

ORIGINAL ARTICLE

EGFR-dependent pancreatic carcinoma cell metastasis through Rap1 activation

M Huang¹, S Anand¹, EA Murphy¹, JS Desgrosellier¹, DG Stupack¹, SJ Shattil², DD Schlaepfer³ and DA Cheresch¹

¹Department of Pathology, Moores University of California San Diego Cancer Center, La Jolla, CA, USA; ²Department of Medicine, University of California San Diego, La Jolla, CA, USA and ³Reproductive Medicine, Moores University of California San Diego Cancer Center, La Jolla, CA, USA

Tyrosine kinase receptors have an essential role in various aspects of tumor progression. In particular, epidermal growth factor receptor (EGFR) and its ligands have been implicated in the growth and dissemination of a wide array of human carcinomas. Here, we describe an EGFR-mediated signaling pathway that regulates human pancreatic carcinoma cell invasion and metastasis, yet does not influence the growth of primary tumors. In fact, ligation/activation of EGFR induces Src-dependent phosphorylation of two critical tyrosine residues of p130CAS, leading to the assembly of a Crk-associated substrate (CAS)/Nck1 complex that promotes Ras-associated protein-1 (Rap1) signaling. Importantly, GTP loading of Rap1 is specifically required for pancreatic carcinoma cell migration on vitronectin but not on collagen. Furthermore, Rap1 activation is required for EGFR-mediated metastasis *in vivo* without impacting primary tumor growth. These findings identify a molecular pathway that promotes the invasive/metastatic properties of human pancreatic carcinomas driven by EGFR.

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Introduction

Although the significance of metastatic burden on mortality from pancreatic carcinoma is well appreciated, the molecular mechanisms that govern such aggressive invasive behavior remain poorly understood. Current efforts have focused on targeted therapies, including inhibitors of epidermal growth factor receptor (EGFR) signaling, a pathway, which is often dysregulated in pancreatic adenocarcinoma (Rivera *et al.*, 2009). How-

ever, resistance to anti-EGFR therapies frequently occurs through mechanisms that activate downstream mediators independent of EGFR activation (Faller and Burtneis, 2009). This could include signaling through other growth factor receptors, such as *recepteur d'Origine nantais* (RON) and insulin-like growth factor (IGF)-1 receptor (Guix *et al.*, 2008). Both receptors have been shown to cross-talk with EGFR (Peace *et al.*, 2003; Morgillo *et al.*, 2006) and their expression correlates with disease progression (Thomas *et al.*, 2007; Wolpin *et al.*, 2007).

The small GTPase Ras-associated protein-1 (Rap1) is activated downstream from EGFR and is a regulator of integrin activation, cell adhesion and migration (Shimomura *et al.*, 2003; Bos, 2005; Jenei *et al.*, 2005; Shattil *et al.*, 2010). Rap1 acts as a signaling switch that cycles between an inactive GDP-bound form and an active GTP-bound form with the assistance of guanine exchange factors (GEFs) and GTPase-activating proteins (GAPs). Because of its low intrinsic GTPase activity, Rap1 relies on GTP hydrolysis by GAPs such as Rap1GAP, which has been identified as a putative tumor suppressor deficient in pancreatic carcinoma. Ectopic expression of Rap1GAP inhibits migration in pancreatic carcinoma cells and serves as a metastasis suppressor, which suggests that Rap1 activity is a critical determinant of tumor cell invasiveness (Zhang *et al.*, 2006).

Previously, we described two distinct pathways of tumor cell migration: one requiring a cross-talk between growth factor receptors and integrin- α v β 5, whereas the other independent of growth factor receptors and using one or more β 1-integrins (Klemke *et al.*, 1994; Brooks *et al.*, 1997). We have also reported that growth factor induced carcinoma cell migration on the α v β 5 substrate vitronectin correlates with the cell's metastatic properties (Klemke *et al.*, 1998). In fact, EGFR-induced activation of Src-family kinases is necessary and sufficient for induction of migration on vitronectin *in vitro* and metastasis *in vivo*. Subsequent to Src-family kinase activation, the adaptor protein Crk-associated substrate (CAS) is phosphorylated on specific tyrosine residues in its substrate domain, which contains 15 YxxP motifs (Ricono *et al.*, 2009).

Deletion of the CAS substrate domain or mutation of all 15 YxxP motifs to FxxP has been shown to

Correspondence: Dr DA Cheresch, Department of Pathology, Moores University of California San Diego Cancer Center, Room 2344, 3855 Health Sciences Drive, MC 0803, La Jolla, CA 92093-1503, USA.
E-mail: dcheresh@ucsd.edu

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block tumor cell migration, invasion and metastasis (Klemke *et al.*, 1998; Brabek *et al.*, 2005). Tyrosine phosphorylation of CAS on the 15 YxxP motifs in its substrate domain creates docking sites for proteins that contain SH2 domains, including other pro-migratory signaling molecules such as Crk and Nck (Schlaepfer *et al.*, 1997; Klemke *et al.*, 1998; Rivera *et al.*, 2006). In this study, we characterize a novel mechanism of Rap1 activation downstream from EGFR that requires the formation of a CAS/Nck1 complex. Activation of Rap1 is required to promote migration on vitronectin but not on collagen. Furthermore, Rap1-GTP loading is critical for EGFR-induced metastasis, without enhancing primary tumor growth. Our results reveal new insights into the mechanism of EGFR-mediated metastasis and establish a previously unrecognized connection between the CAS/Nck1 signaling module and Rap1 activation.

