UNIVERSITY OF CALIFORNIA, SAN DIEGO

EGFR-dependent pancreatic carcinoma cell metastasis via Rap1 activation

A dissertation submitted in partial satisfaction of the requirements for the degree of Doctor of Philosophy

in

Molecular Pathology

by

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LIST OF ABBREVIATIONS

cAMP	cyclic adenosine monophosphate
CAM	Chorioallantotic membrane
CAS	Crk-associated substrate
DEP	Disheveled, EGL-10, and Pleckstrin
ECM	Extracellular matrix proteins
EGF	Epidermal growth factor
FAK	Focal adhesion kinase
FACS	Fluorescence-activated cell sorting
GAP	GTPase activating protein
GEF	Guanine exchange factor
HEF	Human enhancer of filamentation
IGF	Insulin-like growth factor
MRL	Mig-10/RIAM/Lamellipodin family
MSP	Macrophage stimulating protein
N-WASP	neural WASP
PDZ	post synaptic density protein (PSD95), Drosophila disc large tumor suppressor (DlgA), and zonula occludens-1 protein (zo-1)
PH	Plextrin homology domain
PRO	Proline rich region
RA	Ras association domain
Rap1	Ras-associated protein 1
RIAM	Rap1-GTP interacting adapter molecule

RON	Recepteur d’Origine nantais
SBD	Src binding domain
SH	Src homology domain
SFK	Src family kinase
WASP	Wiskott-Aldrich syndrome protein
WT	Wild Type

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ABSTRACT OF THE DISSERTATION

EGFR-dependent pancreatic carcinoma cell metastasis via Rap1 activation

by

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Professor David Cheresh, Chair

Tyrosine kinase receptors play 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. This has lead to the development of EGFR antagonists

to treat patients with different types of tumors. However, the EGFR signaling cascade that promotes tumor metastasis is still unclear. Therefore, it is imperative to elucidate the mechanism of EGFR-dependent dissemination.

The work in this dissertation characterizes an EGFR-mediated signaling pathway that selectively regulates human pancreatic carcinoma cell invasion and metastasis, without influencing the growth of primary tumors. Pancreatic cancer cells were examined for their invasive properties both *in vitro* and *in vivo* following EGFR stimulation. Biochemical studies were performed to analyze the signaling cascade downstream of EGFR that regulates tumor cell invasion and metastasis. I found that ligation/activation of EGFR induces Src-dependent phosphorylation of two critical tyrosine residues of the adaptor protein, p130CAS (CAS), leading to assembly of a CAS/Nck1 complex that promotes Rap1 signaling. Importantly, GTP loading of Rap1 is specifically required for carcinoma cell migration on vitronectin, but not on collagen. Furthermore, Rap1 activation promotes spontaneous 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 the growth factor receptor, EGFR.

Chapter 1

Introduction

1.1 Pancreatic cancer

1.1.1 Overview of pancreatic cancer

Pancreatic cancer ranks as the tenth most commonly diagnosed cancer and the fourth leading cause of cancer deaths in the United States[1]. More than 94% of patients with this disease die within 5 years of diagnosis, with 75% of the deaths occurring within a year. Since 1975, the 5-year survival rate has remained abysmal, increasing from 3% to 6%. Pancreatic cancer has the highest mortality rate of all major cancers, and is the only one of the top 10 cancer killers that has a 5-year survival rate below 10%. Approximately 43,140 Americans will be diagnosed with pancreatic cancer and 36,800 will die in 2010 of this aggressive disease. The number of new cases and deaths over the years has actually been increasing and it is projected that the number of new cases will increase by 55% from 2010 to 2030 [1].

A major hurdle in the treatment of pancreatic cancer is the lack of early detection/diagnosis methods. Few risk factors are known and most symptoms are vague and could be attributed to a number of other diseases. By the time the disease is diagnosed, 52% of patients will already have advanced stage disease that has metastasized and spread to other organs [2]. Thus, metastasis is a major problem.

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[1] American Cancer Society. *Cancer Facts* & *Figures 2010*. Atlanta: American Cancer Society; 2010. The top 5 cancer killers are (in order): lung, colon, breast, pancreatic, and prostate

1.1.2 Metastasis

Metastasis is an extraordinarily complex process and remains the most common cause of cancer deaths. For a cancer cell to successfully disseminate to a distant site, it must complete a sequence of steps which include: local invasion of host tissue into lymph and blood vessels (intravasation), survival during translocation through the lymphatics and vasculature, exit from the vessels (extravasation), survival and proliferation at a distant organ (colonization) [3]. The multitude of steps presents various checkpoints that could prevent the successful metastasis of the cancer cell. When the cancer cell enters circulation, it is exposed to hemodynamic forces and sheering [4], preventing metastasis. Immune surveillance represents another obstacle which cancer cells must evade, particularly once it attempts to establish micrometastasis in a new tissue. Tumor cells are at risk to undergo apoptosis since they are located in a new foreign microenvironment and must adapt to new extracellular stimuli.

One important aspect of metastasis is the ability of a cell to migrate away from the primary tumor. Epithelial cells normally do not migrate, but can gain the ability to move over a long period of time through genetic mutations that activate promigratory signals or eliminate suppressors of migration [5]. Alternatively, cells can also be exposed to external stimuli that will transmit intracellular signals to promote migration [6]. This can be due to a motogenic response, including chemtaxis (migration towards a higher gradient of cytokines

or chemokines) [7] and chemokinesis (increased random migration) [8, 9]. Migration is also promoted by a haptotactic response (migration towards a higher gradient of a particular extracellular matrix), and all of these responses can cooperate [9].

A critical component of migration is the expression of integrins, which are transmembrane receptors composed of heterodimeric α and β subunits. There are at least 24 distinct integrin heterodimers formed by the combination of 18 α-subunits and 8 β-subunits [10]. They function by linking the actin cytoskeleton to the extracellular matrix (ECM) and provide the traction necessary for cell migration and invasion [11]. Integrins are specific to the types of extracellular matrix proteins based on the sequences seen on ECMs, such as RGD, EILDV and REDV. Upon ligation to the ECM, integrins will cluster and recruit kinases (e.g. FAK and Src) and signaling adapter molecules (e.g. p130CAS) to form focal adhesions. The repertoire of α and β heterodimers expressed by cells dictate which ECMs they can migrate on. For instance, migration on collagen requires integrins such as α1β1, α2β1 and α10β1, while migration on vitronectin can utilize αvβ3 and αvβ5 integrins [11].

Interestingly, migration on vitronectin has been correlated with the metastatic capacity of a tumor cell [12]. Although integrin αvβ3 promotes spontaneous migration on vitronectin, integrin αvβ5 requires the activation of a growth factor receptor, such as epidermal growth factor receptor (EGFR) [13]. Prior to growth factor stimulation cell surface integrin αvβ5 does not localize to

focal contacts and instead, appears as punctate structures on the ventral surface of cells [11, 14]. Stimulation with insulin-like growth factor-1 (IGF-1) promotes a redistribution and colocalization of αvβ5 and the cytoskeletal protein α-actinin at the cell substrate interface [12]. Another major difference between integrins αvβ3 and αvβ5 is their distribution on different cell types. Integrin αvβ3 expression is relatively limited while αvβ5 is the most widely expressed vitronectin receptor [15]. Additionally, all pancreatic tumor lines evaluated in a study showed positive expression for β5 [16]. Thus, migration on vitronectin through αvβ5 provides a relevant system to study downstream effectors of growth factor dependent migration and invasion in pancreatic cancer.

Activation of various growth factor signaling pathways is a common route by which tumor cells can migrate and become metastatic. The different mechanisms to activate the growth factor receptors illustrate how they can be activated over a period of time. For instance, cells may acquire genetic mutations that lead to the constitutive activation of the kinase domain, or to the increased expression of the receptor, which promotes receptor dimerization and transphosphorylation to activate the receptors. Alternatively, tumor cells may encounter growth factors to transiently stimulate its cognate receptor and lead to downstream signaling.

1.1.3 Growth factor receptors relevant to pancreatic cancer

While the significance of metastatic burden on mortality from pancreatic carcinoma is appreciated, the molecular mechanisms that govern such aggressive invasive behavior remain poorly understood. Expression of several growth factor ligands and their receptors have correlated with disease progression in pancreatic cancer progression. These include epidermal growth factor (EGF) with EGFR, insulin like growth factor (IGF) with IGF-1R, and macrophage stimulating protein (MSP) with “Recepteur d’Origine nantais” (RON) [17]. Each of these growth factor receptor signaling pathways have been shown to promote migration [13, 18, 19] and metastasis [12, 20, 21]. Currently, antibodies and small molecule inhibitors targeting these receptors are being developed and tested in clinical trials of a range of different types of cancers [18, 22-26]. However, tumor cells can develop multiple resistance mechanisms against inhibitors that target receptor tyrosine kinases such as EGFR. For instance, a T790M mutation in EGFR reduces affinity for the inhibitor gefitinib by twofold and increases competitive ATP binding [27]. Alternatively, activation of other growth factor receptors, such as IGF-1R, can also bypass the inhibition of EGFR signaling [28]. This is supported by the fact that EGFR is known to cross-talk with both IGF-1R and RON [29, 30] which suggests that these signaling pathways may share similar downstream effectors. Therefore, elucidation of the signaling cascade for EGFR may reveal insights into shared mechanisms of migration common to multiple growth factor

receptors. This could identify therapeutic targets to circumvent the acquisition of resistance.

1.1.4 A new pathway of pancreatic cancer metastasis

Recently, a new mechanism of EGFR-mediated metastasis that does not impact primary tumor growth was revealed in the Cheresh laboratory [31]. In this model, EGF stimulation of FG human pancreatic carcinoma cells activates Src Family Kinases (SFKs) leading to the phosphorylation of the adapter protein p130CAS (CAS). Phosphorylation of specific tyrosine residues on CAS is required for the activation of Rap1, a small GTPase involved in integrin activation. This results in migration, invasion and metastasis through the integrin αvβ5, a receptor for the extracellular matrix vitronectin (Figure 1.1).

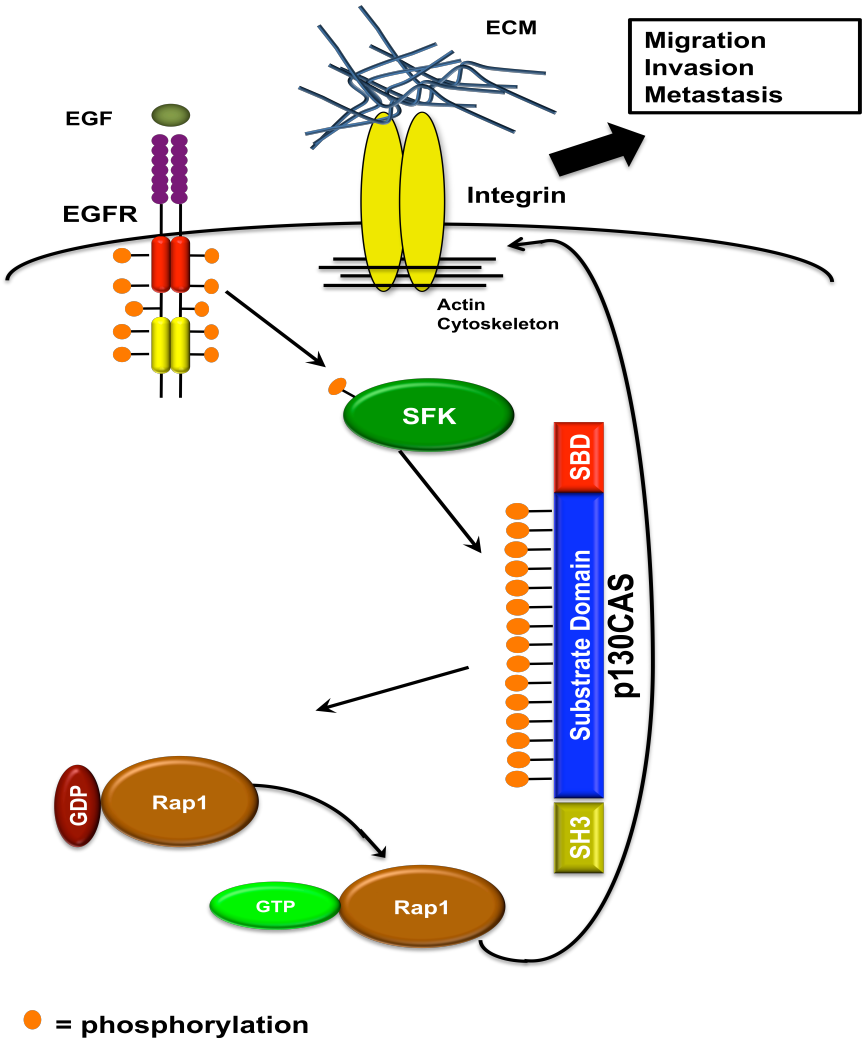


Figure 1.1 EGFR-mediated metastatic pathway in pancreatic cancer

EGF stimulation of FG human pancreatic carcinoma cells results in the activation of Src family kinases (SFK). Subsequently, SFK phosphorylates tyrosine residues in the substrate domain of p130CAS leading to activation of Rap1, migration on vitronectin and metastasis *in vivo*

1.2 EGFR pathway

The EGFR family consists of 4 main members: EGFR/ErbB1, ErbB2, ErbB3, and ErbB4. EGFR is expressed in all adult human tissue except for hematopoietic cells [32] and consists of an extracellular ligand binding domain, a hydrophobic transmembrane domain and an intracellular tyrosine kinase domain. Activation of EGFR can occur via binding of EGF to an EGFR monomer, inducing conformational change and leading to receptor dimerization (either homodimerization or heterodimerzation with another ErbB family member). Dimerization promotes the activation of the tyrosine kinase domain and autophosphorylation of several key tyrosine residues, which promote the binding of other signaling molecules. The activated receptor further phosphorylates other proteins, such as Ras/Raf/MAPK, JAK-STAT, and PI3K-Akt which eventually lead to cell proliferation, survival, migration and metastasis [33-36].

In addition to pancreatic cancer, EGFR expression has been detected in varying degrees of a wide array of solid tumors including head and neck, renal, lung, breast, colon, ovarian, prostate, glioma, and bladder cancer [37]. Activation of EGFR signaling can promote multiple aspects of tumor biology including proliferation, survival, migration, invasion and metastasis. Human pancreatic cancer is known to have elevated levels of EGFR [38] and patients with overexpression of EGFR and its ligands have poor prognosis [39]. High

levels of cytoplasmic EGFR plays an important role in invasion and the acquisition of aggressive clinical behavior [40].

Both monoclonal antibodies and small molecular inhibitors against EGFR have shown efficacy in different tumor types. The monoclonal antibody, Cetuximab, has been approved to treat patients with metastatic colorectal cancer and squamous cell carcinoma of the head and neck, while the small molecule inhibitor, erlotinib, has been approved for patients with non-small-cell lung cancer and pancreatic cancer [37]. Although the addition of erlotinib to gemcitabine significantly increased median survival, the improvement was marginal(6.24 vs 5.91 months) [41]. This could be due to the fact the majority of pancreatic cancer patients already have advanced metastases by the time they are diagnosed, which renders most therapies useless. Additionally, in spite of the presence of erlotinib, other growth factor receptors may be activated to promote the same downstream effectors as EGFR signaling. Thus, there's a need to develop a clearer understanding of what occurs downstream of EGFR activation. Combination therapy with inhibitors targeting downstream effectors of EGFR may improve the efficacy of erlotinib.

1.3 Src family kinases

Src family kinases (SFK) are a family of 9 non-receptor tyrosine kinases commonly activated downstream of EGF stimulation. SFKs are structurally similar and consist of a C-terminal tail with a negative-regulatory tyrosine

residue Y527 and four 'Src homology' (SH) domains. The four SH domains are: SH1 kinase domain, which contains the autophosphorylation Y416 site; SH2 which can interact with Y527 when it's phosphorylated leading to autoinhibition of the kinase; SH3 which also promotes autoinhibitory interactions with the kinase domain; SH4, the myristoylation site required to localize to the membrane for full activation [42, 43]. C-terminal Src Kinase (CSK) is well known regulator of Src activity by phosphorylating the Y527 site and promoting an autoinhibitory interaction with the SH2 domain. This covers the kinase domain and prevents substrate binding. In contrast, protein tyrosine phosphatase-1B (PTP-1B) counteracts CSK by dephosphorylation of the Y527 site to keep Src in an open conformation [44]. Additionally, Src is activated by integrin ligation as well as activation of growth factor receptors, such as EGFR [35-37].

Of the SFK members – Src, Fyn, Yes, Blk, Yrk, Fgr, Hck, Lck, Lyn – Src is the most often implicated in cancer. Src has been implicated in colorectal, hepatocellular, gastric, oesophageal, breast, ovarian, lung, and pancreatic cancer [45-47]. Interestingly, while Src is required for fibroblast cell division and proliferation of precancerous cells [48], Src activity does not correlate with colon cancer proliferation *in vitro* or tumor growth *in vivo.* Instead, Src regulates integrin adhesion and cell spreading on a extracellular matrices [49]. Indeed, cooperation between Src and EGFR affects the invasiveness of colon cancer cells, but does not have an effect on proliferation [50, 51].

The link between Src and EGFR is observed not only in colon cancer, but also in breast cancer. Src and members of the EGFR family are overexpressed in approximately 70% of breast tumors and in a majority of these tumors, Src expression is upregulated along with at least one member of the EGFR family. This would suggest that Src and EGFR functionally cooperate which is supported by the fact that biological synergy is seen when both Src and EGFR are ectopically expressed [52]. Physically, Src interacts with activated forms of EGFR [53] and ErbB2 [54]. Furthermore, Src has been shown to potentiate EGFR signaling [53] and phosphorylates EGFR on multiple tyrosine sites, most notably on Y845 which promotes full catalytic activation of EGFR [55, 56]. Since Src, but not EGFR, leads to the phosphorylation of Y845, EGFR signaling could be mediated by extracellular stimuli other than EGF, which activates Src. Indeed, extracellular matrix proteins, cytokines, and G-protein coupled receptors all transactivate EGFR [52, 57].

Similar to EGFR, elevated expression of Src has been found in human pancreatic carcinoma [58]. Increased levels of Src have been reported in approximately 70% of patients with pancreatic adenocarcinoma and more than 60% of pancreatic tumors exhibit increased Src activity [58, 59]. Patients with higher levels of Src expression or phosphorylation of Src generally had lower overall survival [59]. Animal studies involving the Src/Abl inhibitor, dasatinib, has shown promise inhibiting pancreatic cancer growth and metastasis [59, 60]. In fact, the triple combination therapy of dasatinib, erlotinib and gemcitabine has

provided the best results in abrogating cell migration and invasion compared to using either agent individually or as a double combination [61]. Thus, elucidation of other downstream effectors of EGFR may reveal new promising therapeutic targets.

1.4 p130 Crk Associated Substrate (CAS)

1.4.1 Overview of p130CAS

Crk-associated substrate (p130CAS or CAS) belongs to a family of CAS adapter proteins, which includes CAS, human enhancer of filamentation (HEF), Embryonal Fyn-associated substrate (EFS), and CAS scaffolding protein family member 4 (CASS4) [62]. Of these family members, CAS is the most ubiquitously expressed and has been well characterized to play a role in migration and metastasis. CAS is a large 130kDa protein that contains numerous protein-protein interaction domains including an N-terminal SH3 domain, an internal substrate domain (15 YxxP motifs), and a Src-binding domain (a proline rich region with RPLPSPP motif, and YDYV motif) at the carboxyl terminus. The multitude of binding domains allows CAS to interact with a wide range of kinases and scaffolding proteins. CAS was originally identified as the predominant tyrosine phosphorylated protein in cells expressing v-Crk and it can bind to both v-Crk and v-Src [63]. In fact, expression of activated Src in CAS-deficient fibroblasts resulted in incomplete transformation [64], which suggests that CAS has a functional role in cellular

transformation. While CAS is highly phosphorylated in transformed cells in an adhesion-independent manner, integrin ligation to extracellular matrices in nontransformed cells resulted in an increase in CAS phosphorylation [65, 66].

1.4.2 CAS phosphorylation

Because of the role Focal Adhesion Kinase (FAK) plays in integrin-mediated signaling events, groups have studied a possible relationship between FAK and CAS. Indeed, FAK associates with CAS through the binding of the proline rich region on FAK and the SH3 domain of CAS and promotes the localization of CAS to focal adhesions [67-69]. This interaction likely facilitates the ability of FAK to phosphorylate tyrosine residues on CAS. Much like FAK, Src is also able to interact with CAS and likely has a stronger connection since the Src SH3 domain and the Src SH2 domain can interact with CAS on its C-terminal proline rich region and Src binding domain (SBD), respectively [70].

Interestingly, fibroblasts lacking Src, but not FAK, resulted in the disruption of integrin-mediated CAS phosphorylation, whereas fibroblasts lacking CSK, a negative regulator of Src, have hyperphosphorylation of CAS [71, 72]. Because the autophosphorylation site on Y397 of FAK can bind to the SH2 domain of Src to prevent the autoinihibition of Src, FAK's role in CAS phosphorylation could simply be maintaining Src in the active state. Thus, FAK may have a scaffolding role instead of a kinase role in promoting CAS phosphorylation. However, it was also shown that induced FAK expression in

FAK null fibroblasts increased adhesion-dependent CAS phosphorylation [73]. This suggests that another kinase may be compensating for the loss of FAK, such as the FAK related protein, pyk2. Several mechanisms of how FAK and Src might regulate CAS phosphorylation have been explored. Src was concluded to phosphorylate CAS independently of FAK by creating its own binding sites via phosphorylation of tyrosines in the Src binding domain. When FAK is present, it can act as a bridge to recruit Src to CAS and promote CAS phosphorylation [74] (Figure 1.2a).

In addition to being resistant to Src-mediated transformation, CAS deficient mouse embryonic fibroblasts (MEFs) were also observed to have defects in actin bundling and cell migration [64, 75]. A comprehensive study investigated the role of each CAS domain and motif in actin stress fiber formation, cell migration and Src transformation by expressing various deletion mutants of CAS back into CAS knockout MEFs [76]. CAS constructs without the Src binding domain were unable to rescue CAS phosphorylation, Src transformation and cell migration. Mutation of the proline rich region (RPLP) had a more significant impact in the promotion of Src binding and cell migration than the Y762F mutant. Interestingly, the SH3 domain of CAS appeared to be dispensible for actin stress fiber formation, cell migration and Src transformation, in spite of the fact it can recruit pro-migratory proteins such as FAK, Pyk2, C3G, DOCK180 and PTP-1B [77]. Instead, the SH3 domain, along with the C-terminus, is required to localize CAS to focal adhesions [78]. Lastly,

cells expressing a CAS mutant with the entire substrate domain deleted appeared to have significant defects in actin reorganization and cell migration [76, 79].

