Q1 *Tumor and Stem Cell Biology*

Cancer Research

P120RasGAP-Mediated Activation of c-Src Is Critical for Oncogenic Ras to Induce Tumor Invasion

AU Po-Chao Chan[1,4], and Hong-Chen Chen[1,2,3,4]

Abstract

Ras genes are the most common targets for somatic gain-of-function mutations in human cancers. In this study, we found a high incidence of correlation between Ras oncogenic mutations and c-Src activation in human cancer cells. We showed that oncogenic Ras induces c-Src activation mainly in the Golgi complex and endoplasmic reticulum. Moreover, we identified p120RasGAP as an effector for oncogenic Ras to activate c-Src. The recruitment of p120RasGAP to the Golgi complex by oncogenic Ras facilitated its interaction with c-Src, thereby leading to c-Src activation, and this p120RasGAP-mediated activation of c-Src was important for tumor invasion, induced by oncogenic Ras. Collectively, our findings unveil a relationship between oncogenic Ras, p120RasGAP, and c-Src, suggesting a critical role for c-Src in cancers evoked by oncogenic mutations in *Ras* genes. *Cancer Res; 1–11. ©2012 AACR.*

Introduction

The human Ras proteins, H-Ras, N-Ras, K-Ras (K-Ras4A and K-Ras4B), are the most well-known members of the Ras family. They have been extensively studied for more than 30 years, partly because of their high frequency of activating mutations in human cancers (1). Ras proteins function as GDP/GTP molecular switches that regulate diverse signaling pathways and cell functions (1). Cycling between GDP- and GTP-bound states of Ras is controlled by 2 classes of regulatory molecules: guanine nucleotide exchange factors (GEF) and GTPase-activating proteins (GAP; ref. 2). GEFs facilitate the intrinsic GDP/GTP exchange activity of Ras, whereas GAPs stimulate the relatively slow intrinsic GTP hydrolysis activity of Ras. GTP-bound Ras regulates a complex signaling network by binding to and activating diverse classes of effector molecules, such as Raf, phosphoinositide 3-kinase (PI3K), and RalGEFs (3).

Ras emanates signals not only from the plasma membrane but also from organelles, including the Golgi complex, the endoplasmic reticulum (ER), mitochondria, and endosomes (4). The biologic significance for compartmentalized Ras signaling was initially described in T lymphocytes, in which the activation of Ras/mitogen—activated protein kinase (MAPK) signaling at different subcellular compartments could lead to either negative or positive selection of T lymphocytes (5). The compartmentalized Ras signaling is also conserved in fission yeasts. Ras1p, a single Ras protein in *Schizosaccharomyces pombe*, regulates mating from the plasma membrane and cell morphology from the endomembranes (6). Therefore, the biologic functions elicited by Ras are closely related to its subcellular localizations.

Cellular Src (c-Src), a non–receptor protein tyrosine kinase, has been implicated in the regulation of a variety of cellular functions (7, 8). To exert such diverse biologic activities, c-Src interacts with and phosphorylates a wide range of cellular proteins (7). Crystallographic analysis has revealed that the Src-homology (SH)2 and SH3 domains of c-Src bind to its own tyrosine 527 and a short polyproline type II helix between the SH2 and kinase domain, leaving c-Src in a close, autoinhibited state (9). Interactions of c-Src with other cellular proteins via its SH2 and/or SH3 domains could disrupt the intramolecular inhibitory interactions, resulting in c-Src autophosphorylation on tyrosine 416 in the activation loop, within the kinase domain for its full activation (7). Although c-Src is frequently found to be hyperactivated in human cancers, its genetic mutations are rarely observed (10).

It has been known for years that oncogenic Src (v-Src) can lead to Ras activation through different modes. For example, v-Src can phosphorylate the adaptor protein Shc, which then recruits Grb2/Sos complexes for Ras activation (11). In addition, Ras has been shown to be essential for v-Src–stimulated cell transformation (12). However, it is unclear whether oncogenic Ras could lead to the activation of c-Src. In particular, as activation of Ras and c-Src are frequently found in human cancers, clarification of their causal relationship will be of importance for clinical implication. In this study, we found that oncogenic Ras induces c-Src activation predominantly on the Golgi complex and ER. In addition, p120RasGAP was identified as an effector for oncogenic Ras to induce c-Src activation. Importantly, we showed that the p120RasGAP-mediated

Authors' Affiliations: [1]Department of Life Sciences, [2]Agricultural Biotechnology Center, National Chung Hsing University; [3]Department of Nutrition, China Medical University, Taichung; and [4]Center of Infectious Disease and Signaling Research, National Cheng Kung University, Tainan, Taiwan Q2

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Corresponding Author: Hong-Chen Chen, Department of Life Sciences, National Chung Hsing University, 250 Kuo-Kuang Road, Taichung 40227, Taiwan, Phone: 886-4-22854922; Fax: 886-4-22853469; E-mail: hcchen@nchu.edu.tw

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activation of c-Src is critical for oncogenic Ras to promote tumor invasion.

Materials and Methods

Materials

Plasmids, antibodies, and other reagents are described in the Supplementary Materials and Methods.

Cell culture and transfection

HEK293, Cos, NIH3T3, MDCK, mouse embryo fibroblasts (MEF), and SYF (*src*$^{-/-}$ *yes*$^{-/-}$ *fyn*$^{-/-}$) cells were kindly provided by Dr. Jun-Lin Guan (University of Michigan Medical School, Ann Arbor, MI). The NCI-60 cancer cell lines (A549, HOP-62, NCI-H23, NCI-H460, and HCT-116) were purchased from the National Cancer Institute (Bethesda, MD) and kindly provided by Dr. Jeremy J.W. Chen (National Chung Hsing University, Taichung, Taiwan). Other human cancer cell lines including T24, HT-1080, SW480, MDA-MB-231, MIA PaCa-2, PANC-1, AsPC-1, NCI-H358, and LoVo were purchased from the American Type Culture Collection. HEK293, Cos, NIH3T3, MDCK, MEFs, SYF (*src*$^{-/-}$ *yes*$^{-/-}$ *fyn*$^{-/-}$) cells, and the human cancer cell lines described in Table 1 except MIA PaCa-2, PANC-1, and AsPC-1 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen) supplemented with 10% FBS (HyClone). MIA PaCa-2, PANC-1, and AsPC-1 cells were maintained in RPMI-1640 supplemented with 10% FBS. The NIH3T3 cells with a Lac operon–driven H-Ras^{V12} expression were kindly provided by Dr. H.-S. Liu (National Cheng Kung University, Tainan, Taiwan) and are described previously (13). Reintroduction of c-Src into SYF cell line (SYF/c-Src) was established by retroviral infection. The SYF/H-Ras^{V12} and SYF/c-Src, and H-Ras^{V12} cell lines were established by cotransfection with pBabe-puro and pcDNA3-HA-H-Ras^{V12} plasmids. Cells were selected and maintained in DMEM containing 10% FBS (HyClone) and 1 μg/mL puromycin. Transient transfection was carried out by Lipofectamine (Invitrogen) or FuGENE HD (Roche).

Short hairpin RNA

The lentiviral expression system for short hairpin RNA (shRNA) was provided by the National RNAi Core Facility (Academia Sinica, Taiwan). The production and infection of lentiviruses and the targeted sequences of shRNAs are described in the Supplementary Materials and Methods.

Immunoprecipitation, immunoblotting, and Src kinase assay

Immunoprecipitation, immunoblotting, and Src kinase assay were conducted as described in the Supplementary Materials and Methods.