Results

Rap1 activation is required for EGFR-mediated metastasis

Hyper-activation of EGFR induces the metastasis of a wide range of carcinoma cells (Kim and Muller, 1999; Papouchado *et al.*, 2005; Matsumoto *et al.*, 2006; Ueno *et al.*, 2008). EGF stimulation also results in the selective induction of migration of these cells on a vitronectin substrate *in vitro*, suggesting that EGFR-induced migration on vitronectin might recapitulate specific aspects of metastatic invasion in the context of EGFR stimulation *in vivo* (Klemke *et al.*, 1994; Brooks *et al.*, 1997; Ricono *et al.*, 2009). Previously, we implicated a role for Rap1 specifically in EGFR-dependent migration on vitronectin in the fast growing variant of COLO 357 (FG) human pancreatic tumor cells (Ricono *et al.*, 2009). To determine whether Rap1 is also required for EGFR-mediated metastasis, FG cells stably expressing either a non-silencing short-hairpin RNA (shRNA) or a Rap1 shRNA were treated with or without EGF *ex vivo* and subsequently implanted on the chorioallantoic membrane (CAM) of 10-day-old chick embryos. Primary tumor growth and spontaneous pulmonary metastasis were quantified as described by Ricono *et al.* (2009). Consistent with our previous finding that Rap1 is required for EGFR-mediated migration on vitronectin of FG cells (Ricono *et al.*, 2009) and with BxPC3 pancreatic tumor cells (data not shown), silencing Rap1 expression blocks EGFR-induced metastasis without affecting primary tumor growth (Figure 1a). Furthermore, we assessed the role for Rap1 activation in EGFR-mediated metastasis. To address this, we expressed Rap1GAP in FG cells to block EGFR-dependent Rap1 activation. Inhibition of Rap1 activity suppresses the EGFR-induced metastatic response, without influencing the primary tumor size (Figure 1b). Thus, Rap1 activation is required for EGFR-mediated metastasis. These findings are consistent with previous results implicating Rap1 activation in tumor cell invasion and metastasis (Mitra *et al.*, 2008;

Bailey *et al.*, 2009; Furstenau *et al.*, 2010). To determine whether Rap1 activation is sufficient to induce metastasis in the absence of EGFR stimulation, FG cells stably expressing either an empty vector, wild-type (WT), constitutively active Rap1 (Rap1 G12V) or an inactive Rap1 (Rap1 S17N) were implanted on the chick CAM. Compared with cells expressing either the empty vector or inactive Rap1, cells expressing active Rap1 showed a significant increase in spontaneous pulmonary metastasis yet did not show an increase in primary tumor growth *in vivo* (Figure 1c). These findings support the notion that Rap1 has a critical role in the metastatic properties of these cells and is an important mediator of metastasis downstream from EGFR.

We next considered whether Rap1 activation, which induces spontaneous carcinoma metastasis *in vivo*, might initiate carcinoma cell migration on a vitronectin substrate *in vitro*. FG cells stably expressing an empty vector, active, inactive or WT Rap1 were allowed to migrate on either a vitronectin or a collagen matrix. We found that active Rap1 was sufficient for migration on vitronectin, but to our surprise did not influence FG cell migration on collagen (Figure 1d). These findings closely mirrored the effects of EGF stimulation as reported previously (Klemke *et al.*, 1994).

In addition to EGF, other growth factors are known to induce Rap1 activity. Therefore we examined FG cell migration and Rap1 activation after a brief exposure (10–30 min) to various growth factors implicated in pancreatic cancer, including EGF, IGF or macrophage-stimulating protein (MSP). While each growth factor induced Rap1 activity and induced FG cell migration on vitronectin, none stimulated their migratory response on collagen (Figures 1e and f). Therefore, distinct growth factors induce Rap1-GTP loading and tumor cell migration on a vitronectin substrate.

Next, we sought to identify which RapGEF(s) promotes migration and Rap1-GTP loading in response to EGF stimulation. Of the six known Rap-specific GEFs, FG cells express RapGEF2 and RapGEF6 (data not shown). Knockdown of both RapGEFs blocks EGFR-induced migration on vitronectin (Supplementary Figure S1A) and EGFR-dependent Rap1-GTP loading (Supplementary Figure S1B). Altogether, these results might explain in part how EGF and other growth factors promote the invasive properties of carcinoma cells *in vitro* and *in vivo*.

Src and CAS are required for EGFR-dependent migration and Rap1 activation

EGF stimulation promotes the activation of Src kinase, a known contributor to tumor cell invasion (Summy and Gallick, 2003; Trevino *et al.*, 2006). Previously, we showed that Src activation is required for EGFR-mediated spontaneous metastasis of carcinoma cells (Ricono *et al.*, 2009). In fact, stimulation of FG cells with EGF, IGF or MSP leads to the phosphorylation of the Y416 activation site on Src (Figure 2a). Transient knockdown of Src in FG cells disrupted EGFR-mediated Rap1-GTP loading (Figure 2b) and blocked migration on vitronectin (Figure 2c) but not on collagen

