CYTOSKELETAL SIGNALING PROTEINS; COMPLEX REGULATORY MECHANISMS AND ROLES IN INNATE IMMUNITY

Xinming Cai

A dissertation submitted to the faculty of the University of North Carolina at Chapel Hill in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department or Cell and Developmental Biology, School of Medicine.

Chapel Hill
2007

Approved by:

Professor Michael D. Schaller

Professor Keith Burridge

Professor Ken Jacobson

Professor Lee M Graves

Professor Glenn Matsushima

ABSTRACT

Xinming Cai

Cytoskeletal Signaling Proteins; Complex Regulatory Mechanisms and Roles in Innate Immunity

(Under the direction of Dr. Michael D. Schaller)

In animals, most of the tissues are surrendered by extracellular matrix. The extracellular matrix plays multiple roles in maintaining the function of organs. It not only provides physical support to assemble the cells into an intact tissue but also sends signals to surrounding cells to regulate several cellular behaviors, such as proliferation and anti-apoptosis. The major contact between extracellular matrix and cells is mediated by focal adhesions, a transmembrane protein complex working as a hub to convert the matrix/cell interaction into a chemical signaling. Just like a canonical signaling pathway, focal adhesion signaling cascade is composed of receptors, enzymes and scaffolding proteins. The interplay of these elements precisely transmits the extracellular signal into cells to induce downstream effects. The work described here represents a collection of studies to decipher the detail mechanism of adhesion signaling. The activation of FAK is an essential event in adhesion signal transduction. In chapter II, FRET technology was applied to visualize the spatial and temporal activation of FAK in living cells. PIP2 was identified as a FAK binding partner. That also promotes the activation of FAK via conformational change. In addition to the FAK regulation mechanism, a novel paxillin modification was identified in chapter III**.** Phosphorylation of paxillin on serine 126 and 130, which is mediated by an ERK/GSK-3 dual kinase mechanism, regulates the cytoskeleton reorganization.

Adhesion signaling is critical for multiple biological events, such as angiogenesis, neurite extension, and inflammation. Innate immunity works as the first barrier to fight against microbe infection. It not only directly destroys pathogens but also activates the antigen specific adaptive immune system. The roles of adhesion signaling proteins in innate immunity were also investigated in chapter II and III. The paxillin is phosphorylated by ERK and GSK-3 following LPS stimulation in macrophages, which is required for LPS induced macrophage spreading. In Fcγ receptor meditated phagocytosis, Pyk2 is activated and accumulated to phagocytotic cup during phagocytosis. The deletion or inactivation of Pyk2 impairs phagocytosis in macrophage. The work presented here expands our understanding of how focal adhesion proteins regulate multiple singling pathways.

To my parents, Dingyi Cai and Xia Pang, who have always encouraged me to do my best, and provided me support. And to Wenjie Ye, my wife, who has provided endless love and care to me.

ACKNOWLEDGMENTS

"Life was like a box of chocolates. You never know what you're gonna get." When I migrated across the Pacific Ocean to a American southern college town four years ago, a box of unopened chocolates was waiting for me. Now I have already eaten all of them. The taste is quite good! However, I know I can not enjoy this box of chocolates without everyone who gave me the tremendous supports during my graduate studies.

I would like to extend my appreciation to my advisor, Professor Mike Schaller for his guidance and support on my research. I really learn a lot from him, not only just some technical skills but the way to think and solve the scientific questions. Moreover, I also learn how to face the challenge, frustration and deal with life work balance from Mike. He shows me a way to understand the essence of life in the past four years.

I would also like to thank the current and past members of the Schaller lab for their help for my research. They always turn lab into a place where is filled with fun and happiness. I would like to particularly thank Danielle Smith and Dr. Kavita Vadali for their kind help for thesis proofreading.

I would like to express my appreciation to my committee members. They give me invaluable scientific advice and career guidance. They make me feel that UNC is just like a big and warm family. You can always get what you want from every kind member.

I am grateful for all my colleagues at UNC or somewhere else, especially to Julie Williams and Dr. Andrei Karginov, for their self-giving support and intellectual guidance. I

also want to extend my thanks to Ms. Janice Warfford and Ms. Gina Werner. Their excellent student service makes me never need to worry about anything about class registration and graduation application.

Finally, I would like to thank all my family members and friends for their encouragement and friendship throughout my graduate studies.

TABLE OF CONTENTS

LIST OF FIGURES

Figure

LIST OF ABBREVIATIONS

A	Alanine
APC	Adenomatous polyposis coli
ArfGAP	Arf GTPase-activating protein
ASAP1	ARF- GAP containing SH3, ANK repeats, and PH domain1
ATP	Adenosine 5'-triphosphate
BAEC	Bovine aortic endothelial cell
BODIPY	DIPYrromethene BOron Difluoride
CaMK	Calcium/calmodulin-dependent protein kinase
CDK	Cyclin-dependent kinase
CFP	Cyan fluorescence protein
CRMP-2	Collapsin response mediator protein-2
CSF	Colony stimulating factor
Cys	Cysteine
D	Aspartic acid
DMEM	Dulbecco's modified eagle's medium
DOCK180	180-kDa protein downstream of CRK
DTT	Dithiothreitol
EGF	Epidermal growth factor
eNOS	Endothelial Nitric Oxide Synthase
ERK	Extracellular signal regulated kinases
F	Phenylalanine
FACS	Fluorescence-activated cell sorting

FAK	Focal adhesion kinase
FAT	Focal adhesion targeting
FBS	Fetal bovine serum
FERM	4.1 protein/ ezrin/radixin/moesin
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
FIP200	Focal adhesion kinase family interacting protein of 200 kDa
FlAsH	Fluorescein arsenical hairpin binder
FRET	Fluorescence resonance energy transfer
FRNK	FAK related non kinase
GAP	GTPase Activating Protein
GFP	Green fluorescent protein
Gly	Glycine
GPCR	G protein-coupled receptor
Grb2	Growth factor receptor-bound protein 2
GSK-3	Glycogen synthase kinase 3
GST	Glutathione S-transferase
HEK293	human embryonic kidney cell 293
HeLa	Henrietta Lacks
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HGF	Hepatocyte growth factor
HSF-1	Heat shock factor-1

HUVEC	human umbilical vein endothelial cells
ICAM-1	Intercellular adhesion molecules 1
IL	Interleukin
ILK	Inhibition of integrin-linked kinase
IP3	Inositol 1,4,5-trisphosphate
IRAK	Interleukin-1 receptor-associated kinase
IRF3	Interferon regulatory factor 3
JNK	Jun N-terminal kinase
K	Lysine
KD	Dissociation constant
kDa	KiloDalton
LAD	Leukocyte adhesion deficiency
LBP	Lipopolysaccharide binding Protein
LD	Leucine/aspartate
Lef1	Lymphoid enhancer-binding factor 1
LIM	Lin11/IsI1/mec3
LIMK	LIM domain kinase
LPA	Lysophosphatidic acid
LPS	Lipopolysaccharide
LRR	leucine-rich-repeat
M	Methionine
MAP1B	Microtubule-associated protein 1b

MAPK	Mitogen-activated protein kinases
MEF2	Myocyte enhancer factor-2
MEK	MAP kinase/ERK kinase
Met	Mesenchymal epithelial transition factor
MHC	Major histocompatibility complex
MIP-1	Macrophage inflammatory protein 1
MKK4	Mitogen activated protein kinase kinase 4
MLCK	Myosin light chain kinase
MyD88	Myeloid differentiation primary response protein (88)
NF-κB	Nuclear factor-kappa B
NGF	Nerve growth factor
N-WASP	Neural Wiskott-Aldrich syndrome protein
p130CAS	p130 Crk-associated substrate
PAK	p21-activated kinase
PAMP	Pathogen associated molecular patterns
PBS	Phosphate buffered saline
PC	Phosphatidylcholine
PCR	Polymerase chain reaction
PDGF	Platelet derived growth factor
PE	Phosphatidylethanolamine
PH domain	Pleckstrin homology domain
PI	Phosphatidylinositol

PI3K	Phosphoinositide-3 kinase
PIP2	Phosphatidylinositol(4,5)-bisphosphate
PIP3	Phosphatidylinositol (3,4,5)-trisphosphate
PIP5K	Phosphatidylinositol-4-phosphate 5-kinase
PKC	Protein Kinase C
PKL	Paxillin kinase linker
PLCδ	Phospholipase C δ
PMA	p-methoxyamphetamine
Pro	Proline
PRR	Proline rich region
PTEN	Phosphatase and Tensin homolog
PTP1B	Protein tyrosine phosphatase 1B
PTPα	protein tyrosine phosphatase α
Pyk2	Proline-rich tyrosine kinase 2
RIPA	Radio-immunoprecipitation assay
ROCK	Rho-dependent kinase
ROS	Reactive oxygen species
RPMI 1640	Roswell Park Memorial Institute-1640
S	Serine
SDF-1	Stromal cell-derived factor-1
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis

SFK	Src Family Kinase
SH2	Src homology 2 domain
SH3	Src homology 3 domain
SHP-2	Src homology 2-containing tyrosine phosphatase
SNP	Single nucleotide polymorphism
TCR	T cell receptor
TIR	Toll-interleukin 1 receptor
TIRF	total internal reflection fluorescence
TLR	Toll like receptor
TNF-α	Tumor necrosis factor-alpha
TOLLIP	Toll-interacting protein
TRAF6	TNF receptor-associated factor 6
TRIAP	TIR-containing adapter protein
TRIF	TIR domain containing adapter inducing interferon-β
VE-cadherin	Vascular Endothelial Cadherin
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
Wnt	Wg (wingless) and Int
Y	Tyrosine
YFP	Yellow fluorescent protein
ZAP-70	Zeta chain associated protein kinase 70

CHAPTER 1
Introduction

1.1 The Biological Significance of FAK

FAK was first identified in 1992 by three different groups as a 125kD tyrosine kinase in Src transformed cell and breast cancer cell (165,181,389). Since then, a number of studies suggest that FAK is an important regulator that coordinates multiple cell signaling pathways. The *fak*-/- mouse is embryonic lethal and exhibits defects in mesoderm development (215). Recently, conditional knock out technology has shed light on the tissue specific function of FAK. Endothelial cell specific deletion of FAK leads to embryonic death with strong defects in angiogenesis (31,417). Myocyte FAK conditional knockout mice exhibit abnormal responses to pressure induced hypertrophy (113,354). In neurons, FAK conditional knockout mice exhibit failures in axon branching, sorting and netrin induced axon outgrowth and turning (283,308,372,375). The mammary epithelial-specific conditional knockout mice show defects in lobulo-alveolar and mammary gland development (333). Hence, FAK is extensively involved in regulating organogenesis and maintaining regular physiological function.

1.2 The Structure of FAK

FAK contains three major domains, an N-terminal FERM domain, a central kinase domain and a C-terminal FAT domain (Fig 1.1). The FERM domain is connected to the kinase domain with a linker. There are a number of PRRs (Proline rich region) within the hinge areas between domains. Homologues of FAK have been identified in *X. laevis*, *C. elegans*, and *D. melanogaster* (100,143,189,348,506). These homologues are highly conserved in domain arrangement, important motifs and regulatory sites. This high evolutionary conservation implicates the significance of FAK in biological function. Neuron specific FAK isoforms with insertions close to the auto-phosphorylation site were identified

(42). These FAK splice variants exhibit higher kinase activity. However, the detailed function of these isoforms is still unknown.

The FAT domain contains the targeting motif, which is critical for the focal adhesion localization of FAK (190). However, the mechanism of focal adhesion targeting is very complex. It targets to the focal adhesion via its association with a number of focal adhesion proteins, e.g. paxillin and talin (76,434). Focal adhesion targeting is a critical step for FAK activation. Interestingly, FRNK, an endogenous FAK FAT domain variant, works as a natural dominant negative to inhibit FAK activation (374).

The kinase domain of FAK is quite conserved when compared to other tyrosine kinase catalytic domains. There are two tyrosine residues (Y576/Y577) within the kinase domain that are key regulatory sites for FAK activation (50).

The FERM domain in FAK is an atypical FERM domain when compared with other canonical FERM domains (66). For example, the FERM domain lacks the lipid binding groove between the F1 and F3 subdomain when compared to other FERM domain crystal structures. The importance of the FAK FERM domain was revealed in recent studies. First, the FERM domain serves as a scaffold to coordinate a number of important events. The domain interacts with P53 to promote a cell survival signal (158). In the leading edge of migrating cells, the FERM domain associates with Arp2/3 to regulate actin nucleation and cell protrusion (410). Second, a number of studies suggest that FERM domain works as a "mask" to interact with the kinase domain, which blocks its kinase activity (92,97,296,446).

In addition to the major conserved domains, a number of other motifs within FAK also play important roles. Three proline rich regions are found at the junctions of the major FAK domains. A number of proteins containing SH3 domains interact with FAK via these

motifs. PRR1 is between the FERM and the kinase domain and provides a secondary binding site to promote Src/FAK interaction (439). A CDK5 phosphorylation site (S732) has been identified close to PRR2, which regulates neuronal migration by regulating microtubule organization and nuclear movement (490). PRR2 associates with p130CAS, which is a substrate for Src (365). PRR3 associates with ASAP1 and Graf, both of which are GAPs (GTPase Activating Protein) (191,311). A linker connects the FERM domain and kinase domain. An auto-phosphorylation residue (Y397) within the loop provides a docking site for a number of SH2 domain containing proteins, e.g. Src and PI3K (75,393). The tyrosine phosphorylation of Y397 is essential for FAK activation. Recently tyrosine 407, another Src mediated tyrosine phosphorylation residue in the activation loop has been proposed as a negative regulatory site (298).

1.3 The Molecular Mechanism of FAK Regulation

1.3.1 The Definition of FAK Activation

As an important tyrosine kinase in multiple signaling pathways, the "on" and "off" status of FAK plays a critical role in coordinating the signal transduction pathways. However, when compared with other signaling molecules, there are two different definitions of FAK activation. The traditional definition of FAK activation is the enhancement of the kinase activity. The generalized definition of FAK activation is the transition of FAK from its resting form to a modified form that facilities signal transduction. This modification results not only in the enhancement of its kinase activity but also in the promotion of its scaffold function. Actually, a number of important modifications of FAK e.g. phosphorylation of Serine 732 or tyrosine 925 do not directly change its kinase activity; instead they change its binding affinity to a number of binding partners, such as Grb2

(403,489). Here I will review the general definition of FAK activation. At the same time, I will also pay attention to how to "turn off" FAK and FAK inactivation mechanisms are also discussed here.

1.3.2 Phosphorylation Mediated FAK Activation

FAK was first identified as a tyrosine phosphorylated protein. Tyrosine phosphorylation is a major regulatory mechanism of FAK activation. In addition to tyrosine residues, the serine and threonine residues also play an important role in regulation of FAK. Most of the FAK phosphorylation sites are labeled in Fig 1.1. Here I will review the phosphorylation of FAK by upstream kinases.