Biologic assays

Biologic assays including cell proliferation assay, cell survival assay, soft agar colony formation assay, cell invasion assay, tumorigenicity assay, and metastasis assay are described in the Supplementary Materials and Methods.

Confocal microscopy

Cells were fixed, stained, and scanned by a Zeiss LSM 510 confocal microscope as described in the Supplementary Materials and Methods.

Statistical analysis

The quantitative results are shown as mean ± SD. Statistical significance of 2-way difference was assessed by the Student *t* test.

Table 1. Effect of knockdown of Ras or p120RasGAP on Src activity in human cancer cell lines

Cell lines	Origin	Ras mutation[a]	Src inhibition by Ras knockdown	Src inhibition by RasGAP knockdown
T24	Bladder carcinoma	H-Ras G12V	Yes	Yes
HT-1080	Fibrosarcoma	N-Ras Q61K	Yes	Yes
SW480	Colorectal adenocarcinoma	K-Ras G12V	Yes	Yes
NCI-H460	Lung carcinoma	K-Ras Q61H	Yes	Yes
HOP-62	Lung carcinoma	K-Ras G12C	Yes	Yes
NCI-H23	Lung adenocarcinoma	K-Ras G12S	Yes	Yes
MDA-MB-231	Breast adenocarcinoma	K-Ras G13D	Yes	Yes
MIA PaCa-2	Pancreatic carcinoma	K-Ras G12C	Yes	Yes
PANC-1	Pancreatic carcinoma	K-Ras G12D	Yes	No
AsPC-1	Pancreatic carcinoma	K-Ras G12D	Yes	No
HCT-116	Colorectal carcinoma	K-Ras G13D	No[b]	Yes
A549	Lung adenocarcinoma	K-Ras G12S	No	Yes
NCI-H358	Lung carcinoma	K-Ras G13C	No	Yes
LoVo	Colorectal adenocarcinoma	K-Ras G13D	No	No

[a]Ras mutation as described by Davies and colleagues (14).
[b]Src activity is suppressed by H-Ras^{N17}.

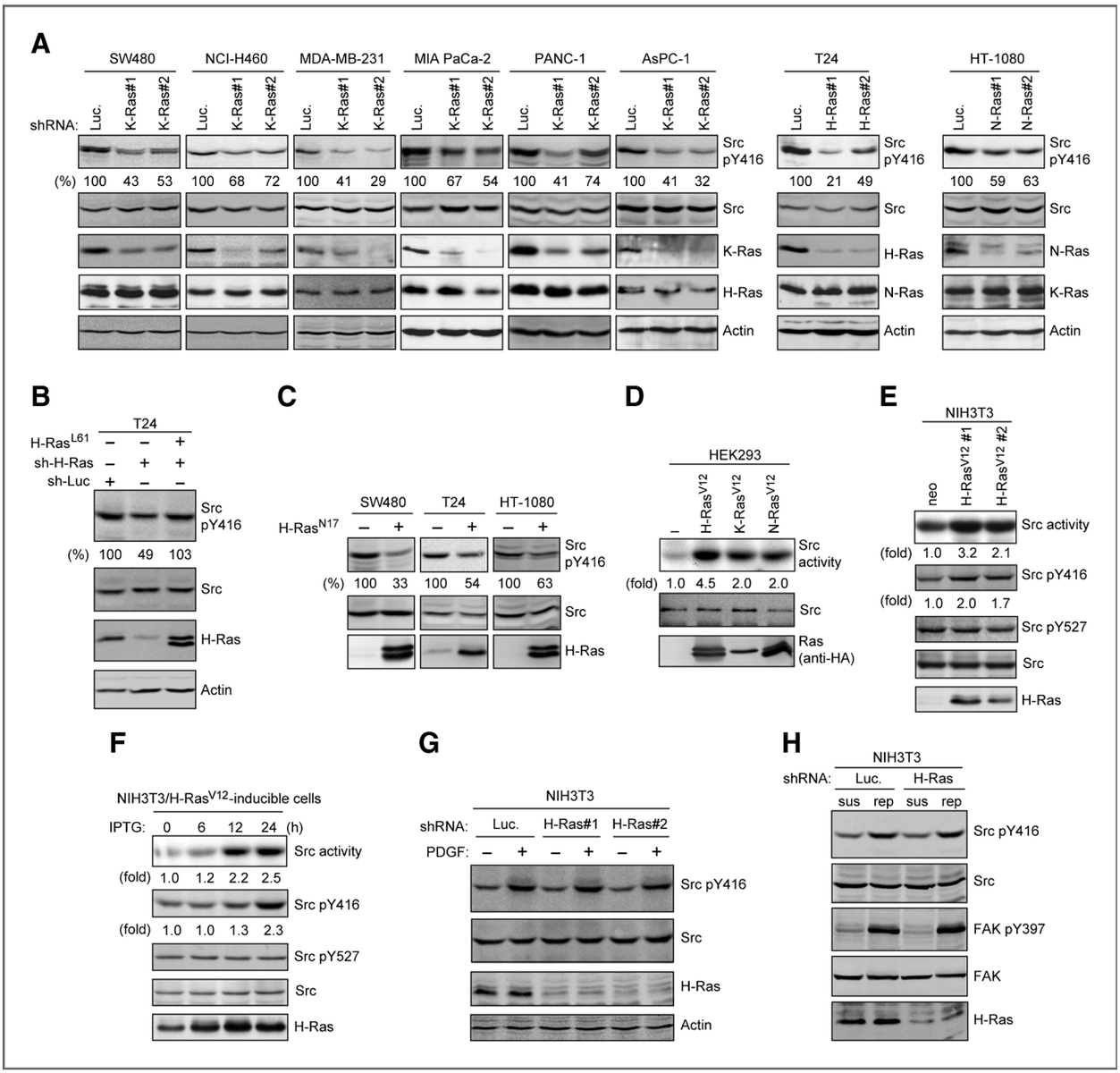

Figure 1. Oncogenic Ras induces c-Src activation. A, human cancer cell lines harboring oncogenic Ras were infected with recombinant lentiviruses encoding shRNAs specific to oncogenic Ras or luciferase (Luc) as a control. Two shRNAs (#1 and #2) were used to knockdown oncogenic Ras. The level of Src pY416 was analyzed by immunoblotting with anti-Src pY416. B, inhibition of Src pY416 by sh-H-Ras in T24 cells was rescued by reexpression of H-Ras^{L61}. C, H-Ras^{N17} was transiently expressed in cancer cells and its effect on Src pY416 was measured. D, HA-tagged Ras^{V12} constructs were transiently expressed in HEK293 cells and their effect on c-Src activity was analyzed by an *in vitro* kinase assay with acid-denatured enolase as a substrate. The expression of HA-tagged Ras was analyzed by immunoblotting with anti-HA. E, cell lysates from 2 NIH3T3 cell clones (#1 and #2) stably expressing H-Ras^{V12} and a neomycin-resistant control clone (neo) were subjected to analysis for the activity and pY416 of c-Src. F, an isopropyl-β-D-1-thiogalactopyranoside (IPTG)-inducible expression system for H-Ras^{V12} was established in NIH3T3 cells. H-Ras^{V12} was allowed to express for various times and its effect on the activity of pY416 and pY527 of c-Src was analyzed. G, endogenous H-Ras of NIH3T3 cells was depleted by shRNA and its effect on platelet-derived growth factor–induced activation of c-Src was measured. H, endogenous H-Ras of NIH3T3 cells was depleted by shRNA and its effect on cell adhesion–induced activation of c-Src was measured. The cells were kept in suspension (sus) and then replated (rep) on fibronectin-coated dishes. Focal adhesion kinase (FAK) pY397 was used as an indicator for cellular responses to cell adhesion.

