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# New Concepts Regarding Focal Adhesion Kinase Promotion of Cell Migration and Proliferation

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**Abstract** Focal adhesion kinase (FAK) is a non-receptor cytoplasmic tyrosine kinase that plays a key role in the regulation of proliferation and migration of normal and tumor cells. FAK associates with integrin receptors and recruits other molecules to the site of this interaction thus forming a signaling complex that transmits signals from the extracellular matrix to the cell cytoskeleton. Crk-associated substrate (CAS) family members appear to play a pivotal role in FAK regulation of cell migration. Cellular Src bound to FAK phosphorylates CAS proteins leading to the recruitment of a Crk family adaptor molecule and activation of a small GTPase and c-Jun N-terminal kinase (JNK) promoting membrane protrusion and cell migration. The relocalization of CAS and signaling through specific CAS family members appears to determine the outcome of this pathway. FAK also plays an important role in regulating cell cycle progression through transcriptional control of the cyclin D1 promoter by the Ets B and Kruppel-like factor 8 (KLF8) transcription factors. FAK regulation of cell cycle progression in tumor cells requires Erk activity, cyclin D1 transcription, and the cyclin-dependent kinase (cdk) inhibitor $p27^{Kip1}$. The ability of FAK to integrate integrin and growth factor signals resulting in synergistic promotion of cell migration and proliferation, and its potential regulation by nuclear factor kappa B (NFκB) and p53 and a ubiquitously expressed inhibitory protein, suggest that it is remarkable in its capacity to integrate multiple extracellular and intracellular stimuli. J. Cell. Biochem. 99: 36–52, 2006. 

**Key words:** CAS; FAK; focal adhesion kinase; HEF1; migration; proliferation

Focal adhesion kinase (FAK) is a non-receptor cytoplasmic tyrosine kinase that plays a key role in several different cell processes largely through its function as a scaffolding molecule [reviewed in Craven et al., 2003; Hanks et al., 2003; Parsons, 2003; Schlaepfer and Mitra, 2004]. This review focus is limited to its role in promotion of cell proliferation and migration. The reader is referred to other recent reviews that cover the recent progress in the interrelated issues of its roles in cell survival and differentiation, as well as its key role during development. In its role as a scaffolding molecule, FAK recruits other signaling molecules to

Abbreviations used: CAS, Crk-associated substrate; Cdk5, cyclin-dependent kinase 5; EGF, epidermal growth factor; EGFr, EGF receptor; FAK, focal adhesion kinase; FAT, focal adhesion targeting; FERM, domain with homology to the ezrin, radixin, and moesin family of proteins; FIP200, FAK-interacting protein of 200 kDa; GSK3, glycogen synthase kinase 3; HEF1, human enhancer of filamentation 1; JNK, c-Jun N-terminal kinase; JSAP1, JNK stress-activated protein kinase-associated protein 1; MEF, mouse embryo fibroblasts; PDGF, platelet-derived growth factor; PDGFr, PDGF receptor; PI-3 kinase, phosphatidylinositol-3 hydroxyl kinase; PLC-γ, phospholipase C-γ; PR, proline rich; SBD, Src binding domain; scid, severe combined immunodeficiency; si, small interfering; Sin/Efs, Src-interacting protein/embryonal Fyn substrate; Skp2, S phase kinase-associated protein 2; TNFα, tumor necrosis factor α; VEGF, vascular endothelial cell growth factor.

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the sub-membranous region of the cell where integrin receptors (also known as cell adhesion receptors) cluster. The signaling complex formed then transmits signals from the extracellular matrix to the cell cytoskeleton through the integrin receptor.

## FAK DOMAIN STRUCTURE

Focal adhesion kinase contains an amino-terminal domain that mediates its interactions with membrane proteins, a centrally located kinase domain, three proline-rich (PR) regions (two in the carboxyl-terminal half of the molecule and one in the amino-terminal half), and the carboxyl-terminal focal adhesion targeting (FAT) domain (see Fig. 1A) [Schaller et al., 1992; Hanks et al., 2003; reviewed in Parsons, 2003; Schlaepfer and Mitra, 2004]. The amino-terminal domain plays a role in the interactions of FAK with integrin receptors, as well as with the platelet-derived growth factor receptor (PDGFr) and the epidermal growth factor receptor (EGFr) [Sieg et al., 2000; Dunty et al., 2004]. This domain has homology with the band 4.1 protein as well as with the ezrin, radixin, and moesin family of proteins and thus is known as the FERM domain. The association of the FERM domain of FAK with integrins is likely mediated through another protein whereas the interaction with the EGFr appears to be direct [Sieg et al., 2000]. It is thought that the FERM domain of FAK likely interacts directly with the PDGFr. The FERM domain also is thought to modulate the kinase activity of FAK; in cell conditions in which FAK is not activated (such as non-tumor cells held in suspension) the FERM domain interacts directly with the kinase domain potentially inhibiting its function [Cooper et al., 2003]. The carboxyl-terminal FAT domain contains binding sites for paxillin and talin which aid in the recruitment of FAK to adhesion complexes [reviewed in Parsons, 2003]. Adhesion complexes form when cells adhere as a monolayer to a matrix protein substrate. The immature adhesion complexes are known as focal contacts and the mature complexes are known as focal adhesions. Paxillin and talin act together with FAK to link integrin receptors to the cytoskeleton [reviewed in Hanks et al., 2003; Parsons, 2003; Schlaepfer and Mitra, 2004]. The FAT domain of FAK also interacts with p190RhoGEF, a RhoA-specific GDP/GTP exchange factor, and this promotes the phosphorylation of p190RhoGEF and is associated with enhanced RhoA activity [Zhai et al., 2003]. A larger region of the carboxyl-terminus, which includes PR1 and PR2 and the FAT domain, is known as the FAK-related non-kinase domain (FRNK). This domain can be expressed independently of the entire FAK protein through alternative splicing of the *FAK* gene in certain mammalian organs, such as the lung and testes [reviewed in Parsons, 2003]. When expressed in cells propagated in vitro, FRNK acts as a negative regulator of FAK. The two PR regions located in the carboxyl-terminal half of the molecule (known as PR1 and PR2) interact with SH3-domaining containing molecules, such as the Crk-associated substrate (CAS) family of docking molecules [reviewed in Polte and Hanks, 1995; O'Neill et al., 2000; Hanks et al., 2003].

## FAK ACTIVATION

The activation of FAK requires autophosphorylation of residue Y397. Such autophosphorylation has been shown to be temporally related to the clustering of integrin receptors in the cell membrane that occurs when these receptors bind their ligand [reviewed in Hynes, 2002; Hanks et al., 2003]. The integrin receptor clustering triggers a conformational change in the associated FAK that alters the interaction of the FERM domain with the kinase domain [Cooper et al., 2003]. FAK also undergoes autophosphorylation with the activation of certain growth factor receptors in adherent cells [reviewed in Craven et al., 2003; Hanks et al., 2003; Parsons, 2003; Schlaepfer and Mitra, 2004]. Phosphorylated (p) Y397 is a high-affinity binding site for Src, and the autophosphorylation of FAK results in the recruitment and binding of cellular Src to pY397 [Schaller et al., 1994]. Cellular Src can then phosphorylate Y576/Y577 in the kinase domain of FAK, in the second step of the autoactivation loop that is essential for maximal FAK kinase activity and activation [reviewed in Calalb et al., 1995; Hanks et al., 2003]. Recent analysis of the crystal structure of the FERM domain suggests that Src binding to the linker region of FAK (adjacent to the FERM domain) through its SH3 and SH2 domains could possibly regulate the protein interactions of the FERM domain [Ceccarelli et al., 2005].