1.4.3 CAS substrate domain

The most intriguing feature of CAS is the substrate domain that contains 15 YxxP motifs (Figure 1.2b). Phosphorylation of these tyrosines creates putative binding sites for proteins containing SH2 domains. Specifically, the addition of an aspartic acid residue in the Y + 1 position (YDxP motifs) presents an increased affinity for SH2 domains [80]. Indeed, deletion of specific motifs within the substrate domain had differential effects. CAS mutants lacking the four YQxPs were able to maintain actin stress fibers, but were impaired in cell migration [76]. Deletion of the nine YDxPs disrupted both actin stress fibers and cell migration. Thus, YDxP motifs, but not YQxP motifs, within the substrate domain of CAS play a significant role in migration.

Although deletion of the CAS substrate domain has been shown to block cell migration [76, 79], it would be difficult to attribute this phenotype only to the loss of the substrate domain as this may result in a conformational change of the CAS protein and would alter its function. Instead, the utilization of CAS mutants that maintain the presence of the substrate domain, but contain tyrosine to phenylalanine mutations at various YxxP motifs would more likely mirror the role of the full length CAS protein. Mutation of all 15 YxxP motifs to

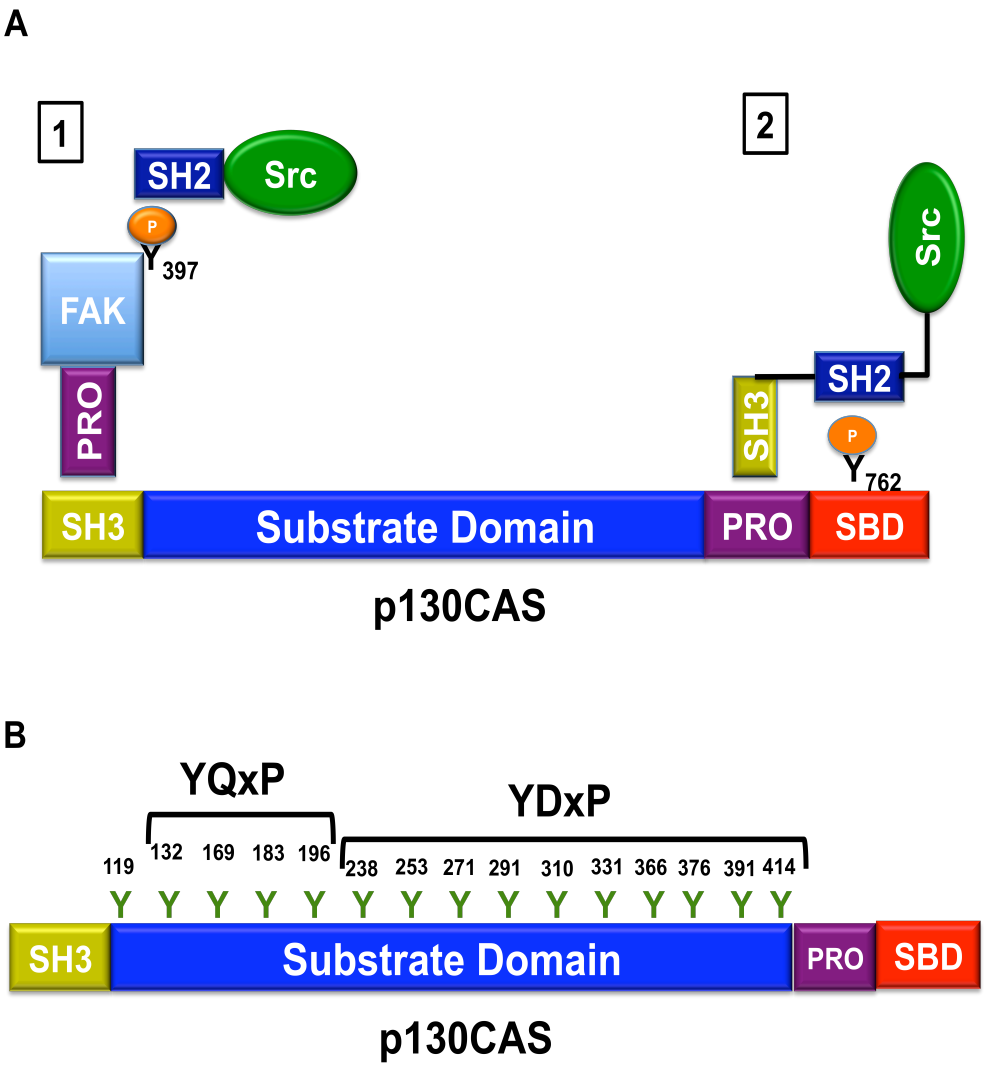


Figure 1.2 CAS phosphorylation and phosphorylation sites

(A) Src phosphorylates CAS through 2 mechanisms. (1) The proline rich region on FAK allows binding to the SH3 domain of CAS. Autophopshorylation of the Y397 site of FAK allows binding of the SH2 domain of Src. (2) The C-terminus of CAS contains a proline rich region that promotes interactions with the SH3 domain of Src. Additionally, the Src binding domain of CAS contains Y762 that promotes binding with the SH2 domain of Src. **(B)** Schematic of the CAS substrate domain containing YQxP motifs and YDxP motifs.

FxxP has been shown to prevent migration in $CAS^{-/-}$ MEFs, metastasis in Src transformed fibroblasts, and EGFR-dependent migration and metastasis [31, 81-83]. While this would indicate that the substrate domain of CAS plays a significant role in migration and metastasis, it is unclear whether specific tyrosine residues may contribute to this phenotype more than the others. Because of Src's prominent role in CAS phosphorylation, several studies examined which sites can be phosphorylated by Src *in vitro* [82-84]. Tryptic peptides incubated with Src and ATP were analyzed by MALDI-TOF mass spectrometry and tyrosine sites 132, 169, 183, 196, 238, 253, 271, 291, 301, 391, and 414 were identified as Src phosphorylation sites. Furthermore, Y253 was the most efficiently phosphorylated by Src and required for Src-dependent migration [84]. A second study utilized site-directed mutagenesis combined with 2D tryptic phosphopeptide mapping to identify all but the first YxxP motif to be Src phosphorylation sites. They suggested that their method would uncover sites missed by mass spectrometry due to its limitations from poor ionization, high mass/charge ratio, or overcrowded spectra. [83]. A third study investigated whether CAS is phosphorylated by Src in a processive manner or a distributive manner and the possibility that there might be an order to which CAS is phosphorylated by Src. Increasing the concentration of various CAS mutant constructs resulted in an elevated level of phosphorylated CAS. This followed the pattern of what would normally occur in a processive phosphorylation event, as increasing substrate concentration would decrease phosphorylation of CAS due to competition. In addition, the generation of four

single-site mutants of CAS in which the most favored Src phosphorylation sites (as defined by synthetic peptide substrates) were mutated resulted in processive phosphorylation by Src, which implies that CAS is not phosphorylated in a specific order [82].

1.4.4 CAS substrate domain binding partners

Since CAS lacks any known kinase activity, it functions by promoting protein-protein interactions and recruiting complexes to different areas within the cell. Interestingly, while many binding partners of CAS have been identified to associate with the SH3 domain, only a few proteins have been known to interact with CAS in its substrate domain, in spite of the fact there are 15 YxxP motifs. These proteins include Crk, Nck, and SHIP2 (SH2-containing inositol 5'-phosphatase). SHIP2 has been associated with increased cell spreading and adhesion [85] while Crk and Nck are known for their effects on migration and the actin cytoskeleton [79, 86, 87] (Figure 1.3).

Crk

Currently, no studies have shown specific CAS tyrosine residues that interact with the SH2 domain of Crk. Although the CAS Y253F mutant blocked Src-mediated migration, the mutation on CAS was not sufficient to block Crk binding [84]. An array of peptides representing each YxxP motif displayed binding to the Crk-SH2 domain on peptides representing sites 1 and 4-15. CAS F6-15 mutant was sufficient to completely block Crk binding to CAS as well as

migration in MEFs in a wound healing assay [83]. Studies have further dissected the mechanism of the CAS/Crk complex in cell migration by noting that DOCK180, a guanine exchange factor (GEF) for the small GTPase Rac1, can interact with the SH3 domain of Crk [88-90].

Nck

Nck family of proteins has not been as well characterized in terms of its interactions with CAS and the role of a CAS/Nck complex in migration. Nck was first observed to associate with CAS-L in T lymphocytes [91] and CAS in fibroblasts stimulated by fibronectin ligation [92]. Nck was also found to associate with CAS in response to extracellular stimuli, such as stromal cell-derived factor-1 alpha (SDF-1a) [93] and platelet derived growth factor B (PDGF-B) [86]. Furthermore, Nck is required for cytoskeletal rearrangement and chemotactic migration towards PDGF-B. Moreover, CAS and Nck both colocalize to membrane ruffles induced by PDGF-B stimulation [86]. One mechanism of migration involving Nck is through the activation of Wiskott-Aldrich syndrome protein (WASP) and neural WASP (N-WASP), both of which regulate the actin cytoskeleton via the Arp2/3 complex [94]. However, as with Crk family of proteins, Nck adapters are also known to activate GTPases, such as Rac1, Cdc42 and Rho [95]

The multiple links between CAS and its binding partner in the activation of a small GTPases suggests that this may be a common mechanism by which

CAS complexes with other adapter proteins is involved in promoting cell migration.

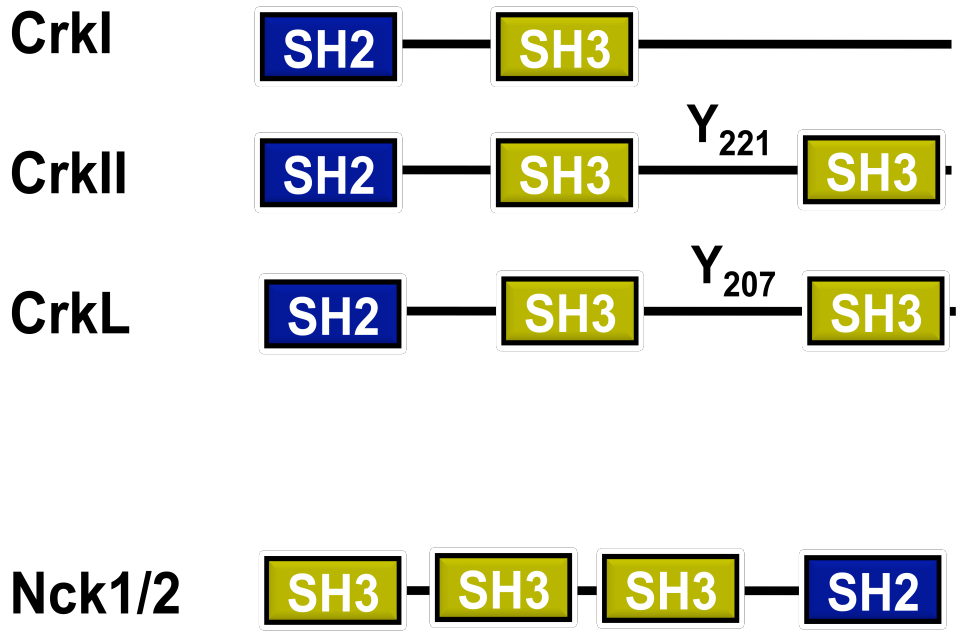


Figure 1.3 Schematic of Crk and Nck family members

(A) Crk family members include CrkI, CrkII and CrkL. All family members contain an SH2 domain and at least 1 SH3 domain. Some also have a Y221 or Y207 site which promote an autoinhibition interaction with its own SH2 domain when phosphorylated. **(B)** Nck1 and Nck2 both share a similar structure. Both contain 3 SH3 domains on the N terminus and an SH2 domain at the C terminus

1.5 Ras associated protein 1 (Rap1)

1.5.1 Rap1 and Ras

Ras associated protein 1 (Rap1) is a small GTPase that was first identified in a screen for proteins which are involved in Ras transformation [96].

Rap1 shares similar homology to Ras, particularly the effector domain. Rap1 consists of 2 isoforms, Rap1a and Rap1b which share 95% homology and differ by 9 amino acids, 6 of which are at the C-terminus [97]. Structural features of Rap1 are the geranylgeranyl modification at the C-terminus that allows for membrane attachment, and a threonine residue at position 61, which most other GTPases have a glutamine. Interestingly, substitution of glutamine for threonine in HRAS confers weak transforming activity which explains why Rap1 GTPase activity is 10 fold lower than that of Ras [98]. Due to the similar effector domains on Ras and Rap1, they share similar binding partners such as Raf-1 and RalGEF. However, binding of Rap1-GTP to these effectors does not activate them, which suggests that this binding competition may explain, in part, how Rap1 antagonizes Ras signaling [99, 100]. In contrast, Rap1-GTP binds and activates BRAF and protein kinase C [101, 102]. While both Rap1 and Ras share similar effectors domains and effectors, they exhibit different binding affinities for the same molecules [103]. Although Ras localizes to the plasma membrane, Rap1 was found to be associated with the Golgi complex [104]. Consistent with this observation, EGF stimulation of COS-1 cells resulted in Ras-GTP formation at the plasma membrane while GTP loading of Rap1 took place at the perinuclear region [105].

1.5.2 Rap1 activity (RapGEFs)

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) (Figure 1.4). 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 [106]. 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.

Rap1 activation can occur through multiple extracellular cues: EGF, PDGF, endothelin and LPA [107, 108]. Second messengers such as cyclic AMP, calcium and diacylglycerol can also activate Rap1[107, 109]. In order to be in the active GTP-bound form, however, Rap1 requires the use of GEF(s)

RapGEF1 (C3G)

Crk SH3-domain-binding guanine-nucleotide releasing factor (C3G) was the first RapGEF to be identified [110]. However, C3G also activates R-Ras which implies that RapGEFs may not be specific to Rap1 [111]. C3G has a catalytic region to promote the GTP exchange and has proline rich regions to bind to SH3 domains of proteins such as Crk. Expression of CrkI in COS cells resulted in phosphorylation of C3G Y504 to activate it [112]

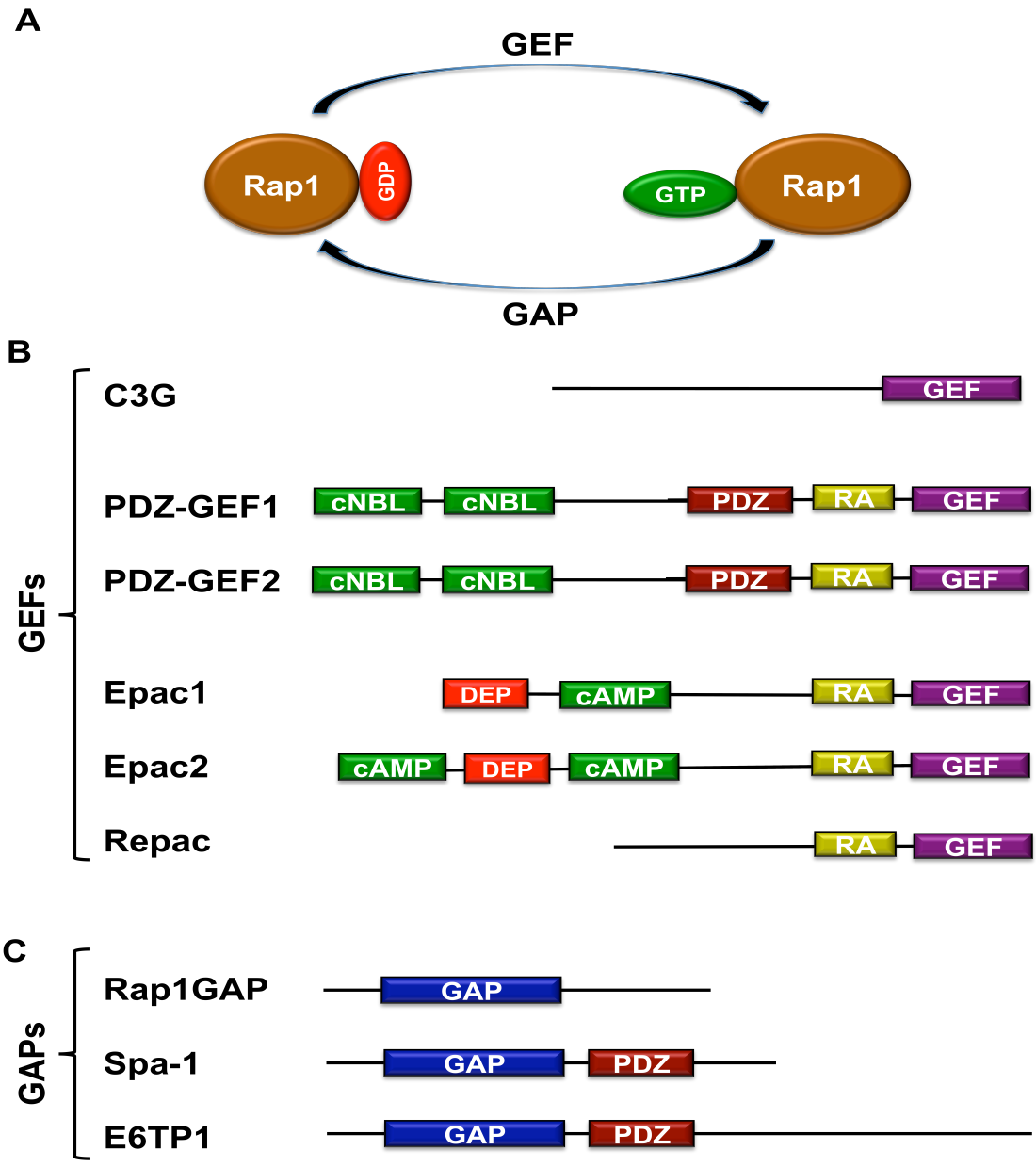


Figure 1.4 Regulation of Rap1-GTP and Rap1-GDP

(A) Rap1 is a Ras-like GTPase that cycles between the inactive GDP bound form and active GTP bound form with the help of GEFs and GAPs. **(B)** Examples of known GEFs that are specific for Rap1. cAMP = cyclic AMP-binding domain; cNBL = cyclic nucleotide binding domain like; RA = Ras association domain; PDZ = post synaptic density protein (PSD95), Drosophila disc large tumor suppressor (DlgA), and zonula occludens-1 protein (zo-1); DEP = Disheveled, EGL-10, and Pleckstrin **(C)** Examples of Rap1 GAPs

RapGEF2 (PDZ-GEF1) and RapGEF6 (PDZ-GEF2)

PDZ-GEF1 was identified as a RapGEF due to its structure which contains a characteristic Ras exchange motif, GEF sequences, proline rich region, PDZ domain, and a structure related to cAMP binding domain (RCBD which has a negative effect on the exchange activity). PDZ-GEF1 can activate Rap1, Rap2, but not Ras, R-Ras, or Ral [113].

PDZ-GEF2 shares a similar structure to PDZ-GEF1, and also exhibits GEF activity to only Rap1 or Rap2 and not any of the Ras family members. Interestingly, PDZ-GEF2 contains a Ras associating (RA) domain, which specifically binds to M-RAS-GTP, while the RA domain of PDZ-GEF1 selectively interacts with Rap1. PDZ-GEF2 appears to activate Rap1 and Rap2 downstream of M-RAS activation at the membrane [114].

RapGEF3 (Epac1), RapGEF4 (Epac2), RapGEF5 (Repac)

Because Rap1 could be activated by cyclic adenosine monophosphate (cAMP), it was thought that protein kinase A (PKA) would be involved. However, some cell types did not require PKA and instead, utilized exchange proteins activated by cyclic AMP 1 (Epac1), which is activated by direct binding with cAMP [115]. Epac1 also has a DEP domain that allows for membrane attachment, Ras-exchange motif, and a GEF domain [115]. Binding of cAMP leads to a conformational change for Epac1 and activates its GEF activity.

Epac2 shares a similar structure to Epac1 except Epac2 has an additional cAMP binding site at the N-terminus. In contrast, Repac only contains a catalytic region and lacks a cAMP dependent regulatory sequence [116]. Repac can interact with Ras-like small GTPases through its Ras binding domain, which upon binding to M-Ras results in a negative feedback loop to inactivate Repac [117]. While Epac proteins are expressed in a range of solid tissues (e.g. ovary, thyroid, kidney, adrenal gland, and brain), they are not found in peripheral blood lymphocytes or leucocytes [115, 118].

Other GEFs

Members of the NSP family of proteins (SH2 domain-containing Eph receptor-binding protein 1 [SHEP1], Cas/HEF1-associated signal transducer [Chat], Breast cancer anti-estrogen resistance 3 [BCAR3]) also promote the activation of Rap1. However, each of them also can activate other GTPases such as Ral, R-Ras and have a lower affinity for Rap1 [119-121]. DOCK4, a member of the CED family of proteins, has also displayed GEF activity for Rap1 and interestingly disrupts growth in soft agar and tumor invasion *in vivo* [122].

1.5.3 Rap1 in "inside-out" signaling

Rap1 was identified based on the fact it promoted a flattened morphology in v-Kras transformed fibroblasts which suggests that it has a role in cell adhesion and spreading [96]. Several lines of evidence support the idea that Rap1 is involved in integrin-mediated cell adhesion. In human T cells,

activated Rap1 promotes adhesion to intercellular adhesion molecule (ICAM) and vascular cell adhesion molecular (VCAM) [123]. Active Rap1 was also a potent activator of leukocyte function associated antigen 1 (LFA-1) to bind ICAM-1 in Jurkat cells [124]. Adhesion that was induced by ligation of the T cell receptor was blocked by the expression of a dominant negative Rap1. In macrophages, expression of active Rap1 promotes the binding of integrin αMβ2 with complement-opsonized phagocytic targets [125]. Conversely, RapGAP Spa-1 blocks HeLa cell attachment to fibronectin [126]. Expression of an inactive form of Rap1 (Rap1 S17N) can also block T-cell Receptor and CD31 induced adhesion [124, 127].