Results

Oncogenic Ras induces c-Src activation

The possibility for oncogenic Ras to activate c-Src was first examined in human cancer cell lines that harbor oncogenic mutations in *ras* genes (14). Depletion of oncogenic Ras (K-Ras, H-Ras, or N-Ras) by shRNA decreased c-Src phosphoY416 (Src pY416) in 71% (10 of 14) of examined cell lines (Fig. 1A; Table 1). The decreased Src pY416 caused by H-Ras knockdown was rescued by reexpression of oncogenic H-Ras^{L61} (Fig. 1B), indicating that the decreased Src pY416 is not because of a

nonspecific off-target effect of shRNA. Moreover, H-Ras^{N17}, which is known to effectively inhibit all 3 Ras isoforms by sequestration of Ras GEFs (15), was capable of suppressing Src pY416 in T24 cells (with oncogenic H-Ras), SW480 cells (with oncogenic K-Ras), and HT-1080 cells (with oncogenic N-Ras; Fig. 1C). Alternatively, an overexpression strategy was undertaken to examine whether the forced expression of oncogenic Ras could lead to c-Src activation. We found that transient, stable, or induced expression of oncogenic Ras stimulated the catalytic activity of c-Src, accompanied by increased Src pY416 (Fig. 1D–F). Together, our results clearly indicate that oncogenic Ras leads to c-Src activation. It is worth noting that c-Src activation by Ras might be specific to oncogenic forms of Ras, as depletion of endogenous Ras did not affect c-Src activation by platelet-derived growth factor (Fig. 1G) or cell adhesion (Fig. 1H).

Oncogenic Ras activates c-Src mainly on the Golgi complex and ER

Ras is known to localize to different membrane compartments via posttranslational modifications, such as farnesylation or palmitoylation, on its carboxyl terminus (4). A selective inhibitor (FTI-277) for farnesyltransferase was found to inhibit the activation of c-Src by H-Ras^{V12} (Supplementary Fig. S1A), suggesting that the association of H-Ras^{V12} with cellular membranes may be important for its ability to activate c-Src. To test this possibility, c-Src activation by H-Ras^{L61} and its variants deficient in association with different membrane compartments was evaluated. The subcellular localization of H-Ras^{L61} and its variants was visualized by GFP-fused Raf Ras-binding domain (Raf-RBD; Supplementary Fig. S1B). H-Ras$^{L61/C186S}$ (defective in farnesylation), which fails to associate with any cellular membranes, lost its ability to activate Src (Fig. 2A). H-Ras$^{L61/C181S/C184S}$ (defective in palmitoylation), which is known to associate with the Golgi complex and ER, but not the plasma membrane (16), retained its ability to activate Src to an extent similar to that activated by H-Ras^{L61} (Fig. 2A). To further confirm that the association of H-Ras^{L61} with the Golgi complex and/or ER is important for it to activate c-Src, H-Ras$^{L61/C186S}$ was engineered to target to the Golgi complex or ER. The Golgi-targeted H-Ras$^{L61/C186S}$ (KDELR-Ras$^{L61/C186S}$) and the ER-targeted H-Ras$^{L61/C186S}$ (M1-Ras$^{L61/C186S}$) substantially activated c-Src to the level comparable with that by Ras^{L61} (Fig. 2A), indicating that the association of H-Ras^{L61} with the Golgi complex and ER is important for it to activate c-Src. Similarly, we found that K-Ras$^{V12/C185S}$, which cannot be farnesylated, failed to activate c-Src (Fig. 2B). In contrast, K-Ras$^{V12/S181E}$, which is known to predominantly associate with endomembranes (17), activated c-Src more potently than K-Ras^{V12} (Fig. 2B). Therefore, our results suggest that association of oncogenic Ras with endomembranes, in particular the Golgi complex and ER, may be crucial for it to activate c-Src.

To detect the subcellular localization where c-Src is activated by oncogenic Ras, MDCK or Cos cells transiently expressing hemagglutinin (HA) epitope–tagged H-Ras^{V12} (HA-H-Ras^{V12}) were stained for active Src with anti-Src pY416. Our results showed that active, endogenous c-Src was detected only in the cells expressing HA-H-Ras^{V12} (Fig. 2C), in which it was colocalized with HA-H-Ras^{V12} at the Golgi complex (Fig. 2D) and ER (Fig. 2E). Notably, although a fraction of HA-H-Ras^{V12} was distributed at the plasma membrane, no active Src was detected at the plasma membrane (Fig. 2C, arrowheads). Our results thus suggest that the Golgi complex and ER may be the major platforms for oncogenic Ras to activate c-Src.

It is known that myristoylation of Src at Gly2 is required for its association with cellular membranes (18). We used c-Src-GFP (with a GSGS-linker between c-Src and GFP) and its G2A mutant to examine whether membrane association is required for c-Src to be activated by oncogenic Ras. We found that c-Src-GFP was retained in an inactive state and mainly resided at the perinuclear region in resting cells (Supplementary Fig. S1C). Upon stimulation by EGF, c-Src-GFP became activated and was recruited to the plasma membrane (Supplementary Fig. S1C), indicating that the activity and subcellular localization of c-Src-GFP can be regulated in response to extracellular stimuli. Unlike c-Src-GFP, c-Src^{G2A}-GFP was diffusely distributed in the cytoplasm and was refractory to be activated by H-Ras^{V12} (Supplementary Fig. S1D). Together, our results suggest that membrane tethering of oncogenic Ras and c-Src on the Golgi complex and ER may be essential for oncogenic Ras to activate c-Src.

Activation of c-Src by oncogenic Ras is not through alterations in reactive oxygen species production, autocrine, or cell adhesion

How does oncogenic Ras induce c-Src activation? First, we examined whether reactive oxygen species (ROS) play a role in this regard. Oncogenic Ras was reported to induce large amount of ROS (19). Because ROS has been shown to stimulate the activity of Src (8, 20), we speculated that oncogenic Ras might activate c-Src via ROS production. However, this possibility was excluded, because elimination of ROS by *N*-acetylcysteine (NAC), a potent ROS scavenger, did not prevent the activation of c-Src by H-Ras^{V12} (Supplementary Fig. S2A and S2B). Second, we speculated an autocrine mechanism might be involved in the Src activation by oncogenic Ras. However, the conditioned medium collected from the cells expressing oncogenic Ras did not stimulate c-Src activity, rendering it less likely that c-Src activation by oncogenic Ras is through an autocrine fashion (Supplementary Fig. S2C and S2D). Third, we examined whether oncogenic Ras activates c-Src through its effect on cell adhesion. We found that oncogenic Ras was able to activate c-Src even when the cells were kept in suspension (Supplementary Fig. S2E and S2F), thus indicating that activation of c-Src by oncogenic Ras cannot be attributed to cell adhesion.