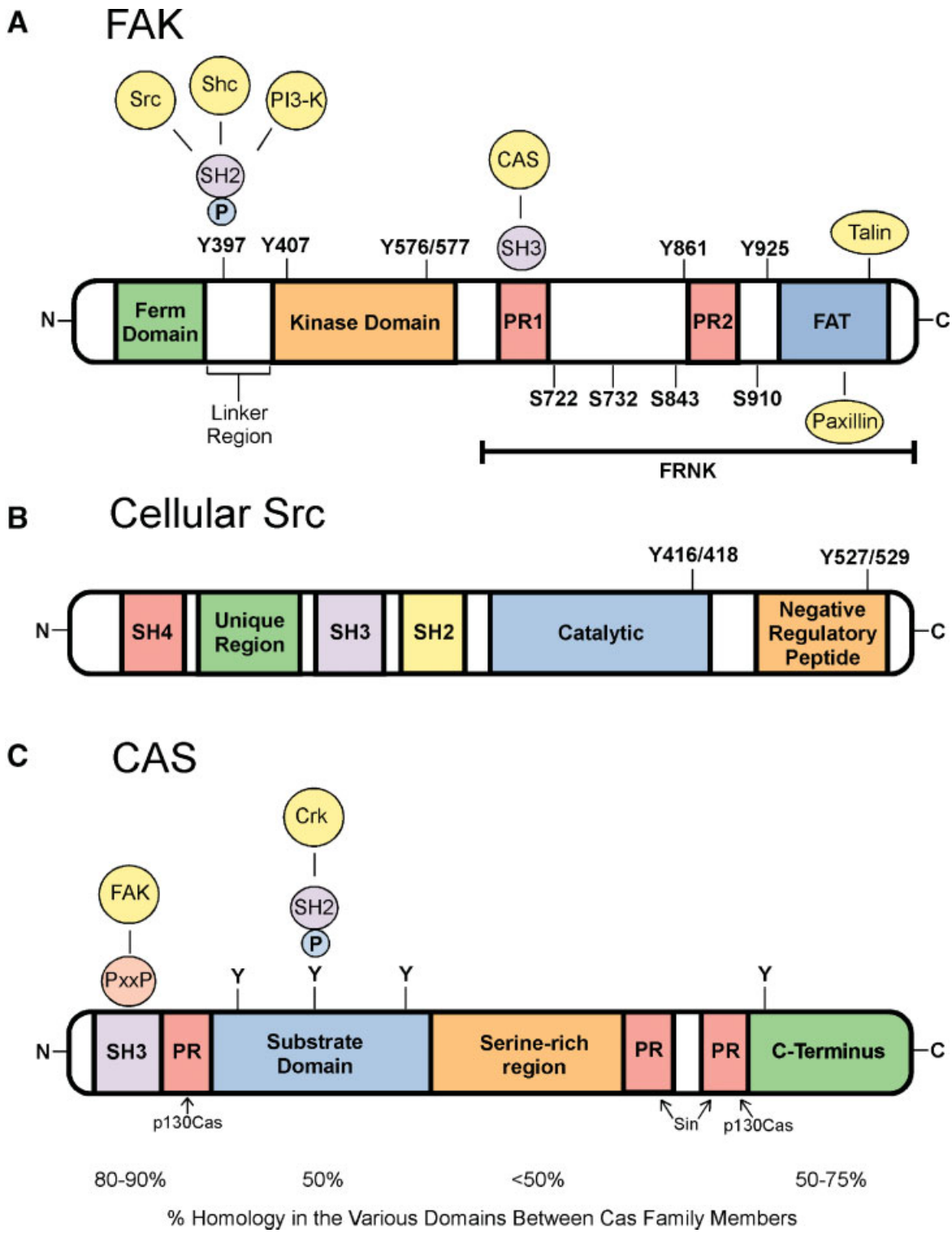



**Fig. 1.** Domain organization of focal adhesion kinase (FAK), cellular Src, and Crk-associated substrate (CAS). **A**: The domain organization of FAK. The FERM domain shares homology to the band 4.1 protein, and the ezrin, radixin, moesin family of proteins. The kinase domain indicates the region of catalytic activity. PR1 and PR2, denotes proline-rich regions 1 and 2 in the carboxyl-terminus. FAT denotes the focal adhesion targeting domain. FRNK denotes the FAK-related non-kinase domain. Sites of tyrosine and serine phosphorylation are indicated. The amino-terminal PR domain is not shown. **B**: The domain organization of cellular Src. The catalytic domain contains the autophosphorylation site Y416/418 in c-Src. The carboxyl-terminal negative regulatory peptide contains Y527/529 which is negatively regulated in mouse/human c-Src. **C**: The domain organization of CAS family members.

## FAK SUBSTRATES

Although FAK function appears to be mediated largely through its action as a scaffolding molecule, FAK also directly phosphorylates several proteins, such as the N-Wiskott Aldrich syndrome protein (N-WASP) [Wu et al., 2004], as well as talin, paxillin, and CAS family proteins [reviewed in O'Neill et al., 2000; Parsons, 2003]. N-WASP is a member of the WASP family of proteins that modulate actin cytoskeletal remodeling. Cellular Src also phosphorylates paxillin, and most likely, cellular Src is the predominant kinase that phosphorylates the tyrosine residues of paxillin [reviewed in Parsons, 2003].

## SH2 DOMAIN BINDING SITES IN FAK

In addition to phosphorylating Y576/577 of FAK, cellular Src can phosphorylate several other tyrosine residues that, when phosphorylated, serve as docking sites for SH2 domain-containing molecules [reviewed in Hanks et al., 2003; Parsons, 2003; Schlaepfer and Mitra, 2004]. These include Y407, Y861, and Y925. To date, however, the autophosphorylation site (Y397) appears to be the main binding site for the molecules that associate with FAK through an SH2 domain, including Src, Shc, phosphatidylinositol-3 hydroxyl kinase (PI-3 kinase), phospholipase C (PLC)-$\gamma$, and Grb7. Although the binding partners of pY407, pY861, and pY925 are not entirely clear, the phosphorylation of these residues may be important for specific cell events. For example, it has been reported that phosphorylation of Y861 is necessary for cell migration in Ras-transformed NIH3T3 cells [Lim et al., 2004] and for the TGF-$\beta$1-induced epithelial-mesenchymal transition of NMuMG murine mammary epithelial cells [Nakamura et al., 2001]. In addition, it has been reported that phosphorylation of Y925 is associated with integrin adhesion and E-cadherin deregulation during Src-induced epithelial-mesenchymal transition in colon cancer cells [Brunton et al., 2005], and with Erk activation in human 293 kidney epithelial cells [Schlaepfer and Hunter, 1997].