1.3.2.1 Auto-Phosphorylation of FAK

Tyrosine 397 was the first identified phophorylated residue within FAK (392). It was shown to be a FAK auto-phosphorylation site and an SH2 domain binding site, especially for SFK (Src family kinase). The kinase activity of the Y397F mutant is greatly impaired both *in vivo* and *in vitro* (345,391). Studies suggest that tyrosine 397 phosphorylation is an early event in FAK activation because tyrosine phosphorylation of other residues is abolished in the mutant (344). This result is reasonable since the SFK/FAK interaction was also greatly impaired in the mutant and SFK is a major group of kinases that phosphorylate other tyrosine residues within FAK. The FAK Y397F mutant shows defects in focal adhesion turn over, cell migration and cell cycle progression (58,467,507). Moreover, the auto-phosphorylation of FAK is not only critical for promoting FAK kinase activity; but it is also required for FAK scaffold function. A number of other signaling molecules, such as PI3K and Grb7, associate with FAK via tyrosine 397 and form a signaling complex (74,176). In addition to SFKs, other FAK binding partners are also critical for FAK related signal transduction. The D395A

mutant is capable of binding Src but not PI3K. Cells expressing the D395A mutant still show defects in cell migration (370).

1.3.2.2 SFK Mediated FAK Phosphorylation

As the first identified FAK binding partners, SFKs are some of the most important FAK activity regulators. When FAK tyrosine 397 is phosphorylated, SFK associates with FAK and phosphorylates other tyrosine residues within FAK. Tyrosine 576, 577, 407, 861 and 925 are all SFK mediated phosphorylation sites. Phosphorylation of tyrosines 576/577 is a key step for FAK to achieve maximal kinase activity (49). Cells expressing FAK Y576/577F mutants exhibit defects in spreading and migration (346). There is no evidence that tyrosine phosphorylation of tyrosine 925 effects the FAK kinase activity. However, the phosphorylated tyrosine 925 creates a binding site for the Grb2 SH2 domain. The assembly of a FAK/Grb2 complex activates the Grb2/MAPK pathway (402). A recent study suggests that tyrosine 925 is required for VEGF induced tumor growth via activation of the MAPK cascade (328). Phosphorylation of tyrosine 861 is also not associated with FAK kinase activity. However, it plays multiple roles in FAK signal transduction. The VEGF induced phosphorylation of tyrosine 861 promotes the assembly of the FAK/Integrin$\alpha_v\beta_5$ complex, which might regulate VEGF induced vascular permeability (126). Other studies also suggests that phosphorylation of tyrosine 861 is related to the regulation of cell survival and migration (5). The function of tyrosine 407 phosphorylation is largely unknown. Interestingly, a recent study suggests that tyrosine 407 is a negative regulatory site for FAK kinase activity (297). However, the detailed molecular mechanism is still unclear. Other studies also suggest that tyrosine 407 is phosphorylated by Pyk2, which promotes VGEF induced cell migration (267). In brief, SFKs induce FAK phosphorylation on multiple sites.

The phosphorylation of each site activates specific downstream pathways, which suggests that FAK precisely coordinates the multiple signal transduction pathways.

1.3.2.3 Other kinases Mediated FAK Phosphorylation

In addition to tyrosine phosphorylation, a number of serine phosphorylation sites have also been identified on FAK. FAK serine 732 is phosphorylated by CDK5 in neuron cells (488). The phosphorylation of serine 732 is important for microtubule organization, nuclear movement, and neuronal migration. Interestingly, serine 732 is also directly phosphorylated by ROCK following VEGF stimulation in HUVECs (266). ROCK induced phosphorylation of serine 732 is required for Pyk2-dependent phosphorylation of tyrosine 407, which promotes endothelial cell migration. Serine 722 was recently identified as a GSK-3 mediated phosphorylation site (23). Similar to other GSK-3 substrates, the phosphorylation of serine 722 depends upon a preexisting phosphorylation at a nearby site. Phosphorylation of FAK at serine 726 by an unknown kinase creates a docking site for GSK-3 promoting serine 722 phosphorylation. Interestingly, FAK kinase activity is inhibited when serine 722 is phosphorylated. Hence the phosphorylation of serine 722 works as a brake to block cell migration. Some other FAK serine phosphorylation sites are also reported, but little is known about the specific physiological functions of these sites (235).

1.3.2.4 FAK and Protein Tyrosine Phosphatases

FAK is also regulated by various phosphatases. There are two different regulatory mechanisms (Fig 1.2). In the direct regulation model, the tyrosine phosphatase, such as SHP-2, impairs FAK activity via direct dephosphorylation of the tyrosine residues within FAK (318,450). SHP-2 associates with FAK via its SH2 domain, which may disrupt the FAK/Src complex. Therefore not only the phosphatase activity of SHP-2 but also its competitive

binding promotes FAK inactivation. However, fibroblasts expressing a SHP-2 mutant lacking phosphatase activity exhibits defects in cell spreading and cell migration. The detailed mechanism is still unclear (501). In the indirect model, some phosphatases, such as PTPα, dephosphorylate Src tyrosine 527 (182). The dephosphorylation of tyrosine 527 activates Src and promotes Src mediated FAK activation. This model explains the migration defects in PTPα null fibroblasts (504). Interestingly, PTP1B is reported to regulate FAK activity via both direct and indirect mechanisms, which suggests that PTP1B is capable of selecting specific substrates in different scenarios (14,307). Another important dual function phosphatase, PTEN is also involved in FAK regulation. An initial study proposed that PTEN directly interacts with and inactivates FAK via dephosphorylation (436). But recent studies suggested that PTEN may not directly regulate FAK. However, PTEN may indirectly block FAK activation via dephosphorylation of PIP3, the PI3K product. Since PI3K is required for FAK activation during cell spreading, the PTEN induced PIP3 hydrolysis may block FAK activation (249).

1.3.3 Conformation Mediated FAK Activation

1.3.3.1 Kinase Conformation Transition

Besides phosphorylation, some kinases also use other strategies to regulate their activities. A conformational switch has been proven to be a very common form of kinase regulation. This regulation strategy is especially important in tyrosine kinases. For Src, kinase activity is blocked when it is in a closed conformation. This auto-inhibited conformation is held when the N-terminal SH2 of Src associates with the corresponding tyrosine residue (tyrosine 527) within the C-terminus of Src, which is phosphorylated by CSK while the cell is in a resting state (16). Structurally, this closed conformation disrupts

the kinase active site and occupies the SH2 and SH3 domains (424,492). Interestingly, this interaction is disrupted in v-Src, a constitutively active mutant identified in Rous sarcoma virus. Other conformational regulatory mechanisms have also been found in other tyrosine kinases. For example, the auto-inhibited conformation of ZAP-70 is held by an interaction between the regulatory segment and the hinge region of the kinase domain (103). This interaction is disrupted when the SH2 domain in ZAP-70 associates with the ITAM motif within the TCR C-tail following TCR activation (185). Conformational transition is also a major regulatory mechanisms in some other serine/threonine kinases, such as PKA and CaMK (155,246).

1.3.3.2 Molecular Mechanism of FAK Conformational Transition

Studies within the last five years have suggested that an intra-molecular interaction may regulate FAK activity. Toutant et al suggested that truncation of the FERM domain may increase FAK kinase activity (445). Cohen et al proposed that the FERM domain of FAK associates with the kinase domain to inhibit FAK activity (96). They also identified lysine 38 within the FERM domain as a critical residue for the FERM/kinase interaction (90). The FAK K38A mutant exhibits higher kinase activity *in vivo* and *in vitro*. Recently, the co-crystallized FERM/kinase structure revealed the interaction mechanism (Fig 1.3 A) (295). The hydrophobic residue phenylalanine 596 projects into a hydrophobic pocket formed within the FERM domain (Fig 3.2E). Introduction of point mutations within these sites greatly increases FAK phosphorylation levels and kinase activity. Moreover, unlike wild type FAK, the activity of these FAK mutants is independent of adhesion signaling. Interestingly, the activation loop is proposed to be embedded underneath the cleft between the FERM and kinase domains. Based upon the structure, a conformation based FAK

activation model has been proposed. In the inactive condition, both the important regulation residues within the linker and activation loop are masked by the FERM domain. Displacement of FERM domain via an unknown mechanism unmasks these residues, which allows auto-phosphorylation followed by Src mediated phosphorylation. In general, FAK conformation change is an initial step for FAK activation.

1.3.4 Other Mechanisms of FAK Regulation

Cleavage of FAK is used as another strategy to regulate FAK activity. Degraded collagen fragments induce calpain dependent cleavage of FAK via the integrin pathway (55). In Src transformed cells, FAK works as a scaffold to assemble a FAK/ERK/calpain complex (57). The PRR1 within the linker associates with calpain and is essential for the cleavage of FAK. ERK activity is also required for calpain mediated cleavage of FAK. Cells expressing the FAK PRR mutant exhibit defects in focal adhesion turn over and cell migration, which is similar to calpain null cells. Calpain dependent cleavage of FAK not only removes FAK out of focal adhesion but also creates FRNK like fragments, which might work as a dominant negative to inhibit the remaining FAK activity in the focal adhesions (54).

FAK is also cleaved by caspase following apoptotic signals, such as HIV envelope induced $CD4^+$ T cell apoptosis (86). However, both focal adhesion turnover and dephosphorylation of FAK are independent of the caspase mediated cleavage of FAK in apoptotic cells, which suggests that the disassembly of focal adhesions precedes the cleavage of FAK. Interestingly, recent studies suggest that the FAK FERM domain shuttles into the nucleus and works as a scaffold to block P53 induced apoptosis (D. Schlaepfer, personal communication). Cleavage of FAK induced by apoptotic signals may promote nuclear

targeting of the FAK FERM domain, which in turn promotes the FERM domain mediated survival.

1.3.5 FAK Activation Model

Our understanding of the molecular mechanism of FAK activation is always developing with the updated collection of data. The new FAK auto-inhibition structure gives us a straightforward view of FAK activation. Based upon the available biochemical and structural data, a novel FAK activation model is proposed (Fig 1.4). FAK holds a "closed" conformation in the resting cells. The N-terminal FERM domain masks the active site and substrate binding. When integrins are activated by the extracellular matrix, FAK is recruited to the nascent focal adhesions where it switches to an "open" conformation. The unmasked kinase domain promotes the auto-phosphorylation of FAK, which provides the binding site for Src. The tyrosine residues within the activation loop are also exposed for phosphorylation, which greatly enhances FAK kinase activity.

Though an elegant FAK activation picture is presented, there are still some gaps in this activation model. The initial conformation switch is the first step for FAK activation. However, we still know little about the factors that trigger this transition. The cytoplasmic tail of integrins is proposed to initiate FAK conformational switch following integrin activation (99). FAK is stretched to an "open" conformation when FAK FERM domain associates with the activated integrin. This hypothesis waits testing in the future.

1.4 FAK Regulates Multiple Signaling Pathways

Over the past fifteen years, extensive research into the functions of FAK has improved our understanding of the roles of FAK in multiple signaling pathways (349). Due to the specific focal adhesion localization, early studies focused on the role of FAK in

regulating integrin signaling, cell adhesion and migration. However, more and more studies suggest that FAK is involved not only in cytoskeleton reorganization but also in most other cell behaviors, such as cell division and survival. Hence, FAK coordinates multiple signaling pathways with integrin pathways to make a comprehensive signaling response (Fig 1.5).

1.4.1 The Role of FAK in Cell adhesion and Cell Migration

In cells seeded on the extracellular matrix coated surface, FAK is activated following integrin activation and regulates cell spreading via multiple mechanisms. For one, FAK mediated substrate phosphorylation is required for cell spreading. FAK induced tyrosine phosphorylation of paxillin promotes cell spreading via the activation of the CrkII/DOCK180/Rac pathway (362,397). On the other hand, though FAK activation depends on RhoA-Rock signaling, activated FAK blocks RhoA activity to attenuate the intracellular tension, which promotes cell spreading (21,195,505).

The role of FAK in cell migration is very complex. It promotes cell movement by regulating multiple biochemical/biological events (325). FAK is implicated in regulating focal adhesion dynamics, which is a key factor for cell migration (466). FAK null cells assemble large focal adhesions and exhibit very low focal adhesion disassembly rates (216). FAK may regulate focal adhesion dynamics via FAK mediated paxillin phosphorylation because similar focal adhesion dynamics were also identified in cells expressing paxillin variant (Y31F/Y118F), which lacks the FAK phosphorylation site (469). FAK also suppresses Rho activity to promote the focal adhesion turn over (408). In addition to the regulation of focal adhesion dynamics, FAK also regulates cell protrusion during migration. It regulates the subcellular localization of N-WASP by interacting with and phosphorylating N-WASP (486). FAK induced tyrosine phosphorylation of N-WASP promotes its

cytoplasmic localization. Cell migration was blocked in cells expressing an N-WASP tyrosine phosphorylation site mutant. Recently FAK has been suggested to promote nascent lamellipodial formation by promoting Arp2/3 mediated actin nucleation (409). Interestingly, evidence suggests that FAK could also regulate cell migration via microtubules. Ezratty et al reported that FAK regulates microtubule induced focal adhesion turnover via dynamin (131). Palazzo et al proposed that FAK maintains cell protrusion via stabilization of microtubules (347). Hence, FAK promotes the two key steps in cell migration: cell protrusion and focal adhesion turn over via a number of different mechanisms.

Interestingly, though it is almost dogma that FAK is essential for cell migration, some studies suggests that FAK may work as a brake to block cell migration under specific conditions. FAK is proposed to block Rac activity in N-cadherin based cell adhesions. HeLa cells treated with FAK siRNA show enhanced Rac activation and cell motility (496). The knock down/rescue assay suggests that tyrosine phosphorylation of tyrosine 861 but not FAK kinase activity or auto-phosphorylation is critical for FAK mediated down regulation of Rac activity in N-cadherin based cell adhesions. Moreover, FAK null endothelial cells show enhanced motility when they are scattered outside of capillaries in the P-Sp explants, which suggests that FAK blocks endothelial cell migration in an organ context(30). The mechanism for this is still unknown.