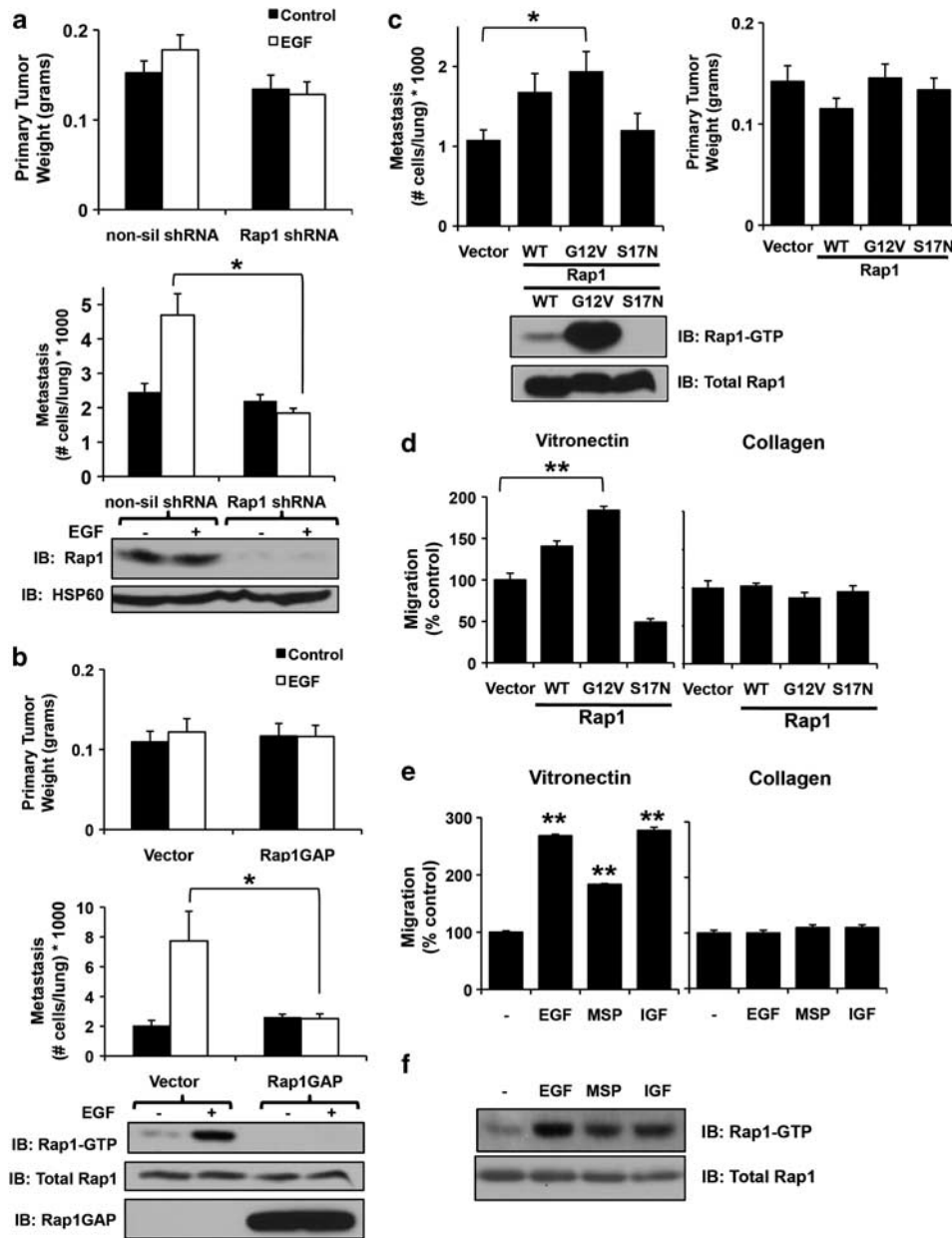


Figure 1 Rap1 activation is required for EGFR-mediated metastasis. **(a)** (Upper) FG human pancreatic carcinoma cells stably expressing a non-silencing or Rap1 shRNA were treated with or without EGF for 10 min and subsequently implanted on the CAM of 10-day-old chick embryos. After 10 days, primary tumors were weighed and pulmonary metastasis was quantified by quantitative PCR for the human *Alu* sequence (see Materials and methods) ($n \geq 15$ in each group). (Lower) Cells were lysed to confirm Rap1 knockdown. **(b)** (Upper) FG cells stably expressing an empty vector or Rap1GAP were treated with or without EGF for 10 min and subsequently implanted on the chick CAM. After 10 days, primary tumors and pulmonary metastasis were quantified ($n \geq 10$ in each group). (Lower) Cells were lysed to analyze for Rap1GAP expression and Rap1 activation status. **(c)** FG cells stably expressing the empty vector, FLAG-tagged WT Rap1, Rap1 G12V (active) or Rap1 S17N (inactive) mutants were implanted on the chick CAM. After 10 days, primary tumors and pulmonary metastasis were quantified ($n \geq 10$ in each group). (Lower) Cell lysates from WT Rap1-, Rap1 G12V- and Rap1 S17N-expressing FG cells were analyzed for Rap1 activation status. **(d)** Migration assay with FG cells expressing an empty vector, WT Rap1, Rap1 G12V or Rap1 S17N on vitronectin or collagen in a Boyden chamber. **(e, f)** FG cells transiently stimulated with EGF (50 ng/ml, 10 min), MSP (10 ng/ml, 10 min) or IGF (100 ng/ml, 30 min) were analyzed for their **(e)** migration on vitronectin or collagen, and for **(f)** Rap1-GTP levels. * $P < 0.05$; ** $P < 0.01$. The data in panels **d** and **e** represent the mean \pm s.e.m. of triplicates, and are representative of three independent experiments.

(data not shown), suggesting that Rap1 activation by EGFR was Src-dependent.

The adaptor protein p130CAS (CAS), a prominent Src substrate, has a key role in cell migration and

invasion (Klemke *et al.*, 1998; Huang *et al.*, 2002; Shin *et al.*, 2004). Indeed, EGF induces the phosphorylation of CAS in an Src-dependent manner as knockdown of Src prevented EGFR-dependent phosphorylation of