Oncogenic Ras activates c-Src independently of PI3K, Raf, RalGEF, or Ral

PI3K, Raf, and RalGEF are the 3 most well-known, immediate effectors for Ras (21). We found that inhibition of PI3K by specific inhibitors (wortmannin and LY294002) or depletion of the p110 catalytic subunit of PI3K by shRNA did not impair the ability of oncogenic Ras to activate c-Src (Supplementary Fig. S3A and S3B). In addition, the inhibition of Raf and its downstream effector MAP/extracellular signal—-regulated kinase (ERK) kinase by the inhibitors ZM336372 and PD98059

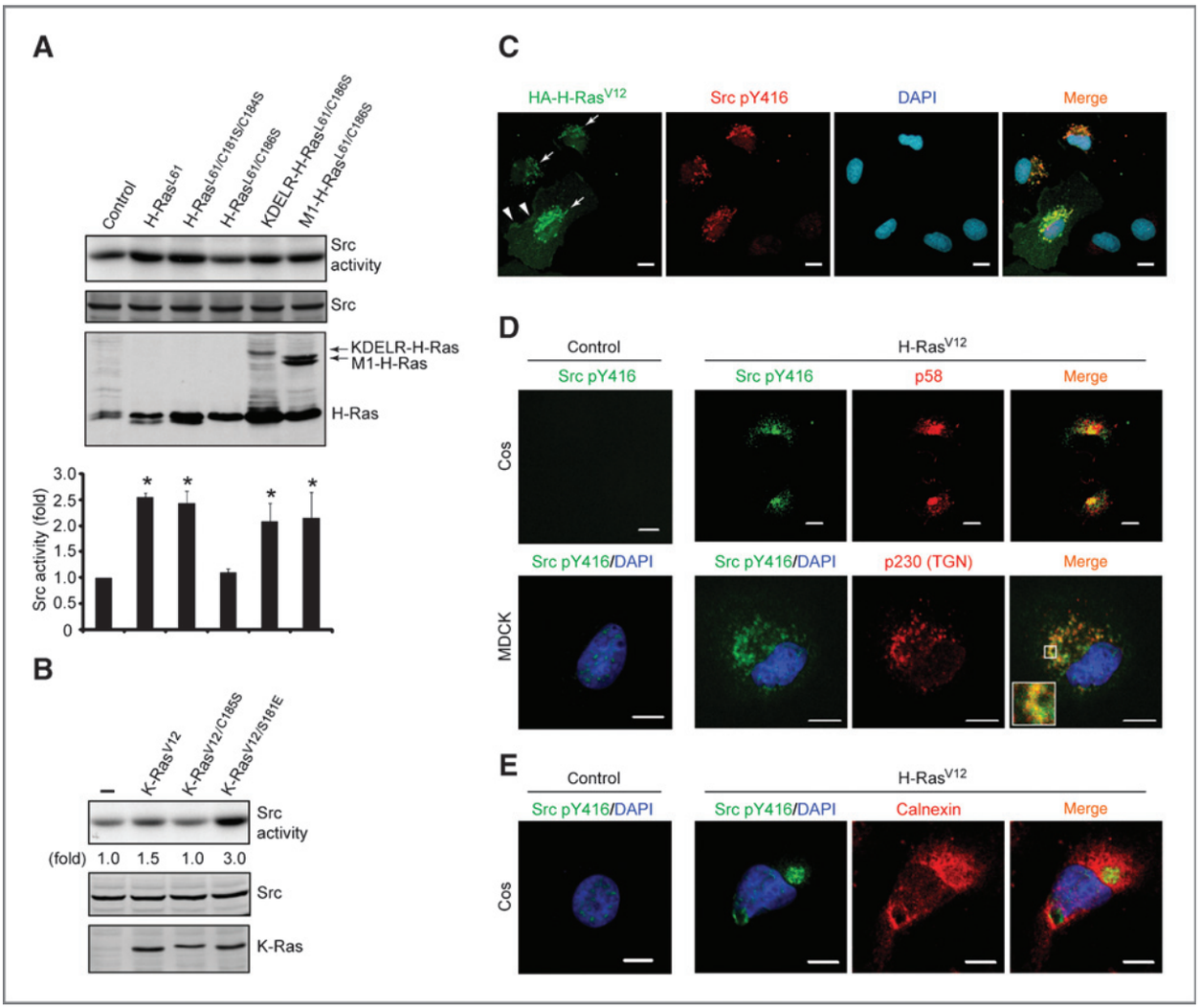

Figure 2. Oncogenic Ras induces c-Src activation mainly on the Golgi complex and ER. A, H-Ras^{L61} and its variants were transiently expressed in HEK293 cells and their effect on c-Src activity was analyzed. *, $P < 0.01$. B, K-Ras^{V12} and its variants were transiently expressed in HEK293 cells and their effect on c-Src activity was analyzed. C, HA-H-Ras^{V12} was transiently expressed in MDCK cells. The cells were subjected to immunofluorescent staining for HA-H-Ras and Src pY416. Note that the endogenous, active c-Src detected by anti-Src pY416 is colocalized with endomembrane-associated H-Ras^{V12} (arrows), but not plasma membrane–associated H-Ras^{V12} (arrowheads). Scale bar, 10 μm. D, H-Ras^{V12} was transiently expressed in Cos cells or MDCK cells. The cells were stained for the Golgi complex and Src pY416, with p58 as a marker for *cis*-Golgi and p230 as a marker for trans-Golgi. The selected area (white box) in MDCK cells was enlarged. Scale bar, 10 μm. E, H-Ras^{V12} was transiently expressed in Cos cells. The cells were stained for the ER and Src pY416, with calnexin as a marker for the ER. Scale bar, 10 μm. DAPI, 4′,6-diamidino-2-phenylindole.

did not prevent c-Src activation by oncogenic Ras (Supplementary Fig. S3C). Moreover, H-Ras$^{V12/S35}$ and H-Ras$^{V12/G37}$, which preferentially activate Raf and RalGEF, respectively (22), activated c-Src to a level similar to that by H-Ras^{V12} (Supplementary Fig. S3D), thus suggesting that activation of Raf and RalGEF by oncogenic Ras does not lead to c-Src activation. The role of Ral in c-Src activation was then further examined. Overexpression of constitutively active RalA or RalB had little effect on c-Src activity (Supplementary Fig. S3E). RLIP76ΔGAP is a dominant-negative construct for both RalA and RalB (23). We found that neither the expression of RLIP76ΔGAP nor depletion of RalA and RalB affected the activation of c-Src by oncogenic Ras (Supplementary Fig. S3F–S3H). Therefore, our results indicate that PI3K, Raf, RalGEF, and Ral are less likely to be involved in Ras-induced activation of c-Src.

P120RasGAP is a key mediator for oncogenic Ras to activate c-Src

To identify the molecule(s) essential for oncogenic Ras to activate c-Src, a collection of shRNAs that target Ras effectors, GEFs and GAPs, was applied in our study. Through an unbiased screening, we identified p120RasGAP (RasGAP) as an effector for oncogenic Ras to activate c-Src. We found that knockdown of p120RasGAP reduced Src pY416 in 79% (11 of 14) of examined cancer cell lines (Fig. 3A; Table 1). Knockdown of both K-Ras and p120RasGAP had more profound inhibition in