## SITES OF SERINE PHOSPHORYLATION

Focal adhesion kinase contains four sites of potential serine phosphorylation, S722, S732, S843, and S910. S722 is phosphorylated by glycogen synthase kinase 3 (GSK3) during cell spreading thereby decreasing the kinase activity of FAK; conversely, inhibition of GSK3 decreases phosphorylation of S722 and increases FAK activity in spreading cells [Bianchi et al., 2005]. In adherent and spreading cells, S722 is targeted for dephosphorylation by serine/threonine protein phosphatase type 1 [Bianchi et al., 2005]. Consistent with these findings, in migrating cells phosphorylation of S722 is decreased and GSK3$\beta$ is inactive. These data suggest a mechanism by which FAK activity is regulated during cell spreading and migration, that is, the phosphorylation of S722, and hence the activity of FAK is dictated by the competing actions of GSK3$\beta$ and serine/threonine phosphatase type 1. Phosphorylation of S732 by cyclin-dependent kinase 5 (Cdk5) is important for microtubule organization, nuclear movement, and neuronal migration in cultured neocortical neurons [Xie et al., 2003]. It has been shown that FAK phosphorylated on S732 (pS732) is enriched along a centrosome-associated microtubule fork that abuts the nucleus in these cells [Xie et al., 2003]. Expression of a mutant FAK (S732A) results in disorganization of the microtubule fork and impairment of nuclear movement in vitro, and neuronal position defects in vivo [Xie et al., 2003]. These observations suggest that Cdk5 phosphorylation of FAK is critical for neuronal migration and acts through regulation of a microtubule fork important for nuclear translocation. Phosphorylation of S843 and S910 has been reported to occur during cell mitosis and is further discussed in Part IV below [Ma et al., 2001].

## CELLULAR Src, A FUNCTIONAL PARTNER OF FAK

Like FAK, cellular Src is a non-receptor cytoplasmic tyrosine kinase that plays a key role in regulating multiple cellular functions, including cell migration and proliferation. Cellular Src associates with multiple cell surface receptors, including some integrins, and with the organized actin cytoskeleton [reviewed in Thomas and Brugge, 1997], as well as with FAK. There are nine known Src family members: c-Src, Fyn, Lyn, c-Yes, Hck, Fgr, Blk, Lck, and Yrk [reviewed in Thomas and Brugge, 1997]. All of the Src family members share a similar domain structure which consists of six distinct functional domains (see Fig. 1B). As

with FAK, it has been shown that the activation of cellular Src occurs upon ligand binding and clustering of the integrin, and with the activation of certain growth factor receptors in adherent cells [Thomas and Brugge, 1997]. FAK and cellular Src act to activate each other. Thus, the association of the SH2 domain of cellular Src with pY397 of FAK and cellular Src phosphorylation of Y576/577 in the autoactivation loop of FAK described above also increases cellular Src activity by opening the closed autoinhibited conformation of cellular Src resulting in increased autophosphorylation [reviewed in Hanks et al., 2003]. These reciprocal roles of FAK and cellular Src in mutual activation are supported by studies of MEFs derived from the FAK-null mouse in which reduced cellular Src activity is found [reviewed in Hanks et al., 2003]. Maximal activation of cellular Src promotes its interaction with, and phosphorylation, of two major targets, CAS family members and paxillin, both of which bind FAK [reviewed in Hanks et al., 2003]. Cellular Src also may play a direct role in phosphorylating Y397 (the autophosphorylation site) of FAK, as cells lacking Src family kinases exhibit reduced phosphorylation of Y397 of FAK, and v-Src has been shown to phosphorylate Y397 of FAK [reviewed in Hanks et al., 2003]. Thus, cellular Src may function both upstream and downstream of FAK.

## SPECIFIC FUNCTIONS OF Src FAMILY MEMBERS

A growing body of evidence indicates that individual Src family members have distinct or specific functions. The specificity of the function of the Src family members is likely regulated by multiple factors, including the cell type, the receptor engaged, the cooperation of receptors, and the sub-cellular localization of the Src family member, as well as other as yet unidentified factors [Thomas and Brugge, 1997; Osterhout et al., 1999; Ding et al., 2003; Colognato et al., 2004]. For example, Fyn promotes the differentiation of neuroglial progenitor cells into oligodendrocytes [Osterhout et al., 1999], whereas Lyn promotes the proliferation of the neuroglial progenitor cells [Colognato et al., 2004]. Also, we have shown that Lyn, but not Fyn, is necessary for the migration promoted by the cooperation of integrin $\alpha v\beta 3$ and the PDGFr$\beta$ in human glioblastoma cells [Ding et al., 2003]. Notably, we found a specific requirement for Lyn in this interaction despite the fact that Fyn mRNA and protein are expressed at higher levels than Lyn in these cells [Ding et al., 2003].

## THE REGULATION of FAK

The regulation of FAK can occur through several different mechanisms including amplification of the gene, alternative splicing, and dephosphorylation by phosphatases [reviewed in Craven et al., 2003; Hanks et al., 2003; Parsons, 2003; Schlaepfer and Mitra, 2004]. Most recently, a novel inhibitor known as FAK inhibitory protein of 200 kDa (FIP200) and transcriptional regulation of the promoter also have been shown to regulate the activity of FAK. FIP200, a cellular protein that was identified by yeast two-hybrid screening [Abbi et al., 2002], binds to the kinase domains of FAK and Pyk2 (a FAK family member) and inhibits their functions, although the inhibitory mechanism is not yet entirely clear. As it is expressed in many tissues and cell lines, it may play an important role in inhibiting some cellular processes involving FAK. Studies of the function of the various domains of FIP200 have shown that the amino-terminal domain associates with the kinase domain of FAK and inhibits its kinase activity, as well as inhibiting FAK promotion of cell migration and proliferation in fibroblasts propagated in vitro [Abbi et al., 2002]. The middle domain of FIP200 also associates with the kinase domain of FAK but inhibits FAK activity to a lesser extent than the amino-terminal domain, whereas the carboxyl-terminal domain of FIP200 binds to the amino-terminal (FERM) domain of FAK but does not affect FAK activity [Abbi et al., 2002]. Recently, FIP200 overexpression in MCF-7 breast cancer cells was shown to inhibit cell cycle progression, proliferation, and clonogenic survival [Melkoumian et al., 2005]. The overexpression of FIP200 decreased the level of cyclin D1 protein, likely through inhibition of FAK function [Melkoumian et al., 2005]. These data suggest the possibility that FIP200 may function as a tumor suppressor molecule in normal cells. The second recently identified regulatory mechanism was revealed on cloning of the 5′ promoter region of the *FAK* gene when a 600 bp region was shown to be necessary for maximal FAK promoter activity [Golubovskaya et al., 2004]. Sites for the binding of several transcriptional factors, including nuclear factor

kappa B (NFκB) and p53, have been identified [Golubovskaya et al., 2004]. The NFκB transcription factor likely plays a role in regulating FAK transcription, as inhibition of NFκB function was shown to decrease FAK luciferase activity, whereas stimulation with tumor necrosis factor α (TNFα) increased FAK luciferase activity.

## NEW FINDINGS REGARDING FAK AND CELL MIGRATION (CAS FAMILY MEMBER SPECIFIC SIGNALING)

Focal adhesion kinase is known to regulate cell migration through its promotion of membrane protrusion and focal adhesion turnover and, potentially, other mechanisms [reviewed in Craven et al., 2003; Hanks et al., 2003; Parsons, 2003; Schlaepfer and Mitra, 2004]. Membrane protrusion and focal adhesion turnover are necessary for cell migration and invasion. The focal adhesions are highly dynamic and heterogenous structures that occur at sites of close membrane contact with the extracellular matrix substrate and anchor a highly bundled and cross-linked actin stress fiber network. Key studies indicating a role for FAK in promotion of cell migration include those demonstrating that FAK-null MEFs migrate poorly in response to chemotactic and haptotactic signals and exhibit an increased number of focal adhesions in adherent cells [Ilic et al., 1995], with re-expression of wild-type FAK restoring the migratory responses [reviewed in Hanks et al., 2003]. Similarly, expression of FAT, FRNK, or mutant FAK(397F) constructs, which typically act as dominant interfering forms of FAK, in various cell types usually results in partial inhibition of FAK activation and partial inhibition of cell migration [Hauck et al., 2001; reviewed in Hanks et al., 2003; Schlaepfer and Mitra, 2004; Ding et al., 2005]. Conversely, the overexpression of wild-type FAK in various different cell types enhances cell migration [Wang et al., 2000; reviewed in Schlaepfer and Mitra, 2004; Natarajan et al., 2005].