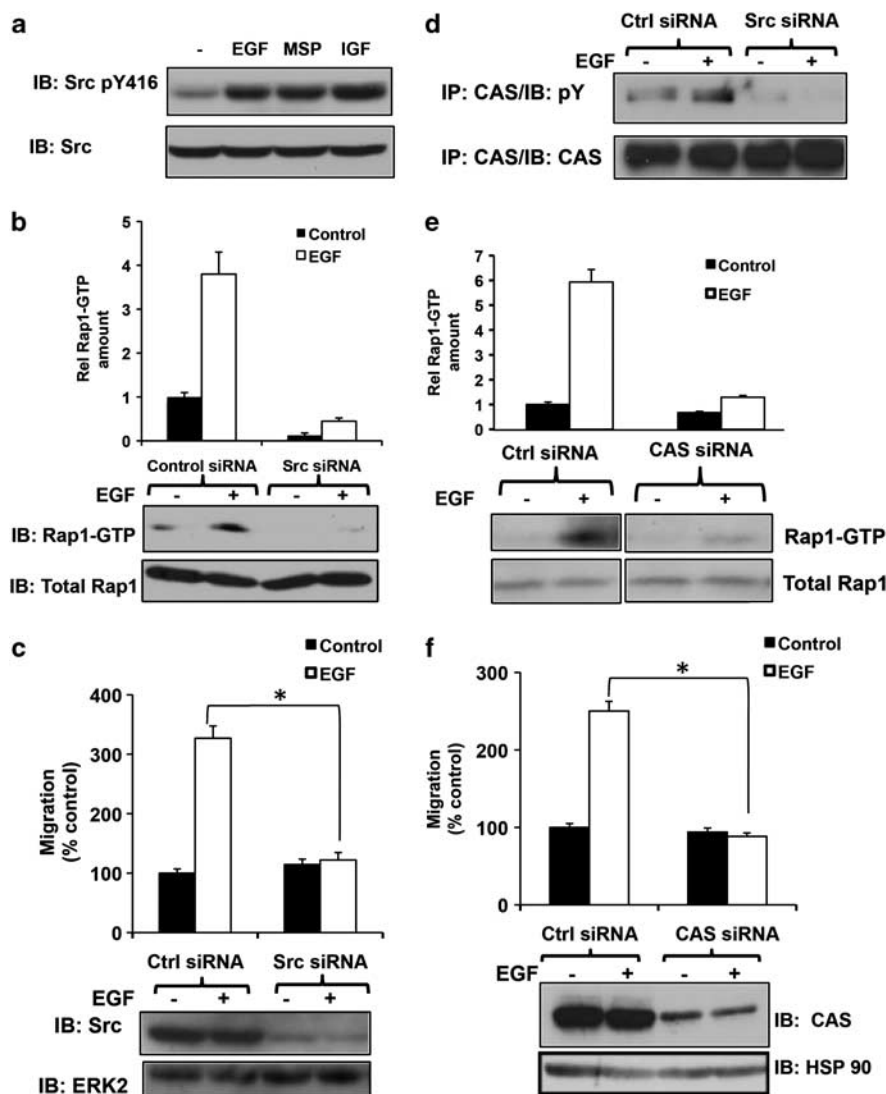


Figure 2 Src and CAS are required for EGFR-dependent migration and Rap1 activation. (a) Lysates from FG cells stimulated with EGF, MSP or IGF were immunoblotted for pSrc Y416 and subsequently re-probed for total Src (loading control). (b) FG cells were transiently transfected with control siRNA or Src siRNA. After 24 h, the cells were serum-starved overnight, followed by treatment with or without EGF, and analyzed for Rap1-GTP. (c) (top) migration on vitronectin (bottom) Src knockdown and (d) CAS phosphorylation. (e) FG cells were transiently transfected with control siRNA or CAS siRNA. After 24 h, the cells were serum-starved overnight, treated with or without EGF, and analyzed for Rap1-GTP. (f, upper) migration on vitronectin and (f, lower) CAS knockdown. $*P < 0.01$. The data in panels c and f represent the mean \pm s.e.m. of triplicates and are representative of three independent experiments. The quantification of Rap1-GTP levels in panels b and e was performed by normalizing total Rap1 levels by densitometric evaluation (ImageJ).

CAS (Figure 2d). Furthermore, downregulation of CAS also blocked EGFR-induced Rap1-GTP loading (Figure 2e). Lastly, knockdown of CAS abolished EGFR-mediated carcinoma cell migration on vitronectin (Figure 2f), whereas it had no effect on cell migration on collagen (data now shown). These results indicate that Src and CAS are both required for growth factor-mediated Rap1 activation and the resulting cell migration response on a vitronectin substrate.

Nck1, a CAS-binding partner, is necessary for EGFR-mediated migration, metastasis and Rap1-GTP loading
The role of CAS in cell migration is linked to its capacity to recruit a range of signaling molecules, including the

adaptor proteins Crk and Nck (Klemke *et al.*, 1998; Rivera *et al.*, 2006). To assess whether Crk and Nck were associated with the induction of EGFR-mediated carcinoma cell migration, lysates from FG cells treated with or without EGF were subjected to immunoprecipitation using anti-CAS followed by immunoblotting for Nck1, Nck2, CrkL and CrkI. EGF stimulation increased the association of both Nck1 and Nck2 with CAS but not CrkL or CrkI with CAS (Figure 3a). We next evaluated the significance of these proteins in EGFR-dependent migration on vitronectin. Interestingly, knockdown of Nck1 but not Nck2 selectively suppressed EGFR-induced cell migration on vitronectin (Figure 3b). However, knockdown of either CrkL or