1.4.2 The Role of FAK in Cell Survival

Though first identified as a tyrosine kinase that regulates the cytoskeleton rearrangement, more and more studies suggest that FAK is an important regulator of cell survival. Braren et al proposed that cell apoptosis is a major reason for the angiogenesis defect in the FAK conditional knockout endothelial cells (29,416). This implicates FAK in

promoting a survival signal that plays a critical role in cardiovascular system development. FAK regulates multiple survival pathways. Constitutively activated FAK prevents "anoikis", which is a kind of apoptosis induced when anchorage-dependent cells detach from the surrounding extracellular matrix (142). This study suggests that FAK is required for regulating the integrin dependent cell survival signal. Two possible mechanisms are proposed. Ilic et al proposed that FAK suppresses P53 induced apoptosis via inhibition of the PKC/phospholipase A2 pathway (214). Almeida et al proposed a different model where the FAK/p130CAS complex activates JNK via a Ras/Rac1/Pak1/MKK4 pathway (10). The above two mechanisms may work together to maintain cell survival in cells growing on a specific extracellular matrix under different stress conditions. When cells are serum starved, the FAK/p130CAS/JNK pathway is activated to promote the cell survival. When cells are stimulated by some "death" cytokines, e.g. TNF-α, FAK blocks the death signal transduction via inhibition of P53. FAK also regulates the activation of the PI3K/AKT pathway, which is another important survival signal in some specific scenarios. FAK is required for PI3K/AKT activation to prevent hydrogen peroxide-induced apoptosis in HL-60 cells (429). Recently, a novel FAK mediated cell survival mechanism has been proposed. The FAK FERM domain directly associates with P53, which suppresses P53 dependent transcription activation (157) (D. Schlaepfer, personal communication). Compared to the other mechanisms, the new model suggests that FAK may promote cell survival only via the FERM domain but not the kinase or FAT domains. Since the FAK FERM domain is reported to localize to the nucleus, it is possible that FAK may regulate apoptosis related transcription in the nucleus instead of the cytoplasm (237,430). These studies raise the possibility that FAK shuttles between cytoplasm and nucleus to propagate different signals

1.4.3 The Role of FAK in Mechanotransduction

In addition to chemical stimuli, physical stimuli are another important source that regulates the cell behavior (280). Focal adhesions connect the extracellular matrix to internal signaling molecules. They not only bear the cellular traction force but also sense extracellular physical changes. Hence, focal adhesions play a critical role in cellular mechanotransduction. As a major tyrosine kinase within focal adhesions, FAK is essential for focal adhesion mediated mechanotransduction.

1.4.3.1 The Role of FAK in Fibroblast Mechanotransduction

FAK is required to maintain normal cell protrusion and directional migration. When normal fibroblasts meet an external force, the cells will turn around and form prominent focal adhesions. FAK null fibroblasts fail to make these responses following physical stimulation. This mechanotransduction defect is rescued via the re-expression of FAK (460). Interestingly, cells expressing the FAK auto-phosphorylation site mutant (Y397F) only exhibit normal focal adhesion dynamics following stimulation by an external force. However, the cells still show defects in reorientation. Another study also suggests that auto-phosphorylation of FAK is critical to maintain cell directional sensing (441).

Normal fibroblasts prefer a rigid matrix to maintain a suitable cell tension. They will move away when they sense that the matrix is too soft. This response to substrate flexibility is greatly impaired in FAK null fibroblasts. This defect is rescued in cells expressing wild type FAK or FAK Y397F (459).

In addition to force and substrate flexibility, matrix topography also influences the fibroblast motility. Normal fibroblasts exhibit multiple protrusions and erratic migration in pillar substrates where migrating cells encounter irregular surface. These responses are

greatly impaired in FAK null fibroblasts. This suggests that FAK is critical for cells to sense matrix topography (141).

1.4.3.2 The Role of FAK in Endothelial Cell Mechanotransduction

As a barrier of vessel, endothelial cells directly bear the physical stimuli applied by blood flow, including shear stress, pressure and circumferential stretch. In response, they regulate cell behavior, such as cytoskeleton rearrangement, cell proliferation and apoptosis (286). A number of receptors and channels are reported to play an important role in sensing mechanical signals. Integrins and the associated focal adhesion proteins work as feet to anchor cells to the extracellular matrix. They are key sensors for endothelial mechanotransduction (423). FAK has also been implicated as a key protein in integrin mediated endothelial mechanotransduction. FAK activation was identified following mechanical stimulation in endothelial cells. An enhancement of FAK tyrosine phosphorylation was identified in both BAECs and HUVECs in shear stress condition (229,278). Shikata et al suggested that the cyclic stretch and shear stress induce FAK phosphorylation via different mechanisms (422). Furthermore, FAK also responds differently to steady pressure and pulsatile stretch (273). All these studies suggest that FAK is capable of make different responses according to the stimuli. In addition to activation, upregulation of FAK synthesis following shear stress and dual ligands (streptavidin-biotin and fibronectin) treatment in endothelial cell was reported by Chan et al (68). Mechanotransduction also regulates FAK spatial distribution. Yano at al proposed that mechanical strain induces not only tyrosine phosphorylation but redistribution of FAK. FAK was recruited to the basal level of cells and aligned with the long axis of the stretched

endothelial cell (498). Li et al suggested that FAK is recruited to the lamellipodial protrusion induced by shear stress in endothelial cells (279).

Little is known about how FAK receives mechanical signals. Both integrin and Src are required for FAK activation in mechanical signal transduction (272). RGD peptides abolish FAK auto-phosphorylation and Src mediated FAK phosphorylation. Src inhibitors also block the activation of FAK. Cyclic stretch induced FAK activation is dependent on mitochondrial oxidants (7). Since the conformational transition is an initial step for FAK activation, the physical stimuli may activate FAK via conformation switch. Interestingly, the conformational transition of some integrin signaling proteins is involved in mechanotransduction. For example, p130CAS is stretched to expose some major phosphorylation sites following physical stimulation (384,435). The exposed tyrosine residues are phosphorylated by Src. The phosphorylated p130CAS activates Rap1 to initiate downstream signaling. FAK is possibly activated via a similar mechanism (Fig 1.6). Just like a spring, FAK may be stretched to the "open" conformation by a relative balance between the external force and the internal cytoskeleton traction. FAK conformational transition might be a direct response to the external force. This model implicates FAK not only as an intermediate but also as a sensor to directly detect physical stimulation, which may explain the quick FAK response (1 min) to shear stress (282). Visualization of the FAK conformational status following mechanical stimulation will be helpful to test this hypothesis. FRET technology is a possible tool to visualize conformational activation in living cells (71,312,464). Wang et al successfully visualized temporal and spatial activation of Src following mechanical stimulation in endothelial cells using Laser-tweezers and Src FRET biosensor (463).

It is important to understand the downstream effects of FAK mediated endothelial cell mechanotransduction. FAK is proposed to regulate ERK and JNK activation following shear stress in endothelial cells (281). However, the detailed mechanism is still unknown. Also, inhibition of flow induced FAK activation by FAK antibody blocks AKT and eNOS activation (257). This result suggests that FAK is required for flow induced NO generation. Expression of GFP-FRNK, a FAK dominant negative, blocks the stretch induced up-regulation of connexin43, N-cadherin and other junction proteins, which implicates FAK in regulating cell-cell junctions in mechanotransduction (493).

Most of the data regarding FAK mediated mechanotransduction is from *in vitro* physical stimulation studies. Little is known about the *in vivo* function of FAK in endothelial cell mechanical transduction. The critical angiogenesis defect in the FAK endothelial conditional knockout mouse poses a technical problem for investigating FAK function *in vivo* (28,415). A novel strategy should be developed to inactivate FAK in endothelial cells in the late stage of animal development, which may help us to understand the function of FAK in mechanotransduction *in vivo*. Interestingly, a recent study suggests that osteoblast-specific knockout of FAK abolishes the osteogenic response following physical stimulation *in vivo* (275).

1.4.3.3 The Role of FAK in Cardiomyocyte Mechanotransduction

The function of cardiomyocyte is to pump blood through the circulatory system by contracting. Physical stimuli are very important signals that regulate the function of myocytes. For one, hypertension induced pressure is a major reason for myocyte hypertrophy. Humans develop cardiac hypertrophy greatly increases the risks of heart attack.

For another, stretch will not only induce hypertrophy but also cardiomyocyte apoptosis, which is also a major reason to induce heart failure.

FAK is a major regulator of cardiomyocyte mechanotransduction. Cardiomyocyte stretch induces FAK activation via integrinβ1 signaling (262). Just like the classical integrin signaling, stretch induced FAK activation is dependent on Rho/Rock activity (443). FAK not only induces ERK activation but also that of MEF2 and c-Jun in cell stretch signaling. This suggests that FAK may not only regulate the transit cytoskeleton rearrangements but also the longer term transcriptional response to mechanical stimulation (263,332). Interestingly, the stretch induced chloride channel is also regulated via integrin/Src/FAK signaling (32).

Recent development of two FAK cardiomyocyte conditional knock out mouse models suggests that FAK is required for pressure overload induced hypertrophy (112,353). The detailed mechanism remains unclear. FAK is activated by pressure over load. Src, Grb2 and PI3K are recruited into a complex following pressure stimulation, which suggests that FAK may regulate the MAPK and PI3K/AKT signaling pathways upon the overload stimulation (140). Importantly, FAK is required for the restoration of the myocyte sarcomere length after uniaxial static strain, which implicates that FAK in involved in strain induced hypertrophy (319). Interestingly, FAK interacts with the C-terminus of the myosin heavy chain in cardiomyocytes. The assembly of this complex is regulated by pressure, implicating FAK in regulating myosin mediated myocyte contractility (138).

1.4.4 The Role of FAK in Angiogenesis

1.4.4.1 FAK as a Key Regulator in Angiogenesis

Angiogenesis is a process involving the growth of new blood vessels from pre-existing vessels, which is a critical step for development. It is also related to the wound

healing and tumor malignant. Several lines of evidence support FAK as a key regulator in endothelial cell angiogenesis. Overexpression of FAK promotes angiogenesis both in vivo and in vitro (256,356). The FAK knock out endothelial cells exhibits defects in vessel formation. Similar defects are also seen in HUVECs treated with FAK anti-sense oligos or a dominant negative form of FAK (217). Most important of all, two recent studies convincingly showed that FAK is a key component in angiogenesis. FAK endothelial conditional knockout mice are embryonic lethal and exhibit serious defects in angiogenesis *in vivo* (27,412). However, there are some discrepancies between these two reports. Though both suggest FAK is required to maintain cell survival in angiogenesis, they have inconsistent conclusions about the role of FAK in endothelial cell proliferation and migration. Further investigation is required to solve this discrepancy. In short, FAK plays an important and comprehensive role in angiogenesis. It regulates not only cell migration, but also cell proliferation, survival, and maybe matrix degradation. Moreover, FAK does not seem to be a separate component in the integrin signaling pathway. Rather, it coordinates the crosstalk between integrin signaling with other pathways in angiogenesis. Here I will review various aspects of the molecular mechanism of FAK in regulating angiogenesis (Fig 1.7).

1.4.4.2 FAK, in Command of the Different Stimuli in Angiogenesis

1.4.4.2.1 FAK in VEGF Signaling Pathway

When endothelial cells receive angiogenesis signals, they enter into a highly proliferative state from the quiescent state. FAK is activated by VEGF stimulation through VEGFR2 (199). VEGF signaling first induces Src activation. Activated Src then induces FAK phosphorylation, especially on tyrosine 861, which promotes the interaction between FAK and integrin$\alpha_V\beta_5$. However, the role of the interaction is still unknown. It is possible

that the FAK/integrin$\alpha_V\beta_5$ interaction is required for VEGF induced vascular permeability since both the Src and Integrin β_5 deficient mice show defects in vascular permeability in response to VEGF (125,352). VEGF induced FAK activation is required for the activation of the Ras-Raf-ERK pathway (198). Activated ERK is a key component in regulating endothelial cell proliferation, survival and migration during angiogenesis. This mechanism may explain the defects in FAK conditional KO mice where ERK activation is impaired in KO cells. The deficiencies exhibited by KO cells might be dependent on ERK activation. However, Bryant et al proposed another novel downstream effect of FAK activation (40). In this model, FAK promotes the degradation of cyclin-dependent kinase inhibitors (CDKIs) p21/Cip and p27/Kip following VEGF signaling, which accelerates endothelial cell proliferation. In addition to the above mechanisms, FAK may regulate PI3K activation following VEGF stimulation (366). Since PI3K/AKT pathway is required to prevent apoptosis, FAK may regulate cell survival through this pathway.

In addition to Src-dependent FAK activation, a Src independent FAK activation pathway is also proposed. VEGF simulation recruits HSP90 to the VEGFR2 and activates Rho and Rho kinase (320). Serine 732 in FAK is then phosphorylated by Rho Kinase, which promotes Pyk2 mediated FAK tyrosine 407 phosphorylation (268). The Tyrosine 407 phosphorylation recruits vinculin and paxillin and promotes the VEGF mediated endothelial cell migration (265). Interestingly, this Src independent pathway is dependent on integrin$\alpha_V\beta_3$. VEGF stimulation promotes the VEGFR2/integrin$\alpha_V\beta_3$ complex formation and transduces the signal (320). This mechanism is similar to the integrated interaction between EGFR/PDGFR and integrins (426). FAK may also work as an adaptor here to integrate two different kinds of receptors. Interestingly, a direct interaction between the C-

terminus of FAK and extracellular domain of VEGFR3 is identified both in vitro and in breast cancer cells (148). Disruption of this interaction blocks cancer cell proliferation and promotes apoptosis. VGEFR-3 is involved in lymphangiogenesis (421). Hence this novel interaction provides another mechanism for FAK to regulate atypical VGEF signaling.

In addition to outside-in signaling, two different mechanisms implicate FAK in a positive feedback mechanism to amplify the VEGF signal. In Src transformed cells, FAK is phosphorylated on tyrosine 925, which activates the Src/FAK/Grb2/MAPK cascade and enhances VEGF expression (327). FAK is also required for epithelial cell contact induced VEGF expression, which provides another possibility for FAK to boost VEGF signaling (419).

1.4.4.2.2 FAK in Other Angiogenesis Related Signaling

FAK is involved in some of the other signaling pathways that regulate angiogenesis. In the FGFR pathway, activated FAK is required for ERK activation (197). However, the FAK activation mechanism here is different when compared to the VEGF signaling pathway. First, FAK mediated ERK activation is independent of Ras activation. Second, Src is not required for FAK mediated ERK activation in the FGFR pathway. Third, activated Raf prevents mitochondria induced apoptosis in an ERK-independent manner, which provides a distinct anti-apoptotic mechanism when compared to VEGF signaling. Serine 910 in FAK however is also phosphorylated following FGF stimulation in an ERK dependent manner (213). The function of this phosphorylation is still unknown.

Angiopoietin-1(Ang-1) is a ligand of the Tie-2 receptor. The Ang-1/Tie-2 pathway is required for the early stage endothelial cell sprouting in angiogenesis. Integrin$\alpha 5\beta 1$ forms a constitutive complex with Tie-2 and promotes Tie-2 activation (61). FAK is recruited to this

complex and activated following Ang-1 stimulation. PI3K is also recruited to this complex and is required for FAK activation. FAK may regulate cytoskeleton rearrangement following Ang-1 signaling because both integrin$\alpha_5\beta_1$ and PI3K is required for Ang-1 dependent cell spouting.

In short, FAK plays multiple roles in angiogenesis following activation by different stimuli. Integrins directly sense the external matrix condition. Other ligands bring the information from the surrounding tissue. These signals are fine tuned via different modifications of FAK, which allows the receptors to cross talk with different integrins resulting in "comprehensive" downstream effects.