These studies suggest that Rap1 functions through “inside-out” signaling. Specifically, Rap1 promotes activation of integrins via increasing integrin affinity and avidity [124, 128]. Integrin-mediated cell adhesion induced by Mn^{2+} required Rap1 activation [129]. In addition to LFA-1, αMβ2, Rap1 also regulates the activation of integrin α4β1, α5β1 and αIIbβ3 [130, 131]. Thus, Rap1 influences cell migration by the selective activation of specific integrin receptors.

An important Rap1 effector in integrin activation is the Rap1-GTP-interacting adapter molecule (RIAM), which is a member of the MRL (Mig-10/RIAM/Lamellipodin) family of adapter proteins [132]. RIAM contains Ras association (RA) and pleckstrin homology (PH) domains and proline-rich regions. Rap1-GTP will bind to the RA domain of RIAM, while talin binds to a

stretch of 103 amino acids in the N-terminus of RIAM. Talin is then recruited to the tail of β integrins to alter its conformation and promote the activation of integrins [132]. Furthermore, a Rap1-GTP/RIAM complex can also play a role in actin cytoskeleton rearrangements by binding to Profilin and Ena/VASP [133]. Alternatively, Rap1 may promote actin cytoskeleton rearrangements by the activation of GEFs for GTPases known for actin dynamics such as Vav2 (Rac GEF) and Frg (Cdc42 GEF) [134, 135].

1.5.4 The role of Rap1 in pancreatic cancer

The role of Rap1 in cell migration, invasion and metastasis is dependent on the cell type. For instance, Rap1 inhibits invasion in mouse osteosarcoma cells and Rap1GAP expression in squamous cell carcinoma results in a more invasive phenotype [122, 136]. In contrast, increased Rap1 activity promotes migration in cells such as lymphocytes [137], melanoma [138], prostate cancer [139], thyroid cancer[140], colon cancer [141] and pancreatic cancer[106]. Rap1GAP expression is lost in 60% of invasive pancreatic cancers, while the other 40% were considered “weak positive” for Rap1GAP. Transfection of pancreatic cancer cells MiaPaCa-2 and Panc1 with Rap1GAP reduces cell motility [106]. In BxPC3 cells, Rap1GAP reduces adhesion to collagen 1 and prevents cell scattering and the upregulation of the N-cadherin, a mesenchymal marker [142]. This suggests that increased Rap1 activation would lead to more migration and possibly metastasis in pancreatic cancer.

1.6 Open questions

Several questions still remain unanswered about the EGFR pathway we previously described [31]. Which specific SFK has a role in this pathway? Which specific sites on CAS are required to be phosphorylated? How does phosphorylation of CAS promote Rap1 activation? Is Rap1 activation significant to promote metastasis? Do other growth factor receptors utilize this pathway of metastasis as well? These questions were investigated in this dissertation to develop a better understanding of how this signaling cascade promotes metastasis (Figure 1.5)

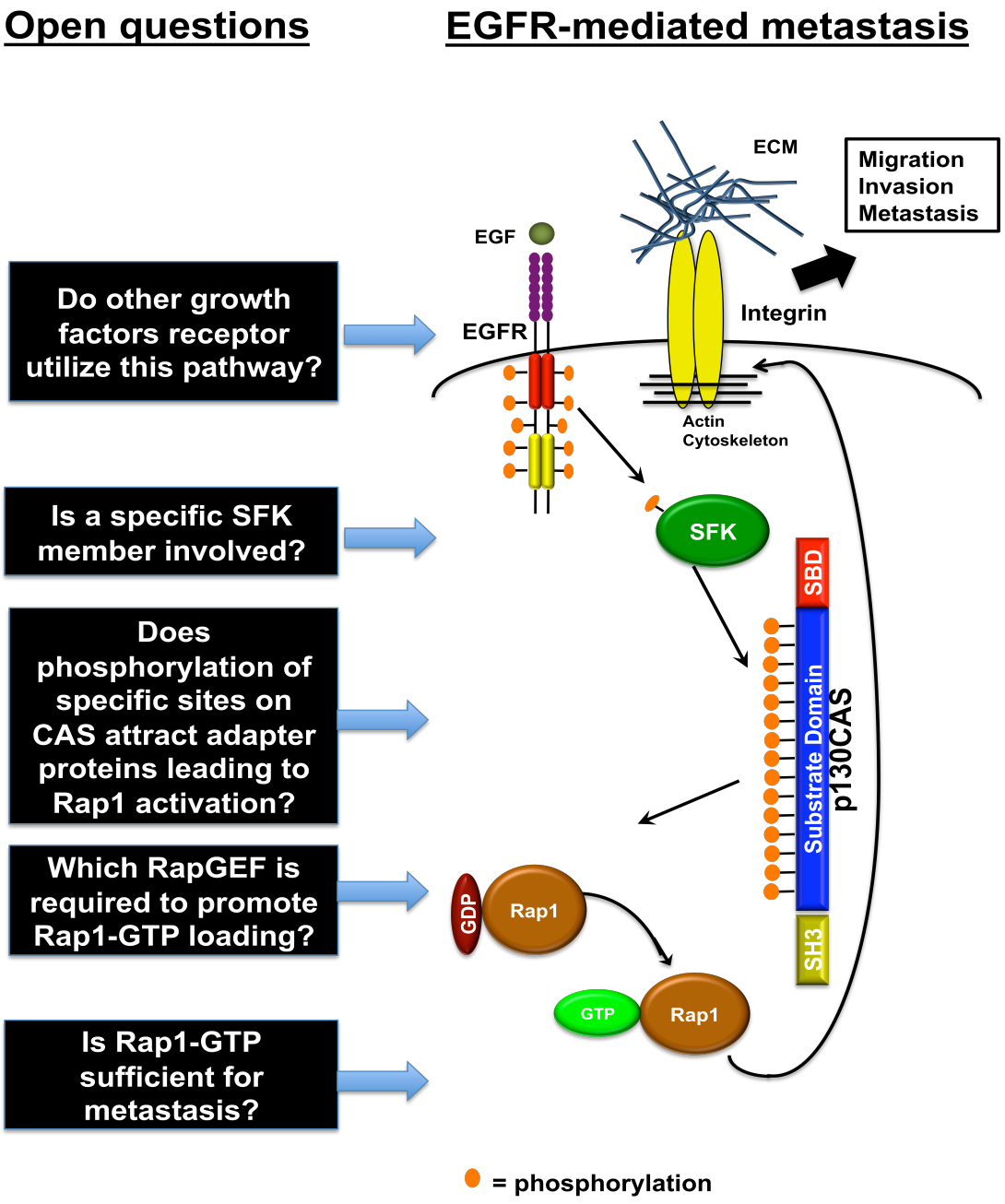


Figure 1.5 Open questions in EGFR-mediated metastasis

EGF stimulates the activation of SFKs, which phosphorylate p130CAS leading to the activation of Rap1 and metastasis. Questions that remain include: Do other growth factors share this mechanism of metastasis? Which specific SFK member is involved? Does phosphorylation of specific sites on CAS attract adapter proteins leading to Rap1 activation? Which RapGEF is required for Rap1-GTP loading? Is Rap1-GTP sufficient for metastasis?

1.7 Hypotheses

Main hypothesis: Growth factor receptors activate Rap1 in pancreatic cancer leading to metastasis.

Sub hypothesis #1: Rap1 activation promotes migration on vitronectin and metastasis.

FG human pancreatic carcinoma cells expressing Rap1 mutants were evaluated for their effect on migration on vitronectin and metastasis in the chick CAM model. Other growth factors known to promote migration were also assessed in their ability to stimulate Rap1-GTP loading.

Sub hypothesis #2: EGFR-dependent phosphorylation of specific tyrosine residues on CAS promotes migration, Rap1 activation, and metastasis

Phenylalanine scan of the CAS substrate domain identified individual tyrosine residues crucial for EGFR-dependent migration. Analysis of known substrate domain binding partners reveal their influence in migration, Rap1 activation and metastasis.

Chapter 2

Materials and Methods

2.1 Cell culture

Mycoplasma-negative 293T and FG human pancreatic carcinoma cells [79] were grown in DMEM (Life Technologies, Carlsbad, CA) with 10% fetal bovine serum. FG cells containing Rap1 and CAS mutations were FACS sorted for green fluorescent protein expression, and Rap1 and CAS expression was verified by immunoblotting. FG cells containing Nck1 mutations were FACS sorted for red fluorescent protein expression, and Nck1 expression was verified by immunoblotting.

2.2 Growth factors and antibodies

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

2.3 Plasmids

Templates for cDNA were amplified by PCR using Phusion High-Fidelity DNA polymerase (New England Biolabs) and primers customized with restriction sites (Valuegene). PCR products were purified by DNA Clean and Concentrator Kit (Zymogen) and digested with restriction enzymes (New England Biolabs). Digest products were run on 1% agarose gels, excised and purified by the Gel Extraction Kit (Qiagen). Gel extracts were quantified and ligated with cut vectors by Rapid DNA Ligation Kit (Roche) and transformed in Stbl3 (for lentiviral vectors) or TOP10 (for all other vectors) competent cells. Minipreps were performed with Zippy Plasmid Miniprep Kit (Zymogen) and digested with restriction enzymes as well as sequenced (Retrogen) to validate plasmids.

Nck1 cDNA was tagged with 3xHA and subcloned into the pCDH vector backbone (CD511-B1 from System Biosciences). Mutant CAS cDNA was amplified from pRc/CMV-CAS templates [83], tagged with 3xFLAG and subcloned into the pCDH vector backbone. *Rap1* cDNA was also tagged with 3xFLAG and subcloned into the pCDH vector backbone.

2.4 siRNA and shRNA knockdown

Nck1 and nonsilencing lentiviral shRNAmir in GIPZ expression system were purchased from Open Biosystems, Huntsville, AL. Lentiviruses were produced in 293T cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA).

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 siRNA against Src, CAS, CrkL, CrkI, Nck1, Nck2, RapGEF2, RapGEF6 (Qiagen, Valencia CA). Transfection of siRNA was carried out using Amaxa Nucleofector Kit V (Lonza, CH-4002 Basel, Switzerland), according to the manufacturer's guidelines.

2.5 Immunoprecipitation

Cells were lysed in SDS lysis buffer (50mM Tris pH 7.4, 150mM NaCl, 0.1% SDS, 2mM EDTA, Protease inhibitor cocktail (Roche), 2mM NaF, 1 mM sodium vanadate) to analyze protein complexes or in modified RIPA buffer (50mM Tris pH 7.4, 150mM NaCl, 0.1% SDS, 1% Triton-x, 2mM EDTA, protease inhibitor cocktail (Roche, South San Francisco, CA), 2mM NaF, 1 mM sodium vanadate) to analyze protein phosphorylation. Lysates were pulled down with the indicated conjugated antibody or antibody with protein A/G beads (Pierce Protein Research) overnight in 4° C. Beads were washed three times in PBS, resuspended in Laemmli buffer, boiled and analyzed on 10% SDS-PAGE.

2.6 Immunoblotting

For whole cell lysates, cells were lysed in modified RIPA buffer (see Section 2.5) and quantified by Bicinchoninic Acid (BCA) assay (Pierce). 50ug of lysate were suspended in Laemmli buffer, boiled, ran on 10% SDS-PAGE, and transferred onto PVDF membranes (BioRad). Membranes were blocked in

5% milk (Salanac) or 3% bovine serum albumin (Thermo Scientific) and incubated with indicated primary antibody overnight in 4 deg C. Blots were washed in TBST, incubated with secondary antibodies (HRP or fluorescent), washed in TBST again, and scanned on the LiCOR machine or exposed on Xray film using chemiluminescence (Pierce).

2.7 Migration assays

We performed haptotaxis migration assays on 6.5 mm diameter 8 μm pore size Transwell inserts (B.D. Biosciences) as previously described [13]. Briefly, cells were starved the night before. Transwells were coated on the underside with either 5ug/mL collagen or 10ug/mL vitronectin for an hour at 37 deg C, washed with PBS and 500uL of FBM w/0.5% BSA was put in the lower chamber. For experiments with EGF treatment, cells were either mock treated or EGF (50ng/mL) treated for 10 minutes, washed with PBS, trypsinized, and neutralized in trypsin neutralizing solution (TNS). Cells were counted and resuspended to 1*10^6 cells/mL of FBM w/0.5% BSA and placed on the upper chamber. After 3 hours (collagen) or 16 hours (vitronectin), cells were fixed and stained in 20% methanol/0.1% crystal violet for 10 minutes. Transwells were washed twice in water and a cotton tip applicator was used to remove the cells from the upper chamber. Cells on the underside were counted in 3 separate fields.

2.8 FGM selection

FG cells were starved overnight and allowed to migrate for 16 hours on Boyden chambers coated with vitronectin on the underside. Chambers were briefly dipped in trypsin and cells from the underside were washed into a 6 well TC dish. Harvested cells were amplified in complete medium and the steps were repeated 10 times to obtain a population of FG cells that are migration competent on vitronectin (FGM) [79].

2.9 Chick embryo metastasis

The chick embryo metastasis assay was performed as described [12]. 10 day old Eggs were obtained from a poultry farm in Lakeside, CA. Chorioallantoic membranes (CAM) were dropped and left in an incubator set between 95-100 deg F. Cells were starved the night before. For experiments involving EGF, cells were either mock-treated or treated with EGF (50ng/mL) for 10 minutes, washed with PBS, trypsinized, and neutralized with TNS. Suspended cells were washed once with FBM + 0.5% BSA and resuspended in FBM w/BSA at 10*10^6 cells/50uL. Cotton tip applicators were used to abrade the CAM and 10*10^6 cells were injected onto the CAM. 10 days later, embryos were euthanized on ice for 30 minutes. Primary tumors were resected and weighed. Lungs were extracted and incubated in a 2.0mL eppendorf tube in 800uL digestion buffer (100mM Tris pH 8.0, 100mM NaCl, 25mM EDTA, 0.5% SDS, 10mg/mL Proteinase K) overnight in 50 deg C incubator. 5 * 10^5 FG cells were incubated with a lung from an egg that did not have any tumor

cells on its CAM as a positive control for qPCR. The following day, the extracts were resuspended in phenol:chloroform:isoamyl (25:24:1) twice at 1:1 ratios. Then supernatants were resuspended in 100% cold ethanol at 4 deg C overnight followed by 70% ethanol. Pellets were air dried for 15 minutes in the fume hood and resuspended in 400uL DEPC-treated water. DNA was quantitated by Nanodrop (Thermo Scientific) and 25ug/uL dilutions were made in 200uL of DEPC water. Real-time quantitative PCR was performed to detect the human ALU sequences and measure the amount of metastasis that went to the lungs. Values were normalized against chicken GAPDH.

2.10 Rap1 activity assays

Rap1-GTP pull-down assays were performed according to the manufacturer's instructions (Millipore). Briefly, cells were starved the night before and either mock-treated or treated with the appropriate growth factor as indicated. Cells were then washed twice with ice cold PBS and cells were then transferred to the cold room. Here, the cells were lysed in Rap1 activation buffer (50mM Tris pH 7.4, 500mM NaCl, 1% NP-40, 2.5mM MgCl2, 10% glycerol) for 1 min, harvested and spun at max speed for 2 minutes. Lysates were transferred back to room temp to quantify the protein levels for 5 min using the BCA (Bicinchoninic Acid) protein assay. 800ug of protein lysate were incubated with 25-30uL of the Ral-GDS sepharose beads in 4 deg C for 45 minutes. Beads were washed 3x in lysis buffer and resuspended in 12.5uL of

1x protein loading buffer and boiled for 10 min. Supernatants were loaded and ran on 10% SDS-PAGE.

2.11 Statistics

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

Chapter 3

Rap1 activation promotes migration on vitronectin and metastasis

3.1 Introduction

Pancreatic cancer is still one of the deadliest diseases worldwide. For the majority of pancreatic cancer patients, they already have advanced stage disease and metastasis by the time of diagnosis due to the lack of an early detection method. Understanding how pancreatic cancer metastasizes will allow the development of metastatic inhibitors to prolong the lifespan of pancreatic cancer patients. Current efforts have focused on targeted therapies including inhibitors of EGFR signaling, a pathway which is often dysregulated in pancreatic adenocarcinoma [143]. However, resistance to anti-EGFR therapies frequently occurs through mechanisms that activate downstream mediators independent of EGFR-activation [144]. This includes signaling through other growth factor receptors, such as IGF-1R [28]. IGF-1R and RON have been shown to cross-talk with EGFR [29, 30] and their expression correlates with disease progression [145, 146].

The small GTPase Ras-associated protein 1 (Rap1) is activated downstream of EGFR and is a regulator of integrin activation, cell adhesion and migration [137, 147-149]. 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). A threonine residue at the 61 amino acid position results in reduced intrinsic GTPase activity compared to other Ras family GTPases. Thus, Rap1 relies on GTP hydrolysis by GAPs such as Rap1GAP, which has been identified as a

putative tumor suppressor deficient in pancreatic carcinoma [106]. 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.

FG cells exhibit two distinct pathways of tumor cell migration: one requires a cross-talk between growth factor receptors and the integrin αvβ5, while the other is independent of growth factor receptors and utilizes one or more β1 integrins [12, 13]. Growth factor stimulation of carcinoma cell migration on the αvβ5 substrate vitronectin correlates with the cell's metastatic properties [79]. In fact, knockdown of integrin β5 in FG human pancreatic carcinoma cells was sufficient to block EGFR-mediated metastasis in a chick CAM model [31]. Furthermore, knockdown of Rap1 as sufficient to selectively block EGFR-mediated migration on vitronectin [31]. However, it is unclear if this effect is due to Rap1 acting as a scaffolding protein or its activation.

3.2 Results

3.2.1 Rap1 activation promotes migration on vitronectin and metastasis.

Rap1 activation has been implicated in tumor cell invasion and metastasis [136, 139, 150] yet its role in these processes is not entirely delineated. To determine whether Rap1 activation is sufficient to induce metastasis, human FG pancreatic tumor cells stably expressing either empty vector, WT, constitutively active Rap1 (Rap1 G12V) or an inactive Rap1 (Rap1 S17N) were implanted on the chorioallantoic membrane (CAM) of 10-day-old chick embryos. Primary tumor growth and spontaneous pulmonary metastasis were quantified as described [31]. Compared to cells expressing either 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 3.1).

3.2.2 EGF, MSP and IGF promotes migration on vitronectin and Rap1 activation

Previous studies have documented that hyperactivation of EGFR induces metastasis of a wide range of carcinoma cells [151-154]. EGF stimulation also results in the selective induction of migration of these cells on a vitronectin substrate *in vitro,* suggesting that EGF-induced migration on vitronectin might serve as a surrogate for metastatic invasion *in vivo* [12, 13, 31]. Therefore, Rap1 activation, which induces spontaneous carcinoma

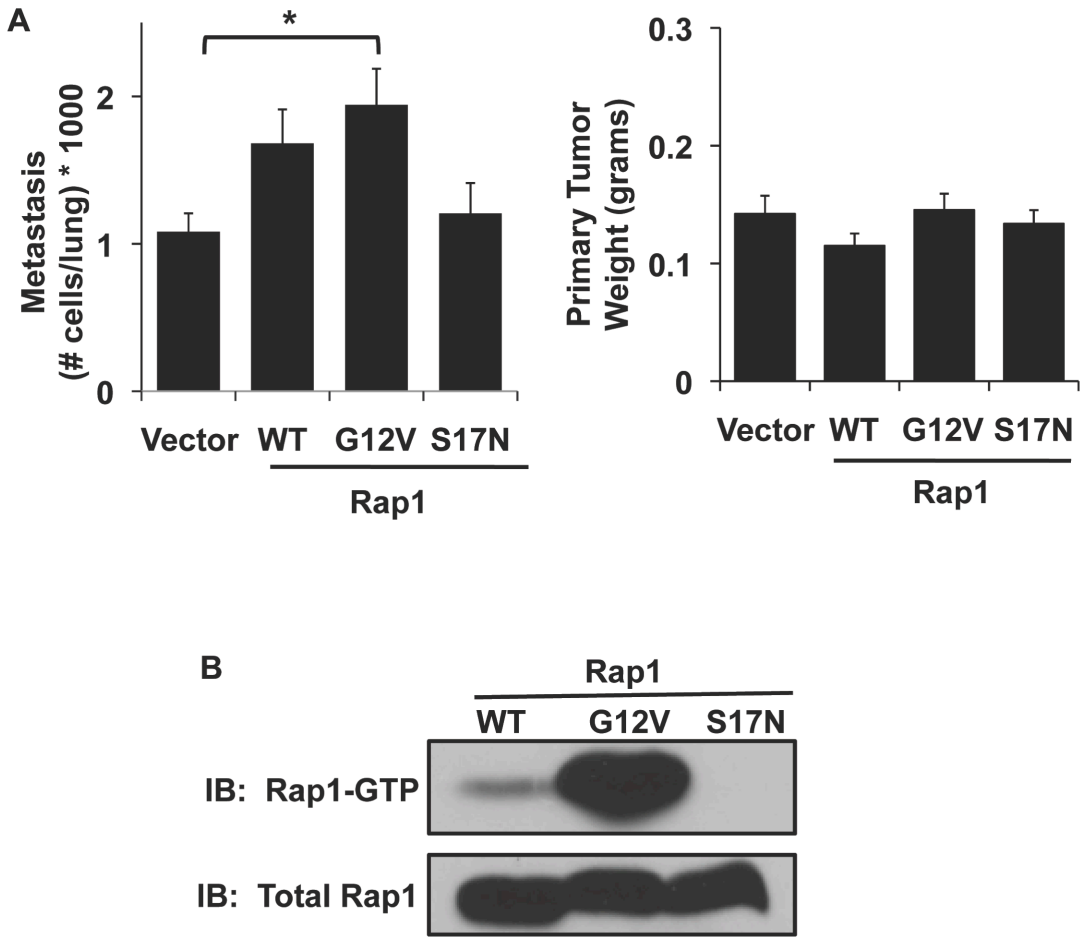


Figure 3.1 Rap1 activation promotes metastasis, but not primary tumor growth.

(**A**) FG human pancreatic carcinoma cells stably expressing the empty vector, FLAG-tagged WT Rap1, Rap1 G12V (active) or Rap1 S17N (inactive) mutants were implanted on the chorioallantoic membrane (CAM) of 10-day old chick embryos. After 10 days, primary tumors were weighed and pulmonary metastasis was quantified by qPCR for human *Alu* sequence (see Materials and Methods) n ≥ 10 in each group. (**B**) Cell lysates from FLAG-tagged WT Rap1, Rap1 G12V, Rap1 S17N expressing FG cells were incubated with GST-RalGDS beads. Bound proteins were subsequently analyzed by immunoblotting with anti-FLAG antibodies. Total FLAG-Rap1 in whole-cell lysates was used as loading control. *$p < 0.01$

metastasis *in vivo*, might initiate carcinoma cell migration on a vitronectin substrate *in vitro*. FG cells stably expressing empty vector, active, inactive or WT Rap1 were allowed to migrate on either a vitronectin or collagen matrix. Active Rap1 was required for migration on vitronectin, but surprisingly did not influence FG cell migration on collagen (Figure 3.2). These findings closely mirrored the effects of EGF stimulation as previously reported [13].