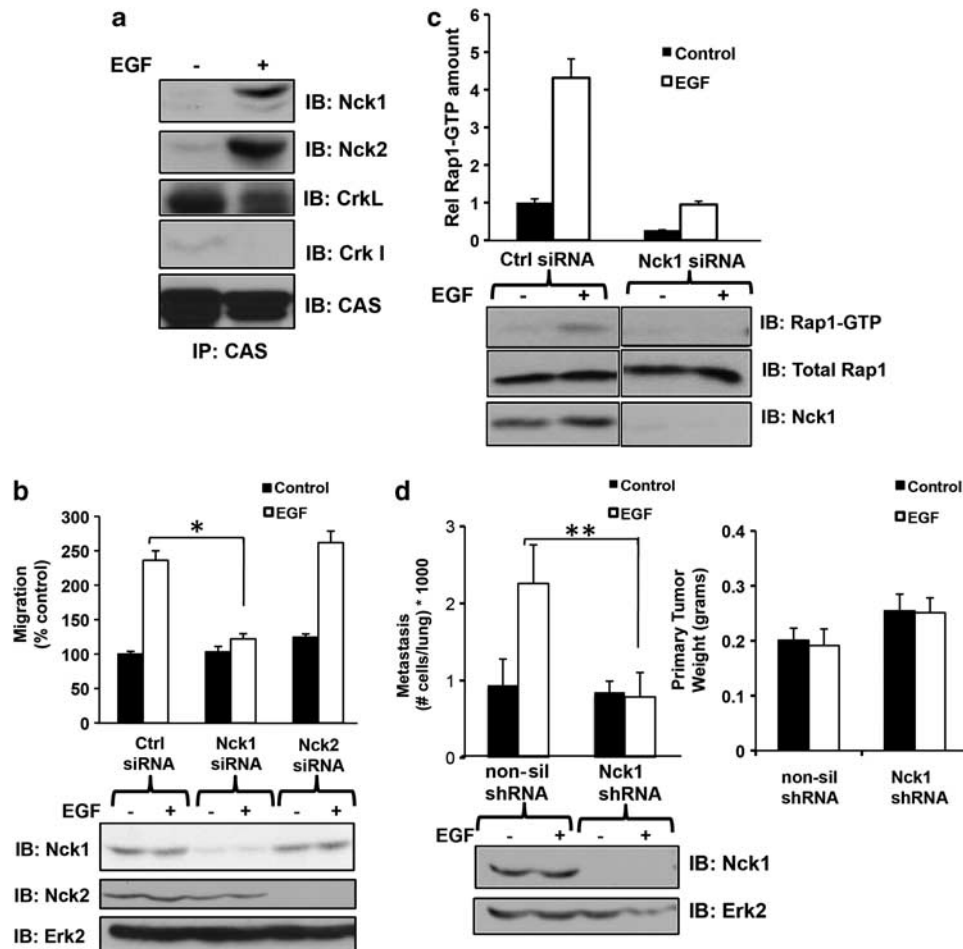


Figure 3 Nck1, a CAS-binding partner, is necessary for EGFR-mediated migration, metastasis and Rap1-GTP loading. (a) Lysates from serum-starved FG cells treated with or without EGF were immunoprecipitated with anti-CAS. The eluted proteins were analyzed by immunoblotting using an anti-CAS, anti-CrkI, anti-CrkL, anti-Nck1 or anti-Nck2 antibody, as indicated. (b) FG cells were transiently transfected with control siRNA, Nck1 siRNA or Nck2 siRNA. After 24 h, cells were serum-starved overnight, subsequently treated with or without EGF, and analyzed for migration on vitronectin (upper) and for confirmation of Nck1 and Nck2 knockdown (lower). (c) FG cells were transiently transfected with control siRNA or Nck1 siRNA. After 24 h, the cells were serum-starved overnight, subsequently treated with or without EGF, and analyzed for Rap1-GTP levels. (d) (Upper) FG cells stably expressing control shRNA or Nck1 shRNA were serum-starved, treated with or without EGF, and implanted on the CAM of 10-day-old chick embryos. After 10 days, primary tumors were weighed and pulmonary metastasis was quantified by quantitative PCR for the human *Alu* sequence ($n \geq 10$ in each group). (Lower) Nck1 knockdown was confirmed by western blot analysis using the indicated antibodies. * $P < 0.01$; ** $P < 0.05$. The data in panel b represent the mean \pm s.e.m. of triplicates and are representative of three independent experiments. The quantification of Rap1-GTP levels in panel c was performed by normalizing total Rap1 levels by densitometric evaluation (ImageJ).

CrkI inhibited cell migration in general, blocking both EGFR-mediated cell migration on vitronectin as well as the constitutive cell migration on collagen (Supplementary Figure S2). In support of these observations, knockdown of Nck1 also disrupted EGFR-mediated Rap1-GTP loading (Figure 3c). These findings indicate that Nck1 is specifically required for the EGFR-mediated carcinoma cell migration response, whereas both Crk-family members appear to have a more generalized role in carcinoma cell migration. These results also suggest that EGF can lead to Src-mediated phosphorylation of CAS, recruitment of Nck1 and induction of Rap1 activation. Thus, Nck1 through its coupling to CAS appears to be required for EGFR-

mediated cell migration on vitronectin. Therefore we considered whether Nck1 was also required for spontaneous metastasis of carcinoma cells *in vivo*. FG cells subjected to Nck1 knockdown and *ex vivo* stimulation of EGF were compared to control cells and analyzed for their primary tumor growth and spontaneous metastasis in the chick CAM model. Consistent with our *in vitro* observations, Nck1 shRNA was sufficient to block the EGFR-induced metastasis, while having no effect on the primary tumor size (Figure 3d). Altogether our data implicate Nck1 and its association with CAS as a key signaling module that regulates EGFR-mediated Rap1 activation, tumor cell invasion and metastasis.

EGFR-induced metastasis, CAS/Nck1 association and Rap1-GTP loading require CAS Y253/Y271

To characterize the mechanism by which Nck1 associates with CAS and thereby influences tumor cell metastasis, we considered the fact that CAS contains a substrate domain characterized by 15 YxxP motifs, which serve as putative docking sites for SH2 domain-containing proteins, including Nck1 (Shin *et al.*, 2004). A number of these sites have been linked to cell migration and metastasis, and are known to represent substrates for Src (Goldberg *et al.*, 2003; Brabek *et al.*, 2004, 2005). Therefore, we performed a mutational analysis of CAS to identify possible Src tyrosine phosphorylation sites on CAS that might serve to recruit Nck1, which initiates pro-metastatic signals. In a previous study we reported that one or more of the first nine tyrosines among the 15 found within the CAS substrate domain were required for EGFR-dependent metastasis (Ricono *et al.*, 2009). Therefore, we considered whether one or more tyrosines within the first nine YxxP motifs in the CAS substrate domain might have a role in EGFR-mediated carcinoma cell invasion and metastasis. We stably expressed a range of Y-F point mutations within the substrate domain of CAS and found that CAS Y253F (referred to as 'F7') or Y271F (referred to as 'F8') or the CAS Y253F/Y271F double mutant (referred to as 'F7/F8') was sufficient to block EGFR-mediated migration on vitronectin (Figures 4a and b) without affecting cell migration on collagen (data not shown). As reported previously (Ricono *et al.*, 2009) the CAS F1-15 mutant (mutation of all 15 YxxP motifs in the substrate domain) blocks EGFR-induced migration; however, restoration of Y253 and Y271 (referred to as 'Y7/Y8') within the CAS F1-15 construct was sufficient to enhance EGFR-dependent migration similarly to WT CAS (Figure 4b). Furthermore, the F7/F8 CAS mutant inhibits EGFR-induced Rap1 activation, whereas the Y7/Y8 CAS mutant allows EGFR-mediated Rap1-GTP loading (Figure 4c).

Y253 and Y271 are putative Src phosphorylation sites (Goldberg *et al.*, 2003; Shin *et al.*, 2004) that are phosphorylated in response to EGF stimulation (Supplementary Figure S3A). To confirm a role for Src in the phosphorylation of these tyrosine residues, we transiently transfected WT CAS, F1-15, F7/F8 or Y7/Y8 CAS in FG cells stably expressing an empty vector or a constitutively active Src construct (Src Y527F or 'SrcA'). CAS was immunoprecipitated from these cells and immunoblotted for CAS phosphorylation. The F7/F8 mutant showed a significant decrease in CAS phosphorylation compared with WT CAS, whereas the Y7/Y8 mutant rescued Src-dependent phosphorylation (Supplementary Figure S3B). To evaluate the importance of phosphorylation of these sites on migration, we compared SrcA cells co-transfected with each of the CAS mutants. SrcA cells expressing WT CAS showed an increased migration response on vitronectin compared with SrcA alone. However, SrcA cells expressing the CAS F7/F8 mutant, like those expressing CAS F1-15, failed to show increased cell migration on vitronectin. Importantly, expression of the Y7/Y8 within the

context of CAS F1-15 reversed the phenotype, suggesting these sites are sufficient to account for the role that Src and CAS have in carcinoma cell migration on vitronectin (Supplementary Figure S3C).