1.4.4.2.3 FAK May Regulate the Matrix Degradation in Angiogenesis

Matrix degradation is one of the early events in angiogenesis, which regulates endothelial cell invasion and migration (179). FAK is proposed to regulate matrix degradation via different mechanisms. Angiopoietin2 interacts with integrin$\alpha_V\beta_1$ and activates the FAK/MAPK pathway. The activated MAPK induces MMP-2 expression and glioma cell invasion (201). In Src transformed cells, FAK works as a scaffold to promote the interaction between Src and endophilin A2. Src phosphorylated endophilin A2 inhibits the endophilin/dynamin interaction and abolishes MT1-MMP endocytosis (485). Although the above data was collected from other systems, a similar mechanism may also exist in endothelial cells to regulate angiogenesis.

1.5 Pyk2

Pyk2 is a member of the focal adhesion kinase family and it shares a number of same characteristics with FAK. First, just like FAK, Pyk2 contains three domains: an N-terminal FERM domain, a central kinase domain, and a C-terminal FAT domain (Fig 1.1). The

tyrosine phosphorylation sites are almost the same between FAK and Pyk2. They also share the same binding partners. Second, both Pyk2 and FAK are activated by a number of common stimuli, such as integrins, growth receptors and GPCRs. Third, functional assays suggest that Pyk2 is also required for cytoskeleton rearrangement (231). Both Pyk2 and FAK regulate cell migration and cell spreading. However, there are a number of differences between FAK and Pyk2. First, unlike FAK, Pyk2 is not well localized to focal adhesions (399). Second, FAK is expressed in almost all tissues, whereas Pyk2 is expressed only in some specific tissues, including the neurons, endothelial cells and hematopoietic cells. Interestingly, macrophages predominantly express Pyk2 but not FAK (123). Third, while FAK and Pyk2 may compensate each other in cytoskeleton regulation, they play antagonistic roles in cell survival(427). FAK promotes the cell survival via multiple pathways while Pyk2 is required to induce efficient cell apoptosis(156,491). In knock out animal models, FAK null mice are embryonic lethal while *pyk2*-/- mice are viable. However, Pyk2 null mice exhibit absence of marginal zone B cells and increased bone density (41,151,167). Macrophages isolated from Pyk2 null mice exhibit defects in chemotaxis and cytoskeleton reorganization (338).

1.6 Other Kinases in Adhesion Signaling

In addition to FAK, a number of other kinases are also involved in adhesion signaling. Some of these kinases, such as ILK and PAK, localize to focal adhesions via the interaction with focal adhesion components. Other kinases, such as ERK and PKC, transiently translocate to focal adhesions and induce the phosphorylation of focal adhesion proteins following multiple stimuli. These kinases not only regulate adhesion induced cytoskeleton reorganization but also other cell behavior, such as survival.

1.6.1 Src

Src is another critical tyrosine kinase in adhesion signaling. Unlike FAK, Src does not predominantly target to focal adhesion. However, the activated form of Src was observed in focal adhesions (241). Interestingly, a number of focal adhesion proteins including FAK, paxillin and p130CAS were identified as Src substrates in v-Src transformed cells, which suggests that Src plays a key role in regulating adhesion signaling. Since FAK and Src form a complex in focal adhesions and activate each other following integrin activation, sometimes they target the same substrate. For an example, paxillin is phosphorylated both by Src and FAK *in vitro*. Moreover, a decrease in paxillin tyrosine phosphorylation is observed in both SFK and FAK null cells(253,414). Both the SFK null cells and fibroblasts treated with SFK inhibitors show strong defects in focal adhesion turn over, cell spreading and migration, which is similar to the phenotype observed in FAK null cells (465). However, Src is definitely not redundant with FAK in adhesion signaling. For one, some substrates are phosphorylated only by Src but not by FAK. For example, p130CAS is only phosphorylated by Src following integrin activation (457). Tyrosine phosphorylated p130CAS switches to an open conformation and recruits a binding partner to activate the downstream pathway (385). Furthermore, the activation of Src does not always depend on FAK. Src is recruited to the cytoplasmic tail of integrinβ_3 and activated following cell adhesion (13). Biochemical assays suggest that the SH3 domain of Src directly associates with the C-terminus of integrinβ_3, which may facilitate the Src conformational switch. In addition, Src is activated in a FAK independent mode following the activation of integrin$\alpha_4\beta_1$ (200). Activated Src induces tyrosine phosphorylation of p130CAS, which finally activates Rac and promotes cell migration.

1.6.2 Abl

Abl was also first isolated as an oncogene and then identified as a tyrosine kinase. Interestingly, Abl directly interacts with F-actin, which implicates Abl in the regulation of cytoskeleton reorganization (455,461). Abl is required for cell spreading and maintaining cell protrusion (476). However, Abl inhibits integrin mediated cell migration (240). In fibroblasts seeded on the fibronectin coated dish, Abl is transiently recruited to nascent focal contacts and is activated (276). Abl phosphorylates a number of focal adhesion proteins, including paxillin, p130CAS and CrkII (475). Interestingly, Abl phosphorylates CrkII at tyrosine 221, which is a negative regulation site for CrkII activity (261). Abl induced CrkII phosphorylation disrupts the CrkII/p130CAS/paxillin complex. The cells expressing CrkII Y221F not only promote the assembly of CrkII/p130CAS/paxillin complex but also increase the activation of Rac (1). This may explain why Abl inhibits the cell migration.

1.6.3 MAPK Family

MAPKs, including JNK, p38 and ERK are activated when cells attach to extracellular matrix. Activation of MAPKs induces the phosphorylation of focal adhesion proteins, which regulate the dynamics of focal adhesions and cell survival.

ERK is required for cell adhesion and cell migration (207). A number of focal adhesion proteins are phosphorylated by ERK. ERK phosphorylates FAK at serine 910, which impairs the interaction between FAK and paxillin (212). However, the physiological function of ERK induced FAK phosphorylation is still unknown. ERK also phosphorylates paxillin at serine 83 following HGF stimulation (228). Phosphorylation of serine 83 promotes the interaction between FAK and paxillin and activates FAK. FAK dependent activation of PI3K and Rac following HGF stimulation is greatly impaired in cells expressing

paxillin S83A mutant (221). Spreading and migration defects have also been identified in these cells. Interestingly, besides being an ERK substrate, paxillin also works as a scaffold protein to facilitate the MAPK cascade activation (227). This may explain the defective ERK activation in paxillin null fibroblasts (172). In addition to phosphorylation of serine83, phosphorylation of serine126 and serine 130 are also dependent on ERK activation (482). ERK also regulates calpain mediated proteolysis of FAK (56). This is critical for focal adhesion turnover and cell migration. Besides the above mechanisms, ERK also regulates the dynamics of focal adhesions via MLCK (468).

JNK is a well known regulator of gene transcription. Several lines of evidence indicate that JNK is also involved in regulating cytoskeleton rearrangement. JNK inhibitors impair random migration of the fish keratocytes (210). Activated JNK is localized within focal adhesions (11). Paxillin was identified as a JNK substrate. JNK directly phosphorylates paxillin at serine 178 following stimulation by growth factors, which promotes cell migration possibly by regulating focal adhesion dynamics. Another study also suggests that JNK induced paxillin phosphorylation is required for neurite extension (494).

In addition to ERK and JNK, P38 also phosphorylates paxillin at serine 83 in neuronal cells following NGF stimulation (206). Neurite outgrowth is blocked in cells expressing paxillin S83A mutant. Hence MAPKs work together to coordinate cytoskeleton reorganization.

1.6.4 PKC Family

PKC is activated following integrin activation. PKC is proposed to regulate tyrosine phosphorylation of FAK following the integrin$\alpha_v\beta_5$ signaling (277). An important focal adhesion protein, vinculin is regulated via conformational switch (236). PKC interacts with

open vinculin and phosphorylates vinculin at serine 1033 and serine 1045 (470,510). Deletion of the critical motif which is required for the lipid mediated vinculin conformation switch blocks PKC mediated vinculin phosphorylation.

1.6.5 PAK

PAK plays a critical role in regulating cytoskeleton dynamics (25). It localizes not only to the cells leading edge but also to focal adhesions. PAK regulates actin dynamics via multiple mechanisms, such as the activation of the LIMK/cofilin/ADF pathway. PAK also promotes focal adhesion turnover via the phosphorylation of paxillin (335). PAK phosphorylates paxillin at serine 273 to promote the association between paxillin and GIT1, which in turn stabilizes the GIT1/PIX/PAK signaling complex at the leading edge. The paxillin S273A mutant exhibits evident defects in cell migration, protrusion, and focal adhesion turnover.

1.6.6 ILK

ILK localizes within focal adhesions and forms a protein complex with Pinch and Parvin (484). The ILK/Pinch/Parvin complex associates with integrins and regulates adhesion signaling. The ILK null mouse is embryonic lethal. ILK null fibroblasts exhibit defects in cell spreading, focal adhesion biogenesis and stress fiber formation (381). Interestingly, the kinase domain of ILK is atypical when compared to the traditional serine and threonine kinases. Some studies propose that ILK phosphorylates AKT and GSK3 to regulate their activities (106). However, these conclusions are still controversial since the phosphorylation of these kinases is not impaired in ILK null cells (382).

1.6.7 Lipid Kinases

Phospholipids are very important messengers regulating in actin nucleation and cytoskeleton reorganization. Both PIP2 and PIP3 are major lipid molecules involves in adhesion signaling (218). PIP3 dependent GTPase activation is required for actin polymerization. PIP2 also regulates actin polymerization and focal adhesion dynamics. However, unlike PIP3, PIP2 associates with a number of scaffold proteins to induce the conformation switch of these proteins. For example, PIP2 is required for N-WASP to switch to the open conformation, which promotes the Arp2/3 dependent actin polymerization (324). In cos7 cells, the levels of PIP2 and PIP3 are greatly increased following the attachment to fibronectin (252). This implicates these phospholipids in adhesion signal transduction. As the most important kinases to control phospholipid levels, PI3K and PIP5K, play a critical role in adhesion signaling.

1.6.7.1 PI3K

Though PI3K is not constitutively accumulated within focal adhesions, the regulatory p85 subunit of PI3K directly associates with the auto-phosphorylation site of FAK (77). Activated FAK might recruit PI3K to focal adhesions following attachment to fibronectin. However, another study suggests that adhesion induced PI3K activation is independent of FAK activity (456). Adhesion induced PIP3 production is inhibited when cells are treated with a PI3K inhibitor (250). Moreover, adhesion induced activation of MEK, ERK and AKT also depends on PI3K activity. Pharmacological studies suggest that PI3K is required for cell attachment, adhesion and motility (369).

1.6.7.2 PIP5K

Depletion of PIP2 by a PIP2 specific antibody induces the disassembly of focal adhesions (153). PIP2 associates with multiple focal adhesion proteins to regulate their activities (301).

A PIP2 binding site was identified within the talin FERM domain (149). PIP2 promotes binding of talin to integrinβ1, which in turn facilitate the talin focal adhesion targeting. A PIP2 binding site was also identified within the vinculin tail domain. PIP2 dissociates the head-tail interaction of vinculin and promotes its interaction with other binding partners, such as talin (152). Interestingly, one of the PIP5K isoforms, PIP5Kγ 661 localizes within focal adhesions by an association with talin (108,300). PIP5Kγ 661 is tyrosine phosphorylated and activated via FAK signaling, which provides a mechanism for PIP2 generation following attachment to extracellular matrix.

1.7 Adhesion Signaling Proteins in Innate Immunity

1.7.1 Innate Immunity System

When pathogens attack a host, the innate immune system provides a quick and sensitive defense against the invasion (234). When compared with the adaptive immune system, the innate immunity responds to the pathogens in a very short time, e.g. several hours. The immune cells specifically interact with a range of molecular structures of a given type which exists only on the pathogens. These "markers" induce a series of innate immune responses to destroy the pathogens and activate the adaptive immune system. Cells stimulated with the pathogens produce specialized chemokines and cytokines, which recruit immune cells to the sites of infection. The stimulated cells also activate the complement cascade to mark and destroy the microbes. Most importantly, the phagocytotic cells identify and clear the pathogens by phagocytosis, a receptor mediated internalization process. The internalized pathogens are finally destroyed in phagosomes. The debris of the pathogens is then presented on the surface of phagocytotic cells via MHC, which activates the specific T

cells with matching TCRs. The process of phagocytosis-antigen presentation-T cell activation builds a bridge to connect adaptive immunity with the innate immune response.

1.7.2 TLR Signaling

Although the innate immune response has been identified for more than one century, the mechanism was a big puzzle until the 1990s. The discovery of toll like receptors shed some light on innate immunity (270). TLR4, an IL-1R family receptor, was first identified to be required for gram negative bacteria induced inflammation response. LPS, the cell wall component of the gram negative bacteria, also proved to be the TLR4 ligand. A series of TLR family receptors and their ligands were identified in the following years. In general, different TLRs recognize discrete sets of PAMPs to activate the innate immune responses. Interestingly, the TLRs are not only expresses in the hematopoietic cell lineage but also exist in some other tissues, such as endothelial and epithelial cells. This indicates that the innate immune response might be induced immediately following the pathogens invasion. Hence, TLR signaling is the most important mechanism to recognize and destroy the pathogens in innate immunity.

Due to its essential role in innate immunity, defects in TLR signaling will induce multiple diseases (78). Animal model studies suggest that TLR signaling is involved in inflammation, cancer, airway diseases and autoimmune disease. SNP analysis also suggests that genetic changes of TLRs are also associated with the above diseases (147). Thus therapeutic modification of TLR signaling is a novel approach to many innate immunity related diseases.

1.7.2.1 Molecular Mechanism of TLR Signaling

When a host is infected with pathogens, the PAMP of pathogens interacts with specific TLRs, which activate the intracellular pathway and induce gene transcription. Ligand specificity is the most important issue in TLR signaling since defects in PAMP recognition may lead to auto-immune defects or hyporesponse to pathogen invasion. The LRRs within the N-terminus of TLRs are critical for receptor ligand specificity. In addition to the arrangement of LRRs, the cooperation between different receptors also contributes to the specificity of TLRs binding affinity. Combinations of different TLRs greatly increase the pathogen reorganization diversity. Some non-TLR family receptors also form a receptor complex with TLRs to promote TLR signaling (270).

Intracellular signal transduction of TLR signaling is generally defined as MyD88 dependent pathway and MyD88 independent pathways (6). In MyD88 mediated signal transduction, activated TLRs recruits MyD88 via the TIR domain and induce the assembly of MyD88/IRAK/TRAF6 complex. This signal complex subsequently activates NF-κB dependent gene transcription and the MAPK cascade. In the MyD88 independent pathway, another scaffold protein, TRIF is required for the IRF3 dependent gene transcription. Interestingly, the TLR signaling mediated NF-κB activation is dependent on MyD88 in the early stage of stimulation. However, the TRIF dependent pathway is critical for delayed NF-κB activation. Different pathways are utilized to make specific responses to pathogen diversity. For example, LPS induced TLR4 signaling activates the downstream pathway via both MyD88 dependent and independent pathways. However, the viral dsRNA induced TLR3 signaling only activates the TRIF dependent pathway.