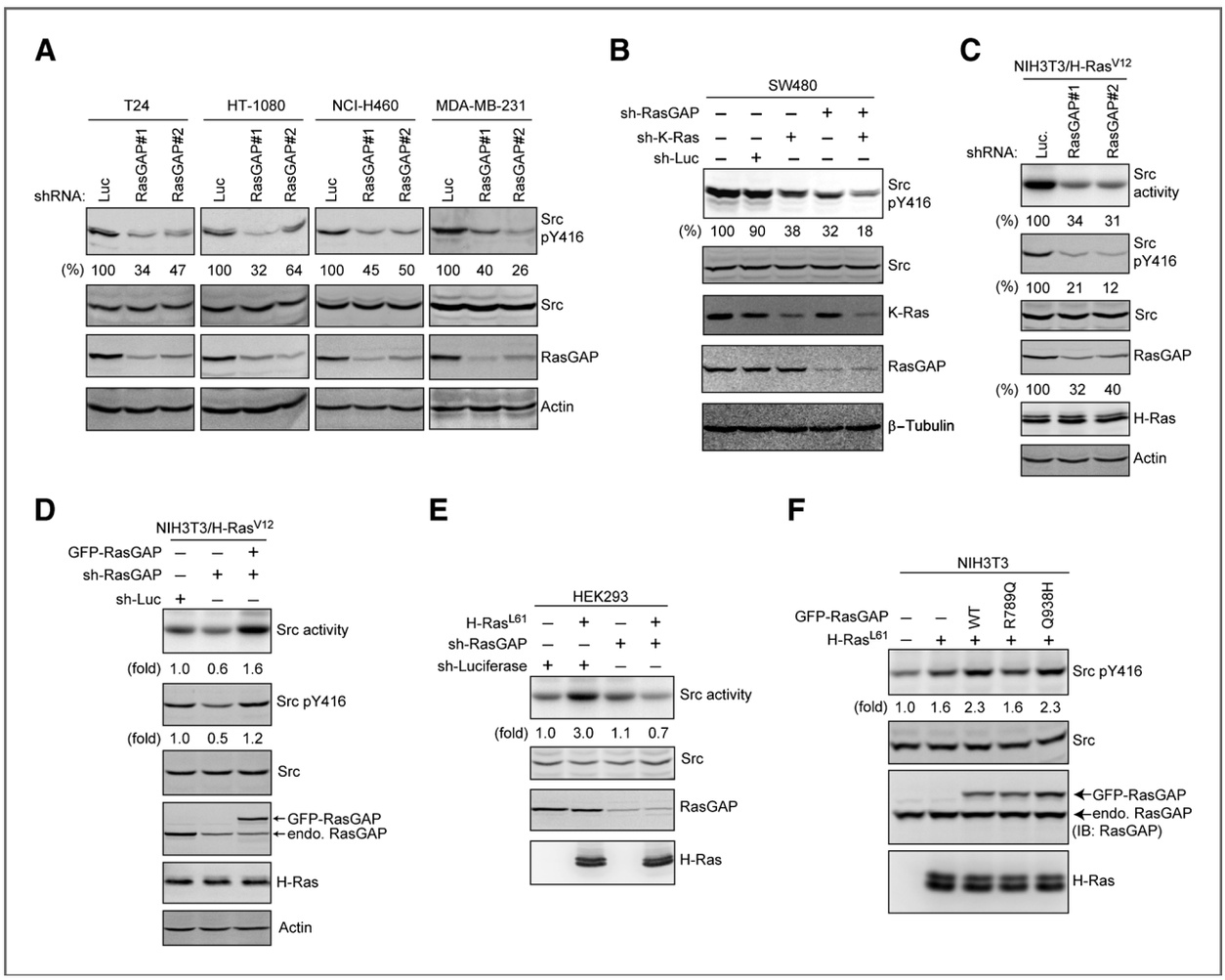

Figure 3. p120RasGAP is a key mediator for oncogenic Ras to activate c-Src. A, p120RasGAP was depleted by shRNAs in human cancer cell lines and its effect on Src pY416 was analyzed. Two shRNAs (#1 and #2) specific to p120RasGAP were used. An shRNA to luciferase (Luc) was used as a control. B, K-Ras and/or p120RasGAP were depleted by shRNAs in SW480 cells and the effect on Src pY416 was analyzed. C, p120RasGAP was depleted in H-Ras^{V12}–transformed NIH3T3 cells and its effect on the pY416 and activity of Src was measured. D, inhibition of Src pY416 and activity by p120RasGAP shRNA (sh-RasGAP) was rescued by expression of GFP-p120RasGAP (GFP-RasGAP). E, H-Ras^{L61} failed to stimulate c-Src activity in the cells expressing p120RasGAP shRNA. F, GFP-p120RasGAP or its mutants were transiently expressed in NIH3T3 cells and their effect on Src pY416 was measured. The R789Q mutant is defective in Ras binding. The Q938H mutant lacks the GAP activity. IB, immunoblotting; WT, wild-type

c-Src activation than knockdown of either one in SW480 cells, (Fig. 3B), supporting that oncogenic Ras, RasGAP, and c-Src are in the same signaling axis. The reduced c-Src activity by p120RasGAP knockdown was rescued by expression of GFP-fused p120RasGAP in H-Ras^{V12}–transformed NIH3T3 cells (Fig. 3C and D). Moreover, knockdown of p120RasGAP impaired the ability of H-Ras^{L61} to activate c-Src (Fig. 3E). Conversely, overexpression of p120RasGAP, but not its R789Q mutant deficient in Ras binding (24), potentiated the ability of H-Ras^{L61} to activate c-Src (Fig. 3F), indicating that binding of p120RasGAP to oncogenic Ras is important for c-Src activation. Of note, the Q938H mutant lacking GAP activity (24) was able to potentiate the ability of H-Ras^{L61} to activate c-Src (Fig. 3F), indicating that the GAP activity of p120RasGAP is not required for it to promote the activation of c-Src by oncogenic Ras.

Oncogenic Ras interacts with c-Src via p120RasGAP

H-Ras^{L61}, but not H-Ras$^{L61/C186S}$, forms stable complexes with endogenous c-Src and p120RasGAP in intact cells (Fig. 4A). The interaction between H-Ras^{L61} and c-Src was specific because c-Src was not co-precipitated with constitutively active Rho^{V14} or Rac^{V12} (Supplementary Fig. S4A). As GTP-bound H-Ras does not directly bind to c-Src *in vitro* (Supplementary Fig. S4B), it is possible that p120RasGAP may mediate the interaction between oncogenic Ras and c-Src. Indeed, depletion of p120RasGAP by shRNAs markedly reduced the association of c-Src with Ras^{L61} (Fig. 4B) and the Golgi-targeted Ras$^{L61/C186S}$ (Fig. 4C). Overexpression of p120RasGAP, but not its R789Q mutant defective in Ras binding, promoted the interaction between Ras^{L61} and c-Src (Fig. 4D).