Because CAS tyrosines 7 and 8 appear to have a significant role in EGFR-induced migration as well as Rap1 activation, we considered whether they serve as docking sites for Nck1. To investigate this, we stably expressed FG cells with a hemagglutinin (HA)-tagged Nck1 construct containing point mutations at each of the three SH3 domains (W38K, W143K, W229K) to ensure CAS binds to the SH2 domain instead of the SH3 domains of Nck1. These cells were then transfected with FLAG-tagged WT CAS, F7/F8 CAS, F1-15 CAS or Y7/Y8 CAS constructs. EGF stimulation of WT CAS- or Y7/Y8 CAS-expressing cells resulted in a specific increase in the HA-Nck1/FLAG-CAS complex, whereas no increase was seen in the F7/F8- or the F1-15 CAS-expressing cells (Figure 4d). These findings indicate that Y7 and Y8 serve as docking sites for Nck1 on CAS, enabling the assembly of a scaffold required for Rap1 activation leading to tumor cell invasion and metastasis. To substantiate this possibility, we treated WT CAS and F7/F8 CAS with or without EGF, implanted them on the chick CAM and monitored their primary tumor growth and spontaneous pulmonary metastasis. As expected, EGF stimulation promoted a significant increase in the lung metastasis of cells expressing WT CAS, whereas EGF stimulation of cells expressing F7/F8 CAS had no effect on tumor growth or metastasis (Figure 4e). These results demonstrate that the Y253 and Y271 sites in CAS are able to recruit Nck1 and thereby have a significant role in EGFR-dependent CAS/Nck1 association, Rap1 activation, migration and metastasis (Figure 5).

Discussion

While mutations in EGFR are rare in pancreatic ductal adenocarcinoma, overexpression of EGFR occurs in over half of all pancreatic cancers, and correlates with poor prognosis (Morgan *et al.*, 2008) and metastasis (Pryczynicz *et al.*, 2008). In animal models of pancreatic cancer, gemcitabine treatment in combination with anti-EGFR therapy significantly inhibited metastasis to the liver and lymph node (Bruns *et al.*, 2000). EGF-induced activation of EGFR stimulates cells to rapidly acquire an invasive or metastatic phenotype (Lotz *et al.*, 2003; Lu *et al.*, 2003). In fact, we recently found that carcinoma cells transiently exposed to EGF gain the capacity to invade and metastasize *in vivo* (Ricono *et al.*, 2009).

In this study, we characterized the effectors involved in EGFR-mediated cell invasion and metastasis of human pancreatic carcinoma cells. We examined the role of the GTPase Rap1 in this process as Rap1 is activated downstream from EGF stimulation (Mochizuki *et al.*, 2001; Ohba *et al.*, 2003) and has been associated with induction of integrin activation (Retta

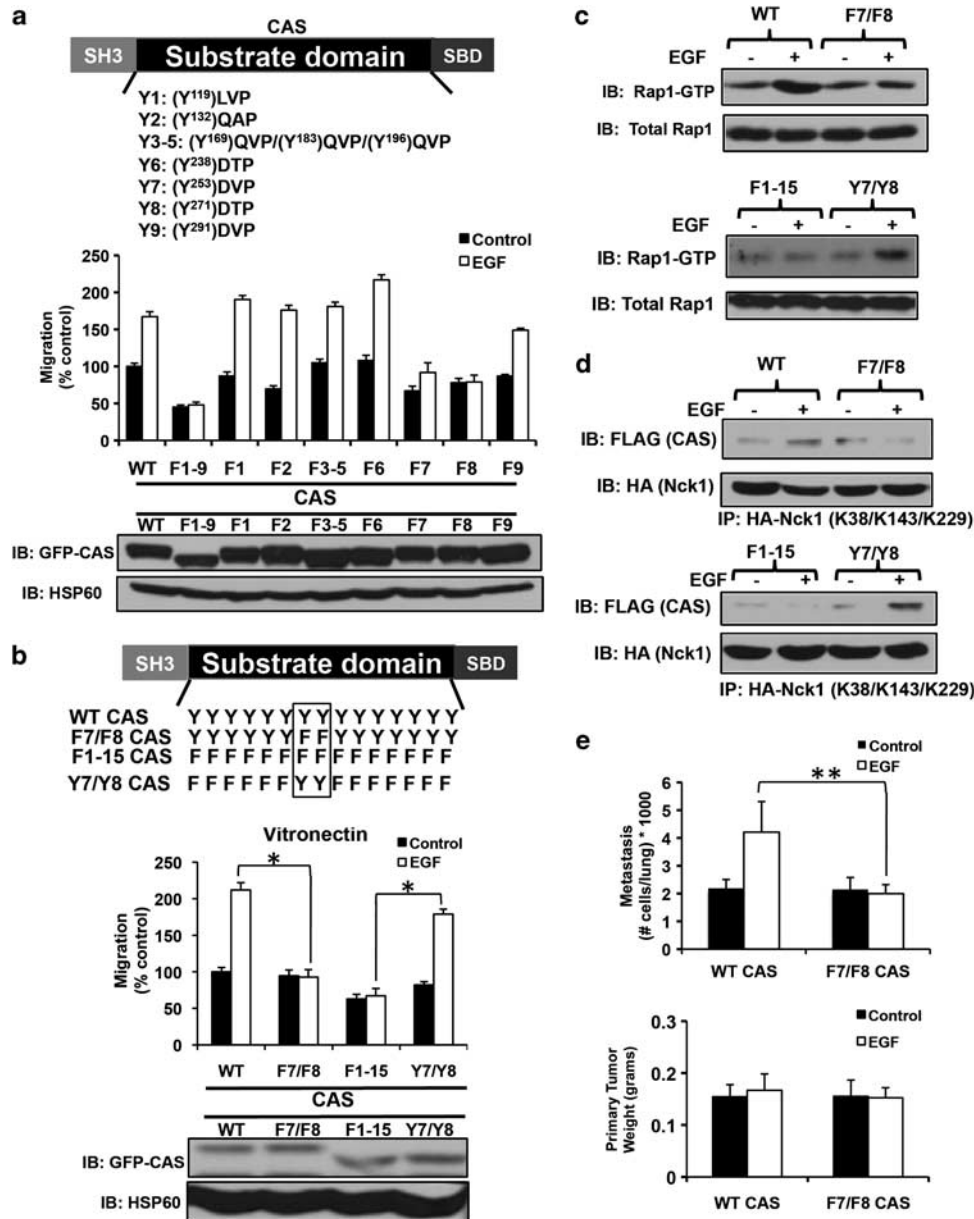
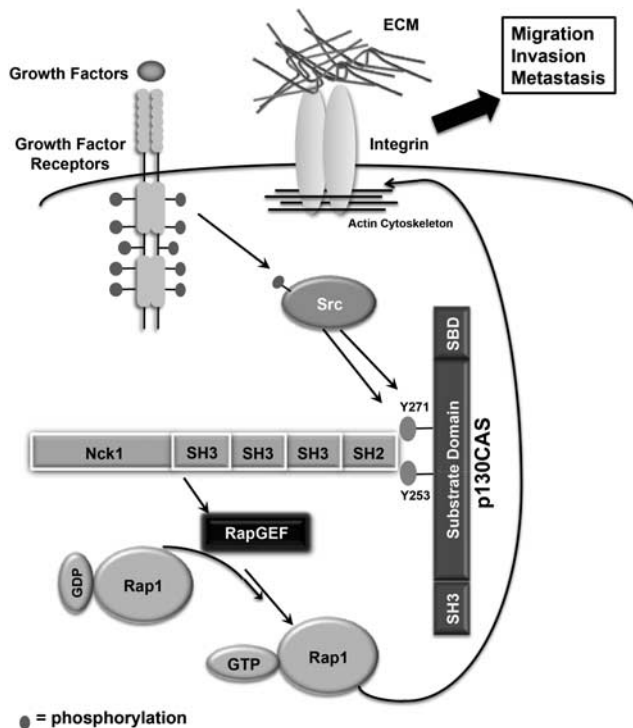