TLR mediated gene transcription is also regulated via epigenetic modification. A number of studies suggest that chromosome modification plays a key role in selectively

turning on or off gene transcription (471). Interestingly, the differential regulation of pro-inflammatory mediators and antimicrobial effectors is controlled by distinct patterns of LPS-induced chromatin modifications, which might explain the stimulation tolerance in LPS signaling (139).

TLR signaling is also negatively regulated by some proteins, such as TOLLIP and IRAK-M (471). TOLLIP associates with activated TLRs and blocks the activation of IRAK. An IRAK inactive variant, IRAK-M works as a dominant negative to blocks the phosphorylation of IRAK and impairs the assembly of IRAK-1/TRAF6 complex. Though the biological function of these negative regulatory proteins is still unclear, these mechanisms might play an essential a role in preventing the hyperimmune response in the host.

1.7.2.2 Adhesion Signaling Proteins in LPS/TLR4 Signaling

LPS was first known to be an endotoxin, which induces a strong immune response in host. LPS activates monocytes and macrophages to produce proinflammatory cytokines, such as TNF-α, IL-1, and IL-12. LPS also induces the generation of oxidants, such as H_2O_2 and NO. The molecular mechanism of LPS signaling has been investigated in recent years . TLR4 has been identified as the LPS specific receptor. LPS associates with LBP. The LBP/LPS complex binds to the GPI-anchored receptor CD14 and TLR4 to activate them. This recruits the adaptor protein MyD88 and protein kinase IRAK to form a complex, which induces the activation of NF-κB and other kinases such as ERK, JNK and PKC, which regulate transcription of immune response genes and other aspects of the immune response.

LPS induced TLR4 activation leads to the formation of filopodia, lamellipodia, and membrane ruffles in Bac1 mouse macrophages (472). LPS induced cell spreading may

promote monocyte adhesion to the endothelial layers, which is a critical step for leukocyte transmigrate to the inflammatory site. LPS also regulates other cytoskeleton related immune responses, such as phagocytosis (163). Adhesion signaling proteins are involved in LPS signaling. Tyrosine phosphorylation of Pyk2 and Paxillin is enhanced in monocytes and macrophages following LPS stimulation (473). LPS induced paxillin tyrosine phosphorylation is mediated by both the MyD88 dependent and independent pathways (186). However, activation of FAK following LPS stimulation is independent of MyD88 (502). Interestingly, FAK forms a complex with TLR4 in enterocytes (269). LPS stimulation induces serine phosphorylation of FAK. The LPS/FAK signaling works as a brake to block cell migration. In addition to cytoskeleton rearrangement, the TLR4 signaling mediated gene transcription is also regulated by adhesion molecules. FAK is necessary for LPS induced IL-6 production (503). A similar defect in IL-6 production was also identified in cells stimulated with TNF-α (144,404). This suggests that FAK regulates a common pathway related to IL-6 production, such as MAPK cascade.

A novel interplay between integrin signaling and TLR4 signaling was proposed recently. Integrin induced PIP2 synthesis is critical for LPS signaling (239). TLR4 signaling is blocked following PIP2 depletion. PIP2 is also required for TRIAP membrane translocation. Membrane targeting of TRIAP facilitates the association between MyD88 and TLR4, which is a critical step for TLR4 signaling. Accordingly, TLR4 signaling is abolished in TRIAP null cells expressing a TRIAP PIP2 binding site mutant.

1.7.3 Phagocytosis

Phagocytosis is a receptor mediated internalization process, which ubiquitously exists in different kinds of organisms (453). In innate immunity, phagocytosis is involved in the

clearance of the microbes. Phagocytosis is also required for clearing apoptotic cells, which is critical for prevention of autoimmune diseases and inflammation. As an important infection defense mechanism, microbe mediated phagocytosis plays multiple roles in both innate immunity and adaptive immunity. The phagocytes not only take up pathogens but also destroy them via superoxide and proteases in phagosomes. In some specific receptor mediated phagocytotic mechanism, the phagocytotic signaling activates cytokine and chemokine production, which are critical for inflammatory responses. Most important of all, phagocytosis directly connects the innate immunity to adaptive immunity.

As in TLR signaling, phagocytosis receptors recognize the microbe via certain specific markers. However, unlike the TLR signaling, most of phagocytosis receptors associate with some opsonins which are coated on the surface of microbe. The phagocytosis of a microbe is mostly mediated by two sets of opsonin/receptor combinations. Internalization of immunoglobulin opsonized microbes is mediated by Fc receptors; internalization of complement opsonized microbes is mediated by complement receptors. Phagocytosis of a microbe is also mediated by some other receptors, such as CD14 and Mannose receptors, which recognize PAMPs instead of opsonins.

1.7.3.1 Fc Receptor Mediated Phagocytosis

Fc receptor belongs to a family of proteins that exhibit high affinity to the immunoglobulins. However, not all Fc-receptors are involved in phagocytosis, e.g. FcγRIIB inhibits phagocytosis. Upon stimulation, SFK are activated and induce the tyrosine phosphorylation of the ITAM motif in the cytoplasmic tail of the FcγR. Then Syk associates with the tyrosine phosphorylated ITAM and activates downstream signals, which includes Rho GTPase activation and PI3 kinase signaling. In the Rho GTPase family, CDC42 and

Rac regulate phagocytotic cup formation and closure respectively. However, the function of RhoA in FcγR mediated phagocytosis is still controversial due to the inconsistent results from different kind of cells. PI3K signaling is critical for the phagosome formation. It could be also involved in other stages of phagocytosis, such as pseudopod extension (150,431).

1.7.3.2 Adhesion Proteins in Fc Receptor Mediated Phagocytosis

Cytoskeleton rearrangement is a critical process in Fc Receptor mediated phagocytosis. Just like focal adhesions, actin and tyrosine phosphorylated proteins are enriched within the phagocytotic cup in a Fc receptor mediated phagocytosis (8). A number of focal adhesion proteins, such as paxillin, α-actinin and vinculin are found in the phagocytotic cup. Tyrosine phosphorylation of paxillin and Pyk2 is greatly enhanced following the activation of Fc receptors (244). All these studies suggest that adhesion signaling proteins might regulate Fc receptor mediated phagocytosis. However, the function of these proteins in Fc receptor mediated phagocytosis is still unclear.

Figure 1.1 Domain structure of FAK/Pyk2. The organization of FAK/Pyk2 domains are shown. Both the major tyrosine phosphorylation sites and PRRs are denoted. The black residues are for FAK and the red ones for Pyk2.

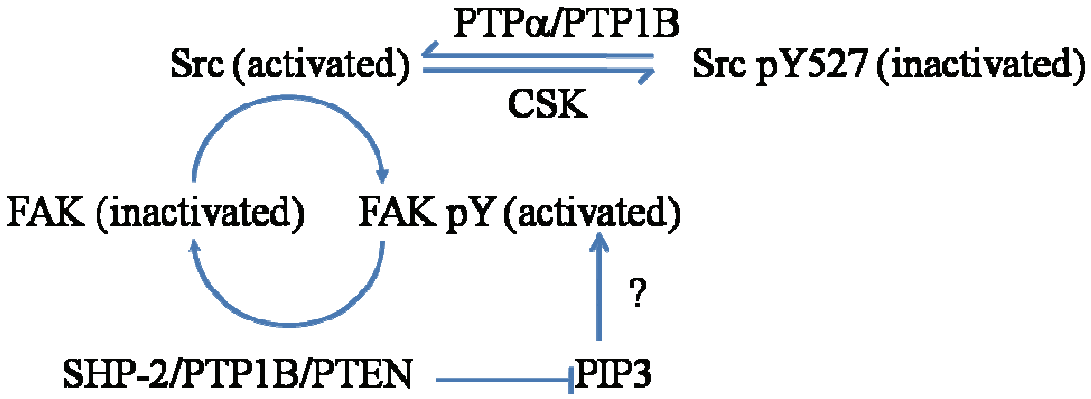


Figure 1.2 Regulation of FAK by protein tyrosine phosphatases. FAK is directly or indirectly regulated by protein tyrosine phosphatases. Moreover, it is possible that FAK is regulated by PIP3 level controlled by PTEN.

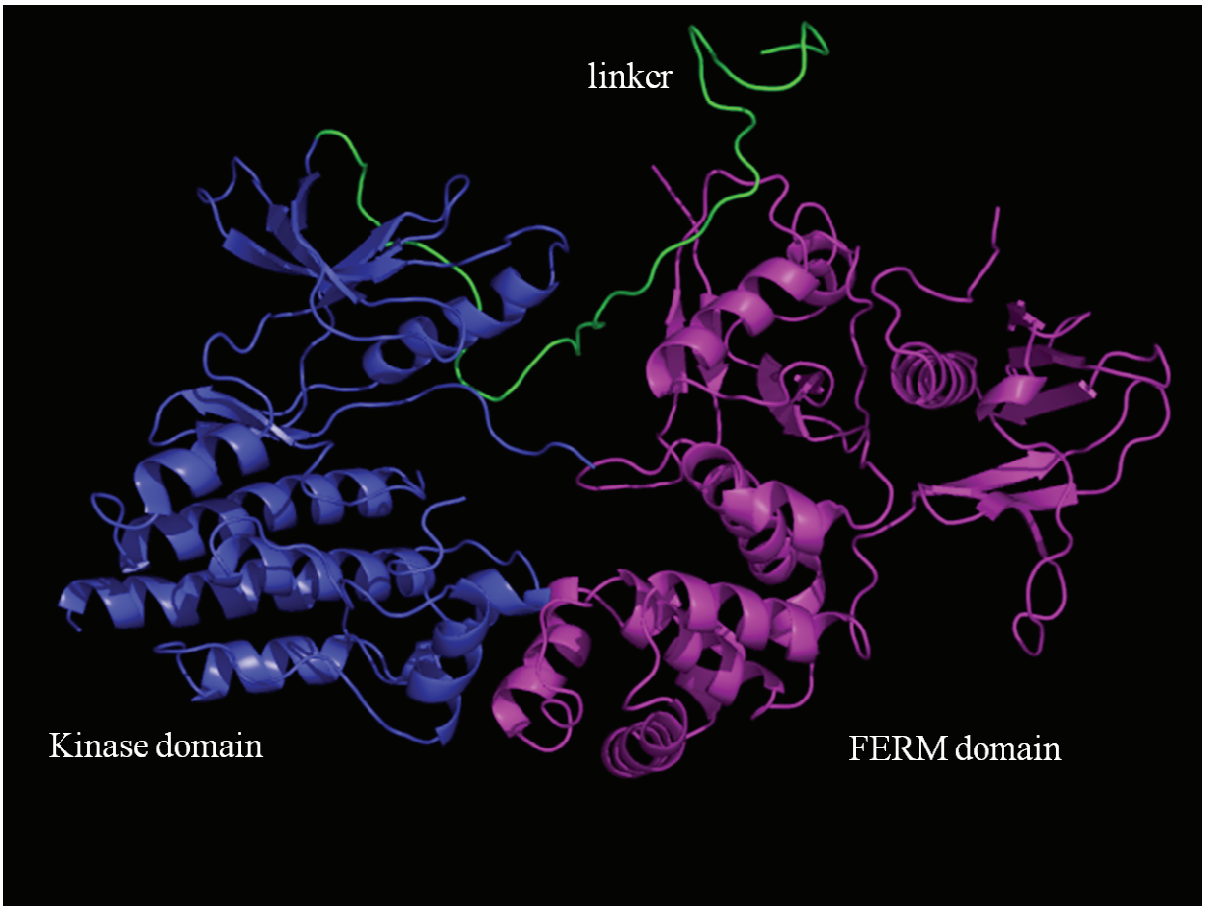


Figure 1.3 The crystal structure of auto-inhibited FAK.

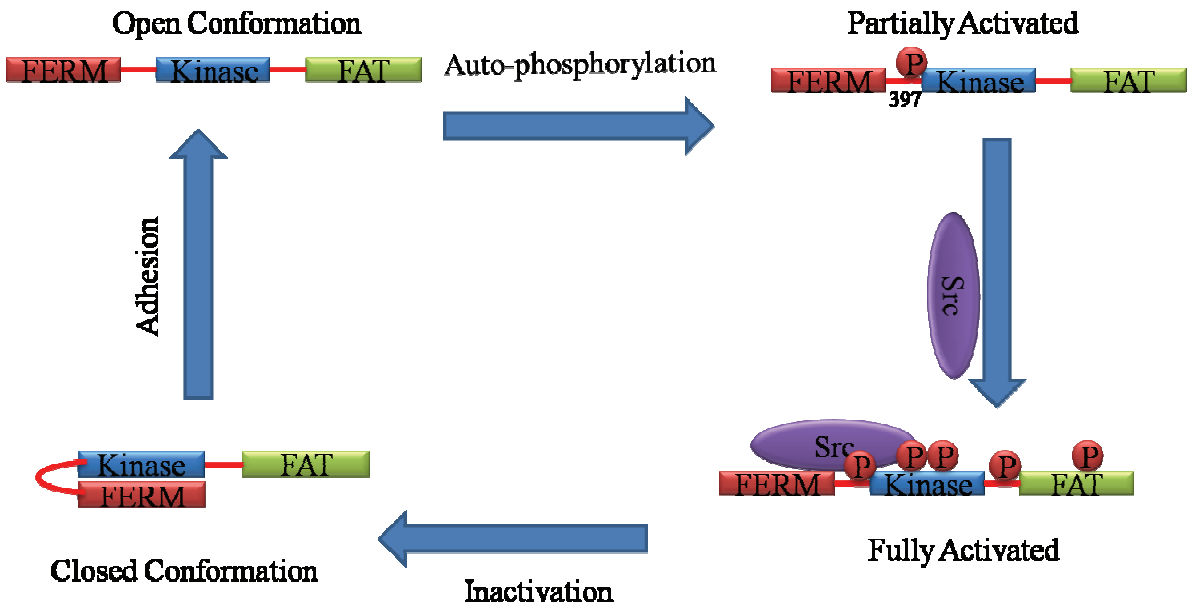


Figure 1.4 The FAK regulation model. In resting conditions, the kinase domain of FAK is masked by N-terminal FERM domain (Closed Conformation). The FERM/Kinase domain interaction is disrupted following cell attachment to the extracellular matrix, which exposes the linker for auto-phosphorylation. Then Src is recruited and induces the phosphorylation of other tyrosine residues, which makes FAK fully activated.