### CAS Protein Structure and Family Members

Focal adhesion kinase promotes cell migration through more than one signaling pathway [reviewed in Hanks et al., 2003]. One signaling pathway utilizes the CAS family of proteins as downstream effectors of FAK. The CAS family of proteins include p130CAS, identified initially as a highly tyrosine phosphorylated protein in v-Src and v-Crk transformed cells [Sakai et al., 1994; reviewed in Parsons, 2003], human enhancer of filamentation 1 (HEF1) identified in a yeast two-hybrid screen as a protein homologous to p130CAS that promotes a morphologic change [Law et al., 1996; Minegishi et al., 1996], and Src-interacting protein/embryonal Fyn substrate (Sin/Efs) that was first identified as a Fyn-interacting protein [Ishino et al., 1995; Alexandropoulos and Baltimore, 1996]. p130CAS, HEF1, and Sin/Efs share a relatively conserved architecture, with an amino-terminal SH3 domain, followed by a substrate domain, a serine-rich region, and a carboxyl-terminal region that promotes dimerization of HEF1 [reviewed in O'Neill et al., 2000; Hanks et al., 2003] (see Fig. 1C). The substrate domain contains multiple tyrosine residues that are a consensus binding site for the SH2 domain of the docking molecule Crk. In the carboxyl-terminal half of the molecule is a peptide containing a tyrosine residue that, when phosphorylated by FAK, is recognized by the SH2 domain of cellular Src and is known as the Src binding domain (SBD) [Tachibana et al., 1997]. In addition to the domains described above, p130CAS and Sin/Efs contain two PR regions that are not found in HEF1. Although there is substantial domain homology among the three CAS family members, their expression, utilization, and function differ [Manie et al., 1997; Almeida et al., 2000; Cho and Klemke, 2000; Law et al., 2000; Natarajan et al., 2005].

### The FAK/CAS Signaling Pathway

Crk-associated substrate proteins bind through their SH3 domain to the PR1 and/or PR2 region(s) of FAK or Pyk2 [reviewed in Polte and Hanks, 1995; O'Neill et al., 2000; Hanks et al., 2003]. Cellular Src optimally phosphorylates CAS proteins in the substrate domain when bound to FAK, leading to the recruitment of a Crk family adaptor molecule, activation of a small GTPase, such as Rac1 or Cdc42 and c-Jun N-terminal kinase (JNK), and the subsequent promotion of membrane protrusion and cell migration [Cary et al., 1998; Dolfi et al., 1998; Kiyaokawa et al., 1998; Klemke et al., 1998; Cho and Klemke, 2000; Fashena et al., 2002; Natarajan et al., 2005]. Consistent with a pivotal role for CAS family proteins in cell

motility, fibroblasts isolated from p130CAS null mice exhibit defects in cell migration [Honda et al., 1998]. Knockout mouse models of HEF1 and Sin/Efs have not yet been reported. Recently it was shown that a scaffold protein for JNK known as JNK stress-activated protein kinase-associated protein 1 (JSAP1) cooperates with FAK to regulate JNK activation and cell migration [Takino et al., 2005]. JSAP1 complexes with FAK and p130CAS in the U-87MG glioblastoma cells upon fibronectin stimulation, resulting in increased FAK activity and the phosphorylation of JSAP1 and p130 CAS [Takino et al., 2005].

The interactions of FAK, cellular Src, p130CAS, and paxillin promote focal contact/focal adhesion turnover at the leading edge of the cell in fibroblasts [Webb et al., 2004]. The promotion of focal contact/focal adhesion turnover by FAK in these cells is due, at least in part, to the ability of FAK to promote the localization of calpain to focal contacts/focal adhesions [Carragher et al., 2003]. Calpain cleaves talin and FAK [reviewed in Schlaepfer and Mitra, 2004]. Focal adhesion disassembly appears to be triggered by localized elevation of the levels of calcium [Giannone et al., 2004], and by the localization of microtubules to focal adhesions [Ezratty et al., 2005]. Both FAK and the GTPase dynamin are necessary for focal adhesion disassembly induced by microtubules [Ezratty et al., 2005].

### CAS Phosphorylation and Localization

Crk-associated substrate proteins are modified by tyrosine kinases in response to integrin ligation, cell adhesion, and oncogenic transformation [Sakai et al., 1994; Petch et al., 1995; Polte and Hanks, 1995; Harte et al., 1996; Minegishi et al., 1996; Vuori et al., 1996; Manie et al., 1997; Fashena et al., 2002]. The phosphorylation status of CAS proteins contributes to their sub-cellular localization. Thus, p130CAS and HEF1 localize to focal adhesions [Sakai et al., 1994; Polte and Hanks, 1995; Fashena et al., 2002], with the SH3 domain of p130CAS being necessary for its localization in focal adhesions [Nakamoto et al., 1997]. Non-phosphorylated p130CAS is localized mainly in the cytosol, but when phosphorylated p130CAS is localized predominantly in an insoluble fraction associated with focal adhesions, membranous regions, and the nucleus [Sakai et al., 1994; reviewed in Hanks et al., 2003].

### CAS Family Tissue Distribution

Although the CAS family of proteins exhibits high levels of homology, the tissue distribution of the individual family members differs. p130CAS is expressed ubiquitously [Sakai et al., 1994], whereas HEF1 is expressed most abundantly in epithelial and lymphoid cells and in the developing hindbrain [Law et al., 1996; Minegishi et al., 1996; Merrill et al., 2004], and Sin/Efs is expressed in embryonic tissues, the thymus, adult muscle, and brain [Ishino et al., 1995]. The ubiquitous nature of p130CAS suggests a fundamental role for this molecule in normal cell function, whereas the apparently restricted tissue distribution of HEF1 and Sin/Efs may reflect their specific functions.

### CAS Family Member Specific Signaling Downstream of FAK

We have shown recently that HEF1 acts as a necessary and specific downstream effector of FAK in the migration and invasion of glioblastoma cells [Natarajan et al., 2005]. In studies aimed at determining whether a CAS family member is a downstream effector of FAK in promoting glioblastoma cell migration, we utilized conditions in which cells overexpressing FAK were stimulated with PDGF. These conditions simulate the in vivo situation in glioblastoma tumor biopsies, as FAK expression is elevated and a PDGF autocrine loop has been identified [Hermanson et al., 1992; Wang et al., 2000; Zagzag et al., 2000; Hecker et al., 2002]. We found that both FAK overexpression and PDGF stimulation promoted the phosphorylation of HEF1 on tyrosine residues but not of p130CAS or Sin/Efs, in glioblastoma cells adherent to rec-osteopontin or vitronectin [Natarajan et al., 2005]. Similarly, FAK overexpression and PDGF stimulation promoted the migration of glioblastoma cells adherent to rec-osteopontin or vitronectin, and the invasion of glioblastoma cells through detergent-free normal brain homogenates. To determine the requirement for HEF1, we downregulated HEF1 or p130CAS using small interfering (si) RNA. We found that the downregulation of HEF1, but not of p130CAS, inhibited the basal, FAK-promoted, and PDGF-stimulated migration of the glioblastoma cells as well as their invasive potential [Natarajan et al., 2005] (see Fig. 2). Using a second glioblastoma cell line, we confirmed that HEF1 was necessary for basal