Figure 4 EGFR-induced metastasis, CAS/Nck1 association and Rap1-GTP loading require CAS Y253/Y271. (a) (Upper) A schematic representation of the tyrosine residues of the first 9 YxxP motifs in the substrate domain of CAS. (Lower) FG cells stably expressing CAS mutants with individual or a subset of Y/F mutations in the first 9 YxxP motifs were serum-starved, treated with or without EGF, and analyzed for their migratory capacity on vitronectin. (b, c) (b, upper) A schematic representation of the tyrosine residues of the YxxP motifs in the substrate domain of WT CAS, F7/F8 CAS, F1-15 CAS or Y7/Y8 CAS. The boxed residues represent the tyrosine residues of the seventh and eighth YxxP motifs. (b, lower) FG cells stably expressing WT CAS, F7/F8 CAS, F1-15 CAS or Y7/Y8 CAS were serum-starved, treated with or without EGF, and assessed for (b) their migration phenotype on vitronectin and (c) Rap1-GTP statuses. (d) FG cells stably expressing HA-tagged Nck1 (W38K, W143K, W229K) were transiently transfected with WT CAS, F7/F8 CAS, F1-15 CAS or Y7/Y8 CAS. After 24 h, cells were serum-starved overnight, followed by treatment with or without EGF, and lysed. The lysates were immunoprecipitated with anti-HA and analyzed by immunoblotting for FLAG and HA (loading control). (e) FG cells stably expressing WT CAS or F7/F8 CAS were serum-starved, treated with or without EGF, and implanted on the CAM of 10-day-old chick embryos. After 10 days, primary tumors were weighed and pulmonary metastasis was quantified by quantitative PCR for the human *Alu* sequence ($n \geq 10$ in each group). * $P < 0.01$; ** $P < 0.05$. The data in panel b represent the mean \pm s.e.m. of triplicates and are representative of three independent experiments.

et al., 2006). Rap1 has also been associated previously with the invasive properties of a wide range of tumor cells, including: melanoma (Gao *et al.*, 2006), prostate

cancer (Bailey *et al.*, 2009), thyroid cancer (Tsygankova *et al.*, 2007), colon cancer (Tsygankova *et al.*, 2010) and pancreatic cancer (Zhang *et al.*, 2006). Indeed, we



● = phosphorylation

Figure 5 EGFR activates migration and metastasis through a CAS/Nck1/Rap1 signaling module. EGF stimulation of EGFR activates Src kinase, leading to phosphorylation of CAS Y253 and Y271, which binds to the SH2 domain of Nck1. The assembly of the CAS/Nck1 complex along with RapGEFs leads to GTP loading of Rap1. This facilitates actin remodeling, cell migration, invasion and metastasis.

found that EGFR-mediated metastasis requires the EGFR-dependent activation of Rap1 (Figure 1b). Interestingly, EGF, IGF or MSP stimulation of FG cells results in increased cell migration on vitronectin as well as Rap1-GTP loading (Figures 1e and f). Thus, Rap1 appears to be an important regulator of tumor cell metastasis downstream from multiple growth factor receptors.

Knowing that EGFR could promote Rap1 activation leading to increased metastasis of carcinoma cells, we sought to characterize the effectors leading to the activation of Rap1 to gain a better understanding of how EGFR leads to tumor invasion and metastasis. In previous studies, we determined that EGFR-mediated Src activation leads to the tyrosine phosphorylation of CAS, a focal contact-localized scaffolding protein previously linked to cell migration and invasion (Klemke *et al.*, 1998; Huang *et al.*, 2002; Brabek *et al.*, 2004). CAS phosphorylation is known to recruit a wide range of adaptor molecules and kinases linked to cell invasive behavior, where it appears to have a critical role in the migratory property of various cells (Klemke *et al.*, 1998; Shin *et al.*, 2004). However, we observed that genetic knockdown of CAS inhibited EGFR-induced carcinoma cell migration, yet to our surprise did not disrupt migration on collagen (Figure 2f). Furthermore,

CAS knockdown also blocked EGFR-mediated Rap1-GTP loading. These findings suggest that CAS, once phosphorylated in pancreatic cancer cells, promotes the assembly of a signaling complex that leads to the activation of Rap1 and metastasis. Indeed, this pathway was found to depend on the Src phosphorylation of 2 of 15 tyrosine sites (Y253 and Y271) within the CAS substrate domain. While Src phosphorylates CAS on multiple tyrosine residues (Goldberg *et al.*, 2003; Shin *et al.*, 2004), CAS Y253 and Y271 together are critical for EGFR-dependent migration, metastasis and Rap1 activation.

CAS has a prominent role in migration owing to its binding with the adaptor protein Crk as well as a number of other effectors. Specifically, studies have identified the assembly of a CAS/Crk/Dock180/Rac1 signaling axis as having a key role in the cell migration response (Cheresh *et al.*, 1999; Gu *et al.*, 2001; Payne *et al.*, 2006). In fact, the CAS/Crk complex has been described as a 'molecular switch' in a general role for cell migration (Klemke *et al.*, 1998). However, our current study demonstrates that EGF stimulation of FG pancreatic carcinoma cells does not increase the association of CAS with either CrkL or CrkI, consistent with their generalized role in migration on either collagen or vitronectin substrates. By contrast, Nck1, but not Nck2, is specifically involved in EGFR-dependent migration and metastasis as EGF stimulation of FG cells specifically enhances the association of Nck1 with CAS, which uses CAS Y253 and Y271 as docking sites for the Nck1 SH2 domain.