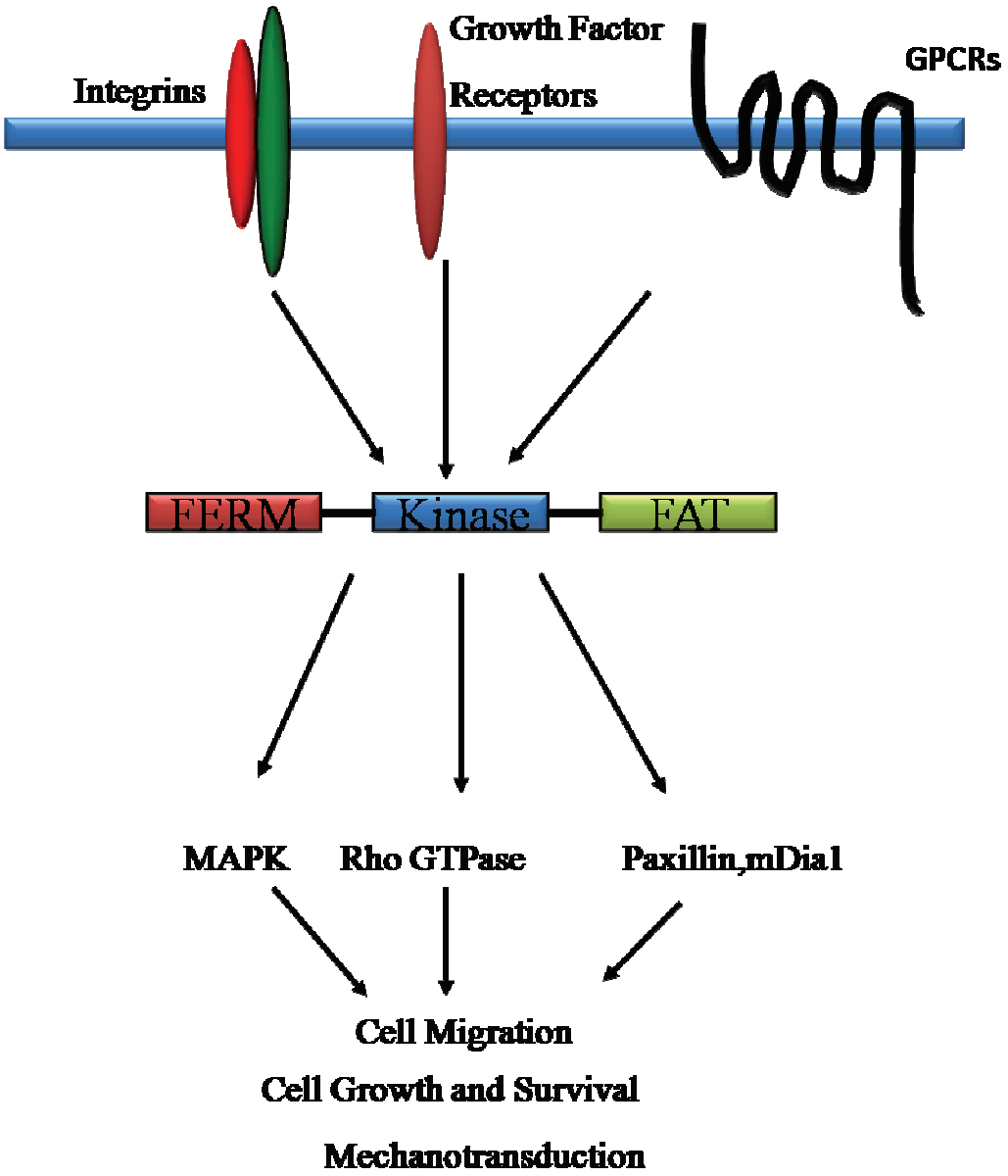


Figure 1.5 The role of FAK in multiple signaling. FAK is activated by the signals from integrins, growth receptor and GPCRs. The activation of FAK regulates cell behavior via multiple pathways.

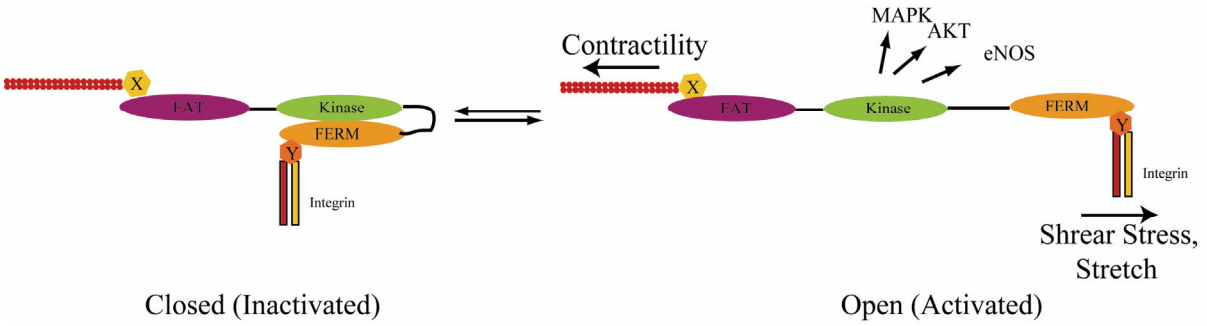


Figure 1.6 Stretch model of FAK activation. FAK is in a closed (inactive) conformation prior to stimulation. The N-terminus of FAK interacts with the receptors via an undefined mechanism (Y) and the C-terminus is tethered to the actin cytoskeleton via adaptor proteins (X, likely paxillin). When physical stimuli are applied, FAK is stretched to an open (active) conformation and transmits downstream signals.

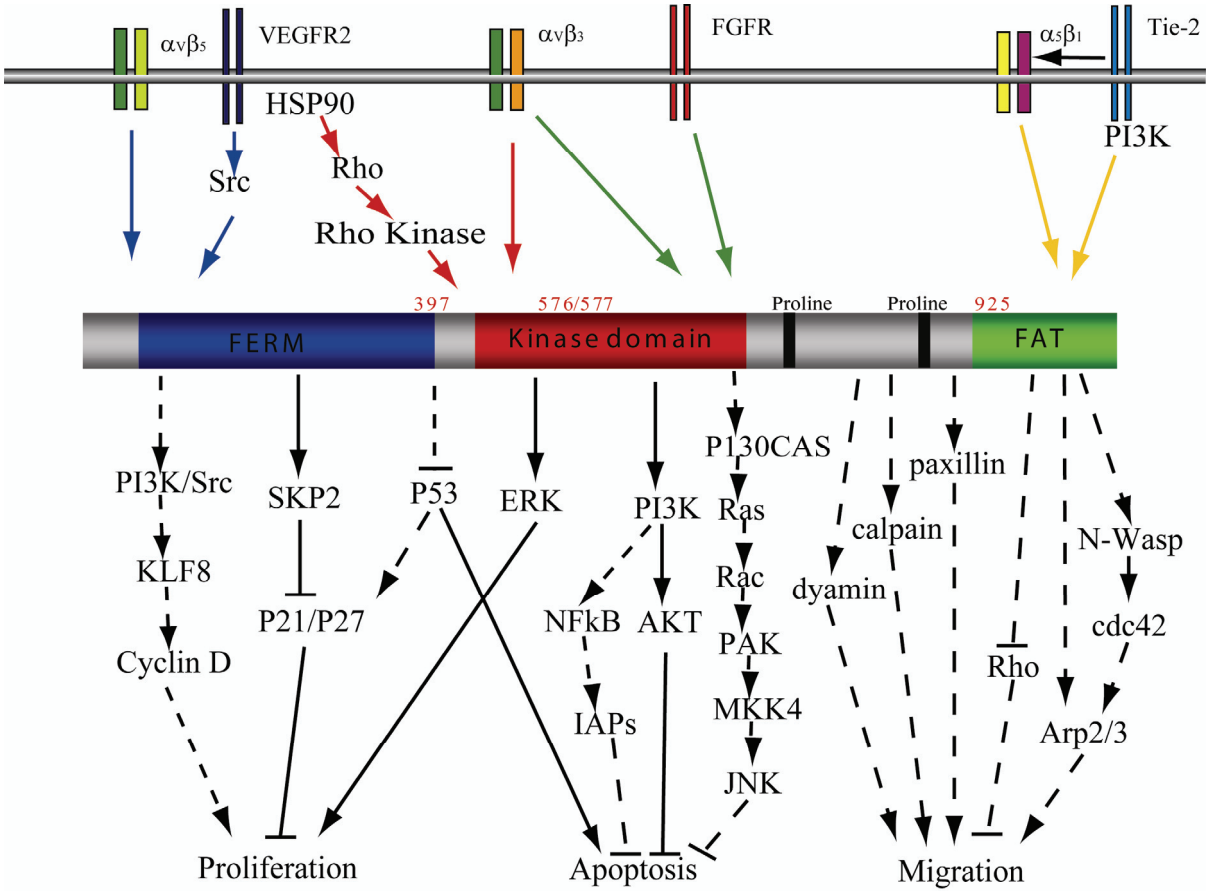


Figure 1.7 FAK and angiogenic signaling pathways. Multiple signals induce FAK activation in angiogenesis. Colored arrows denote FAK activation pathways, with pathways that act in a concerted fashion to activate FAK indicated in the same color.FAK coordinates cell survival, proliferation and migration to promote vessel formation. Solid arrows indicate survival and proliferation signaling pathways demonstrated in endothelial cells. Dotted lines represent signaling pathways demonstrated in other cell types.

CHAPTER 2
GSK-3- and ERK-Dependent Phosphorylation of Paxillin Regulates Cytoskeletal Rearrangement

This chapter has previously been published under the same title as : Cai X, Li M, Vrana J, and Schaller MD Mol Cell Biol. 2006 Apr;26(7):2857-68

2.1 Abstract

Paxillin is a 68-kDa focal adhesion associated protein that plays an important role in controlling cell spreading and migration. Phosphorylation of paxillin regulates its biological activity and thus has warranted investigation. Serine 126 and serine 130 were previously identified as two major ERK-dependent phosphorylation sites in Raf transformed fibroblasts. Here serine 126 is identified as a phosphorylation site induced by LPS stimulation of RAW264.7 cells. A number of other stimuli, including adhesion and CSF induce serine 126 phosphorylation in RAW264.7 cells, and NGF treatment induces serine 126 phosphorylation in PC12 cells. The kinase responsible for phosphorylation of this site is identified as GSK-3. Interestingly, this GSK-3-dependent phosphorylation is regulated via an ERK-dependent priming mechanism, i.e. phosphorylation of serine 130. Phosphorylation of S126/S130 was required to promote spreading in paxillin null cells and LPS induced cell spreading of RAW 264.7 cells was inhibited by expression of the paxillin S126A/S130A mutant. Furthermore, this mutant also retarded NGF induced PC12 cell neurite outgrowth. Hence, phosphorylation of paxillin on serine 126 and 130, which is mediated by an ERK/GSK-3 dual kinase mechanism, plays an important role in cytoskeletal rearrangement.

2.2 Introduction

Paxillin is a 68-kDa focal adhesion associated protein that functions as a scaffolding protein assembling signaling molecules into complex downstream of integrins (38,388). It plays an important role in regulating cell spreading and migration. The paxillin knock out mouse exhibits embryonic lethality, which suggests that paxillin plays an essential role in development (171). Paxillin contains five LD motifs in the N-terminal half of the molecule. These peptide motifs mediate protein-protein interactions and bind a number of proteins

including FAK and vinculin (37,451). Four LIM domains are found in the C-terminal half of paxillin, two of which are required for the discrete localization of paxillin to focal adhesions (33).

Multiple stimuli induce phosphorylation of paxillin, including growth factors, integrin-dependent cell adhesion to extracellular matrix and other ligands (36,388). Two major tyrosine phosphorylation sites, Y31 and Y118, have been identified in the N-terminal half of paxillin (396). Phosphorylation of these sites modulates docking of SH2 domain containing proteins, such as CRK, and is important for regulation of cell motility (360,497). In addition to tyrosine phosphorylation, serine and threonine phosphorylation sites have been identified in paxillin. Serine residues 188 and 190 are phosphorylated following integrin ligation (22). Threonines 398 and 403 in LIM2 and serines 457 and 481 in LIM3 are phosphorylated following cell adhesion and stimulation with angiotensin II (34,35). Phosphorylation of these LIM domain residues regulates focal adhesion localization of paxillin and/or cell adhesion to fibronectin. Though the upstream kinases responsible for phosphorylation of many of these sites remain unidentified, several kinases have been shown to directly phosphorylate paxillin. JNK phosphorylates threonine 178 and phosphorylation of this site functions in the regulation of cell migration (209). Two kinases, p38 MAP kinase and ERK, have been reported to phosphorylate serine 83 in murine/rat paxillin (205,223). This site is not precisely conserved in human paxillin, but p38 phosphorylates a similar sequence at serine 85 in the human homologue. P38-dependent phosphorylation of this site regulates neurite outgrowth in PC12 cells and ERK-dependent phosphorylation of the site regulates epithelial morphogenesis. Two additional serine phosphorylation sites in the N-terminal domain of paxillin, serines 126 and 130, were identified in Raf transformed cells

and phosphorylation is apparently mediated by the Raf/MEK/ERK pathway (483). However, it is unclear whether ERK directly phosphorylates these two sites and the function of phosphorylation of these sites has not been determined.

Glycogen synthase kinase 3 (GSK-3) was first identified as the enzyme that phosphorylates and regulates glycogen synthase (127). The two isoforms, GSK-3α and GSK-3β, share high similarity in structure but are not redundant in function (116). GSK-3 is now known to phosphorylate a broad range of substrates and control many processes in addition to glycogen metabolism. GSK-3 plays a key role in regulating the Wnt signaling pathway and the control of cell proliferation (351). GSK-3 has also been suggested to regulate microtubule stability through phosphorylation of three microtubule/tubulin associated proteins, Tau, MAP 1B and CRMP-2 (161,180,499). Regulation of GSK3 activity, via regulation of microtubule dynamics is believed to play an important role in the regulation of neuronal cell axon polarity (508). GSK-3 has also been suggested to control actin cytoskeleton rearrangement since it can regulate formation of long lamellipodia in human keratinocytes (255).

Unlike many other kinases, GSK-3 is constitutively active in cells and initiation of downstream signaling is not modulated by activation of the kinase, but by modification of the substrate resulting in its interaction with GSK-3 (24). GSK-3 prefers a primed substrate, which has been previously phosphorylated by a priming kinase, and the priming phosphorylation increases the efficiency of substrate phosphorylation of most GSK-3 substrates by 100-1000-fold (135). In several instances the detailed mechanism of GSK-3 substrate phosphorylation has been elucidated. For example, casein kinase-2 is required to prime glycogen synthase to promote the sequential multi-site phosphorylation by GSK-3

(134), and casein kinase-1 was identified as a priming kinase promoting GSK-3 mediated β-catenin phosphorylation (306).

Here we identify paxillin, a focal adhesion associated protein, as a GSK-3 substrate. Serine 126 is identified as a phosphorylation site induced by LPS stimulation of RAW264.7 cells. Phosphorylation of this site is regulated by ERK, but is directly mediated by GSK-3. LPS induced cell spreading was partially inhibited in cells expressing the paxillin S126A/S130A mutant and this mutant was defective for promoting fibroblast spreading on fibronectin. Furthermore, we found ERK/GSK-3 mediated phosphorylation of paxillin is also involved in NGF induced PC12 cell neurite outgrowth. These data suggest that phosphorylation of paxillin at serine residues 126 and 130 plays an important role in the control of cytoskeleton rearrangements and provides insight into the molecular mechanism via which GSK-3 controls remodeling of the actin cytoskeleton.