and PDGF-stimulated migration [Natarajan et al., 2005] and further demonstrated that FAK acts to promote migration through the activation of the Rac1 effector molecule. Consistent with the proposed role of HEF1 in glioblastoma cell migration, we found that in glioblastoma cells adherent to rec-osteopontin or vitronectin, HEF1 was localized predominately in the 0.1% Triton X-100 insoluble fraction. This is consistent with a localization at focal adhesions. In the two glioblastoma cell lines used in these studies (U-251MG and U-87MG) the relative levels of p130CAS, HEF1, and Sin/Efs proteins were not significantly different; thus, assuming that the polyclonal antibodies used were of relatively similar affinity, the differences in the relative levels of expression cannot account for the specific phosphorylation of HEF1 under these cell conditions. Taken together, these data suggest that signaling through CAS family members, in this case HEF1, promotes migration and invasion of tumor cells. FAK overexpression was shown previously to promote the phosphorylation of p130CAS and cell migration in fibroblasts, CHO cells, and COS cells [Cary et al., 1998; Klemke et al., 1998] and the phosphorylation of HEF1 and cell migration in T cells [van Seventer et al., 2001].

Signaling through specific CAS family members had previously been reported in B cells. In this case, stimulation of the β1 integrin resulted in HEF1 and p130CAS phosphorylation, whereas stimulation with anti-IgM or anti-IgG $F(ab')_2$ antibody resulted in HEF1 (not p130CAS) phosphorylation [Manie et al., 1997]. Similarly, the response to apoptotic stimuli appears to be affected by the CAS family member activated. The overexpression of p130CAS in fibroblasts protects cells from apoptosis induced by serum starvation, whereas the overexpression of HEF1 in MCF7 breast epithelial cells increases their sensitivity to TNFα, which is a pro-apoptotic stimulus [Almeida et al., 2000; Cho and Klemke, 2000; Law et al., 2000].

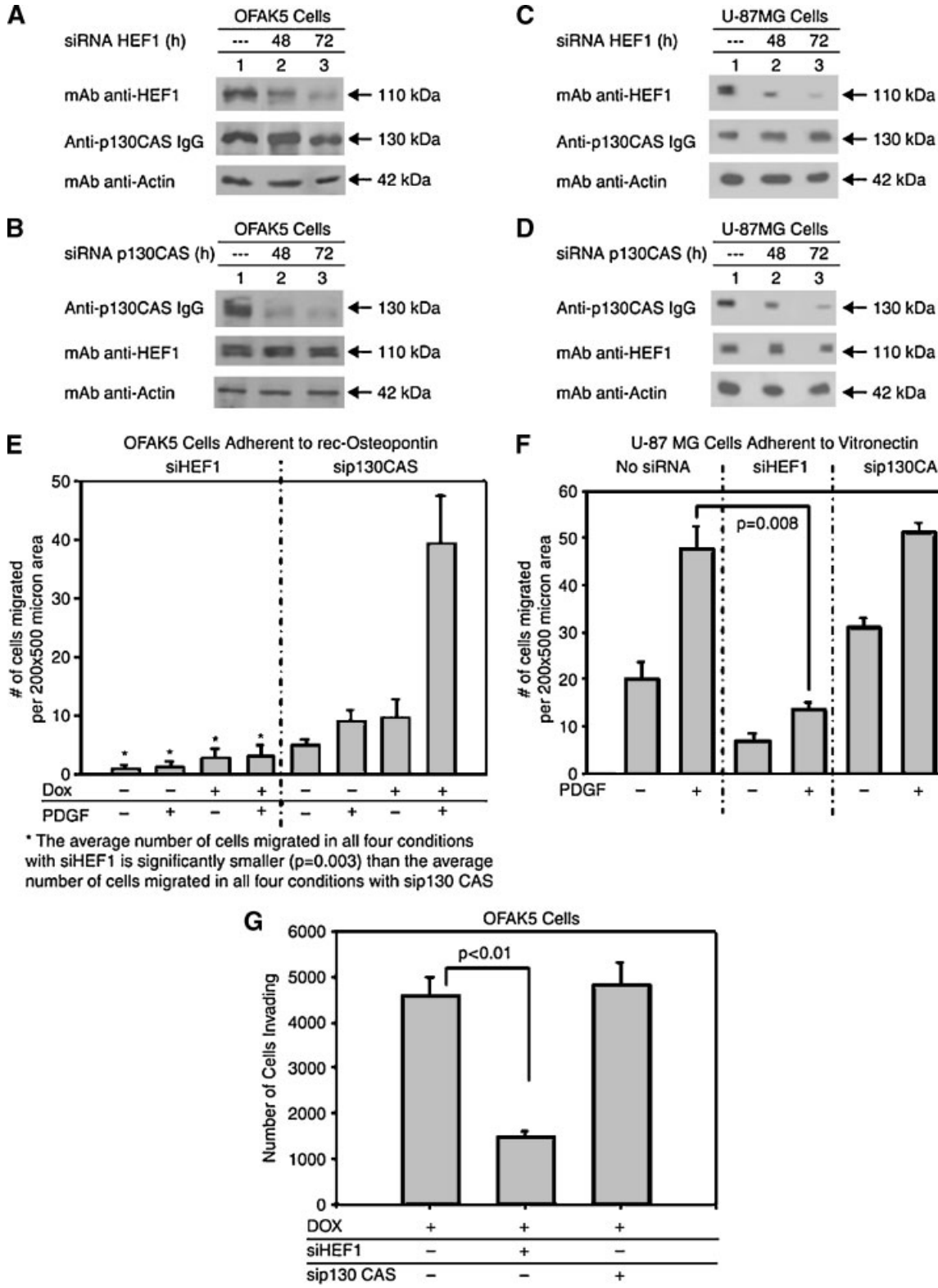
## NEW FINDINGS REGARDING FAK AND CELL PROLIFERATION (A ROLE FOR $p27^{Kip1}$)

### FAK Promotes Cell Proliferation by Promoting Cyclin D1 Transcription

Accumulating evidence suggests that FAK plays an important role in regulating cell cycle progression by integrating signals from integrins and growth factor receptors. Previous studies have shown that inhibition of FAK function by microinjection of the dominant-interfering FAK construct—FRNK results in decreased cell proliferation, as measured by BrdU labeling, in non-tumor cells [reviewed in Craven et al., 2003; Parsons, 2003]. Building on these studies, Zhao et al. [1998] using NIH3T3 fibroblasts demonstrated that the overexpression of wild-type FAK increased cell proliferation by accelerating cell cycle progression through the $G_1$ to S phase, whereas the expression of a FAK mutant construct lacking the 14 carboxyl-terminal amino acids (ΔC14) and acting as a dominant-interfering construct inhibited cell cycle progression. Cell cycle progression at the $G_1$ to S phase is regulated by cdk inhibitors, cyclins, and the Ink4 family of tumor suppressor genes [reviewed in Slingerland and Pagano, 2000; Ivanchuk et al., 2001; Pietenpol and Stewart, 2002]. In studies dissecting the mechanism whereby FAK regulates cell cycle progression in NIH3T3 fibroblasts, Zhao et al. [1998, 2001] demonstrated that expression of the mutant FAK (ΔC14) construct resulted in a decrease in cyclin D1 expression, whereas overexpression of wild-type FAK increased cyclin D1 expression. Also, cyclin D1 expression was necessary for wild-type FAK promotion of cell cycle progression, and FAK increased transcription of the *cyclin D1* gene by enhancing the binding activity of an Ets transcription factor to the Ets B element in the cyclin D1 promoter [Zhao et al., 2001]. Furthermore, Erk activity was necessary for FAK promotion of cell proliferation. These results are consistent with previous reports that active Erk phosphorylates Ets transcription factors [Wasylyk et al., 1998] and induces transcription of cyclin D1 [Lavoie et al., 1996].

