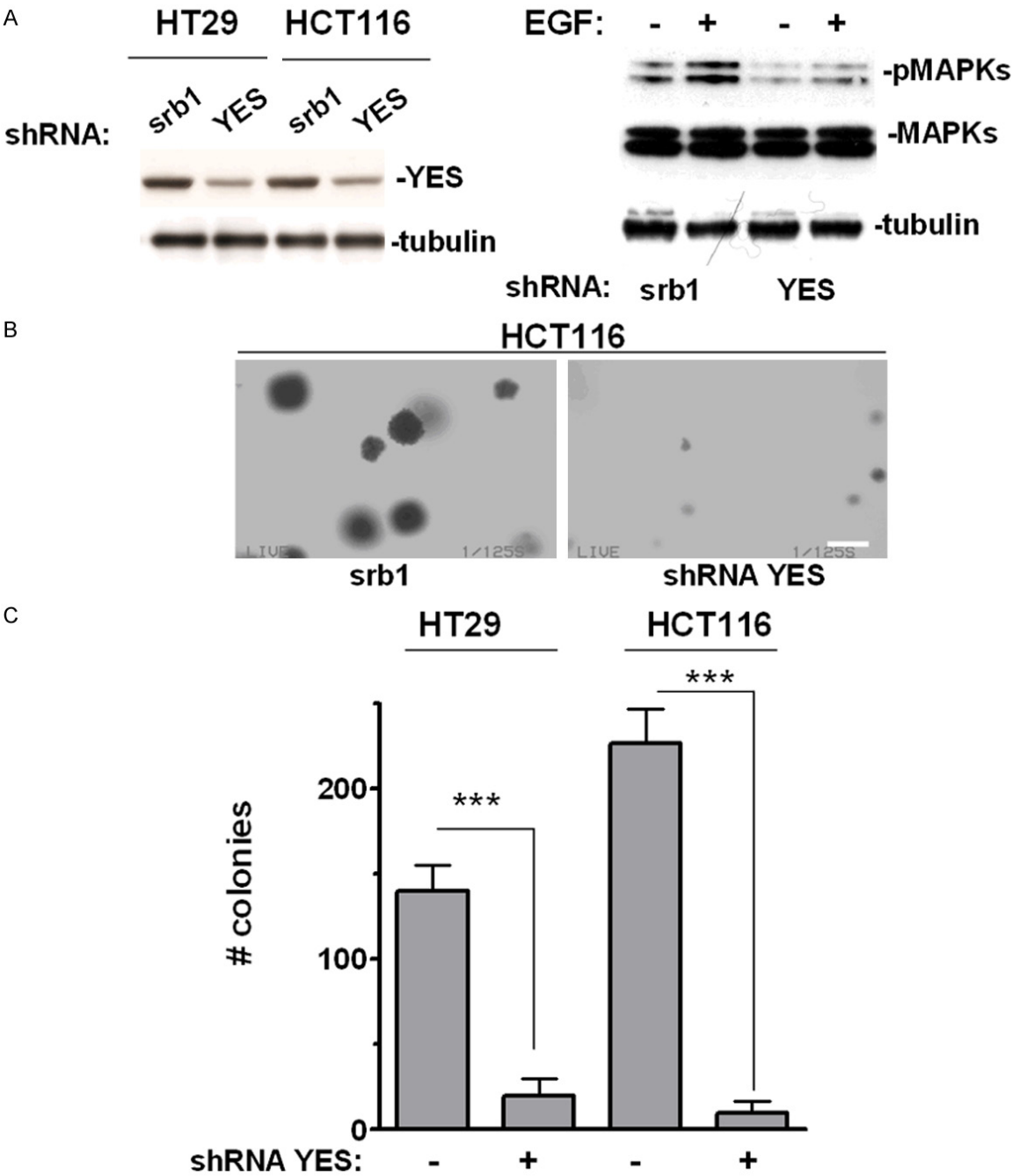

Figure 6. YES oncogenic signaling in HCT116 cells. A. *YES* silencing in HCT116 cells. Western blotting showing YES levels in lysates of indicated CRC cells infected with retroviruses that express YES or srb (negative control) shRNAs. Tubulin was used as loading control. B. MAPK signaling defect in *YES*-silenced HCT116 cells. MAPK activity and expression in the indicated cells stimulated or not with 50 ng/ml EGF for 5 min. C. Anchorage-independent growth defect in *YES*-silenced HCT116 and HT29 cells. Size (top panel) and number (bottom panel) of colonies obtained from the indicated CRC cell lines in which *YES* was silenced or not. The mean ± SD (n > 3) is shown. ***P ≤ 0.001. Scale bar, 500 μm.