2.3 Materials and Methods

Cells. RAW264.7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS). HEK293T cells were maintained in DMEM-F12 containing 10% FBS. PC12 cells were maintained as described previously (204). Primary murine peritoneal macrophages, isolated following thioglycollate injection (a kind gift from Dr. Glenn Matsushima), were maintained in RPMI 1640 containing 5% FBS for 1 week prior to LPS stimulation. Paxillin null cells were a generous gift from Dr. Sheila Thomas and were maintained in DMEM containing 15% FBS. In some experiments, RAW264.7 cells were starved in DMEM without serum overnight, then stimulated with LPS (1 μg/ml) (Sigma, St. Louis, MO), PMA (100 ng/ml) (Calbiochem, San Diego, CA), or M-CSF (1.32 nM) (PeproTech, Rocky Hill, New Jersey). PC12 cells were starved in DMEM containing 1%

FBS for 6 hr. The cells were then stimulated with 100 ng/ml NGF (Calbiochem, San Diego, CA). In some experiments cells were pretreated with the indicated doses of LiCl, U0126 (Calbiochem, San Diego, CA), GSK-3 inhibitor IX (Calbiochem, San Diego, CA) or GSK-3 inhibitor I (Biosource, Camarillo, CA) for 1 hr before stimulation. In some experiments PP2 (Calbiochem, San Diego, CA) was added 20 min before stimulation.

Molecular biology. The MEK constructs were gifts from Dr. Channing Der. The EGFP-paxillin β plasmid was a gift from Dr. Ken Jacobson (208). EGFP-paxillin derived point mutations were created by PCR using the Quik-Change mutation kit (Stratagene, La Jolla, CA). Sequence analysis was performed on each mutant to verify the intended point mutations and that no unintended mutations were present. These analyses were performed in the UNC-CH Genome Analysis Facility on a model 3730 DNA Analyzer (Perkin Elmer, Applied Biosystems Division) using the ABI PRISM^{TM} Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq DNA Polymerase, FS (Perkin Elmer, Applied Biosystems Division). The control siRNA and siRNA targeting GSK-3β (SMARTpool®) were from Dharmacon (Lafayette, CO).

Stable and transient transfection. RAW264.7, PC 12, HEK293T and paxillin null cells were transfected using Lipofectamine plus according to the manufacturer's instructions (Invitrogen Carlsbad, CA). To establish stable transfectants, RAW264.7 cells were incubated in fresh medium containing 10% FBS for 48 hr and then selected with 400 μg/ml G418. After 10 days, surviving cells were cultured in medium containing 10% FBS with 200 μg/ml G418. GFP expressing cells were further enriched by cell sorting by FACS. Despite this double selection procedure, the resulting population was heterogeneous, containing both GFP positive and negative cells. Further, EGFP-paxillin expression was lost upon passaging.

For this reason, studies with these cells were restricted to biochemical studies examining regulation of phosphorylation of the exogenous EGFP-paxillin constructs and single cell biological assays where expression of the EGFP-paxillin constructs could be validated by fluorescent microscopy. Stable populations of paxillin null cells re-expressing wild type or S126A/S130A paxillin were established by infection with pBABE retroviral vectors encoding these constructs followed by selection with puromycin. RAW264.7 cells were transfected with 75nM siRNAs using TransitTKO according to the manufacturer's instructions (Mirus Madison, WI). After 24 hours, the cells were starved in serum-free DMEM overnight, then stimulated with LPS.

Cell Spreading Assay. Paxillin null cells and variants re-expressing wild type and mutant paxillin were serum starved overnight, trypsinized and plated onto fibronectin coated dishes in serum-free medium. After various times, the cells photographed and the relative area of individual cells (>50 per experiment) was determined using Image J software. RAW264.7 cells expressing wild type or mutant EGFP paxillin fusion proteins were plated on 35-mm petri dishes and cultured overnight, then starved in serum free medium for 12 hr. The cells were then stimulated with 1 μg/ml LPS and cultured for 3 hr. Cells were examined using a Zeiss Axiovert 200 microscope. Transfected cells were identified as green cells by fluorescent microscopy and the morphology of the transfected cells scored by phase contrast microscopy. Round phase bright, refractile cells were scored as unspread. Phase dark and nonrefractile cells, and cells that had obviously become elongated (exhibiting a ratio of length to width of greater than 2:1) were defined as spread. At least 100 green cells were scored in each experiment.

Neurite outgrowth assay. The PC12 cell neurite outgrowth assay was performed as described previously (203). In brief, PC12 cells transiently transfected with constructs expressing wild type or mutant EGFP paxillin fusion proteins were plated on 35-mm petri dishes precoated with 10 μg/ml collagen I (BD Biosciences, San Jose, CA USA) and cultured overnight. The cells were starved in DMEM medium containing 1% FBS for 6 hr. The cells were then stimulated with 100 ng/ml NGF (Calbiochem, San Diego, CA) and cultured for 36 hr at 37°C. Cells were examined using a Zeiss axiovert 200 microscope. Transfected cells were identified as green cells by fluorescent microscopy and the morphology of the transfected cells scored by phase contrast microscopy. More than 100 green cells were examined in each experiment and the length of neurites was scored as described previously (109).

Protein purification and in vitro phosphorylation assay. The expression and purification of the N-terminal GST fusion proteins, GST-N-C3 and GST-N1-C1A, were performed as described previously (437). For *in vitro* kinase assays, GST fusion proteins were washed twice with reaction buffer (25 mM Hepes, pH7.5, 10 mM $MgCl_2$, 1 mM EDTA, 0.1 mM DTT) and aliquoted. 2 μg of substrate was incubated in reaction buffer containing 40 μM ATP with ERK1 (UBI, Lake Placid, NY) and/or active GSK-3 (Biosource, Camarillo, CA) at 30°C for 1 hr. The reactions were stopped by the addition of sample buffer. The samples were boiled and analyzed by Western blotting. To further examine phosphorylation by ERK, 2 μg of GST-N1-C1A was incubated in reaction buffer containing 40 μM ATP (2 μCi γ-[^{32}P]ATP) with ERK1 at 30°C. The reactions were stopped by the addition of sample buffer. The samples were boiled and analyzed by SDS-PAGE. The gel was stained with Coomassie blue before autoradiography.

Cell Lysis, Protein Analysis, and Immunoprecipitation. Cells were lysed in ice-cold modified RIPA buffer (418). Lysates were clarified, and protein concentrations were determined using the bicinchoninic acid (BCA) assay (Pierce, Rockford, IL). For immunoprecipitations, the paxillin antibody (BD Biosciences, San Jose, CA) or the PS126 antibody was incubated with 500 μg of cell lysate at 4°C for 1 hr or 16 hr. Immune complexes were precipitated at 4°C for 1 hr with protein A-Sepharose beads (Sigma, St. Louis, MO). For immunopreciptiations using the paxillin monoclonal antibody, the beads were coated with AffiniPure rabbit anti-mouse IgG (Jackson ImmunoResearch Labs, West Grove, PA). Immune complexes were washed twice with ice-cold lysis buffer, and once with ice-cold PBS. Beads were resuspended in sample buffer, boiled to elute the proteins, and the samples were analyzed by Western blotting. The paxillin phosphospecific antibodies, PS126, PY31, PY118 and pERK antibodies were from Biosource (Camarillo, CA). The ERK and ERK2 antibodies were from Santa Cruz Biotech (Santa Cruz, CA). The PS83 antibody was a generous gift from Drs. Ishibe and Cantley (Yale University).

Immunofluorescence. Paxillin null fibroblasts were transiently transfected with plasmids encoding GFP-paxillin fusion proteins and cultured overnight. The cells were then trypsinized, held in suspension for 45 minutes and plated onto fibronectin coated coverslips for 60 minutes prior to fixation. PC12 cells were plated on collagen coated coverslips and stimulated with NGF for various times prior to fixation. Cells were fixed in 3.7% formaldehyde and permeabilized with 0.5% Triton X-100. Serine 126 phosphorylation was detected using PS126 and a rhodamine or fluorescein conjugated anti-rabbit antibody (Molecular Probes Eugene, OR) as previously described (93). The cells were visualized using a Nipkow-type spinning disk confocal scan head attached to an IX81inverted

microscope (Olympus Dulles, VA) equipped with a 60X 1.45 NA objective, a CCD camera, and controlled by AQM Advance 6 software.

2.4 Results

LPS induces paxillin phosphorylation in macrophages. Paxillin, a major focal adhesion-associated protein, becomes tyrosine phosphorylated upon LPS stimulation of human monocytes and other macrophage cell lines (474). To examine phosphorylation of paxillin in RAW264.7 cells, the cells were serum starved overnight, and then stimulated with 1 μg/ml LPS for 60 minutes prior to lysis. Paxillin was immunoprecipitated from cell lysates and the immune complexes analyzed by Western blotting. The most striking observation from this experiment was the evident retardation of the relative mobility of paxillin following LPS stimulation (Fig 2.1A), which suggests that paxillin may be phosphorylated following LPS stimulation. To determine if the LPS induced shift in gel mobility of paxillin was in fact due to phosphorylation, paxillin immune complexes from LPS stimulated RAW264.7 cells were incubated with calf intestine alkaline phosphatase for half an hour. Phosphatase treatment decreased the apparent molecular weight of paxillin to the original level and lower, suggesting that the LPS induced molecular weight shift was due to phosphorylation and that basal levels of phosphorylation of paxillin also produced a small shift in the apparent molecular weight of the protein. To directly examine tyrosine phosphorylation of paxillin, site-specific antibodies were used to probe Western blots of cell lysates from LPS-stimulated and unstimulated RAW264.7 cells. The two major tyrosine phosphorylation sites of paxillin, tyrosine 31 and tyrosine 118, were phosphorylated in response to the LPS stimulation (Fig 2.1B). In addition to tyrosine phosphorylation, it was also likely that LPS induced serine/threonine phosphorylation of paxillin. In particular, serine 126 was an excellent

candidate as an LPS induced site of phosphorylation since phosphorylation of this site has been correlated with a shift in the electrophoretic mobility of paxillin (478). A phosphorylation site-specific antibody recognizing PS126 was used to probe lysates of stimulated and unstimulated cells. Whereas paxillin from unstimulated cells was weakly recognized by the PS126 antibody, LPS stimulation induced a dramatic increase in signal (Fig 2.1B). Phosphorylation of serine 83 was also induced in response to LPS. In order to confirm the specificity of the PS126 antibody, its ability to recognize wild type paxillin and an S126A mutant of paxillin was examined. RAW264.7 cell line derivatives stably expressing EGFP-paxillin or EGFP-paxillinS126A were established by drug selection using G418. GFP expressing cells were further enriched by cell sorting by FACS. These two cell populations were challenged with LPS and cell lysates blotted with the PS126 antibody (Fig. 2.1C). As observed with endogenous paxillin, the wild type EGFP-paxillin was recognized weakly by this antibody in unstimulated cells and the signal was profoundly increased upon LPS stimulation. In contrast, EGFP-paxillinS126A was not recognized by the PS126 antibody under either set of conditions. These results demonstrate that this antibody specifically recognizes paxillin that is phosphorylated on serine 126. To establish the generality of the observation that serine 126 phosphorylation was induced by LPS, primary cultures of peritoneal macrophages and two additional macrophage cell lines, J774 and Bac1, were also analyzed. After LPS stimulation serine 126 was phosphorylated in each of these cell types (Fig. 2.1D), which suggests that this phosphorylation event is a general response to LPS signaling in macrophages. To estimate the stoichiometry of phosphorylation at serine 126, the PS126 antibody was used to immunoprecipitate paxillin following LPS stimulation of RAW264.7 cells. The amount of paxillin recovered in the immune complex and the

amount remaining in the supernatant was examined by Western blotting. From these studies it is estimated that 30-40% of paxillin is phosphorylated on serine 126 following LPS stimulation.

Serine 126 phosphorylation is ERK dependent. Serine 126 and serine 130 were first identified as paxillin phosphorylation sites in Raf transformed NIH 3T3 cells (480). In this scenario, additional evidence demonstrated that this phosphorylation event occurred downstream of the Raf-MEK-ERK pathway. It is well established that ERK is activated in macrophages after LPS stimulation (420). To begin to address the role of ERK in LPS-induced paxillin phosphorylation, serine 126 phosphorylation was temporally compared with ERK activation following LPS stimulation of RAW264.7 cells. ERK was strongly activated at 40 and 60 minutes following LPS stimulation, as assessed by blotting with a phosphorylation specific ERK antibody (Fig 2.2A). Phosphorylation of serine 126 was increased after 40 minutes and more dramatically increased following 60 minutes of stimulation. Thus the kinetics of ERK activation is consistent with a role in controlling phosphorylation of paxillin at serine 126. Furthermore, treatment of cells with two different MEK inhibitors, PD98095 and U0126, efficiently blocked the LPS induced phosphorylation of paxillin on serine 126 (Fig 2.2B, C). These findings demonstrate that the LPS induced activation of ERK is required for the induction of paxillin phosphorylation on serine 126. To determine if activation of the ERK signaling pathway is sufficient to induce serine 126 phosphorylation, wild type MEK1 or constitutively active MEK1 were transiently transfected into 293T cells. The latter induced activation of ERK and phosphorylation of paxillin at serine 126 (Fig. 2.2D). These results demonstrate that serine 126 phosphorylation is regulated by ERK signaling.

Serine and tyrosine phosphorylation of paxillin are independent. Recently serine 83 of paxillin was identified as a direct ERK phosphorylation site (224). An intriguing mechanism regulating ERK-dependent phosphorylation of serine 83 has been proposed. Phosphorylation of paxillin at tyrosine 118 creates a docking site for ERK, which recruits ERK into complex facilitating serine 83 phosphorylation (226). In order to investigate whether a similar mechanism is used in the regulation of serine 126 phosphorylation in response to LPS in macrophages, a paxillin mutant was analyzed. RAW264.7 cells were transfected with EGFP-paxillin or EGFP-paxillinY31F/Y118F, a mutant in which the two major tyrosine phosphorylation sites of paxillin have been substituted with phenylalanines. The cells were challenged with LPS and then lysates were blotted with the PS126 antibody. LPS induced phosphorylation of the mutant on serine 126 (Fig 2.3A). While there was an apparent increase in serine 126 phosphorylation of the mutant relative to wild type, this was likely due to the increased expression level of the mutant. Notably the EGFP-paxillinY31F/Y118F mutant was not defective for serine 126 phosphorylation. Furthermore, treatment with PP2, a Src kinase inhibitor, potently blocked the LPS induced phosphorylation of tyrosine 118, but had little effect on serine 126 phosphorylation (Fig 2.3B). These findings demonstrate that serine126 phosphorylation occurs independently of phosphorylation at tyrosine 118. Given the proximity of serine 126 to tyrosine 118, it was also of interest to determine if serine126 phosphorylation could affect phosphorylation at tyrosine 118. This was tested using a pharmacological approach. U0126 blocked serine 126 phosphorylation without affecting phosphorylation at tyrosine 118 (Fig 2.3C). These data suggest that LPS induced paxillin phosphorylation at serine 126 and tyrosine118 are independent and controlled by separate

mechanisms. It further suggests that the mechanisms regulating ERK mediated serine 83 and serine 126 phosphorylation are distinct.