### The Transcription Factor—Kruppel-Like Factor 8 (KLF8) Is Also a Downstream Target of FAK

Zhao et al. [2003] went on to show that FAK regulates expression of KLF8 in NIH3T3 cells; FAK overexpression increased KLF8 expression whereas expression of the mutant FAK (ΔC14) inhibited KLF8 expression. KLF8 directly activates cyclin D1 transcription by binding to the GT box in the cyclin D1 promoter. FAK promotion of cyclin D1 expression in the NIH3T3 cells required KLF8, as the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mAb anti-HEF1
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siRNA HEF1 (h)
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Anti-p130CAS IgG
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mAb anti-HEF1
mAb anti-Actin
130 kDa
110 kDa
42 kDa
E
OFAK5 Cells Adherent to rec-Osteopontin
siHEF1
sip130CAS
# of cells migrated per 200x500 micron area
Dox
PDGF
* The average number of cells migrated in all four conditions with siHEF1 is significantly smaller (p=0.003) than the average number of cells migrated in all four conditions with sip130 CAS
F
U-87 MG Cells Adherent to Vitronectin
No siRNA
siHEF1
sip130CAS
p=0.008
# of cells migrated per 200x500 micron area
PDGF
G
OFAK5 Cells
p<0.01
Number of Cells Invading
DOX
siHEF1
sip130 CAS

Fig. 2.

downregulation of KLF8 with siRNA inhibited FAK-induced cyclin D1 expression and cell cycle progression [Zhao et al., 2003]. These studies suggest that FAK regulates cell cycle progression in normal or non-tumor cells through the transcriptional control of the cyclin D1 promoter by enhancing the binding activity or expression of the Ets B and KLF8 transcription factors, respectively.

### Other Molecules Associated With FAK Promotion of Cell Proliferation in Non-Tumor Cells

Recent studies have shown that the overexpression of FAK in smooth muscle cells is associated with increased expression of S phase kinase-associated protein 2 (Skp2) and increased proliferation [Bond et al., 2004]. Skp2 has been reported previously to be necessary for the ubiquitination and degradation of $p27^{Kip1}$ [Carrano et al., 1999; Sutterluty et al., 1999]. Consistent with these findings, inhibition of Skp2 function by microinjection with an anti-Skp2 antibody resulted in cell cycle arrest at the $G_1$ phase [Zhang et al., 1995]. These results suggest that Skp2 also may be involved in FAK regulation of cell cycle progression in non-tumor cells.

### FAK Regulates the Cell Cycle Through Cyclin D1 and $p27^{Kip1}$ in Tumor Cells Propagated In Vitro

Cell cycle progression through the $G_1$ phase requires upregulation of cyclins and downregulation of cdk inhibitors, allowing cells to pass through the $G_1$ restriction point, enter S phase, and proliferate [Slingerland and Pagano, 2000; Pietenpol and Stewart, 2002]. Cyclin–cdk complexes are regulated by two families of proteins, the INK4 family of tumor suppressor genes (p16, p15, p18, and p19) and the Cip/Kip family of cdk inhibitors ($p21^{Waf1}$, $p27^{Kip1}$, and $p57^{Kip2}$) [Slingerland and Pagano, 2000; Ivanchuk et al., 2001]. In non-tumor cells, specifically NIH3T3 fibroblasts, the ability of the mutant FAK (ΔC14) construct to inhibit cell cycle progression did not require the cdk inhibitor $p21^{Waf1}$, and the level of $p27^{Kip1}$ protein was not altered with overexpression of wild-type FAK or expression of the mutant FAK; thus the necessity of $p27^{Kip1}$ was not tested [Zhao et al., 1998]. However, the activity and regulation of signal transduction pathways controlling cell cycle progression and cell proliferation are frequently aberrant in tumors and this is thought to contribute to their malignancy [Slingerland and Pagano, 2000; Nho and Sheaff, 2003]. We have shown previously that overexpression of wild-type FAK promotes soft agar growth in the U-251MG glioblastoma cells [Wang et al., 2000], whereas expression of mutant FAK(397F) inhibits soft agar growth of these cells [Hecker et al., 2004]. In vivo, we have demonstrated that glioblastoma cells overexpressing wild-type FAK propagated in the brains of C.B.17 severe combined immunodeficiency (scid) mice exhibited higher cell proliferation (~twofold) than wild-type U-251MG glioblastoma cells or control clones propagated similarly [Wang et al., 2000]. Expression of the mutant FAK(397F) in glioblastoma cells inhibited proliferation of these cells on propagation in the brains of C.B.17 scid mice [Ding et al., 2005]. These studies suggest that FAK promotes cell proliferation in malignant astrocytomas/glioblastoma tumors. These animal studies simulate the elevated expression of FAK that has been demonstrated in tumor cells of human tumor biopsies from various types of malignant tumors [reviewed in Craven et al., 2003], including human malignant astrocytoma/glioblastoma [Wang et al., 2000; Zagzag et al., 2000].

**Fig. 2.** Downregulation of human enhancer of filamentation 1 (HEF1), but not p130CAS, inhibits basal, FAK-promoted and platelet-derived growth factor (PDGF)-stimulated migration, as well as invasion of glioblastoma cells. **A–D**: OFAK5 cells and U-87MG cells were transfected with 200 nM siHEF1 or sip130CAS, the transfection repeated at 24 h, and at 48 or 72 h, the cells lysed in RIPA lysis buffer and the lysates (40 μg) from each condition subjected to SDS–PAGE, and immunoblotted with the indicated antibodies. **E**: OFAK5 cells were transfected with 200 nM siHEF1 or sip130CAS, the transfection repeated at 24 h, and at 48 h, treatment with doxycycline or vehicle was begun and continued throughout the experiment. At 72 h, the cells were replated onto rec-osteopontin-coated wells in DMEM-0.4% FBS-1% BSA and subject to the scratch assay. **F**: U-87MG cells were similarly transfected with siHEF1 or sip130CAS, the cells harvested with buffered EDTA, resuspended in DMEM-0.8% FBS-1% BSA and plated onto vitronectin-coated wells. At 24 h the monolayer was subject to the scratch assay and photographed at 48 h. **G**: OFAK5 cells were treated, harvested with buffered EDTA, resuspended in DMEM-0.4% FBS-1% BSA, and plated onto 8 mm pore filters coated with 50 μg/ml normal brain homogenate and allowed to invade for 24 h (37°C, 5% $CO_2$). Cells on the bottom filter surface were fixed, stained, and counted. The data were analyzed and presented as the mean ± SEM for each condition. This figure is reprinted with permission from Oncogene 2006; 25: 1721–1732.