In addition to EGF, other growth factors are known to promote migration such as IGF and MSP. Dose responses for MSP (1ng/mL-200ng/mL) and IGF (10ng/mL-250ng/mL) were first performed with 15 minute pre-treatment prior to the start of the migration assay. After determining the optimal dose of each growth factor, time courses were performed using those doses to determine the best length of time to prestimulate the cells. Altogether, MSP had its strongest increase in migration on vitronectin at 10ng/mL with 10 minute pre-treatment, while IGF promoted the highest migration on vitronectin at 100ng/mL with 30 minute pre-treatment (Figure 3.3 and 3.4).

To evaluate the ability of these growth factors to stimulate Rap1 activity, FG cells were stimulated with EGF, MSP and IGF at the doses and treatment times that optimized for migration on vitronectin. Indeed, the same doses and time points which increased motility on vitronectin also induced activation of Rap1 as well (Figure 3.5). Therefore, distinct growth factors stimulated Rap1-GTP loading and FG cell migration on a vitronectin substrate.

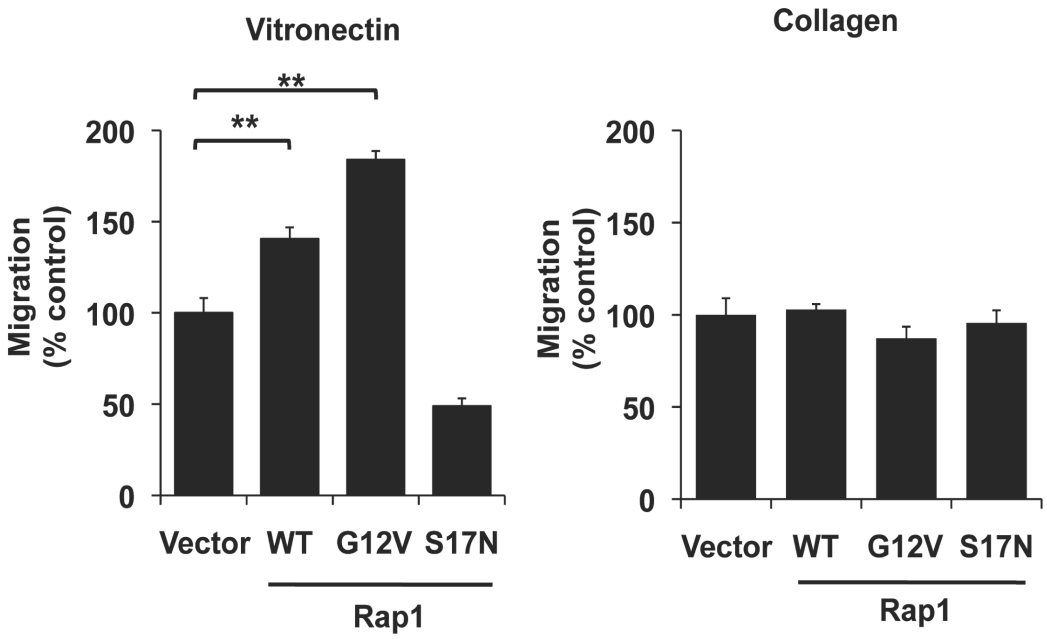


Figure 3.2 Rap1 activation specifically promotes migration on vitronectin but not on collagen

FG cells stably expressing empty vector, WT Rap1, Rap1 G12V or Rap1 S17N were starved overnight and then placed on Boyden chambers coated with vitronectin or collagen on the underside. **p<0.01

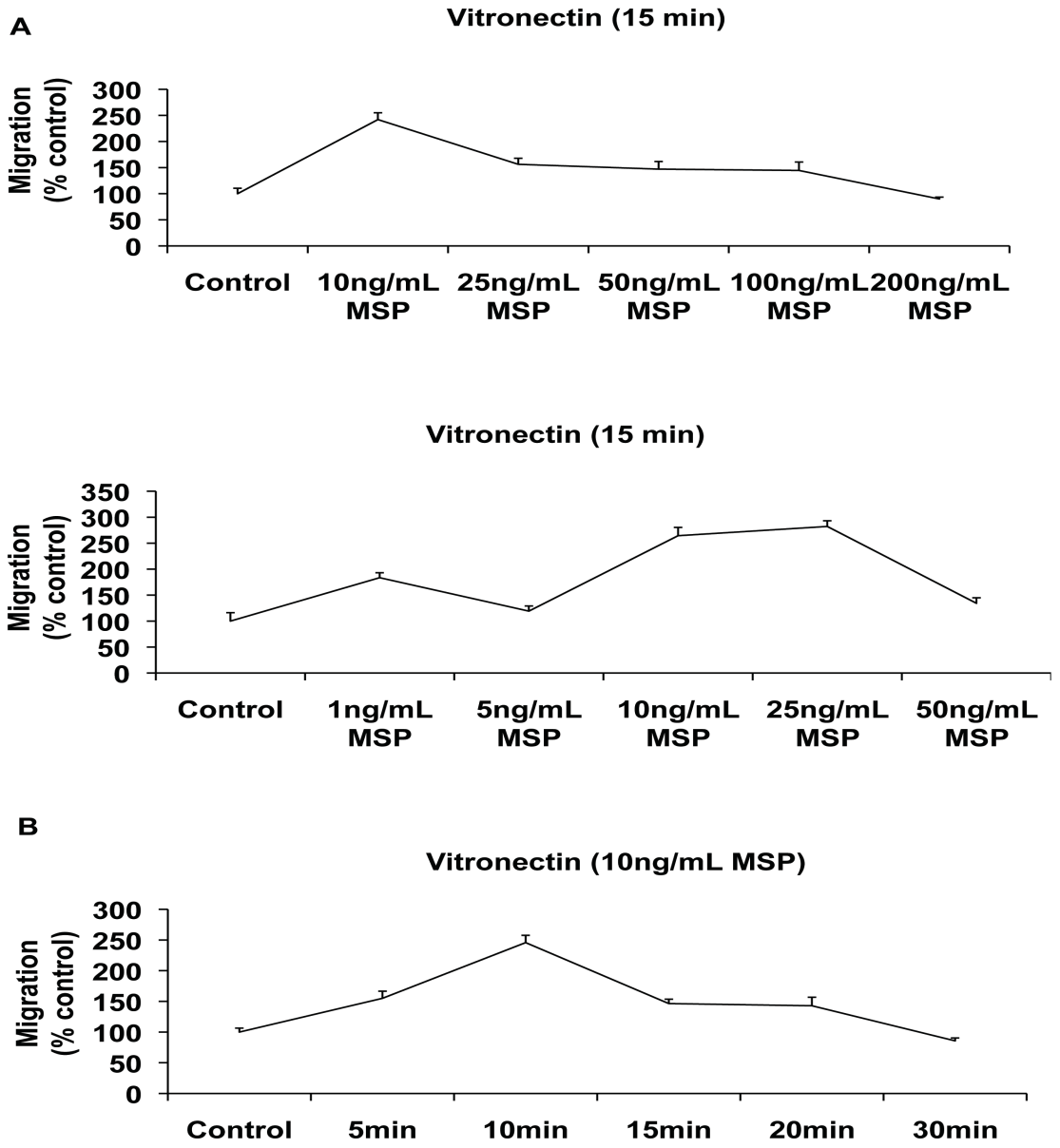


Figure 3.3 MSP stimulation of FG cells (10ng/mL, 10 minutes) promotes migration on vitronectin

(**A**) FG cells were starved and stimulated with a dose range of MSP (1-200ng/mL) for 15 minutes and placed on migration chambers coated with vitronectin on the underside. After 16 hours, cells were fixed in methanol and stained in crystal violet. 3 fields were counted for each transwell and averaged. (**B**) After 10ng/mL appeared to be the optimal dose at 15 minute stimulation, FG cells were starved and treated with 10ng/mL MSP between 5-30 min stimulation prior to washing and trypsinizing before placing cells on transwells coated with vitronectin on the underside

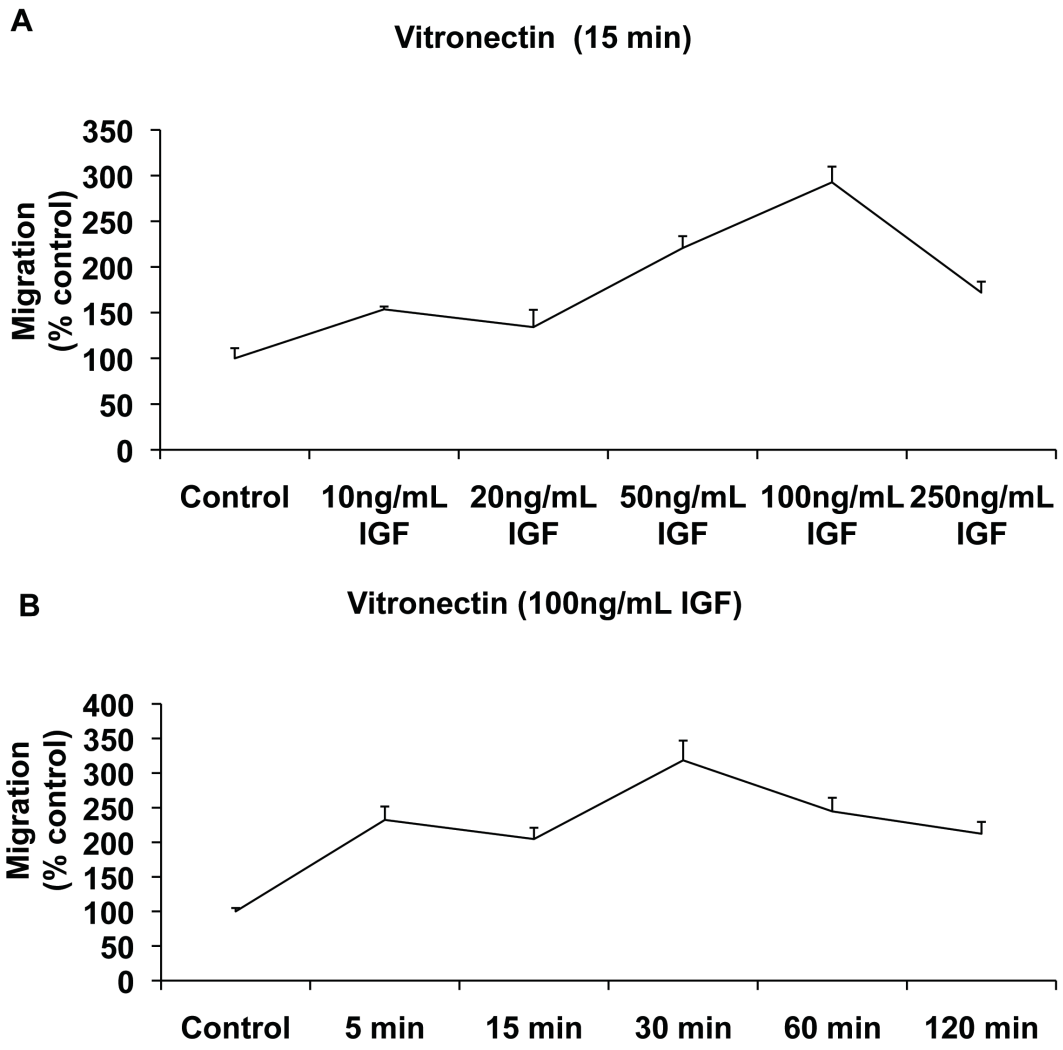


Figure 3.4 IGF stimulation of FG cells (100ng/mL, 30 minutes) promotes migration on vitronectin

(**A**) FG cells were starved and stimulated with a dose range of IGF (10-250ng/mL) for 15 minutes and placed on migration chambers coated with vitronectin on the underside. After 16 hours, cells were fixed in methanol and stained in crystal violet. 3 fields were counted for each transwell and averaged. (**B**) After 100ng/mL appeared to be the optimal dose at 15 minute stimulation, FG cells were starved and treated with 10ng/mL MSP between 5-120 min stimulation prior to washing and trypsinizing before placing cells on transwells coated with vitronectin on the underside

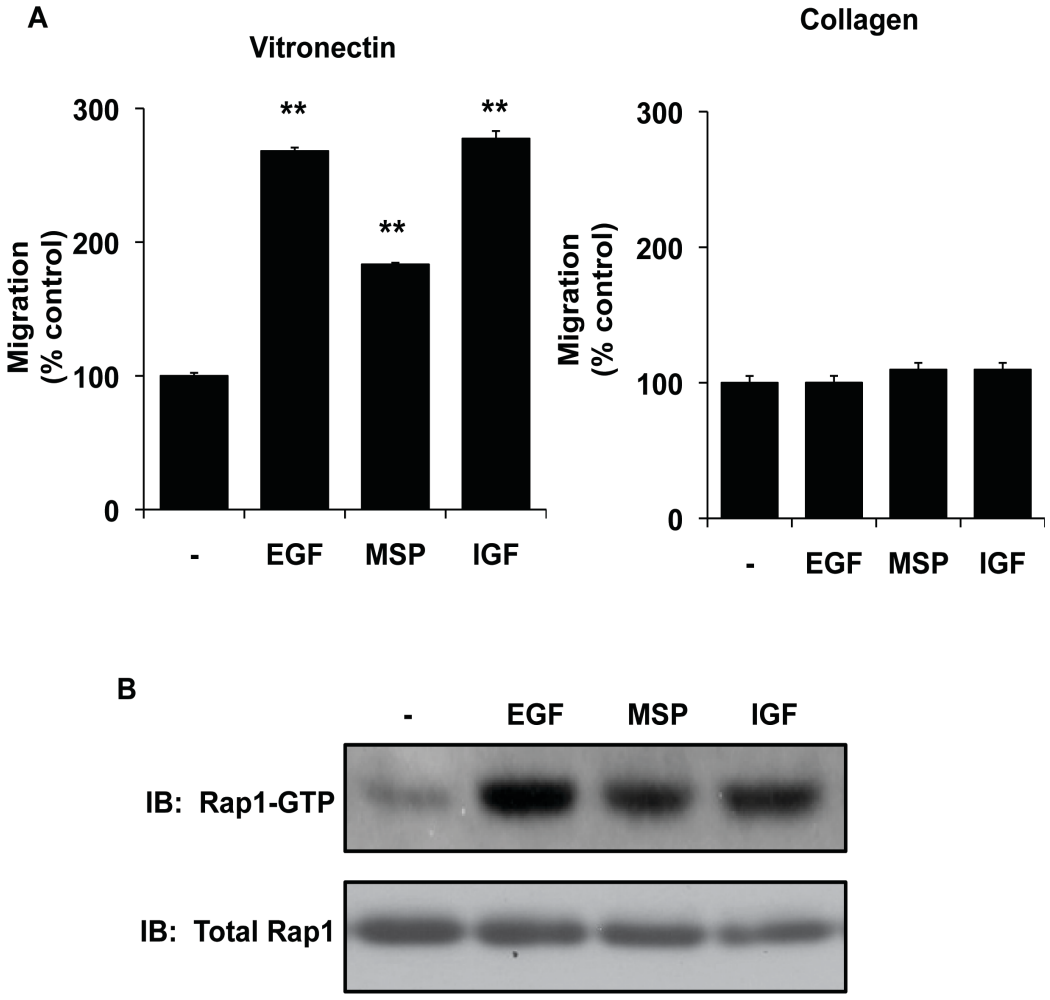


Figure 3.5 EGF, MSP and IGF promote Rap1 activation and selectively increase migration on vitronectin

FG cells transiently stimulated with EGF (50ng/mL, 10min), MSP (10ng/mL, 10min), or IGF (100ng/mL, 30 min) were analyzed for their (**A**) migration on vitronectin or collagen, and for (**B**) Rap1-GTP levels. **p < 0.01.

3.2.3 RapGEF2 and RapGEF6 are required for EGFR-mediated Rap1 activation

Since RapGEFs promote Rap1 activation, growth factor induced-Rap1-GTP loading likely requires one or more RapGEFs. Immunoblotting for the six known specifics RapGEFs revealed that FG cells express only RapGEF2 and RapGEF6. Knockdown of either RapGEF reduces EGFR-dependent Rap1-GTP loading (Figure 3.6). 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*.

3.2.4 Rap1-GTP levels are elevated in FG cells selected for their ability to spontaneously migrate on vitronectin

Although the vast majority of FG cells do not spontaneously migrate on vitronectin, a small population does [79]. By selecting for FG cells that migrate on vitronectin for 10 rounds without growth factor treatment, a population of cells (FGMs) is found to be more metastatic than the parental FG cells [79]. These cells also endogenously express higher levels of Rap1-GTP, which is sensitive to inhibition of EGFR (30 min pre-treatment with erlotinib) and SFK (30 min pre-treatment with dasatinib) (Figure 3.7). These results suggest that active Rap1 indeed plays a role in promoting migration on vitronectin and may explain how FGMs are more metastatic than FG cells.

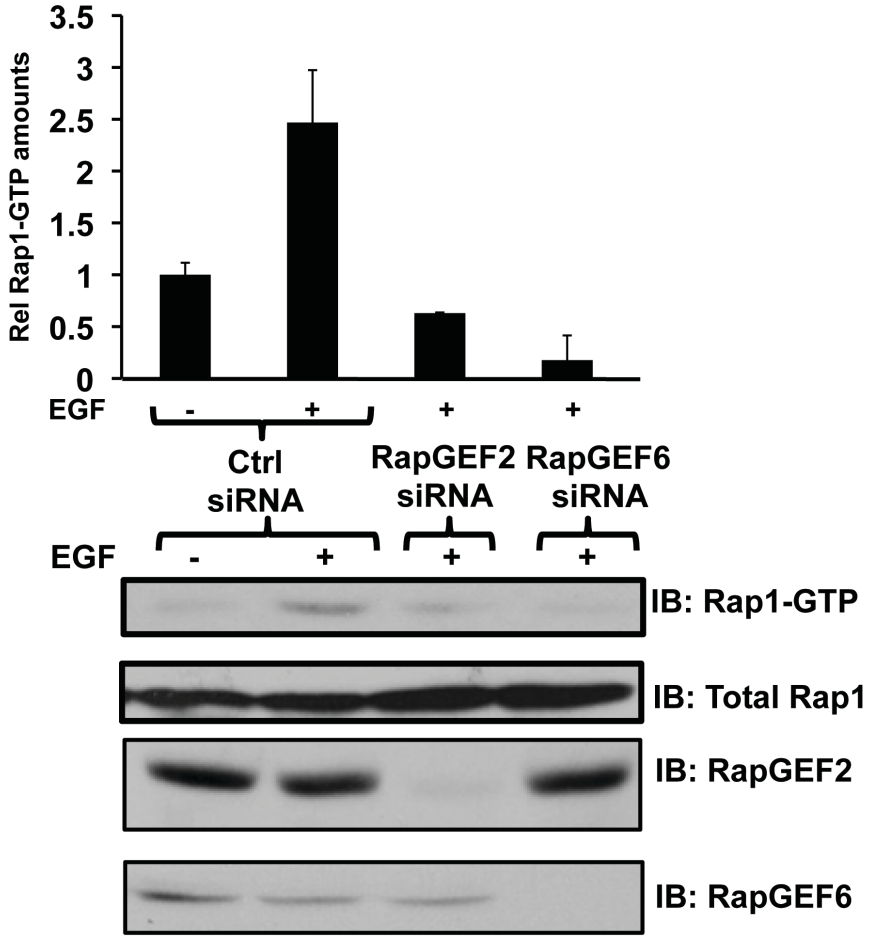


Figure 3.6 EGFR-induced Rap1 activation requires RapGEF2 and RapGEF6

FG cells were transiently transfected with control siRNA, RapGEF2 siRNA, or RapGEF6 siRNA. After 24h, cells were serum starved overnight, subsequently treated with or without EGF and analyzed for Rap1 activation. Quantification of Rap1-GTP levels was performed by normalizing total Rap1 levels by densitometric evaluation (ImageJ).

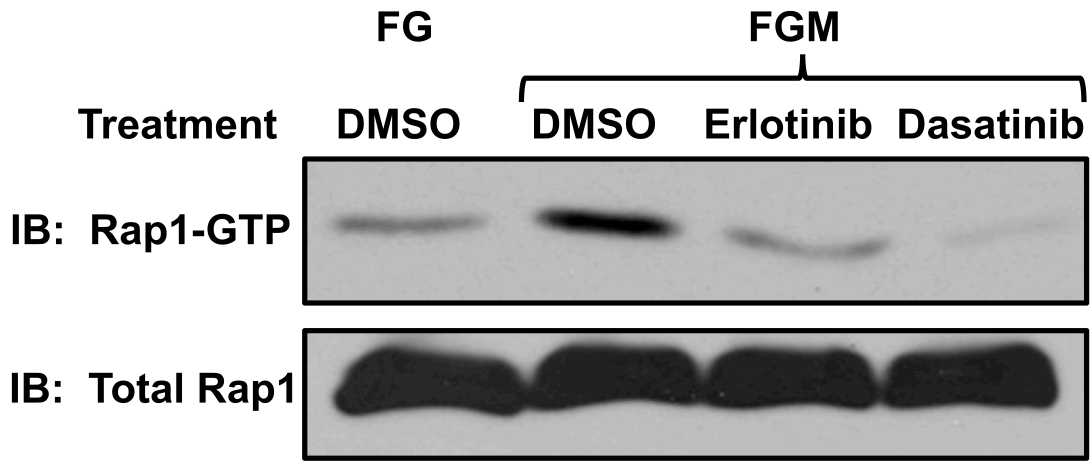


Figure 3.7 Erlotinib and Dasatinib block Rap1 activation in FGMs

FGM, a population of FG cells that migrate spontaneously on vitronectin, were selected by 10 rounds of haptotaxis migration on transwells coated with vitronectin on the underside. FGs were pretreated with DMSO and FGMs were pretreated with DMSO, Erlotinib (EGFR inhibitor) and Dasatinib (SFK inhibitor) for 30 minutes and then lysed in Rap1 activation lysis buffer.
Lysates were incubated with GST-RalGDS beads. Bound proteins were subsequently analyzed by immunoblotting with anti-Rap1 antibody. Total Rap1 in whole-cell lysates was used as loading control.