Moreover, we found that H-Ras^{L61} promoted the interaction between p120RasGAP and c-Src (Fig. 4E). In HT-1080 cancer

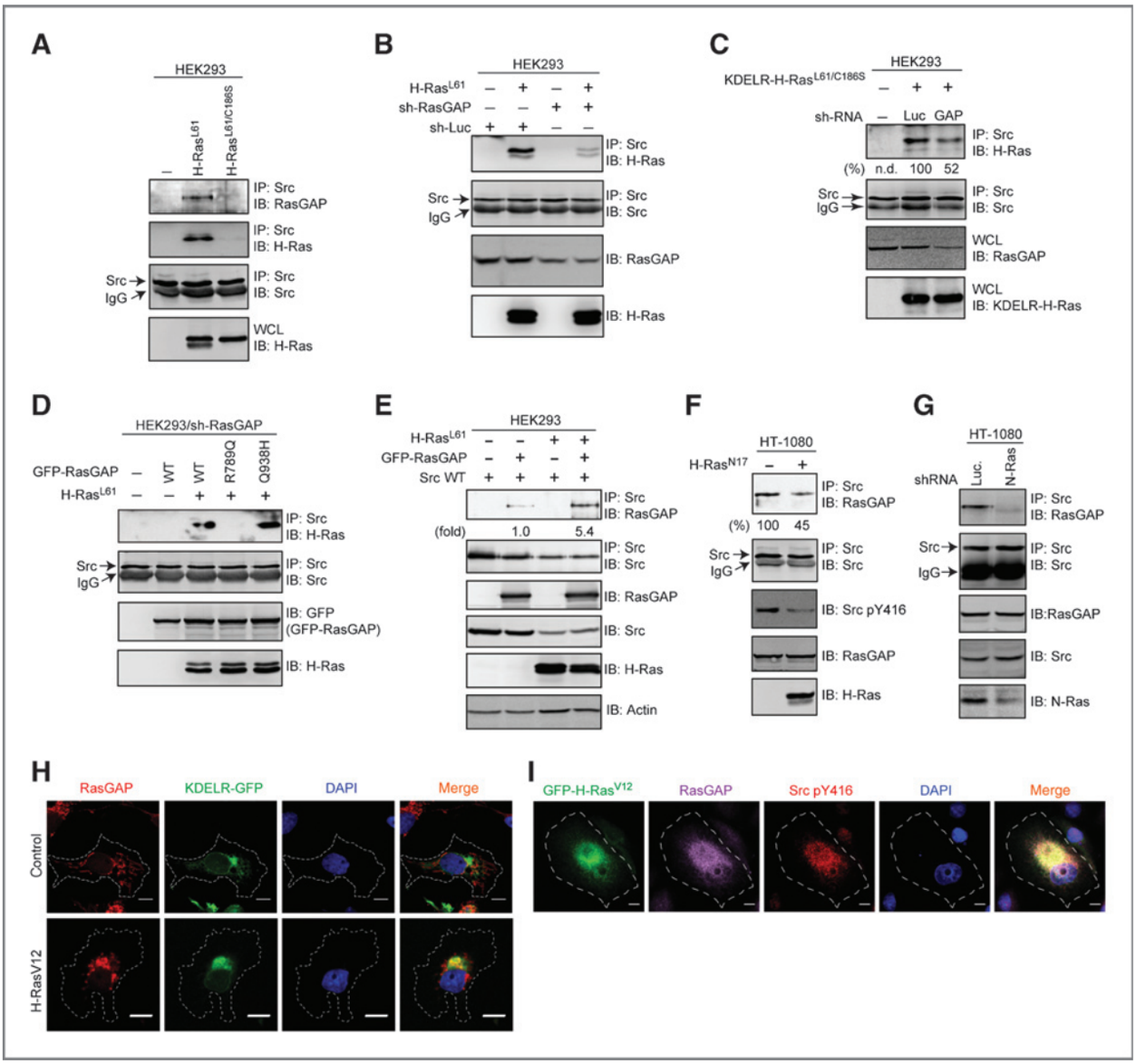

Figure 4. Oncogenic Ras interacts with c-Src via p120RasGAP. A, oncogenic Ras is associated with endogenous c-Src in intact cells. Ras^{L61} or Ras$^{L61/C186S}$ was transiently expressed in HEK293 cells and the lysates were subjected to immunoprecipitation (IP) and immunoblotting (IB) with antibodies, as indicated. WCL, whole-cell lysates. B, knockdown of p120RasGAP by shRNA (sh-RasGAP) suppresses the association of H-Ras^{L61} with endogenous c-Src. H-Ras^{L61} was transiently expressed in HEK293 cells stably expressing shRNAs to p120RasGAP or luciferase (Luc). The association of H-Ras^{L61} with Src was analyzed. C, the association of the Golgi-targeted Ras mutant (KDELR-H-Ras$^{L61/C186S}$) with Src was partially suppressed by p120RasGAP knockdown. D, H-Ras^{L61} and GFP-p120RasGAP were transiently expressed in HEK293 cells stably expressing shRNA to p120RasGAP. The association of H-Ras^{L61} with Src was analyzed. E, H-Ras^{L61} enhances the interaction between p120RasGAP and c-Src. HEK293 cells were transiently transfected with plasmids, as indicated, and the association of Src with p120RasGAP was analyzed. F, H-Ras^{N17} was transiently expressed in HT-1080 cells and its effect on the interaction between p120RasGAP and c-Src was analyzed. G, N-Ras was depleted in HT-1080 cells and the interaction between p120RasGAP and c-Src was analyzed. H, Cos cells and those stably expressing H-Ras^{V12} were transiently transfected with the plasmid encoding KDELR-GFP. The cells were stained for p120RasGAP and the nucleus. KDELR-GFP was used as an indicator for the Golgi complex. Note that whereas p120RasGAP is scatter distributed in the control cells, it is condensed at the Golgi complex in the cells expressing H-Ras^{V12}. The dashed lines mark the outline of the cell. Scale bar, 10 μm. I, GFP-H-Ras^{V12} was transiently expressed in Cos cells. The cells were stained for p120RasGAP and Src pY416. Note that GFP-H-Ras^{V12} colocalizes with p120RasGAP and active Src at the perinuclear region. The dashed lines mark the outline of the cell. Scale bar, 10 μm. DAPI, 4′,6-diamidino-2-phenylindole; n.d., not determined.

cells, suppression of oncogenic Ras by Ras^{N17} or specific shRNA inhibited the binding of p120RasGAP to c-Src (Fig. 4F and G). Thus, our results support a role for oncogenic Ras to facilitate the interaction between p120RasGAP and c-Src. In accordance with this notion, we found that in the absence of oncogenic Ras, endogenous p120RasGAP was distributed throughout the cytoplasm in a tubule-like pattering and did not co-localize with the Golgi marker KDELR-GFP (Fig. 4H). In contrast, in the