CAS phosphorylation has been linked to Rap1 activation through the assembly of a CAS/Crk complex with Crk binding to the RapGEF C3G (Sakakibara *et al.*, 2002; Tamada *et al.*, 2004). Our results, however, indicate a novel requirement for Nck1 in EGFR-mediated Rap1 activation in a CAS-dependent manner. In fact, Nck1 could promote Rap1-GTP loading by recruiting RapGEFs with Rap1. Although knockdown of RapGEF2 and RapGEF6 inhibits EGFR-mediated migration on vitronectin and Rap1 activation, we cannot rule out other GEFs that may also influence this pathway of Rap1-GTP loading (Xu *et al.*, 2007; Yaman *et al.*, 2009; Frische and Zwartkruis, 2010).

Here, we have identified an EGFR/Src/CAS/Nck1/Rap1 signaling axis that promotes migration on vitronectin and spontaneous metastasis of human pancreatic carcinoma cells (Figure 5). We used a chick CAM model, which enabled us to quantify both primary tumor growth and spontaneous metastasis after a brief treatment of cells to EGF *ex vivo*. Our results underscore the importance for Rap1-GTP in tumor cell invasion and metastasis. Importantly, Rap1-GTP loading may also be a convergence point for multiple growth factor-regulated metastatic signaling pathways, as EGF, IGF and MSP stimulation all promote migration on vitronectin and Rap1-GTP loading. These findings suggest that antagonists of Rap1 might be particularly useful therapeutic agents to suppress the progression of various epithelium-derived cancers that are typically exposed to a wide range of cytokines.

Materials and methods

Cell culture

Mycoplasma-negative 293T, FG and BxPC3 human pancreatic carcinoma cells (Klemke *et al.*, 1998) were grown in Dulbecco's modified Eagle's medium (Life Technologies, Carlsbad, CA, USA) with 10% fetal bovine serum. FG cells containing CAS or Nck1 mutations were sorted by fluorescence-activated cell sorting for green fluorescent protein expression, and CAS or Nck1 expression was verified by immunoblotting. FG cells containing Rap1 mutations were sorted by fluorescence-activated cell sorting for red fluorescent protein expression and Rap1 expression was verified by immunoblotting.

Growth factors and antibodies

Growth factor stimulation was performed with EGF (Millipore, Temecula, CA, USA), IGF-1 (Sigma-Aldrich, St Louis, MO, USA) or MSP (R&D Systems, Minneapolis, MN, USA). Antibodies were purchased for CAS, Erk2, RapGEF6, HSP90, CrkL (Santa Cruz Biotechnology, Santa Cruz, CA, USA); HSP60 (Enzo Life Sciences, Plymouth Meeting, PA, USA); Crk1, Nck1 (BD Biosciences, San Jose, CA, USA); 4G10, Rap1, Nck2 (Millipore, Temecula, CA, USA); HA (Abcam, Cambridge, MA, USA); FLAG M2 (Sigma-Aldrich); pSrc Y416, pCAS Y249 (Cell Signaling Technology, Danvers, MA, USA); and RapGEF2 (Abnova, Taipei, Taiwan). Sepharose-conjugated antibodies were also purchased for anti-FLAG-M2 and anti-HA (Sigma-Aldrich).

Plasmids

Rap1GAP and *Nck1* cDNA was tagged with 3 × HA and subcloned into the pCDH vector backbone (CD511-B1 from System Biosciences, Mountain View, CA, USA). Mutant CAS cDNA was amplified from pRc/CMV-CAS templates (Shin *et al.*, 2004), tagged with 3 × FLAG and subcloned into the pCDH vector backbone. *Rap1* cDNA was also tagged with 3 × FLAG and subcloned into the pCDH vector backbone.

siRNA and shRNA knockdown

Rap1, Nck1 and non-silencing lentiviral shRNAmir in a GIPZ expression system were purchased from Open Biosystems (Huntsville, AL, USA). Lentiviruses were produced in 293T cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Cells were selected 48 h after infection with 1 μg/ml puromycin and low-expressing cells were further selected by flow cytometry.

Transient knockdowns were performed with short interfering RNA (siRNA) against Src, CAS, CrkL, Crk1, Nck1, Nck2, RapGEF2, RapGEF6 (Qiagen, Valencia CA, USA). Transfection of siRNA was performed by using the Amaxa Nucleofector Kit V for FG cells and the Nucleofector Kit L for

BxPC3 cells (CH-4002; Lonza, Basel, Switzerland), according to the manufacturer's guidelines.

Immunoblotting and immunoprecipitation

For analysis of protein complexes, cells were lysed in sodium dodecyl sulfate (SDS) lysis buffer (50 mM Tris (pH 7.4), 150 mM NaCl, 0.1% SDS, 2 mM EDTA, protease inhibitor cocktail (Roche, South San Francisco, CA, USA), 2 mM NaF, 1 mM sodium vanadate) and pulled down using the indicated conjugated antibody or antibody with protein-A/G beads (Thermo Scientific, Rockford, IL, USA) overnight in 4 °C. The beads were washed three times in phosphate-buffered saline, resuspended in Laemmli buffer and analyzed by 10% SDS-PAGE. For whole-cell lysates, cells were lysed in modified radioimmunoprecipitation assay buffer (50 mM Tris (pH 7.4), 150 mM NaCl, 0.1% SDS, 1% Triton-X, 2 mM EDTA, protease inhibitor cocktail (Roche), 2 mM NaF, 1 mM sodium vanadate) and analyzed by 10% SDS-PAGE.

Migration assays

We performed haptotaxis migration assays on 6.5-mm-diameter, 8-μm pore size Transwell inserts (BD Biosciences) as described previously (Klemke *et al.*, 1994).

Chick embryo metastasis

The chick embryo metastasis assay was performed as described by Brooks *et al.* (1997).

Rap1 activity assays

Rap1-GTP pull-down assays were performed according to the manufacturer's instructions (Millipore).

Statistics

Unless stated otherwise, bar graphs represent the mean ± s.d. of triplicate samples. All data presented are representative of at least two experiments. *P*-values were generated by two-tailed *t*-test.

Conflict of interest

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on the Oncogene website (<http://www.nature.com/onc>)