3.3 Conclusions

Active Rap1 (G12V) expression was sufficient to induce FG cell metastasis *in vivo* without influencing primary tumor growth. This may be explained by the role of Rap1 activity in migration on a vitronectin substrate. Active Rap1 specifically increased migration on vitronectin, while a dominant negative mutant of Rap1 (S17N) disrupted migration on vitronectin. In contrast, the Rap1-GTP status had no impact in the migration response on collagen. Importantly, EGF, MSP and IGF stimulation of FG cells also increased migration selectively on vitronectin, but not on collagen. Consistent with these results, each growth factor also induces Rap1 activation, with RapGEF2 and RapGEF6 facilitating the GTP loading. In addition, cells that spontaneously migrate on vitronectin (FGMs) display higher levels of active Rap1 than their parental FG cells in an EGFR and SFK dependent manner. Therefore, growth factor induction of Rap1-GTP can account for migration and metastasis and the mechanism of how Rap1 becomes activated downstream of growth factor stimulation is investigated in the next chapter of this dissertation.

Chapter 3, in part, has been submitted for publication of the material as it may appear in Oncogene, 2011, Huang, Miller; Anand, Sudarshan; Murphy, Eric A.; Desgrosellier, Jay S.; Stupack, Dwayne G.; Shattil, Sanford J.; Schlaepfer, David D.; Cheresh, David A. “EGFR-dependent carcinoma cell metastasis via Rap1 activation**”.** The dissertation author was the primary investigator and author of this paper.

Chapter 4

EGFR-dependent phosphorylation of specific tyrosine residues on CAS promotes migration, Rap1 activation, and metastasis

4.1 Introduction

Many mechanisms involved in tumor biology are a dysregulation of systems normally reserved for proper development. For example, the formation of new blood vessels through angiogenesis is crucial for embryonic survival. Tumor cells can hijack these pathways to upregulate angiogenic signaling for their own growth and survival in the host. Similarly, a wound healing response or tissue remodeling needs to occur in a relatively short period of time and hosts cannot wait for cells to undergo genetic events to begin migration and repair the wound. Instead, cells must utilize transient extracellular cues to promote a signaling cascade that promotes cell motility. In addition, immune regulatory cells require signals as well in order to quickly and efficiently detect sites of infections and to respond towards those regions. These signaling cues can include ECMs, growth factors, cytokines and chemokines. Migration through EGFR in particular has been involved in wound healing responses. Unfortunately, many tumors also rely on EGFR signaling to promote their own growth, survival and ability to disseminate throughout the body of a host.

As was described in the previous chapter of this dissertation, FG human pancreatic carcinoma cells exhibit two pathways of migration: one that is independent of growth factor stimulation and a second that requires growth factor signals, such as EGF. This provides an ideal system to identify key players in EGFR-mediated migration, invasion and metastasis. Recently, EGFR-induced activation of Src family kinases (SFK) was found to be

necessary and sufficient for induction of migration on vitronectin *in vitro* and metastasis *in vivo* and this was dependent on integrin αvβ5 [31]. Indeed, the requirement for SFK activity for migration on vitronectin was found not only in pancreatic cancer cell lines, but also in ovarian, colon, and breast. Thus, SFK-dependent migration on vitronectin may signal through the same downstream mechanism for multiple tumor types. Furthermore, total levels of Src and active levels of Src were found to be elevated in 70% and 60% of human pancreatic cancer patient samples, respectively. Patients with higher levels of total or active Src exhibited a lower overall survival rate, validating the importance of Src in human pancreatic cancer [59].

Subsequent to SFK activation, the adapter protein Crk-associated substrate (CAS) is phosphorylated on tyrosine residues in its substrate domain, which contains 15 YxxP motifs [155]. Deletion of the CAS substrate domain or mutation of all 15 YxxP motifs to FxxP has been shown to block tumor cell migration, invasion and metastasis [79, 81]. Tyrosine phosphorylation of CAS on the 15 YxxP motifs in its substrate domain creates dockings sites for proteins that contain SH2 domains, including other pro-migratory signaling molecules such as Crk and Nck [79, 86, 92]. In this chapter, a novel mechanism of Rap1 activation downstream of EGFR is characterized that requires the formation of a CAS/Nck1 complex. As described in Chapter 3 of this dissertation, Rap1 activation is crucial in the migration response on vitronectin and in metastasis.

Therefore, genetic manipulations that influence EGFR-dependent Rap1 activation should also be relevant for migration on vitronectin and metastasis.

4.2 Results

4.2.1 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 [156, 157]. Previously, we showed that Src activation is required for EGFR-mediated spontaneous metastasis of carcinoma cells [31]. In fact, stimulation of FG cells with EGF, IGF or MSP leads to the phosphorylation of the Y416 activation site on SFK (Figure 4.1). Since Src, Yes and Fyn are common SFK members expressed in epithelial cells, they were screened for their role in migration on vitronectin. Transient knockdown of Src and Yes, but not Fyn, in FG cells disrupted EGFR-mediated migration on vitronectin, but not on collagen (Figure 4.2) suggesting Rap1 activation by EGFR was Src and Yes dependent. To further define their roles in this pathway, Src and Yes were transiently knocked down by siRNA in FG cells prior to EGF stimulation. Lysates were immunoprecipitated with anti-CAS antibodies and blotted for anti-phoshotyrosine. While downregulation of Src and Yes both reduced CAS phosphorylation, Src appeared to be the predominant SFK member that blocks EGFR-dependent CAS phosphorylation (Figure 4.3). Although knockdown of Yes also reduces CAS phosphorylation, there still is an increase in CAS phosphorylation in response to EGF stimulation of FG cells which suggests that Yes may be involved in EGFR-dependent migration via phosphorylation of a separate target. Lastly, Src was

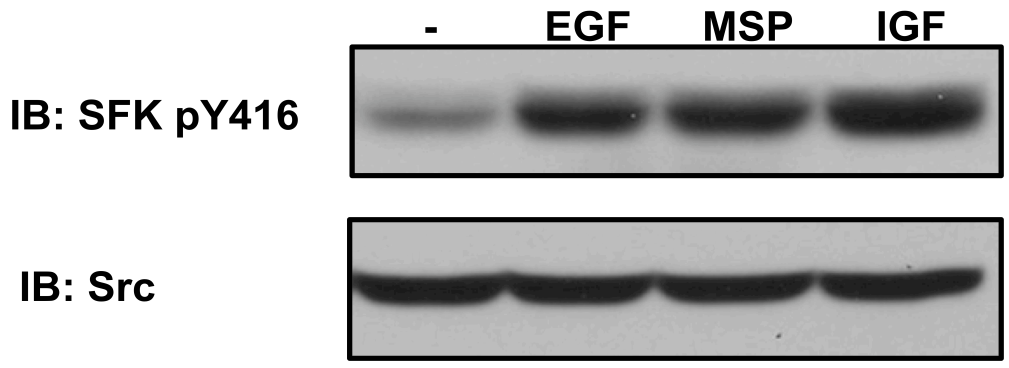


Figure 4.1 EGF, MSP and IGF induce SFK activation in FG cells.

Lysates from FG cells transiently stimulated with EGF, MSP or IGF were immunoblotted for pSFK Y416 and subsequently re-probed for total Src (loading control).

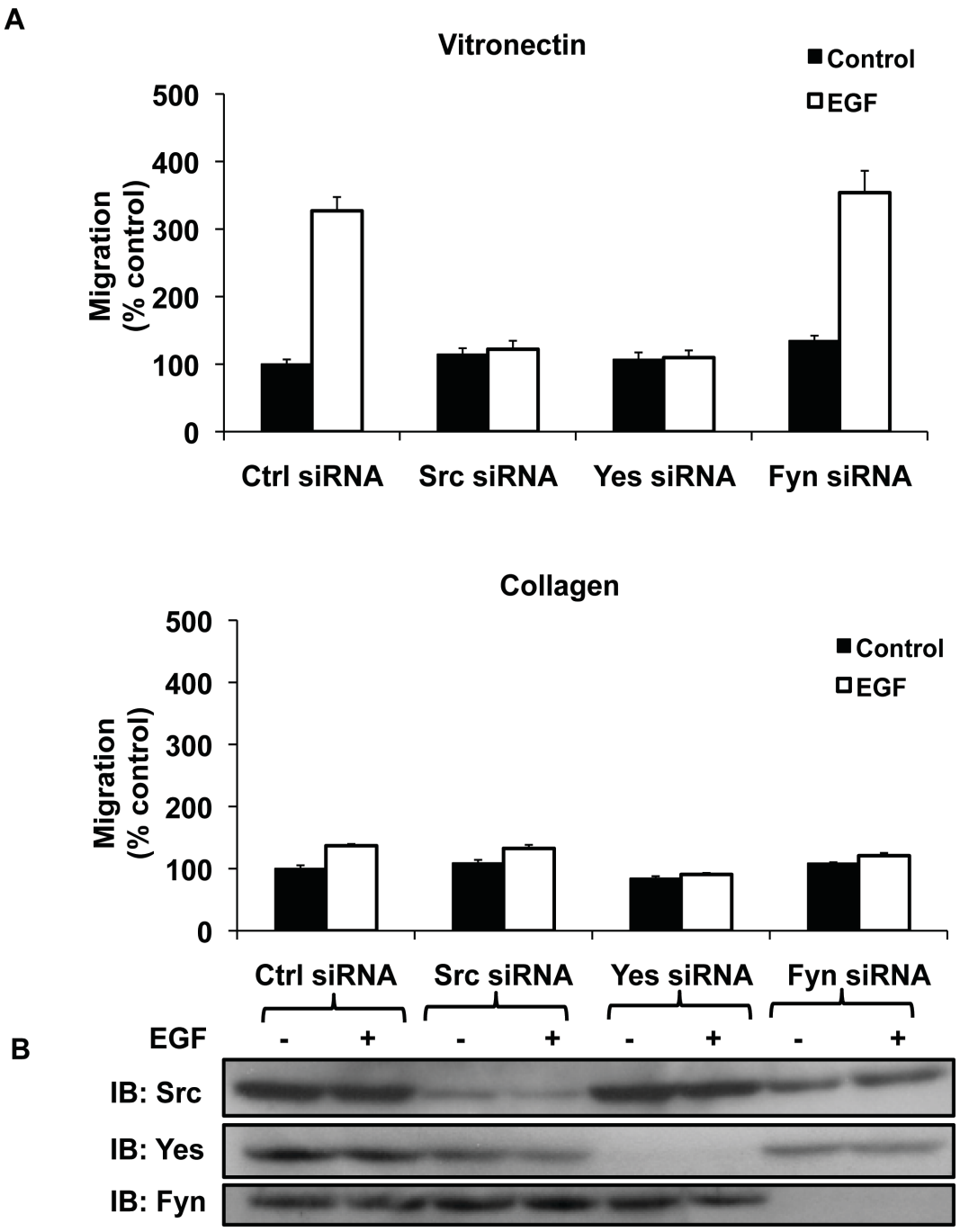


Figure 4.2 Src and Yes are required for migration on vitronectin

FG cells were transiently transfected with control siRNA, Src siRNA, Yes siRNA and Fyn siRNA. After 24h, the cells were serum starved overnight, followed by treatment with or without EGF and analyzed for (**A**) migration on vitronectin or collagen and (**B**) knockdown of Src, Yes and Fyn

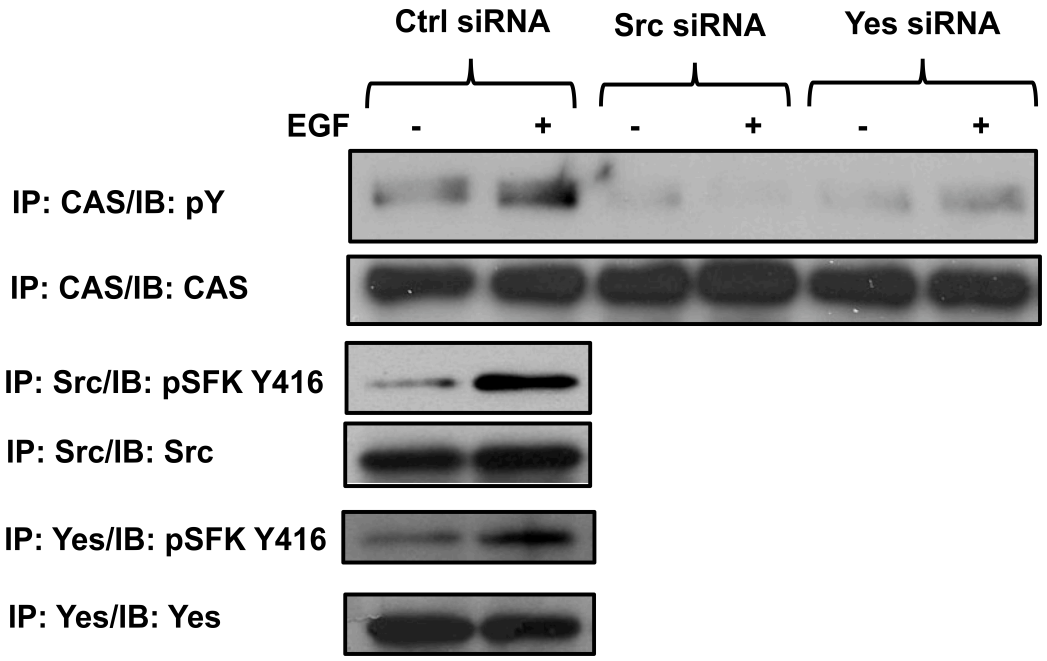


Figure 4.3 Src is the predominant SFK member that phosphorylates CAS in an EGFR-dependent manner

FG cells were transiently transfected with control siRNA, Src siRNA, Yes siRNA. After 24h, the cells were serum starved overnight, followed by treatment with or without EGF and lysed. Lysates were immunoprecipitated with anti-CAS, anti-Src or anti-Yes antibodies and immunoblotted for anti-phosphotyrosine, and anti-SFK pY416. Membranes were stripped and re-probed with total CAS, Src and Yes antibodies for loading control.

evaluated for a role in EGFR-mediated Rap1 activation. Transient transfection of Src siRNA in FG cells was sufficient to block EGFR-dependent Rap1-GTP loading (Figure 4.4)

The adaptor protein p130CAS (CAS), a prominent Src substrate, plays a key role in cell migration and invasion [76, 79, 83]. A closely related family member of CAS, human enhancer of filamentation (HEF), also plays a role in cell migration and metastasis [158]. To determine whether CAS and/or HEF may be involved in EGFR-mediated migration, FG cells were first transiently transfected with siRNA against either CAS, HEF or CAS and HEF. Down-regulation of CAS, but not HEF, blocked EGFR-induced migration on vitronectin, but not collagen (Figure 4.5). Additionally, CAS knockdown in FG cells disrupted Rap1-GTP loading (Figure 4.6). 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.

4.2.2 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 [79, 86]. 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 with anti-CAS followed by immunoblotting for Nck1, Nck2, CrkL and CrkI. EGF stimulation increased the

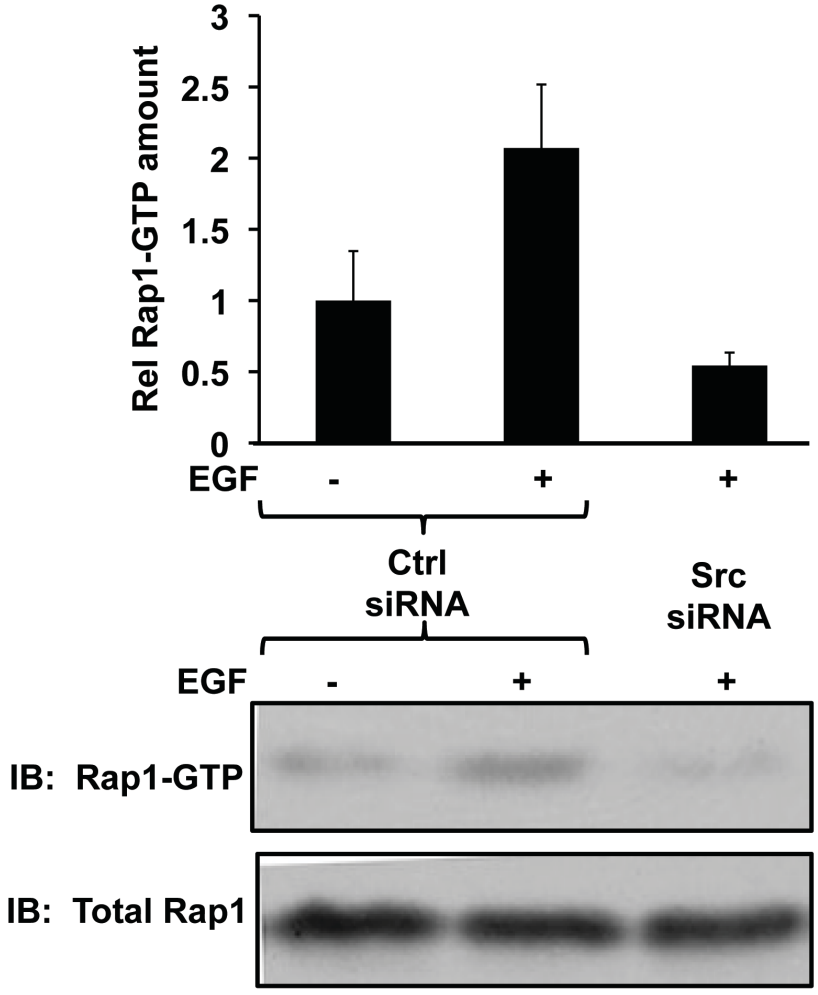


Figure 4.4 Src is required for EGFR-mediated Rap1 activation

FG cells were transiently transfected with control siRNA or Src siRNA. After 24h, the cells were serum starved overnight, followed by treatment with or without EGF and analyzed for Rap1-GTP. Quantification of Rap1-GTP levels was performed by normalizing total Rap1 levels by densitometric evaluation (ImageJ).

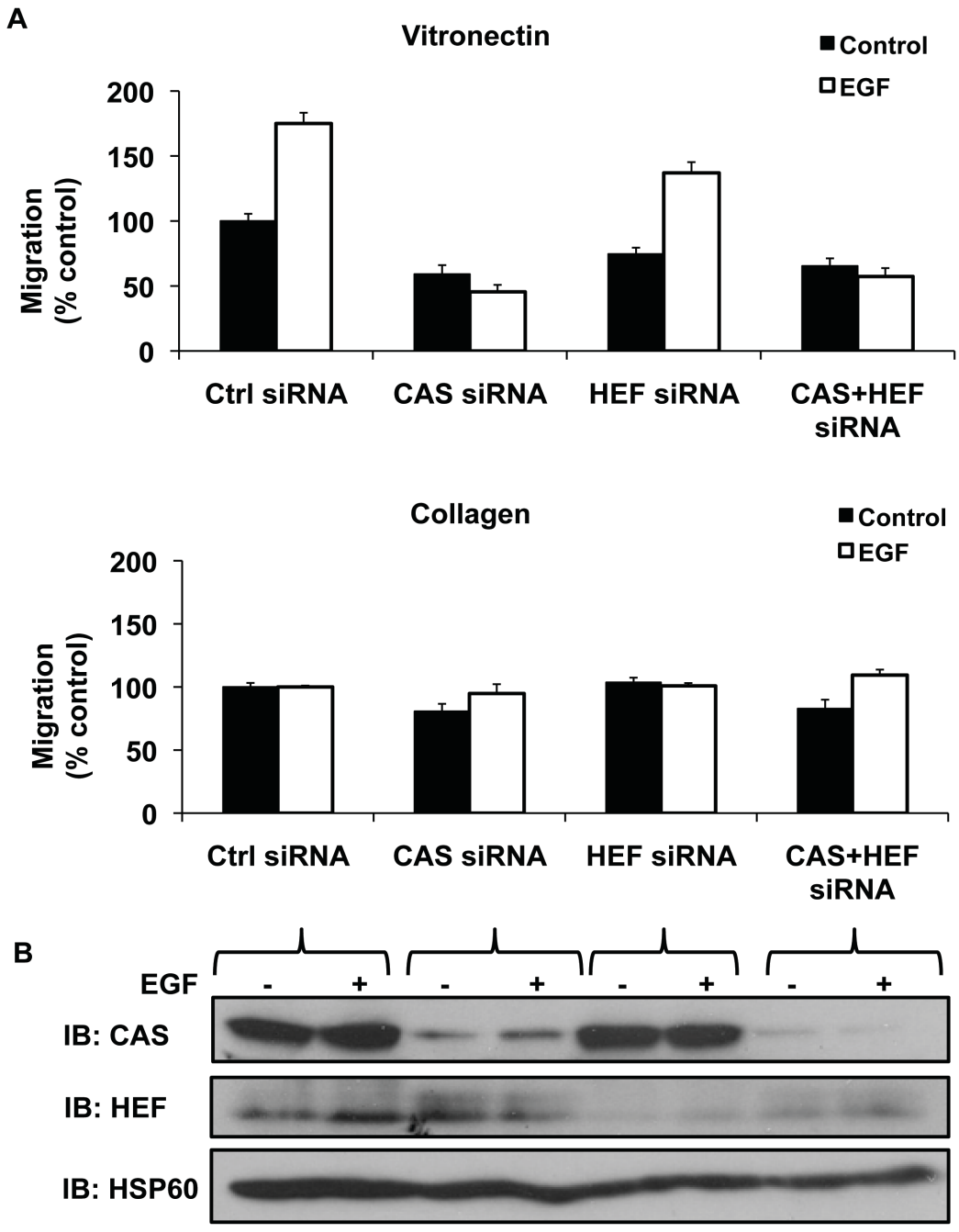


Figure 4.5 CAS, but not HEF, is required for EGFR-induced migration on vitronectin

FG cells were transiently transfected with control siRNA, CAS siRNA, HEF siRNA or CAS + HEF siRNA. After 24h, they were serum starved overnight, treated with or without EGF and analyzed for (**A**) migration on vitronectin or collagen, and (**B**) CAS + HEF knockdown.