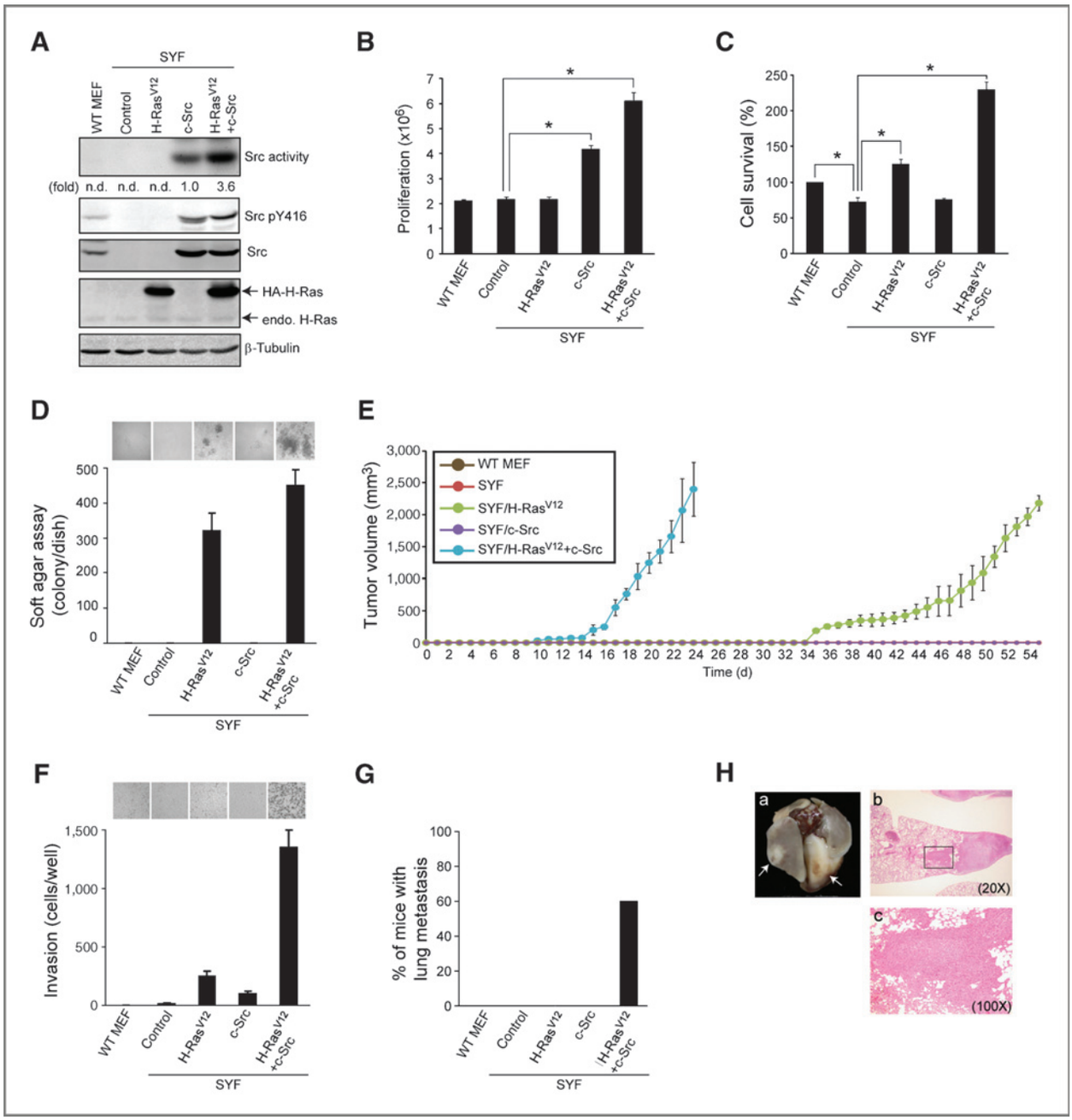

Figure 5. c-Src is essential for H-Ras^{V12} to stimulate cell invasion and metastasis. A, lysates from wild-type MEFs (WT MEF) and SYF cells (src$^{-/-}$ yes$^{-/-}$ fyn$^{-/-}$) stably expressing HA-tagged H-Ras^{V12} (SYF/H-RasV12), c-Src (SYF/c-Src), or both (SYF/c-Src + H-Ras^{V12}) were subjected to analysis for the activity and pY416 of Src. B–E, the stable cell lines as described in (A) were subjected to assays for cell proliferation (B), survival (C), soft agar colony formation assay (D), and tumorigenicity in nude mice (E). Results are presented as mean ± SD. ($n = 3$). *, $P < 0.01$. F and G, the stable cell lines as described in (A) were subjected to assays for cell invasion (F) and lung metastasis (G). Results are presented as mean ± SD. ($n = 3$). H, lungs were excised from the mice injected with the SYF cells expressing both H-Ras^{V12} and c-Src and then fixed in formalin. Representative images for the lung (i) and hematoxylin and eosin (H&E) staining (ii and iii) are shown. Arrows indicate tumor nodules. n.d., not determined.

presence of H-Ras^{V12}, p120RasGAP became condensed and colocalized with KDELR-GFP at the perinuclear region (Fig. 4H), where it was colocalized with active c-Src (Fig. 4I). *In vitro*, p120RasGAP bound to both SH2 and SH3 domains of c-Src (Supplementary Fig. S4C). Deletion of the amino-terminal proline-rich region in p120RasGAP significantly reduced its interaction with the SH3 domain of Src (Supplementary Fig. S4D). Together, our results support a model that recruitment of p120RasGAP to the Golgi complex by oncogenic Ras facilitates the interaction of p120RasGAP with c-Src, which may alleviate