In experiments to determine whether FAK promotes cell cycle progression in these tumor cells, we found that overexpression of FAK decreased the expression of $p27^{Kip1}$ and $p21^{Waf1}$ and increased the expression of cyclins D1 and E, whereas the expression of mutant FAK(397F) enhanced the expression of $p27^{Kip1}$ and $p21^{Waf1}$ and reduced the expression of cyclins D1 and E [Ding et al., 2005]. Furthermore, the expression of $p27^{Kip1}$ was necessary for the inhibition of cell cycle progression that occurs on expression of the mutant FAK(397F), as downregulation of $p27^{Kip1}$ with siRNA blocked the inhibitory effect of the mutant FAK(397F) [Ding et al., 2005] (see Fig. 3). In contrast, although increased expression of $p21^{Waf1}$ protein was seen on expression of the mutant FAK(397F), downregulation of $p21^{Waf1}$ with siRNA did not block the inhibition of cell cycle progression associated with the expression of mutant FAK(397F) [Ding et al., 2005] (see Fig. 3). These data indicate that $p27^{Kip1}$ is required for the inhibition of cell cycle progression by mutant FAK(397F). Similar to results reported for NIH3T3 cells, we also found that cyclin D1, not cyclin E, was necessary for the promotion of cell cycle progression observed on overexpression of wild-type FAK in the glioblastoma cells, and that the overexpression of wild-type FAK enhanced KLF8 expression whereas expression of the mutant FAK(397F) had the opposite effect [Ding et al., 2005].

Importantly, we had shown previously that the overexpression of FAK promoted Ras and Erk activity in the U-251MG glioblastoma cells [Hecker et al., 2002, 2004]. In support of these studies and those of Zhao et al. [1998], we also found that Erk activity was necessary for FAK-promoted glioblastoma cell proliferation [Ding et al., 2005]. Our studies suggest that FAK regulation of cell cycle progression in glioblastoma tumor cells, and perhaps tumor cells in general, requires Erk activity, cyclin D1 transcription, and $p27^{Kip1}$.

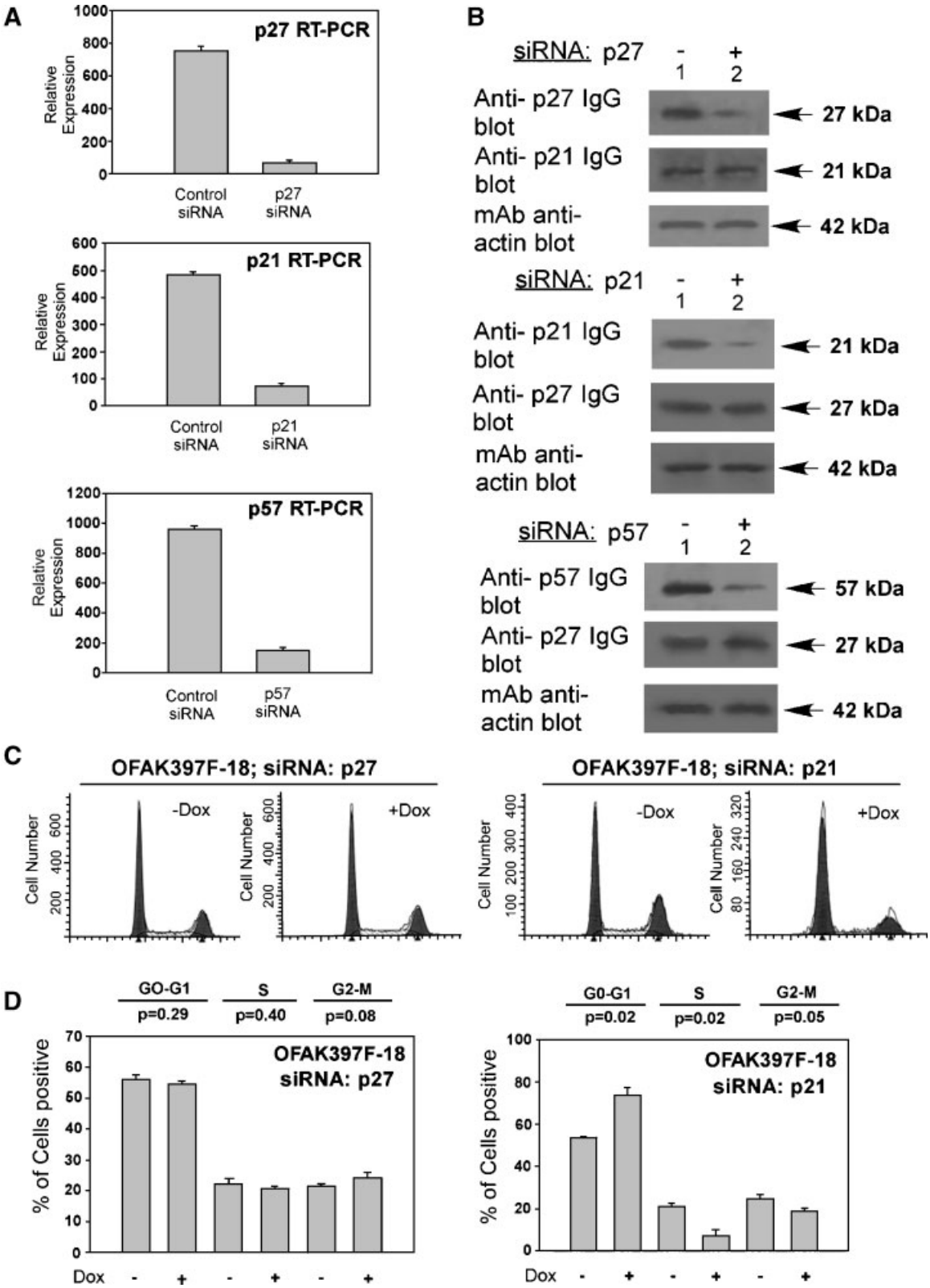
## FAK Promotes Cell Cycle Progression In Vivo: Role for $p27^{Kip1}$ and Cyclin D1

As all prior work regarding FAK regulation of the cell cycle had been performed on cells propagated in vitro, we examined whether FAK also regulated the cell cycle in vivo using an intracerebral xenograft model of human glioblastoma cells propagated in the C.B.17 scid mouse brain. We found that, as our in vitro findings had suggested, the overexpression of wild-type FAK in the glioblastoma cells propagated in vivo promoted cell cycle progression, whereas expression of the mutant FAK(397F) inhibited cell cycle progression [Ding et al., 2005]. The mechanism by which FAK regulates the cell cycle in vivo appeared similar to that observed in vitro, as FAK overexpression inhibited $p27^{Kip1}$ and $p21^{Waf1}$ expression and increased cyclin D1 and E expression, whereas expression of the mutant FAK(397F) increased $p27^{Kip1}$ and $p21^{Waf1}$ expression and reduced cyclin D1 and E expression [Ding et al., 2005]. Also, FAK overexpression in vivo promoted Erk activity and increased KLF8 expression, whereas expression of the mutant FAK(397F) inhibited Erk activity and reduced KLF8 expression. These data taken together with our in vitro findings suggest that FAK regulation of the cell cycle is a physiologic event, and that in tumor cells it requires not only Erk activity and cyclin D1, but also $p27^{Kip1}$.