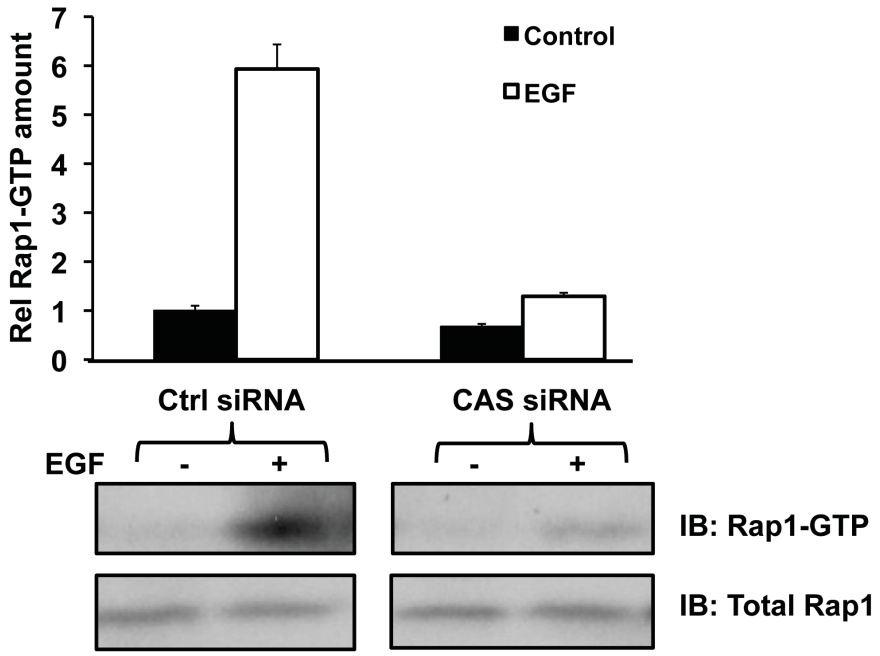


Figure 4.6 CAS is necessary for EGFR-dependent Rap1-GTP loading

FG cells were transiently transfected with control siRNA or CAS siRNA. After 24h, they were serum starved overnight, treated with or without EGF and analyzed for Rap1-GTP. Quantification of Rap1-GTP levels was performed by normalizing total Rap1 levels by densitometric evaluation (ImageJ).

association of both Nck1 and Nck2 with CAS but not CrkL or CrkI with CAS. (Figure 4.7). We next evaluated the significance of these proteins in EGFR-dependent migration on vitronectin. Interestingly, knockdown of either CrkL or CrkI inhibited cell migration in general, blocking both EGFR -mediated cell migration on vitronectin as well as the constitutive cell migration on collagen (Figure 4.8). In contrast, knockdown of Nck1 but not Nck2 selectively suppressed EGFR-induced cell migration on vitronectin, but not on collagen (Figure 4.9). In support of these findings, knockdown of Nck1 also disrupted EGFR-mediated Rap1-GTP loading (Figure 4.10). These findings indicate that Nck1 is specifically required for the EGFR-mediated carcinoma cell migration response while both Crk family members appear to have a broad, generic 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 the EGFR-mediated cell migration on vitronectin. Therefore we considered whether Nck1 was also required for the 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 4.11). Taken together these data implicate Nck1 and its

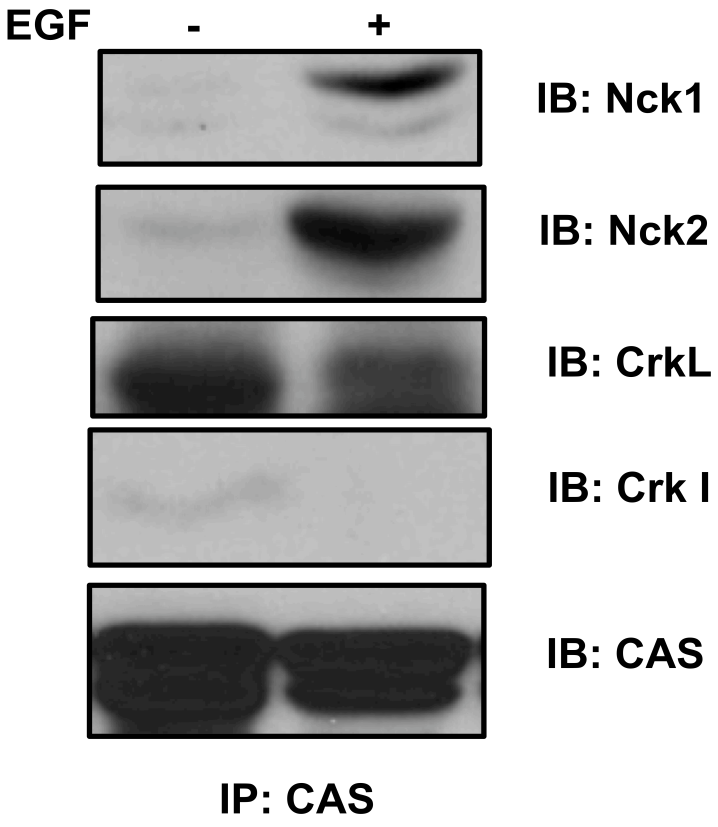


Figure 4.7 EGF stimulation of FG cells induce CAS association with Nck adapters, but not Crk family proteins

Lysates from serum-starved FG cells treated with or without EGF were immunoprecipitated with anti-CAS. Eluted proteins were analyzed by immunoblotting with anti-CAS, anti-CrkI, anti-CrkL, anti-Nck1 or anti-Nck2 antibody, as indicated.

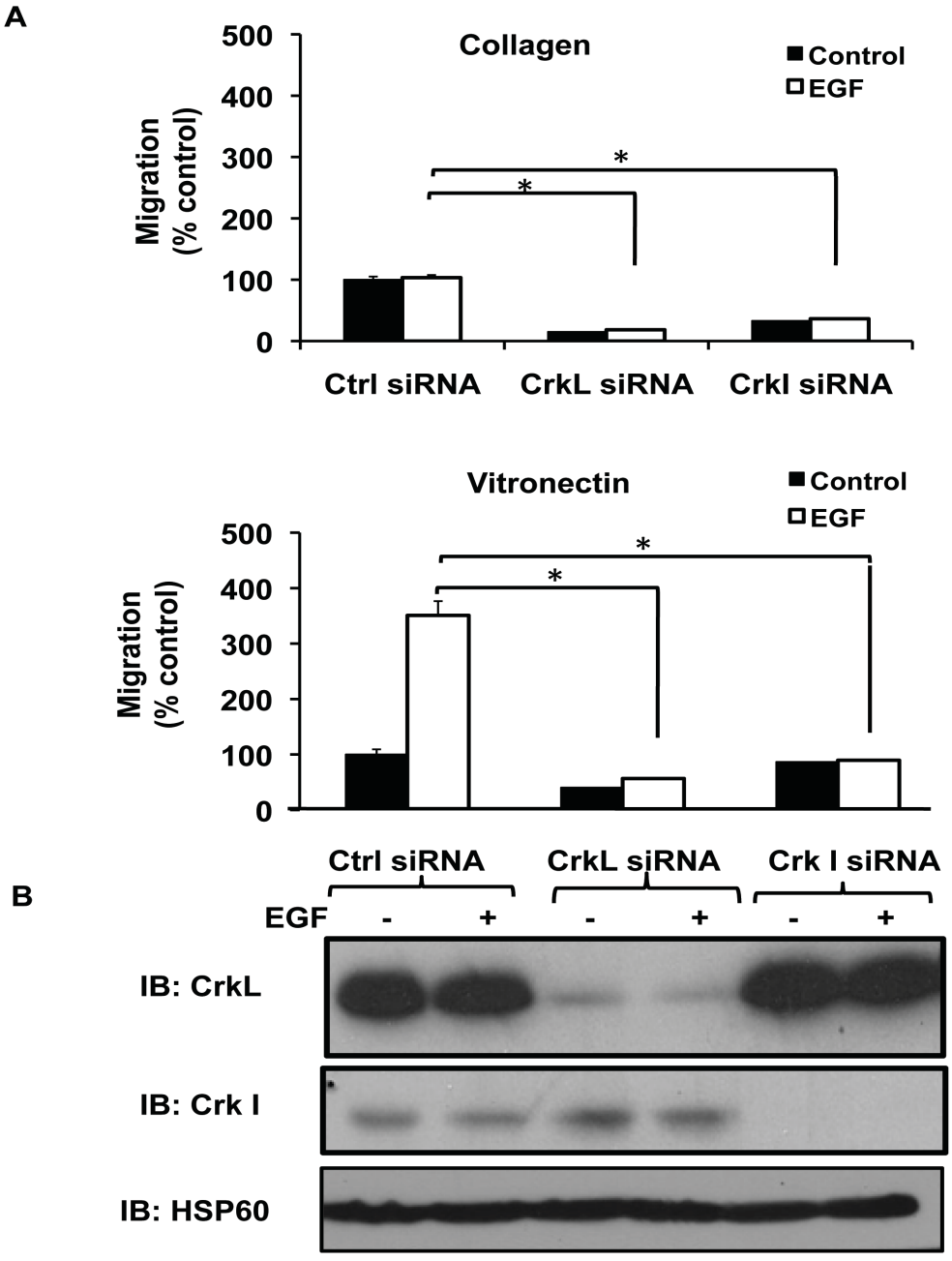


Figure 4.8 Crk family members have a general role in migration

FG cells were transiently transfected with control siRNA, CrkL siRNA, or CrkI siRNA. After 24h, cells were serum starved overnight, subsequently treated with or without EGF and analyzed for (**A**) migration on vitronectin or collagen and (**B**) knockdown for CrkL and CrkI. *$p < 0.01$.

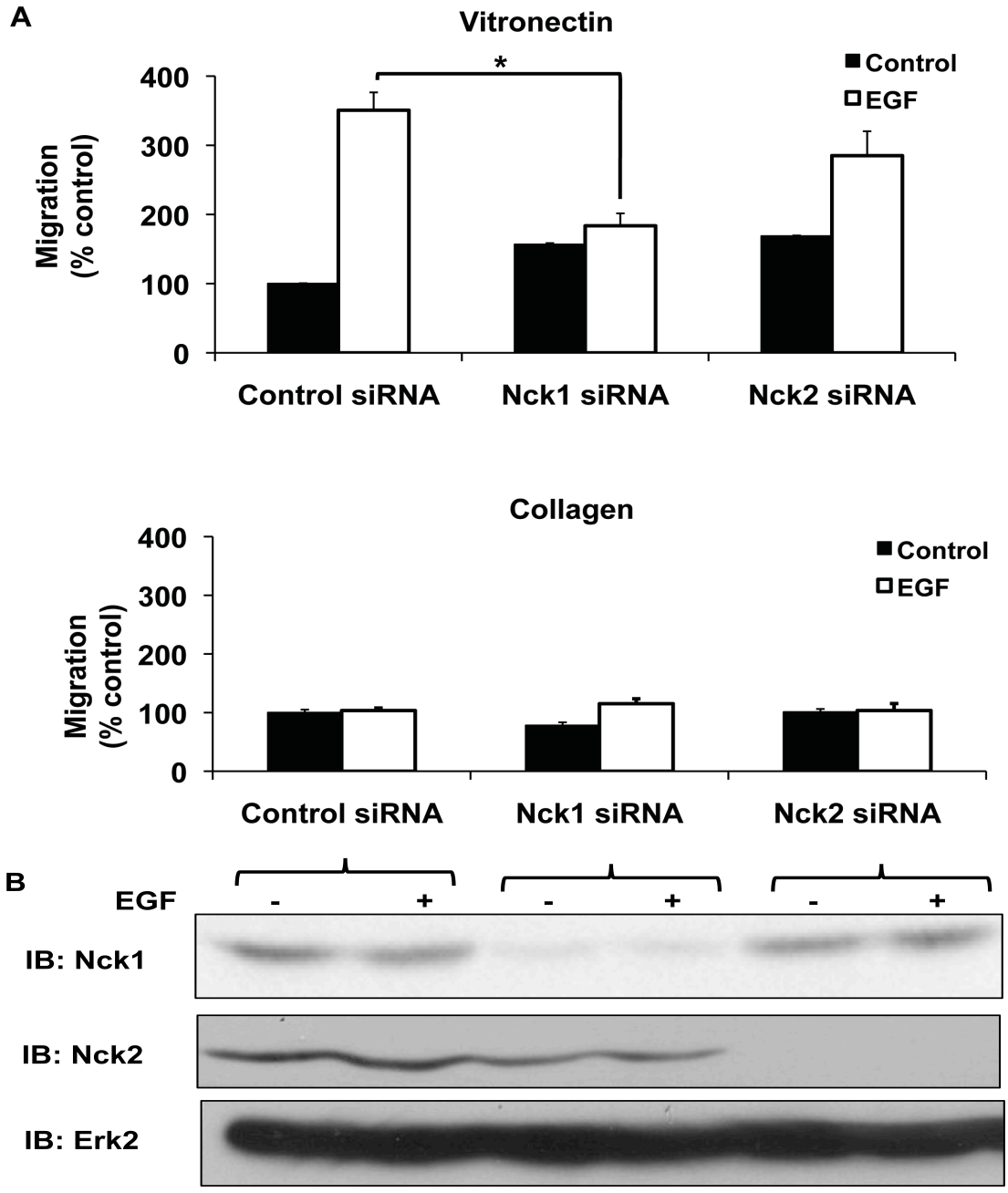


Figure 4.9 Nck1, but not Nck2 is required for EGFR-mediated migration on vitronectin

FG cells were transiently transfected with control siRNA, Nck1 siRNA, or Nck2 siRNA. After 24h, cells were serum starved overnight, subsequently treated with or without EGF and analyzed for (**A**) migration on vitronectin or collagen and (**B**) confirmation of Nck1 and Nck2 knockdown. *p<0.01

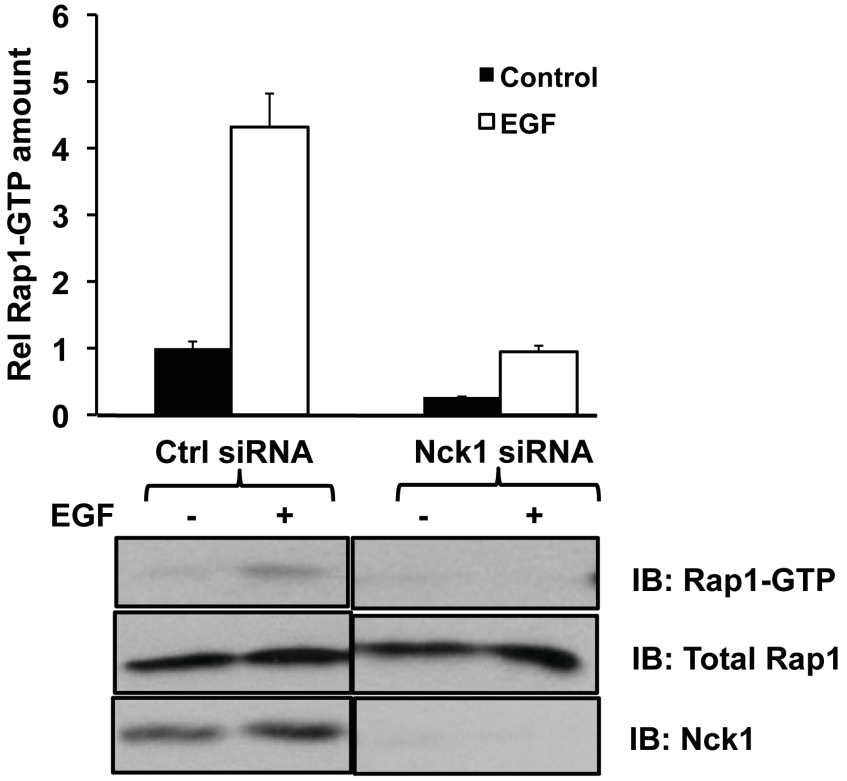


Figure 4.10 Nck1 is necessary for EGFR-mediated Rap1 activation

FG cells were transiently transfected with control siRNA or Nck1 siRNA. After 24h, they were serum starved overnight, subsequently treated with or without EGF and analyzed for Rap1-GTP levels. Quantification of Rap1-GTP levels was performed by normalizing total Rap1 levels by densitometric evaluation (ImageJ)

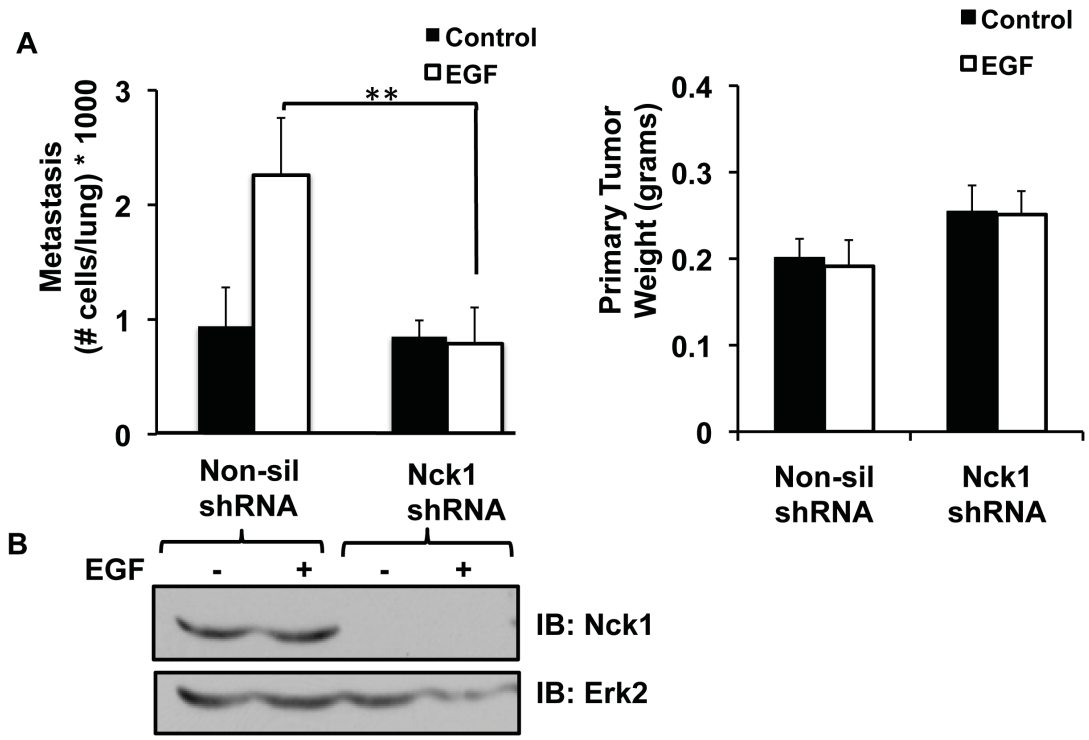


Figure 4.11 Nck1 is necessary for EGFR-dependent metastasis

(**A**) 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 qPCR for human *Alu* sequence and chicken *GAPDH* and normalized to a standard curve. n ≥ 10 in each group. (**B)** Nck1 knockdown was confirmed by western blot analysis using indicated antibodies. **p < 0.05.

association with CAS as a key signaling module that regulates EGFR-mediated Rap1 activation, tumor cell invasion and metastasis.

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

The mechanism by which Nck1 associates with CAS and thereby influences tumor cell metastasis may rely on the CAS substrate domain which is characterized by 15 YxxP motifs and serve as putative docking sites for SH2 domain containing proteins including Nck1 [83]. A number of these sites have been linked to cell migration and metastasis and are known to represent substrates for Src [81, 84, 159].. A previous study reported that one or more of the first nine tyrosines among the fifteen found within the CAS substrate domain were required for EGFR-dependent metastasis [31]. Therefore, one or more tyrosines within the first nine YxxP motifs in the CAS substrate domain might play a role in EGFR-mediated carcinoma cell invasion and metastasis. A range of Y-F point mutations within the substrate domain of CAS were stably expressed in FG cells and the mutants CAS Y253F (referred to as “F7”) or Y271F (referred to as “F8”) was sufficient to block EGFR-mediated migration on vitronectin (Figure 4.12) without affecting cell migration on collagen (data not shown). As previously reported [31] 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

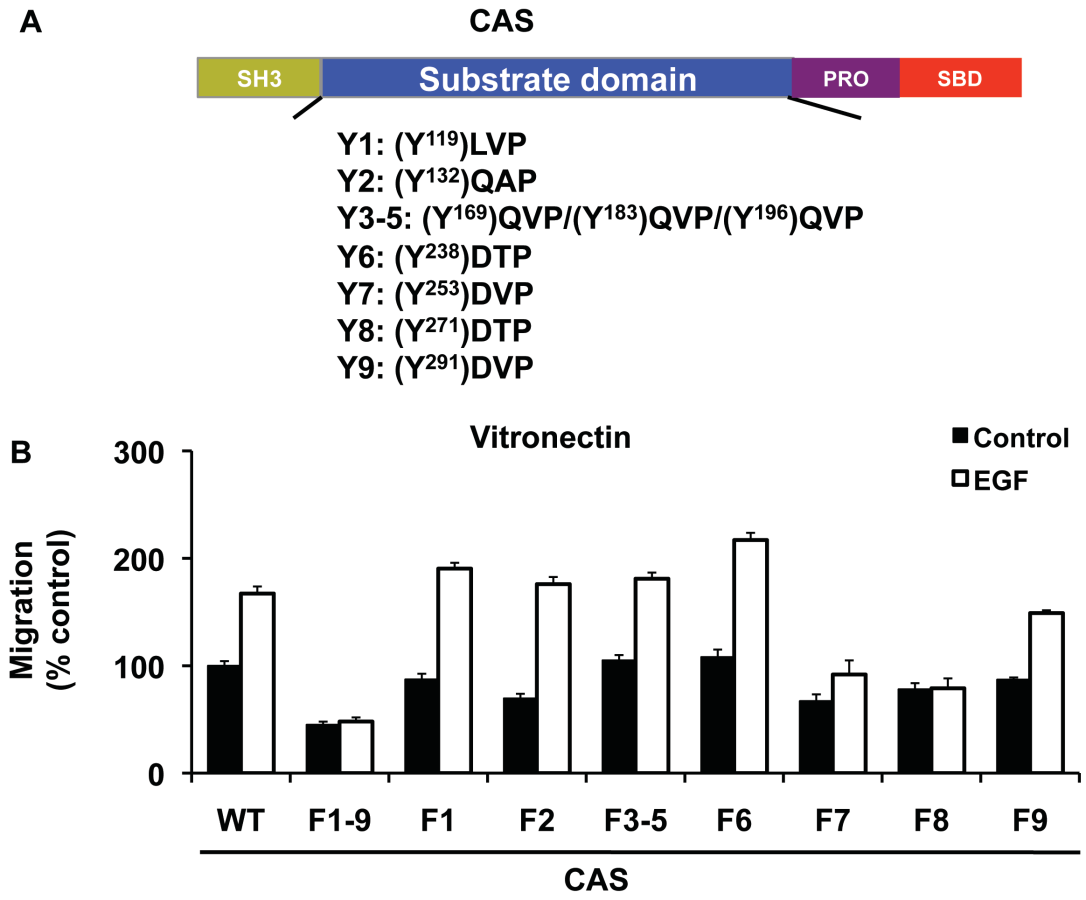


Figure 4.12 CAS Y253 and Y271 are required for EGFR-mediated migration on vitronectin

(**A**) Schematic of the tyrosine residues of the first 9 YxxP motifs in the substrate domain of CAS. (**B**) 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.