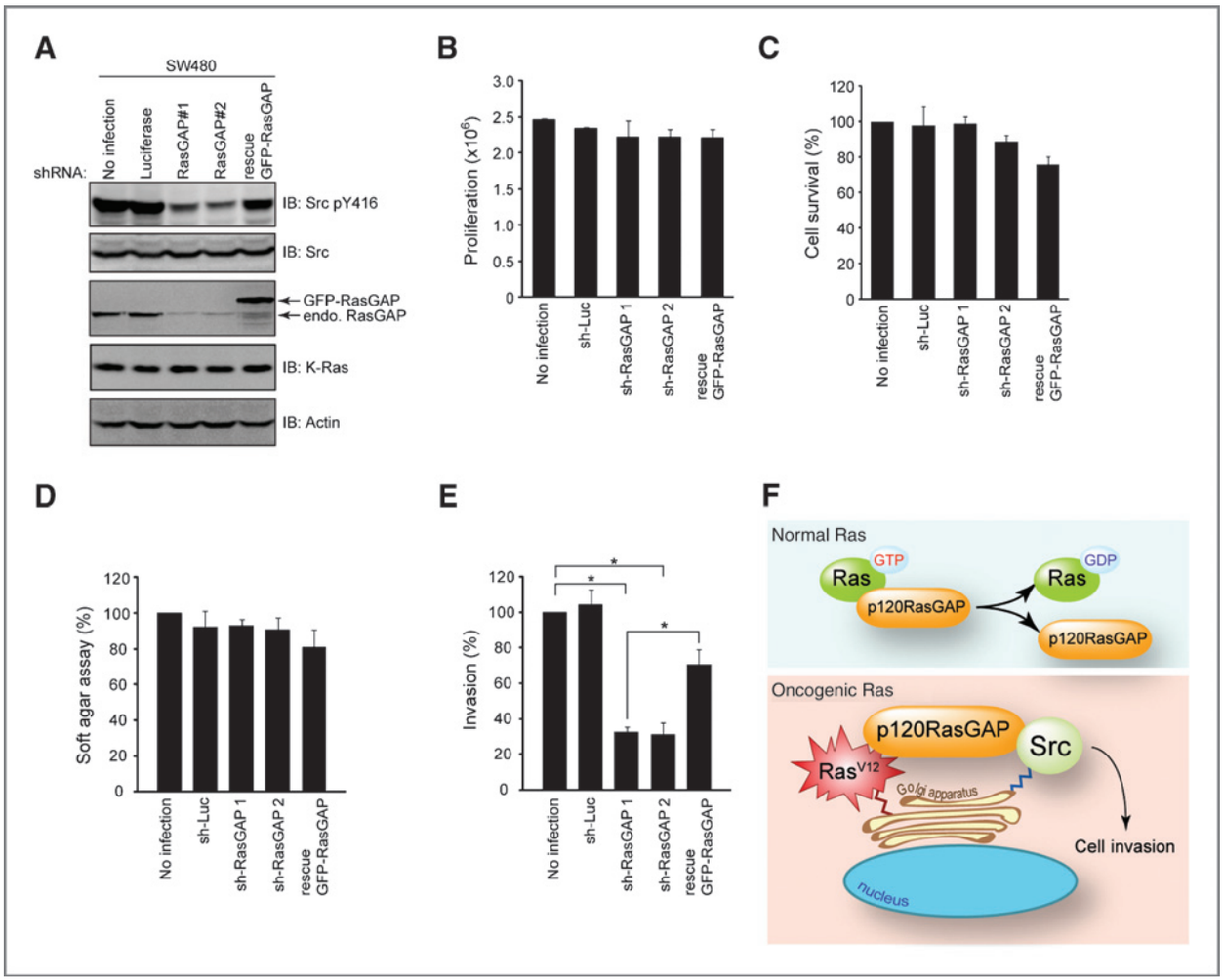

Figure 6. p120RasGAP is essential for oncogenic Ras to promote tumor invasion. A, p120RasGAP was depleted by shRNAs (#1 and #2) in SW480 cells. GFP-p120RasGAP was introduced into the cells expressing shRNA #1 (rescue GFP-RasGAP). The whole-cell lysates were analyzed by immunoblotting (IB) with antibodies, as indicated. B–E, SW480 cells as described in (A) were subjected to assays for cell proliferation (B), survival (C), soft agar colony formation assay (D), and cell invasion (E). Results are presented as mean ± SD. ($n = 3$).*, $P < 0.01$. F, a model illustrating that oncogenic Ras activates c-Src through p120RasGAP on the Golgi complex (bottom), whereas normal Ras is negatively regulated by GTP hydrolysis through p120RasGAP (top).

the intramolecular inhibitory conformation of c-Src, leading to c-Src activation.

P120RasGAP-mediated activation of c-Src is essential for oncogenic Ras to promote tumor invasion

To examine the functional significance of c-Src activation in the transforming potential of oncogenic Ras, H-Ras^{V12} was coexpressed with or without c-Src in SYF (src$^{-/-}$ yes$^{-/-}$ fyn$^{-/-}$) cells (Fig. 5A). We found that H-Ras^{V12} by itself did not promote proliferation of SYF cells (Fig. 5B), but it was sufficient to support anchorage-independent growth of SYF cells in soft agar (Fig. 5D) and allowed them to form tumors in mice (Fig. 5E). However, H-Ras^{V12} by itself failed to stimulate invasive and metastatic capabilities of SYF cells (Fig. 5F–H). H-Ras^{V12} was capable of conferring invasive and metastatic potential to the cells only in the presence of c-Src (Fig. 5F–H), strongly supporting a critical role for c-Src in tumor invasion induced by oncogenic Ras.

To examine the significance of p120RasGAP in oncogenic Ras–induced malignant transformation, p120RasGAP was depleted in human SW480 colon cancer cells that express oncogenic K-Ras. Depletion of p120RasGAP by shRNA significantly decreased c-Src activity (Fig. 6A) and impaired the invasiveness of the cells (Fig. 6E) but had no effect on cell proliferation, survival, and anchorage-independent growth (Fig. 6B–D). Expression of GFP-p120RasGAP restored the c-Src activity and invasiveness in p120RasGAP-depleted SW480 cells (Fig. 6A and E). These results suggest that c-Src activation may be essential for oncogenic Ras to promote cell invasion but not anchorage-independent cell growth. It is worth noting that knockdown of p120RasGAP did not decrease the invasiveness of LoVo cells (Supplementary Fig. S5), in which c-Src activity is independent of p120RasGAP (Supplementary Fig. S5A; Table 1). These results together highlight the significance of the p120RasGAP/Src axis in tumor invasion evoked by oncogenic Ras.

Discussion

Because Ras and c-Src represent 2 major molecular switches for intracellular signal transduction, understanding their interplay will help us not only to realize the intracellular signaling network linked by these 2 molecular switches but also to delineate more effective strategies for future therapeutic intervention. In this study, we uncover a new signaling pathway that links oncogenic Ras to c-Src activation. Through an shRNA-based screening, we identified p120RasGAP as a key mediator for oncogenic Ras to activate c-Src. As summarized in Fig. 6F, p120RasGAP acts as a negative regulator for normal Ras by stimulating GTP hydrolysis. However, in cancer cells, oncogenic Ras facilitates the interaction of p120RasGAP with c-Src on the Golgi complex, which may induce conformational changes in c-Src, leading to its activation. Our results suggest that p120RasGAP-mediated activation of c-Src may be essential for oncogenic Ras to induce tumor invasion but not anchorage-independent cell growth.

Although p120RasGAP serves as a negative regulator for normal Ras, it has been implicated as an effector for oncogenic Ras (25–27). For instance, p120RasGAP was shown to be important for oncogenic Ras to induce cell transformation (25). In particular, interruption of the interaction between oncogenic Ras and p120RasGAP was shown to suppress Ras-induced transformation (26). However, the direct targets for p120RasGAP in Ras-induced transformation were unclear. In this study, we showed that p120RasGAP functions as an effector for oncogenic Ras to activate c-Src. Our results indicated that the Ras-binding capability of p120RasGAP, but not its GAP activity, is required for oncogenic Ras to activate c-Src (Fig. 3F). In addition, we showed that p120RasGAP is recruited to the Golgi complex upon the expression of oncogenic Ras (Fig. 4H). We thus propose that recruitment of p120RasGAP to the Golgi complex by binding to oncogenic Ras may cause conformational changes in p120RasGAP, thereby exposing its binding sites to c-Src. Moreover, we found that the expression level of p120RasGAP is upregulated by oncogenic Ras (Supplementary Fig. S6), which could further contribute to Ras-induced cell transformation. P120RasGAP has been shown to interact with p190RhoGAP (28). In this study, we found that depletion of p190RhoGAP does not affect c-Src activation by oncogenic Ras (Supplementary Fig. S7), suggesting that p190RhoGAP is not involved in c-Src activation in context with oncogenic Ras.

In this study, we showed that c-Src activation is crucial for oncogenic Ras to stimulate tumor invasion. This leads to the question of how active c-Src promotes tumor invasion in context with oncogenic Ras. It is possible that active c-Src on the Golgi complex may enhance the secretion of matrix metalloproteinases through the regulation of exocytosis. It was shown recently that active c-Src phosphorylates and activates dynamin 2 to induce marked Golgi fragmentation and vesicle transport from the Golgi to the plasma membrane during secretory processes (29). In addition, Golgi-associated c-Src may promote cell migration and invasion through its effect on protein glycosylation (30). As alterations in the glycans of membrane proteins could lead to changes in cell adhesion and migration (31, 32), it is possible that the Golgi-associated c-Src may alter protein glycosylation in a way that is beneficial for tumor invasion.

We showed in this study that expression of oncogenic Ras induces c-Src activation. In addition, we found that there is a high correlation between oncogenic Ras and c-Src activation in human cancer cell lines that harbor oncogenic mutations in *Ras* genes (Table 1). In accordance with our findings, Shields and colleagues (33) recently reported that elevated Src activity is detected in more than 60% of patients with pancreatic ductal adenocarcinoma, which is characterized with a high incidence of oncogenic mutations in the *K-Ras* gene. In this study, we found that knockdown of oncogenic Ras or p120RasGAP by shRNAs led to suppression in c-Src activity in 79% of examined cell lines harboring oncogenic Ras mutations. Given that Ras is the most common target for somatic gain-of-function mutations in human cancers, clarification of the role of c-Src in Ras-dependent malignancy is important for determining clinical implication and may be helpful for the development of therapeutic strategies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: P.-C. Chan, H.-C. Chen
Development of methodology: P.-C. Chan, H.-C. Chen
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): P.-C. Chan, H.-C. Chen
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): P.-C. Chan, H.-C. Chen
Writing, review, and/or revision of the manuscript: P.-C. Chan, H.-C. Chen
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): P.-C. Chan, H.-C. Chen
Study supervision: H.-C. Chen

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