Discussion

Here we show that YES unique transforming activity is specified by palmitoylation of its SH4 domain. This post-translational modification present in YES but not in SRC regulates SFK micro-domain distribution, substrate phosphorylation and selective oncogenic signaling. The

contribution of palmitoylation to YES transforming activity is further supported by the significant redundant function between YES and SRC palm+, when targeted to CRC micro-domains. Therefore, regulation of SFK spatial distribution may be the main cause of YES unique signaling observed in CRC cells. This idea is also consistent with the existence of distinct SFK pools controlling various aspects of CRC cell transformation [12]. An important issue related to this notion is the nature and the function of micro-domains in tumor cells. Cholesterol-enriched membrane domains, including caveolae, have been proposed to limit SFK transforming potential in fibroblasts [11]. Conversely, our present report supports a promoting role for CRC micro-domains on SFK transforming activities. The tumor function of these membrane domains may rely on the abundance and the nature of their lipid and protein constituents, such as glycosphingolipids, flotillins and caveolins [29, 30]. CRC micro-domains may also have selective sub-cellular localizations and functions. For instance, these domains are involved in the maintenance of cell-cell adhesion, including adherens and tight junctions, in epithelial cells [42, 43]. Therefore, aberrant SFK activity in CRC micro-domains promotes phosphorylation and disassembly of critical components of adhesive complexes present in these structures, as revealed by proteomics. This leads to cell dissociation and migration, processes involved in the epithelial to mesenchyme transition and required for cell dissemination and metastasis formation [44]. Additionally, CRC micro-domains may play an important role in cell invasion, as recently reported concerning the regulation of invadopodia, membrane protrusions specialized in extracellular matrix degradation [45]. These F-actin-enriched structures secrete proteases at cell contact sites with the extracellular matrix, allowing matrix degradation and local cell dissemination [46].

Our report also brings important molecular insights into the nature of YES oncogenic signaling in CRC cells. Our proteomic analysis suggests that YES might drive cell scattering by direct phosphorylation of the E-cadherin complex. YES may also promote beta-catenin signaling by direct phosphorylation of this oncoprotein. Finally, YES may regulate CRC stem cell properties [27] by phosphorylating critical determinants of this neoplastic process, such as receptors of the EPH family [47]. It should be noted that YAP1 was not recovered in our SILAC approach, probably because in HT29 cells this beta-catenin complex is weakly expressed [27]. Alternatively, YAP1 might be activated by an indirect mechanism that does not involve tyrosine phosphorylation. Proteomics also identified a cluster of TKs as YES targets, thus revealing a novel role for YES in the control of TK activity to promote neoplastic transformation. YES also phosphorylates RTKs and cell-surface receptors without catalytic activity. This raises the counter-intuitive idea that YES behaves as an upstream inducer of cell surface receptor signaling in CRC cells. Therefore, deregulated YES activity could perturb RTK signaling localized in CRC micro-domains, leading to the aberrant tumor cell response to local extracellular cues and favoring tumor progression. For instance, MET has a key role in metastatic progression by mediating the interaction between CRC cells and stroma [48, 49]. YES-induced aberrant MET activity in CRC micro-domains could support tumor cell interaction with the niche for metastatic outgrowth.

Our results also suggest that YES, rather than SRC, is an important inducer of RAS/MAPK signaling in CRC cells. This hypothesis is further supported by the lack of MAPK activity reduction upon SRC silencing and the functional link between RAS and YES during cell invasion. As SFK spatial regulation is the major cause of their non-redundant function in CRC cells, our data suggest that EGFR/RAS/MAPK signaling is initiated in CRC micro-domains where YES resides. The molecular mechanism by which YES regulates this signaling cascade was not investigated in this study, but a link between YES and EGFR has been reported during receptor trafficking, including transcytosis [50] and nuclear transport [23]. Therefore, one mechanistic explanation may rely on EGFR membrane trafficking deregulation. Additionally, the tyrosine phosphatase SHP2 seems to be a critical YES substrate for the regulation of its signaling response. This hypothesis is supported by (i) the strong effect of YES silencing on SHP2 phosphorylation, (ii) the increased pTyr level of the SHP2 target and RAS activator GAB1 upon YES down-regulation, and (iii) the similar regulation of SHP2 and YES on sustained MAPK activation induced by EGF. Mechanistically, SHP2 has been reported to oppose the rasGAP-

dependent reduction of RAS activity by disrupting rasGAP-GAB1 interaction [41]. How YES regulates SHP2 activity for the regulation of RAS/MAPK signaling in CRC cells is currently unknown, but it might involve SHP2 phosphorylation on specific Tyr residues (not shown).

Finally, our results may have significant implications in CRC therapy. They suggest that in addition to SRC, YES is also an attractive target in metastatic CRC. Although most SFK inhibitors developed for the clinic also target YES *in vitro*, they may not target all SFK pools, including YES in CRC micro-domains, due to the lipophilic micro-environment. It would therefore be important to design inhibitors that will also target SFK in micro-domains in advanced CRC tumors. Similarly, an assay that fully measures SFK activity in tumors, including in CRC micro-domains, is also required. For instance, the level of FAK phosphorylation by SRC is often used as surrogate of SFK activity; however it is not a reliable marker of YES activity in CRC, as revealed by our SILAC analysis (i.e., no change in pTyr-FAK level upon YES silencing). Finally, our data suggests that YES, rather than SRC, plays an important role in the induction of EGFR/RAS/MAPK signaling in CRC cells. EGFR is a clinically validated therapeutic target in metastatic CRC and neutralizing antibodies have some clinical benefit, but they failed to significantly extend patient survival [51]. Therefore, our results predict that an inhibitor targeting SFK activity also in CRC micro-domains may increase the tumor response to EGFR-based therapy.

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Disclosure of conflict of interest

None.

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