F1-15 construct was sufficient to enhance EGFR-dependent migration (Figure 4.13) and Rap1 activation (Figure 4.14) equally to WT CAS.

Y253 and Y271 are putative Src phosphorylation sites [83, 84]. To confirm this in FG cells we transiently transfected an empty vector or a constitutively active Src construct (Src Y527F or "SrcA") in FG cells stably expressing WT CAS, F1-15, F7/F8 (Y253F/Y271F) or Y7/Y8 CAS. CAS was immunoprecipitated from these cells and immunoblotted for CAS phosphorylation. The F7/F8 mutant exhibited a significant decrease in CAS phosphorylation compared to WT CAS, while the Y7/Y8 mutant rescued Src-dependent phosphorylation (Figure 4.15). To evaluate the importance of phosphorylation of these sites on migration, SrcA cells co-transfected with the each of the CAS mutants were compared amongst each other in migration on vitronectin. SrcA cells expressing WT CAS showed an increased migration response on vitronectin compared to SrcA alone. However, SrcA cells expressing the CAS F7/F8 mutant, like those expressing CAS F1-15, failed to show the 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 play in carcinoma cell migration on vitronectin (Figure 4.15).

Because CAS tyrosines 7 and 8 appear to have a significant role in EGFR-induced migration as well as Rap1 activation, they may serve as docking sites for Nck1. To investigate this, FG cells were stably expressed

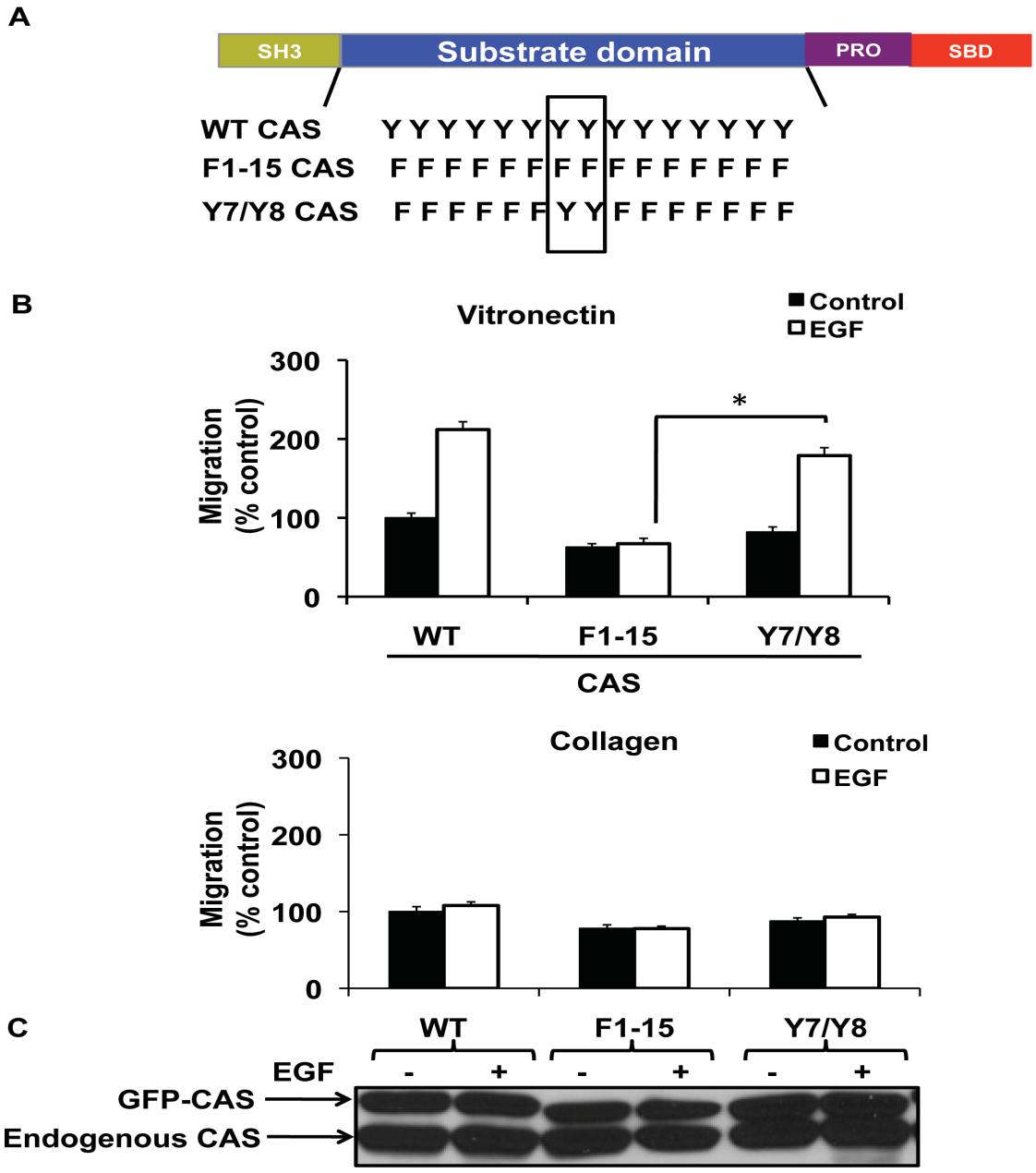


Figure 4.13 CAS Y253 and Y271 restored in CAS F1-15 is sufficient to enhance EGFR-dependent migration compared to WT CAS

(**A**) Schematic of the tyrosine residues of the YxxP motifs in the substrate domain of WT CAS, F1-15 CAS or Y7/Y8 CAS. Box represents the tyrosine residues of the 7th and 8th YxxP motifs. (**B, C**) FG cells stably expressing WT CAS, F1-15 CAS or Y7/Y8 CAS were serum starved, treated with or without EGF and assessed for (**B**) migration phenotype on vitronectin or collagen and (**C**) expression of GFP-CAS mutants. *$p < 0.01$

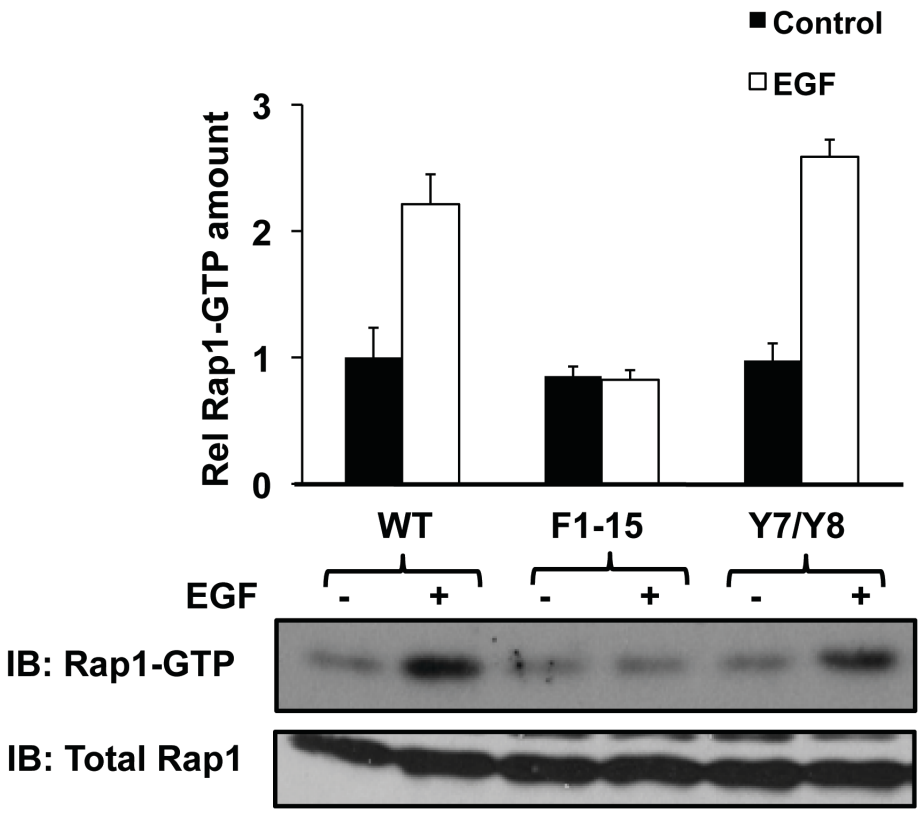


Figure 4.14 CAS Y253 and Y271 restored in CAS F1-15 is sufficient to enhance EGFR-dependent Rap1 activation compared to WT CAS

FG cells stably expressing WT CAS, F1-15 CAS or Y7/Y8 CAS were serum starved, treated with or without EGF and assessed for their Rap1 activation. Quantification of Rap1-GTP levels was performed by normalizing total Rap1 levels by densitometric evaluation (ImageJ)

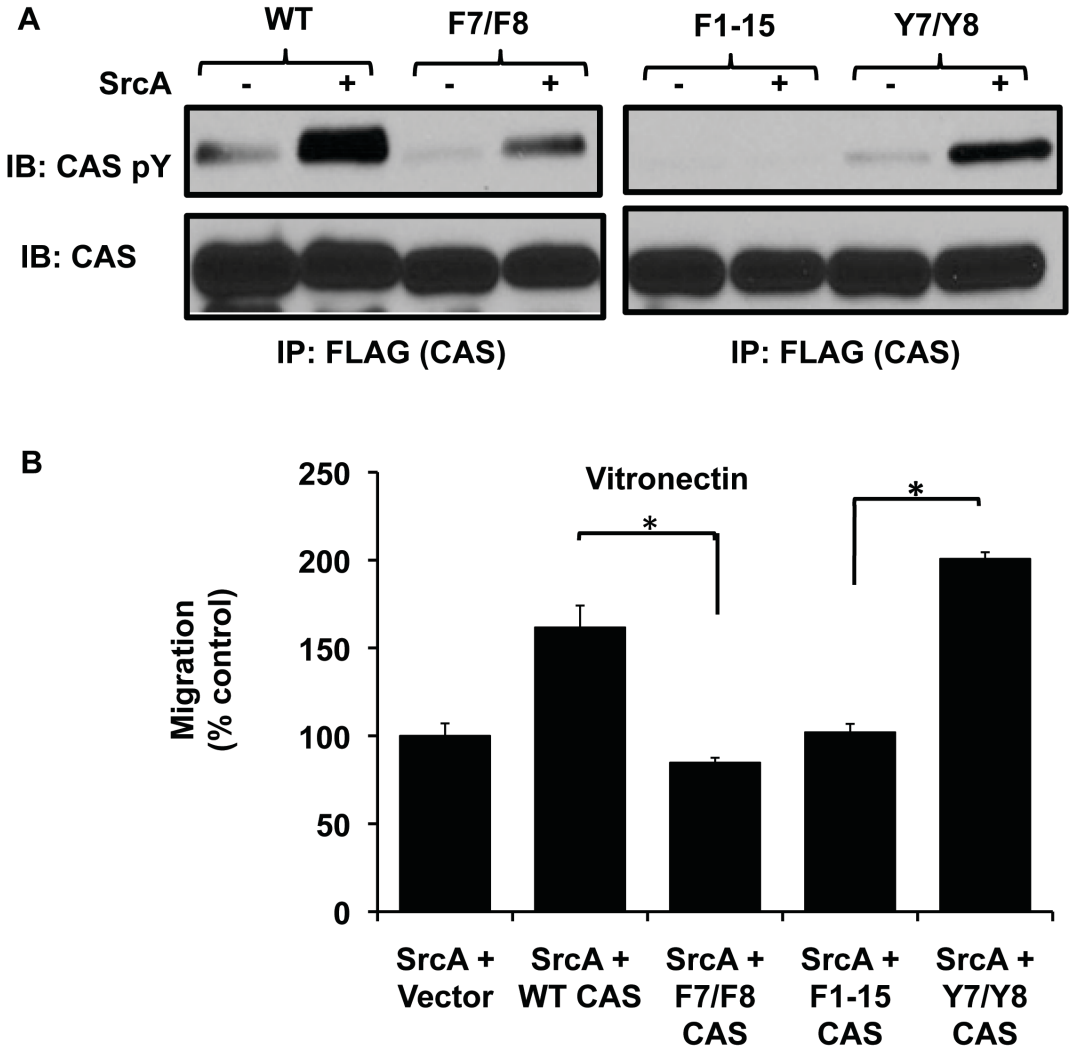


Figure 4.15 CAS Y253 and Y271 are Src phosphorylation sites required for CAS-induced migration on vitronectin

(**A**) FG cells stably expressing SrcA (Src Y527F) were transiently transfected with empty vector, FLAG-tagged WT CAS, F7/F8 CAS, F1-15 CAS or Y7/Y8 CAS and analyzed for their migration phenotype on vitronectin. (**B**) FG cells stably expressing empty vector or SrcA were also transiently transfected with FLAG-tagged WT CAS, F7/F8 CAS, F1-15 CAS or Y7/Y8 CAS. After 24h, cells were serum starved overnight and lysed. Lysates were immunoprecipitated with anti-FLAG and analyzed by immunoblotting for phospho-CAS followed by anti-FLAG (loading control). *$p < 0.01$.

with a 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 the 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 HA-Nck1/FLAG-CAS complex, while no increase was seen in the F7/F8 or the F1-15 CAS expressing cells (Figure 4.16). 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 observation, WT CAS and F7/F8 CAS were mock-treated or treated with EGF, implanted on chick CAM and monitored their primary tumor growth and spontaneous pulmonary metastasis. As expected, EGF stimulation promoted a significant increase in lung metastasis of cells expressing WT CAS cells, whereas EGF stimulation of cells the expressing F7/F8 CAS construct had no effect on tumor growth or metastasis (Figure 4.17). These results demonstrate that Y253 and Y271 sites in CAS are able to recruit Nck1 and thereby play a significant role in EGFR-dependent CAS/Nck1 association, Rap1 activation, migration and metastasis (Figure 4.18).

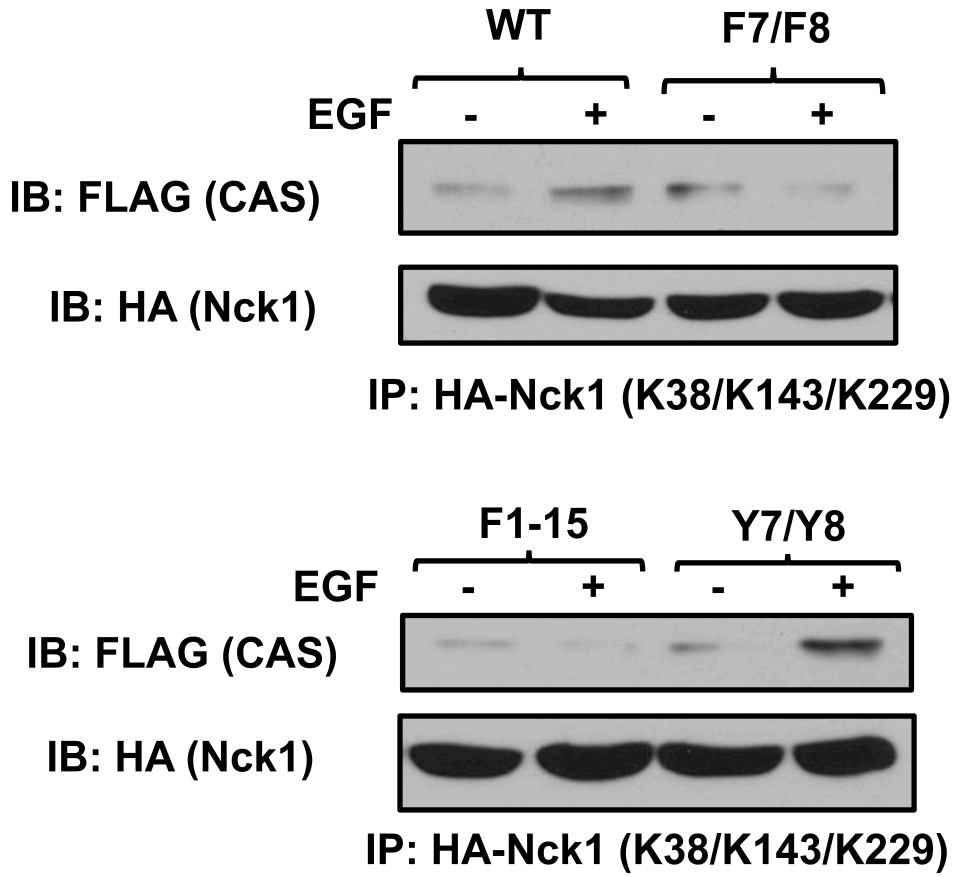


Figure 4.16 CAS Y253 and Y271 are necessary and sufficient for EGFR-induced CAS/Nck1 association

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 24h, cells were serum starved overnight, followed by treatment with or without EGF and lysed. Lysates were immunoprecipitated with anti-HA and analyzed by immunoblotting for FLAG and HA (loading control).

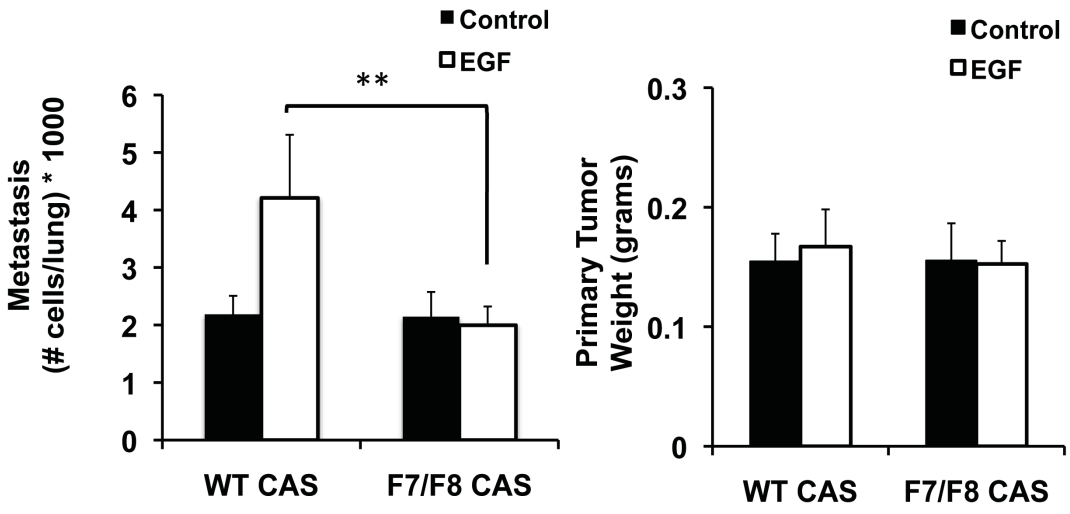


Figure 4.17 CAS Y253 and Y271 is necessary for EGFR-induced metastasis

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 qPCR for human *Alu* sequence and chicken *GAPDH* and normalized to a standard curve. $n \geq 10$ in each group. $^{**}p < 0.05$

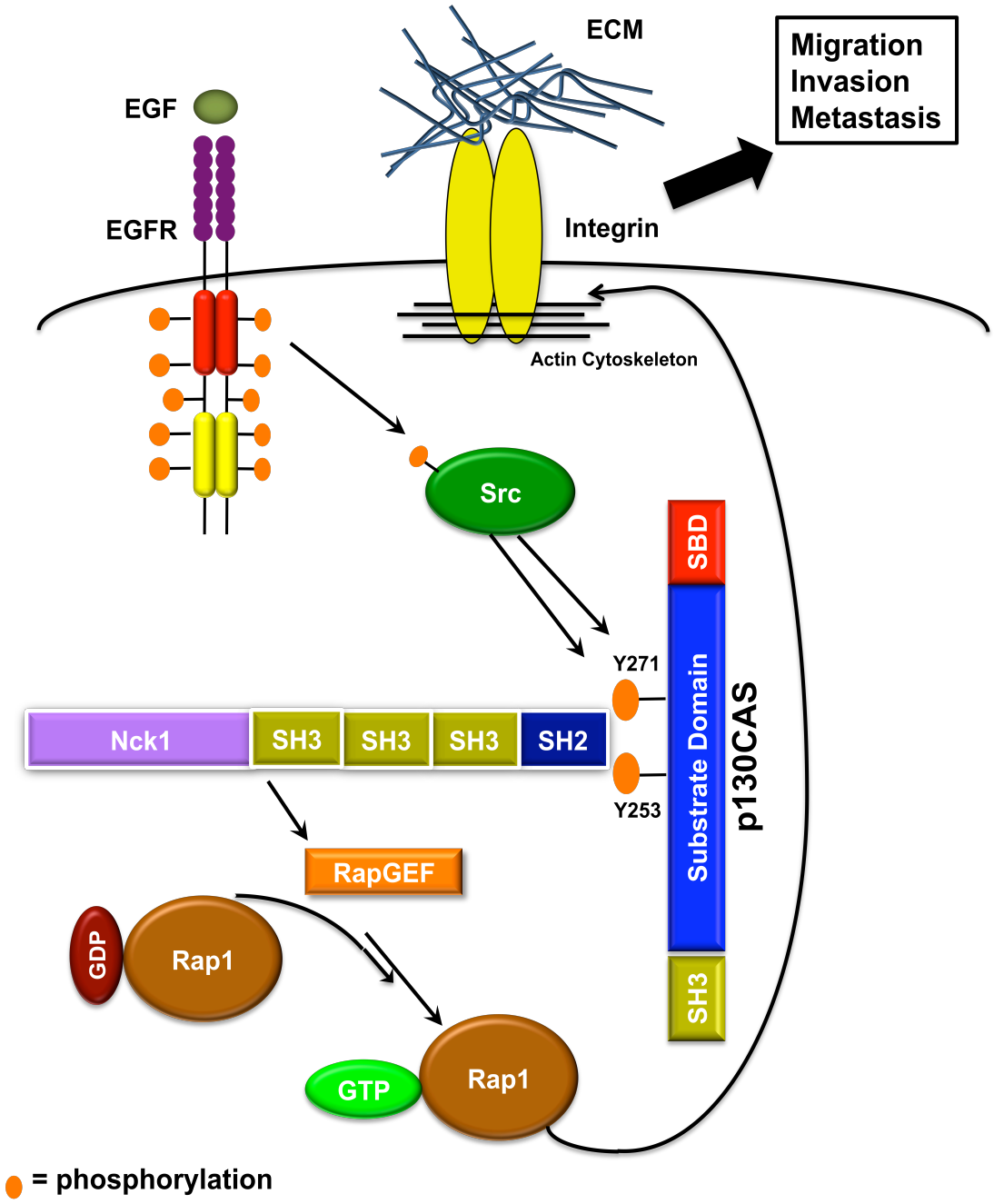


Figure 4.18 EGFR activates migration and metastasis via a CAS/Nck1/Rap1 signaling module.