## During Mitosis FAK Is Phosphorylated on Serine Residues

Investigators have shown that FAK is heavily phosphorylated on serine residues during mitosis in rat and chicken embryo fibroblasts [Yamakita et al., 1999; Ma et al., 2001]. This increase in serine phosphorylation is found on residues 843 and 910 and correlates with a decrease in FAK activation and overall tyrosine phosphorylation [Yamakita et al., 1999; Ma et al., 2001]. In addition, FAK re-distributes to

**Fig. 3.** Downregulation of $p27^{Kip1}$ blocks the inhibitory effect of mutant FAK397F on the cell cycle progression of glioblastoma cells. Parallel cultures of the OFAK397F-18 clone propagated in complete medium were administered 2 μg/ml doxycycline (*Dox*) or vehicle for 4 days and then transfected with duplex siRNA directed toward $p27^{Kip1}$, $p21^{Waf1}$, or $p57^{Kip2}$. **A**: Post-transfection (48 h) the cells were harvested and RNA extracted for real time RT-PCR using forward and reverse primers directed specifically toward $p27^{Kip1}$, $p21^{Waf1}$, or $p57^{Kip2}$. The relative expression of these mRNAs was normalized to actin. **B**: Cells were detergent lysed and the lysate subjected to immunoblotting with the indicated antibodies. **C** and **D**: The cells were harvested, propidium iodide labeled, and the DNA content analyzed by FACS analysis. In (D) the percentage of cells in $G_0/G_1$, S, and $G_2/M$ phases is plotted as a histogram, and the statistical significance of differences in each phase when cells were Dox treated versus vehicle treated is indicated by the *P*-value; $P < 0.05$ was considered significant. This figure is reprinted with permission from the Journal of Biological Chemistry 2005; 280:6802–6815.


A
p27 RT-PCR
Relative Expression
1000
800
600
400
200
0
Control siRNA
p27 siRNA
p21 RT-PCR
600
500
400
300
200
100
0
Control siRNA
p21 siRNA
p57 RT-PCR
1200
1000
800
600
400
200
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Control siRNA
p57 siRNA
B
siRNA: p27
- 1
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Anti- p27 IgG blot
27 kDa
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21 kDa
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42 kDa
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- 1
+ 2
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21 kDa
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27 kDa
mAb anti-actin blot
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siRNA: p57
- 1
+ 2
Anti- p57 IgG blot
57 kDa
Anti- p27 IgG blot
27 kDa
mAb anti-actin blot
42 kDa
C
OFAK397F-18; siRNA: p27
-Dox
+Dox
Cell Number
OFAK397F-18; siRNA: p21
-Dox
+Dox
Cell Number
D
GO-G1
S
G2-M
p=0.29
p=0.40
p=0.08
OFAK397F-18
siRNA: p27
% of Cells positive
70
60
50
40
30
20
10
0
Dox - + - + - +
G0-G1
S
G2-M
p=0.02
p=0.02
p=0.05
OFAK397F-18
siRNA: p21
% of Cells positive
100
80
60
40
20
0
Dox - + - + - +

Fig. 3.

the mitotic spindle, the microtubule-organizing center or centrosomes during mitosis of T lymphocytes [Rodriguez-Fernandez et al., 1999]. These data taken together with the above-discussed role of FAK in promoting exit from the $G_1$ phase of the cell cycle, suggests that FAK is active early on in cell cycle progression and that its localization and function are altered with progression through the $G_2$/M phase of the cell cycle.

### Serine Phosphorylation of FAK May Help to Determine Whether FAK Promotes Cell Migration or Proliferation

Phosphorylation of the S722 residue inhibits the rec-SH3 domain of p130CAS from binding to the PR1 region in FAK [Yamakita et al., 1999; Ma et al., 2001]. Consistent with this observation, serine phosphorylation of FAK correlates with FAK dissociation from p130CAS [Yamakita et al., 1999; Ma et al., 2001].

### HEF1 Regulates the Activation of Kinases Necessary for Centrosome Function

Human enhancer of filamentation 1 redistributes from focal adhesions to mitotic spindle asters in MCF7 breast epithelial cells in the $G_2$/M phase [reviewed in O'Neill et al., 2000]. Most recently, Pugacheva and Golemis [2005] reported that HEF1 was necessary for the activation of two kinases, AuroraA and Nek2, at the centrosome. This suggests that the cell adhesion protein HEF1 can help to regulate both cell migration and cell division.

## FUTURE DIRECTIONS REGARDING FAK-PROMOTED MIGRATION THROUGH CAS- AND FAK-PROMOTED PROLIFERATION

### What Determines CAS Family Member Specificity in the Signaling Downstream of FAK That Promotes Cell Migration?

There is some evidence to suggest that the functions of the CAS family members are cell type specific; for example, the aforementioned differences in the distribution of the individual family members in various different tissues and cell types [reviewed in O'Neill et al., 2000]. Further tissue and cell distribution studies regarding the CAS family members need to be performed; however, the preferential utilization of CAS family members cannot be explained completely in terms of cell type as we observed preferential utilization in cells in which all three CAS family members were expressed at relatively similar levels. There is evidence to suggest that CAS family member signaling could be dependent on the microenvironment, including the specific cytokine or growth factor stimuli. Law et al. [2000] reported that the overexpression of HEF1 in MCF7 breast epithelial cells increased their sensitivity to the pro-apoptotic effect of TNF$\alpha$ stimulation. This concept needs further exploration, as we found that EGF stimulation of the glioblastoma cells adherent to a rec-osteopontin substrate did not promote increased HEF1 phosphorylation, nor did it promote the phosphorylation of p130CAS or Sin [Natarajan et al., 2005]. Consistent with these findings no increase in migration was observed on EGF stimulation of these cells. Rather, we found that PDGF stimulation promoted HEF1-specific phosphorylation in the cells adherent to rec-osteopontin or vitronectin and that this correlated with increased cell migration [Natarajan et al., 2005]. Thus, further defining how changes in the microenvironment affect specific CAS family member activation will likely lead to a better understanding of CAS family member specific functions. Also, importantly, in vivo studies in animal models need to be performed examining the effect of conditionally downregulating a specific CAS family member, followed by studies determining the effect on cell migration or invasion.

### How Does FAK Regulate $p27^{Kip1}$?

The mechanism by which FAK regulates $p27^{Kip1}$ has not yet been elucidated and it will be intriguing to determine whether FAK regulation of $p27^{Kip1}$ occurs through FAK regulation of Skp2. Notably, $p27^{Kip1}$ is aberrantly regulated in many cancer cells. This aberrant regulation is associated with an alteration in its protein binding partners, causing it to be sequestered in the cytoplasm, and increased ubiquitination, which leads to enhanced degradation [Slingerland and Pagano, 2000; Nho and Sheaff, 2003]. Thus, the effect of reduced levels of $p27^{Kip1}$ on FAK-mediated proliferation in cancer cells is also of interest. Similarly, as a p53 binding site has been identified in the FAK

promoter [Golubovskaya et al., 2004] and an absence of p53 function is observed in many tumors [Pietenpol and Stewart, 2002], the effects of p53 on FAK regulation of the cell cycle is of interest.

In conclusion, FAK appears to play a key role in cell migration and proliferation and to coordinate the activities of several molecules that are considered to be associated closely with pathophysiologic proliferative conditions, including cancer, inflammation, and atherosclerosis. As is apparent from this prospect/review, the effects of FAK on cell migration and the pathways associated with these activities have been analyzed in several normal cell types and multiple tumors, whereas the effects of FAK on cell proliferation have been analyzed in only a few normal cell types and a few tumors. The results of the studies to date suggest that analysis of other cell types, tumors that originate from these cells, and pathophysiologic conditions in animal models could be very informative. Importantly, as FAK serves to integrate the effects of signals generated by several different components of the extracellular environment (such as matrix proteins and growth factors/cytokines), such studies should yield data that are of relevance to the behavior of cells in vivo and could lead to the further development and clinical application of FAK-specific inhibitors in various diseases.

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