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.

4.3 Conclusion

Stimulation of FG cells with EGF, MSP and IGF leads to the activation of SFKs. Specifically activation of Src and Yes, are required for migration on vitronectin and phosphorylation of CAS. Src, however, is the predominant SFK member that phosphorylates CAS and promotes Rap1 activation. Mutational analysis of the individual tyrosine residues in the substrate domain of CAS reveals that Y253 and Y271 are required for EGFR-dependent migration on vitronectin. Conversely, restoration of Y253 and Y271 in an F1-15 mutant rescues the phenotype for EGFR-dependent migration on vitronectin. This is likely due to the fact that both tyrosine residues are docking sites for the SH2 domain of Nck1 to form a CAS/Nck1 complex. Nck1 is essential for EGFR dependent migration, metastasis and Rap1 activation. In contrast, Crk family members play a role in migration on vitronectin as well as collagen, which suggests that Crk plays a generic role in migration. Thus, EGFR activation promotes Src phosphorylation of CAS Y253 and Y271 leading to the assembly of a CAS/Nck1 complex to activate Rap1 and induce migration and metastasis.

Chapter 4, in part, has been submitted for publication of the material as it may appear in Oncogene, 2011, Huang, Miller; Anand, Sudarshan; Murphy, Eric A.; Desgrosellier, Jay S.; Stupack, Dwayne G.; Shattil, Sanford J.; Schlaepfer, David D.; Cheresh, David A. “EGFR-dependent carcinoma cell metastasis via Rap1 activation**”.** The dissertation author was the primary investigator and author of this paper.

Chapter 5

Discussion

5.1 EGFR in pancreatic cancer

Pancreatic cancer is an extremely aggressive disease with the highest mortality rate of all major cancers. Current targeted treatments for patients with pancreas cancer are centered around the inhibition of EGFR. 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 [160] and metastasis [17]. In animal models of pancreatic cancer, gemcitabine treatment in combination with anti-EGFR therapy significantly inhibited metastasis to the liver and lymph node [161]. EGF-induced activation of EGFR stimulates cells to rapidly acquire an invasive or metastatic phenotype [21, 162]. In fact, carcinoma cells transiently exposed to EGF gain the capacity to invade and metastasize *in vivo* [31]. However, the addition of the EGFR inhibitor, erlotinib, with gemcitabine leads to only a marginal increase in the lifespan of pancreas cancer patients. One likely explanation for the lack of significant clinical efficacy of EGFR inhibition in pancreatic cancer may be genetic mutations in EGFR that perturbs the binding of erlotinib. It is also likely that other growth factor signaling pathways are activated which share similar downstream effectors with EGFR. As described earlier, EGF, MSP and IGF all can activate SFK and Rap1 in FG cells.

5.2 Rap1-GTP as an *in vitro* measurement for metastasis *in vivo*

This dissertation characterized the effectors involved in EGFR-mediated cell invasion and metastasis of FG human pancreatic carcinoma cells. The role

of the GTPase Rap1 was examined in this process since Rap1 is activated downstream of EGF stimulation [163, 164] and has been associated with the induction of integrin activation [165]. Rap1 has also previously been associated with the invasive properties of a wide range of tumor cells including: melanoma [138], prostate cancer [139], thyroid cancer [140], colon cancer [141] and pancreatic cancer [106]. Indeed Rap1-GTP selectively promotes FG cell migration on vitronectin *in vitro* and metastasis *in vivo,* yet surprisingly did not impact primary tumor growth. Since growth factor stimulation of FG cells also resulted in the specific increase of migration on vitronectin as well as the activation of Rap1, Rap1-GTP may be an important regulator of tumor cell metastasis based on its capacity to induce motility on the αvβ5 substrate, vitronectin. Conversely, FGMs exhibited not only increased metastasis in a chick CAM assay [79], but also had elevated levels of Rap1-GTP compared to FGs. FGMs express higher amounts of EGFR and ErbB2 compared to FG cells, which may explain how erlotinib treatment of FGMs reduced their Rap1-GTP levels (data not shown). Thus, in addition to migration on vitronectin, Rap1-GTP levels may be another *in vitro* readout for metastasis *in vivo.*

5.3 Src and CAS downstream of EGFR activation

Because of a connection between Rap1 activation and EGFR-mediated migration on vitronectin, characterization of effectors leading to the activation of Rap1 may provide a better understanding of how EGFR leads to tumor invasion and metastasis. Previously, EGF stimulation of FG cells has been shown to

promote activation of SFK leading to the tyrosine phosphorylation of CAS, a focal contact localized scaffolding protein previously linked to cell migration and invasion [76, 79, 159]. It was not known, however, if there was a specific SFK member that played a more significant role than the others. Src, Yes and Fyn are structurally very similar and can bind to and phosphorylate many of the same proteins [166, 167] RNA interference experiments targeting Src, Yes and Fyn led to the discovery that Src and Yes but not Fyn are involved in EGFR-induced migration. Furthermore, Src is the predominant SFK member that promotes CAS phosphorylation. Although Yes also appears to phosphorylate CAS, knockdown of Yes failed to suppress an increase in CAS phosphorylation in response to EGF, which implies that while Yes may be necessary for EGFR-dependent migration on vitronectin, it likely signals through a substrate other than CAS.

CAS phosphorylation is known to recruit a wide range of adaptor molecules and kinases linked to cell invasive behavior where it appears to play a critical role in the migratory property of various cells [79, 83]. A close family member of CAS, HEF, also has been shown to promote migration and metastasis [158]. However, we observed that genetic knockdown of CAS, but not HEF inhibited EGFR-induced carcinoma cell migration, yet surprisingly did not disrupt migration on collagen. This would be in contrast to previous reports that claim CAS is significant in a general role for migration [76, 83]. 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 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 [83, 84], CAS Y253 and Y271 together are critical for EGFR-dependent migration, metastasis and Rap1 activation.

5.4 CAS substrate domain binding partners

CAS has a prominent role in migration due to its binding with the adapter 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 playing a key role in the cell migration response [90, 168, 169]. In fact, the CAS/Crk complex has been described as a “molecular switch” in a general role for cell migration [79]. However, this dissertation demonstrates that EGF stimulation of FG pancreatic carcinoma cells does not increase the association of CAS with either CrkL or CrkI, consistent with their generic role in migration on either collagen or vitronectin substrates. In contrast, while EGFR activation results in enhanced Nck1 and Nck2 association with CAS, only Nck1 is specifically required in EGFR-dependent migration. Knockdown of Nck1 was also sufficient to block EGFR-induced metastasis. Moreover, the Nck1 SH2 domain utilizes CAS Y253 and Y271 as docking sites upon EGF stimulation.

Studies have provided conflicting evidence in regards to functional distinctions between Nck1 and Nck2. Nck1 and Nck2 appear to be functionally redundant as mice deficient in either family member remain viable, while knockout of both isoforms leads to developmental abnormalities and death *in utero* [170]. Nck1 and Nck2 share 68% amino acid identity in their overall structure and 82% in their SH2 domains which suggests that their SH2 domains may have similar binding partners. The majority of their sequence differences occur within the linker regions between the SH2 and SH3 domains [171]. However, their SH2 domains bind to separate sites on the same protein, such as PDGFR-B [95]. Others have also shown that Nck1 and Nck2 are both required for full PDGF-B mediated migration and PDGF-B induced actin rearrangements [86, 95]. Interestingly, Nck adapters are also linked to invadopodia formation and ECM degradation through the binding of the scaffolding protein Tks5 in a Src dependent manner [172]. Nck1 specifically has also been found to be required for EGFR-induced invadopodia formation in MTLn3 mammary carcinoma cells [173]. This result, in addition to our finding that Nck1, but not Nck2, is required for EGFR-dependent migration illustrates a specific role for Nck1 in EGFR signaling.

Phosphorylation of the CAS substrate domain has been linked to Rap1 activation [174]. Specifically, mutation of the first 9 tyrosine residues disrupts EGFR-dependent Rap1 activation [31]. Studies have described a mechanism for Rap1 activation through CAS via the assembly of a CAS/Crk complex with

Crk binding to the Rap1 GEF C3G [175, 176]. Results in this dissertation, instead, indicate a novel requirement for Nck1 in EGFR-mediated Rap1 activation in a CAS dependent manner. A recent study also described a role for Nck2 in CAS-mediated activation of Cdc42 to regulate cell polarity, while CrkII was involved in Rac1 activation to promote cell protrusions [87]. This would suggest that different adapter proteins may be specifically required for the activation of particular small GTPases. Interestingly, a time course of EGF stimulation in FG cells revealed that Rap1 associated with CAS at similar time points that Nck1 would associate with CAS, further supporting a connection between Nck1 and Rap1 (data not shown).

5.5 Clinical implications

The novel findings from this dissertation implicate phosphorylation of CAS Y253 and Y271 and Rap1-GTP as potential correlative factors for aggressive and malignant tumors. Blockade of CAS phosphorylation or Rap1 activation prevents EGFR-dependent migration and metastasis. However, current reagents to detect either phosphorylation of CAS Y253/Y271 or Rap1-GTP are not suitable to stain human tissue samples to determine if there is a relationship between those biochemical measurements and metastatic disease. The antibodies for CAS phosphorylation are not specific towards a particular tyrosine site. While an antibody was designed to specifically react with the phosphorylation of CAS Y253, this antibody can cross-react with the phosphorylation of sites other than Y253 [177]. For Rap1-GTP detection, cells

need to be lysed and active Rap1 needs to be immunoprecipitated in a time sensitive manner. Current Rap1 antibodies only detect total Rap1 and do not distinguish between the inactive and active forms. One promising study revealed that cAMP leads to not only activation of Rap1, but also phosphorylation of Rap1b S179, which leads to allosteric effects on the switch domains and effector loop regions [178]. Although phosphorylation of S179 did not necessarily correlate with activation status of Rap1, there may be a post-translational modification of Rap1 that could be immunodetected and used as a means to detect Rap1-GTP in human tissues.

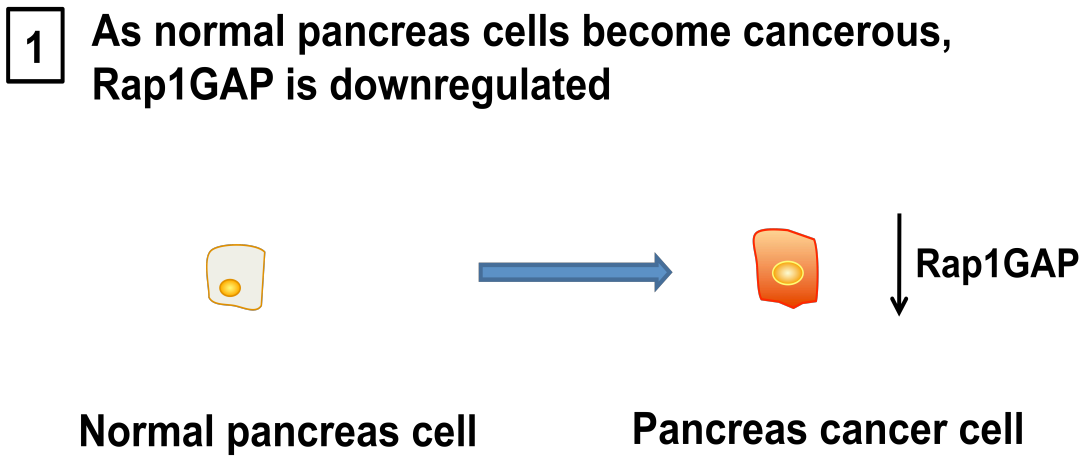
Since knockdown of Nck1 resulted in the suppression of EGFR-induced metastasis and Rap1 activation, competitive binding peptides could be designed against Nck1. Specifically, peptides competitively binding to the SH2 domain of Nck1 would prevent the formation of a CAS/Nck1 complex. Nck1 expression may also be a biomarker that's indicative of the aggressiveness of tumor cells. Future studies should evaluate Nck1 expression in tissues from human patients with or without malignant tumors to determine if there is a correlation.

While this pathway has only been demonstrated in FG human pancreatic carcinoma cells, this may be applicable to other cell types as well. Similar to FG cells, Mia-PaCa (pancreatic), MCF-7 (breast), 2008(ovarian) and HT-29 (colon) cancer cells also exhibited a dependence on Src activity for migration on vitronectin [31]. Since IGF and MSP also activate Src, the CAS/Nck1/Rap1

signaling axis may be therapeutic targets for cancers that depend on those signaling cascades or to overcome resistance against tyrosine kinase inhibitors.

5.6 Physiological relevance

A hallmark of pancreatic cancer is desmoplasia, which is characterized by increases in the expression of extracellular matrices. This process represents a dysregulation of the normal epithelial wound healing response [16]. Human pancreatic adenocarcinomas have been shown to produce ECM including vitronectin which was expressed at levels equivalent to other provisional matrices [179]. Vitronectin has also been found to be upregulated in the stroma surrounding pancreatic cancer [16] and is expressed in human plasma [180]. Importantly, the presence of vitronectin in lymph nodes has been described as a factor that allows these structures to be common sites of dissemination, such as the lymph nodes and liver [181-183]. Since multiple pancreatic tumor cells express the αv and β5 integrins [16], this might explain how pancreatic adenocarcinomas, particularly those that overexpress EGFR (e.g. FGM), would invade the surrounding stroma, intravasate into the lymphatics and metastasize to the liver and lymph nodes (Figure 5.1). In fact, preclinical studies reveal that a monoclonal antibody recognizing the αvβ5 integrin (14C5) is a promising reagent to diagnose as well as to treat pancreatic cancer [184].



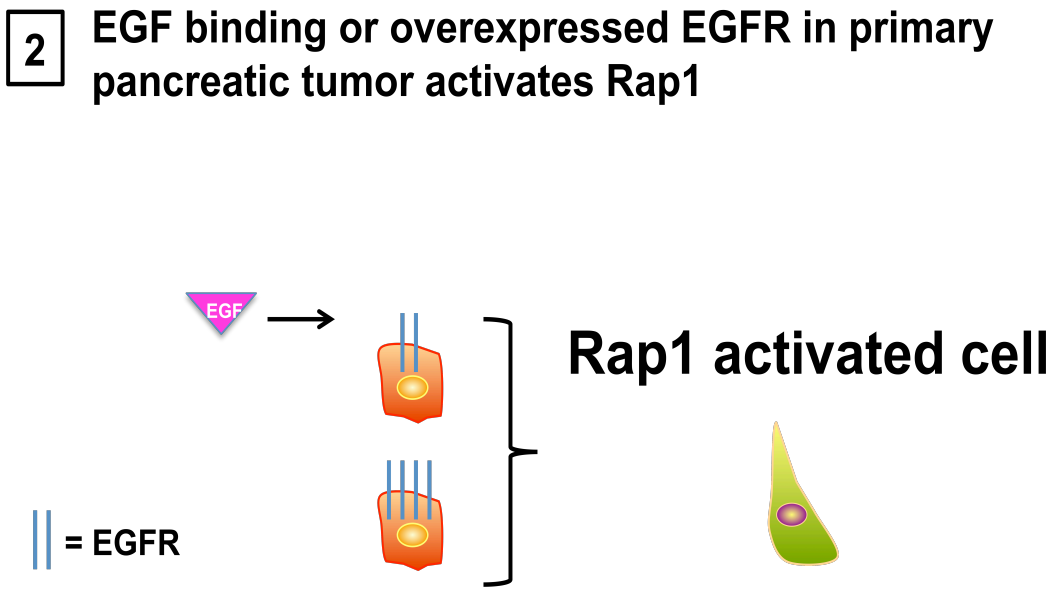


Figure 5.1 Activation of Rap1 in tumor cells promotes invasion through vitronectin and dissemination to sites of vitronectin

(1) Rap1GAP expression is lost in pancreatic tumor cells, which sets up the cells to have elevated levels of active Rap1. (2) EGF, located in the pancreatic juices, could bind to EGFR on pancreatic tumor cells or the tumor cells can overexpress EGFR to activate the signaling cascade in Figure 4.18 to activate Rap1. The loss of Rap1GAP in these cells allow prolonged Rap1 activation in these cells

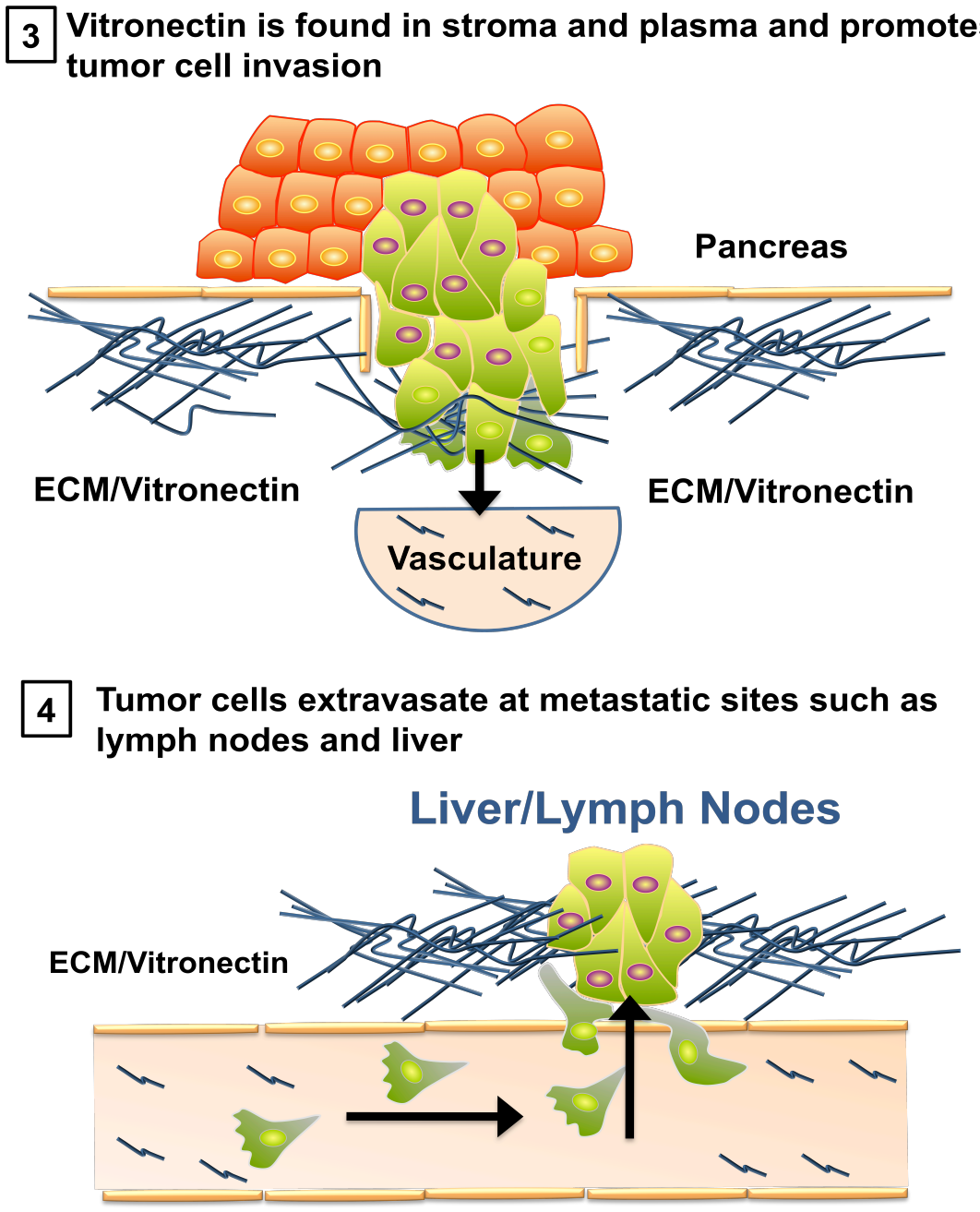


Figure 5.1 Activation of Rap1 in tumor cells promotes invasion through vitronectin and dissemination to sites of vitronectin (continued)

(3) Vitronectin is increasingly found in the stroma due to desmoplasia in pancreatic cancer and is also present in the plasma. The vitronectin attracts tumor cells with activated Rap1 to invade through the stroma and intravasate into the vasculature. (4) The presence of vitronectin at metastatic sites, such as the liver and lymph node, act as a homing signal for the tumor cell to disseminate to those regions.

In both thyroid and pancreatic cancer, Rap1GAP expression is lost during tumorigenesis [106, 140]. Interestingly, Rap1GAP is downregulated by activated Ras [140] in thyroid cancer and activation of KRAS is an early event in pancreatic carcinoma progression. Furthermore, oncogenic KRAS is found in >90% of pancreatic cancers [185] and may therefore explain why Rap1GAP expression is downregulated in this type of carcinoma. The combined loss of Rap1GAP, overexpression of EGFR and Src in pancreatic cancer would suggest that Rap1 activity levels are elevated in this type of cancer, which could explain the aggressive nature of this disease. The data in this dissertation would support this hypothesis.

5.7 Summary

Here, this dissertation has identified an EGFR/Src/CAS/Nck1/ RapGEF/Rap1 signaling axis that promotes migration on vitronectin and spontaneous metastasis of human pancreatic carcinoma cells. A chick chorioallantoic membrane model was utilized which enabled the quantification of both primary tumor growth and spontaneous metastasis following a brief treatment of cells to EGF *ex vivo*. While no differences in primary tumor growth were seen with EGF treatment, it's plausible that this signaling cascade downstream of EGFR activation could promote an increase in survival in order for the metastatic process to be completed.

The results in this dissertation 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, MSP, and IGF 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 epithelial derived cancers that are typically exposed to a wide range of cytokines.

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