Paxillin serine 126 phosphorylation can be induced by different stimuli. Since paxillin phosphorylation at serine 126 is mediated by the ERK pathway, which plays a key role in many signaling pathways, it was of interest to determine whether the ERK mediated paxillin phosphorylation at serine 126 was specific for LPS signaling or occurred in response to other stimuli that activate ERK. ERK is activated upon integrin-dependent cell adhesion to extracellular matrix proteins (401). Further, paxillin was shown to become serine phosphorylated in response to adhesion to extracellular matrix in macrophages (102). To examine cell adhesion-dependent phosphorylation, RAW264.7 cells were trypsinized, incubated in suspension at 37^{o}C for half an hour, and then replated on fibronectin for half an hour at 37^{o}C prior to lysis. Phosphorylation at serine 126 decreased when cells were held in suspension. Upon cell adhesion, phosphorylation of paxillin at serine 126 was induced (Fig 2.4A). Serine 126 was also phosphorylated in RAW264.7 cells following PKC activation by stimulation with PMA (Fig 2.4B). Treatment of RAW264.7 cells with CSF, an important cytokine controlling macrophage function, also modestly induced phosphorylation of serine 126 (Fig 2.4C). Phosphorylation of serine 126 is also likely induced by growth factors that signal via receptor tyrosine kinases (477). Interestingly, the phosphorylation level of S126 was elevated following NGF stimulation in PC12 cells (Fig 2.4D). Each of these stimuli activate ERK and in each case inhibition of this signaling pathway with MEK inhibitors blocks phosphorylation of serine 126 in response to the stimulus. These findings suggest that serine 126 phosphorylation is a general response to activation of the ERK pathway.

Phosphorylation of serine 126 is abolished by GSK-3 inhibitors. Though the data demonstrate that residue 126 is a major serine phosphorylation site in paxillin that is dependent upon ERK activation, there is no evidence that this site is directly phosphorylated by ERK. Further, the sequence flanking serine 126 does not conform to a consensus ERK phosphorylation site. Note however, that serine 130 has also been identified as a paxillin phosphorylation site in Raf transformed NIH 3T3 cells (481). Upon phosphorylation of serine 130, serine 126 resembles a consensus GSK-3 phosphorylation site, SXXX(P)S. It therefore appeared likely that paxillin was phosphorylated by GSK-3 at serine 126. To examine whether serine 126 phosphorylation was dependent upon GSK-3, RAW264.7 cells were incubated with LiCl, which is an inhibitor of GSK-3, for 1 hr prior to simulation with LPS. Phosphorylation at serine 126 was blocked by LiCl in a dose dependent manner (Fig 2.5A). In addition to LiCl, two other pharmacological inhibitors of GSK-3, GSK-3 inhibitor IX and GSK-3 inhibitor I, were tested. Both efficiently inhibited paxillin phosphorylation at serine 126 in RAW264.7 cells in response to LPS (Fig 2.5B, C). Further LiCl also blocked serine 126 phosphorylation in NGF stimulated PC12 cells (Fig 2.5D). It is interesting to note that ERK activation is unattenuated in all samples treated with GSK-3 inhibitors. To further test the role of GSK-3β in regulating serine 126 phosphorylation, an siRNA approach was used. RAW264.7 cells were transfected with a control siRNA or a pool of siRNAs targeting GSK-3β. Western blotting demonstrated knockdown of GSK-3β, but not GSK-3α expression (Fig 2.5E). Transfected cells were stimulated with LPS and inhibition of GSK-3β reduced the induction of serine 126 phosphorylation on paxillin, but had no effect upon ERK activation (Fig 2.5E). The GSK-3β independent phosphorylation of serine 126 is likely mediated by GSK-3α. Although serine 126 phosphorylation downstream of multiple stimuli

is ERK-dependent, it is also GSK-3 dependent, and GSK-3 appears to operate downstream of ERK. These findings are consistent with the hypothesis that ERK phosphorylates paxillin at serine 130, priming paxillin for subsequent phosphorylation by GSK-3 at serine 126.

Phosphorylation of paxillin is mediated by an ERK/GSK-3 dual kinase mechanism. In order to examine whether paxillin can be directly phosphorylated by GSK-3 *in vitro*, the N-terminal half of paxillin was expressed in E. coli as a GST fusion protein, called GST-N-C3. Recombinant GSK-3β was incubated with GST-N-C3 in kinase reaction buffer containing ATP, and the phosphorylation of serine 126 monitored by Western blotting with the phospho-specific antibody. There was little evidence of serine 126 phosphorylation on GST-N-C3 following incubation with GSK-3β (Fig 2.6A). This again is consistent with the hypothesis that this GSK-3 site in paxillin is primed by phosphorylation by another kinase. ERK was a candidate for this kinase as phosphorylation of 126 *in vivo* was ERK-dependent and the sequence around serine 130 resembles an ERK phosphorylation site. ERK failed to directly phosphorylate serine 126. To test whether ERK functions as a priming kinase for GSK-3-dependent phosphorylation of paxillin, GST-N-C3 was pre-incubated with ERK *in vitro*, prior to incubation with GSK-3β in the *in vitro* kinase reaction and phosphorylation measured by blotting with PS126. Under these conditions, serine 126 phosphorylation was dramatically increased (Fig 2.6A). To confirm that GSK-3-dependent phosphorylation *in vitro* was dependent upon serine 130 phosphorylation, a GST-N-C3 variant with a substitution of alanine for serine 130 was also analyzed. While GST-N-C3 was phosphorylated at serine 126 when incubated *in vitro* with ERK and GSK-3, GST-N-C3/S130A was not (Fig 2.6B), demonstrating the requirement of the priming site for GSK-3-dependent phosphorylation *in vitro*. To further determine if ERK could phosphorylate serine

130 *in* vitro, recombinant ERK was incubated with GST-N1-C1A in kinase reaction buffer containing [γ^{32}P] ATP. Wild type GST-N1-C1A was efficiently phosphorylated by ERK *in vitro*, whereas the S130A variant of GST-N1-C1A was marked defective for phosphorylation, exhibiting approximately 50% of the level of phosphorylation of the wild type protein (Fig. 2.6C). Further, the paxillin S130A mutant was expressed transiently as an EGFP-fusion protein in PC12 cells and phosphorylation of serine 126 in response to NGF stimulation measured by Western blotting. While wild type EGFP-paxillin was phosphorylated on serine 126 upon NGF stimulation, the S130A mutant was not phosphorylated in response to NGF (Fig 2.6D). These data suggest that paxillin is phosphorylated by ERK at serine 130, both *in vitro* and *in vivo*, and that this phosphorylation event primes paxillin for phosphorylation by GSK-3 on serine 126 (Fig 2.6E).

Localization of paxillin phosphorylated on serine 126. Paxillin null fibroblasts were used for the initial studies. The cells were transiently transfected with EGFP-paxillin or EGFP-paxillinS126A, then fixed and stained with the PS126 antibody (Fig. 2.7A). Cells expressing the wild type and mutant EGFP fusion proteins were identified by fluorescent microscopy. The cells expressing wild type EGFP-paxillin stained positive with the PS126 antibody, exhibiting nuclear, cytoplasmic and focal adhesion staining. As the untransfected cells in the population also exhibited nuclear staining, this is apparently non-specific (data not shown). Besides this background staining, the PS126 staining was similar to the EGFP-paxillin localization. The localization of the mutant protein was similar to EGFP-paxillin, however, cells expressing EGFP-paxillinS126A exhibited only the background nuclear staining with the PS126 antibody. These data suggest that PS126 exhibits sufficient specificity in

immunofluorescence experiments that it can be used to determine the localization of paxillin that is phosphorylated on serine 126.

NGF stimulated PC12 cells were also fixed and stained with the PS126 antibody (Fig. 2.7B). PS126 staining is dramatically increased after LPS stimulation and was located at the tips of developing neurites several hours after NGF treatment. After 30 hours of NGF stimulation, long neurites had grown and PS126 staining was enriched near the tips of these neurites. These studies demonstrate the localization of paxillin that is phosphorylated on serine 126 at dynamic sites of cytoskeleton remodeling and suggest the potential role of serine 126 and serine 130 phosphorylation of paxillin in controlling NGF induced neurite outgrowth.

ERK/GSK-3 mediated phosphorylation of paxillin is involved in cell spreading. To address the function of phosphorylation of paxillin at residues 126 and 130, paxillin null fibroblasts stably expressing wild type paxillin or the S126A/S130A mutant were established. The pBABE retroviral vector, engineered to encode the paxillin constructs, was used to infect the paxillin null cells and the infected cells selected using puromycin. Western blotting demonstrated expression of the wild type and mutant proteins (Fig. 2.8A). Further the wild type protein was phosphorylated on serine 126 in cells growing in culture, whereas the mutant was not (Fig. 2.8A). Paxillin null cells exhibit a defect in cell spreading on fibronectin that can be rescued by re-expression of wild type paxillin (170). To quantify spreading, cells were serum starved overnight, then plated on fibronectin and photographed at various times. The area of spread cells was determined using the Image J software. The null cells and wild type paxillin re-expressers increase in area over time, but at each time point the average area of paxillin expressing cells was approximately 25% greater that the

average area of the null cells (Fig. 2.8B). In contrast, cells expressing the S126A/S130A mutant spread slower than wild type expressing cells, only exceeding the average area of the null cells after 60 minutes on fibronectin. Thus phosphorylation of these sites is required for the paxillin-dependent spreading of fibroblasts on fibronectin.

LPS induces distinct changes in cell morphology and actin organization in monocytes and macrophages. Serum starved RAW264.7 cells are round and phase bright when examined by phase contrast microscopy, whereas many LPS stimulated cells are phase dark and spread or exhibit an elongated phenotype (data not shown). To begin to address the role of serine 126 phosphorylation in the control of cell spreading, MEK and GSK-3 were inhibited with U0126 and LiCl respectively. LPS stimulated RAW264.7 cell spreading was inhibited by both compounds (data not shown). To directly explore the role of paxillin in regulation of this phenotype, RAW264.7 cells were stably transfected with EGFP-paxillin or EGFP-paxillinS126A/S130A and the cells were stimulated with LPS to induce spreading. Transfected cells were identified as green cells by fluorescent microscopy and the morphology of both the transfected and untransfected cells scored by phase contrast microscopy (Fig 2.8C). Approximately 25% of the cells expressing EGFP-paxillin exhibited a spread/elongated morphology (Fig 2.8D). This frequency of spreading was very similar to that observed for untransfected cells in the same culture. In contrast, cells expressing EGFP-paxillinS126A/S130A exhibited only 10% cell spreading/elongation following LPS stimulation. This mutant is apparently functioning in a dominant negative fashion to inhibit the action of endogenous wild type paxillin, suggesting that phosphorylation at serines 126 and 130 is important for optimal spreading. Therefore, ERK/GSK-3 mediated

phosphorylation of paxillin is involved in the control of LPS induced RAW264.7 cell spreading.

ERK/GSK-3 mediated phosphorylation of paxillin is involved in NGF induced PC12 cell neurite outgrowth. Both GSK-3 and ERK are required for NGF induced neuronal cell neurite outgrowth (43,367). To assess the role of ERK/GSK-3-mediated paxillin phosphorylation in neurite extension, PC12 cells were transiently transfected with EGFP-paxillin or EGFP-paxillinS126A/S130A, and the NGF-induced neurite extension of these cells was examined after 36 hours. Transfected cells were identified as green cells by fluorescent microscopy and neurite extension examined by phase contrast microscopy (Fig 2.9A). Approximately 36% of the PC12 cells expressing EGFP-paxillin exhibited neurites longer than two cell bodies and 29% of cells produced neurites longer than three cell bodies (Fig 2.9B). Neurite extension in nontransfected cells in the same culture exhibited a similar morphological response to NGF. In contrast, cells expressing EGFP-paxillinS126A/S130A exhibited a retardation in neurite extension. Only 23% of the cells expressing EGFP-paxillinS126A/S130A had neurites longer than two cell bodies and 16% of the cells had neurites longer than three cell bodies. Cells transiently transfected with EGFP-paxillinS126D/S130D exhibited similar NGF induced neurite extension as observed in untransfected and EGFP-paxillin transfected cells. These findings suggest that phosphorylation of paxillin at serine residues 126 and 130 is involved in the control of NGF-induced neurite extension.

2.5 Discussion

GSK-3 is a key regulatory component of a large number of cellular processes and aberrant control of GSK-3 regulated pathways plays a role in a number of human diseases,

such as diabetes, Alzheimer's disease and cancer (115,238,350). More than 40 proteins are phosphorylated by GSK-3, and these substrates include metabolic proteins, cytoskeleton proteins and transcription factors (114). Here we identify a new GSK-3 substrate, the focal adhesion associated protein paxillin. GSK-3 phosphorylates paxillin at serine 126 and requires priming by phosphorylation of serine 130. Our findings suggest that GSK-3 mediated serine 126 phosphorylation is a general response to activation of the ERK pathway and is controlled by ERK-dependent priming. Using a mutant of paxillin that cannot be phosphorylated at serines 126 and 130 as a tool to explore the function of these phosphorylation events, we provide evidence that phosphorylation of these sites controls cell spreading and neurite extension in a macrophage and neuronal cell line respectively.

Paxillin phosphorylation at serine 126 is ERK dependent but the site is not a direct ERK phosphorylation site, in contrast to serine 83, which is directly phosphorylated by ERK (220,259). Interestingly, phosphorylation of serine 83 depends upon phosphorylation of paxillin at tyrosine 118, which creates a docking site for ERK and consequently phosphorylation of paxillin at serine 83 (225). The ERK-dependent phosphorylation of serine 126/130 is mediated by a distinct mechanism. Pharmacological inhibitors that block paxillin tyrosine phosphorylation did not block serine 126 phosphorylation. Further, a paxillin mutant with phenylalanine substitutions for the major sites of tyrosine phosphorylation, Y31F/Y118F, was phosphorylated at serine 126 following LPS stimulation. These data suggest that there are two different ERK mediated paxillin phosphorylation events and that these occur via two different mechanisms, one of which is dependent upon paxillin tyrosine phosphorylation (serine 83) and the other independent (serine 126/130). Thus

tyrosine phosphorylation provides an intriguing mechanism to direct site selective phosphorylation of paxillin by ERK.

ERK has been identified as a priming kinase for GSK-3 mediated paxillin phosphorylation at serine 126. In addition to paxillin, this ERK/GSK-3 dual kinase mechanism of phosphorylation has been reported for HSF-1, and results in the inactivation of HSF-1 (85,187). Another ERK dependent mechanism of GSK-3 activation has also been reported, in which ERK can operate through a downstream kinase to activate GSK-3 (159). However, this mechanism is unlikely to control ERK/GSK-3 mediated paxillin phosphorylation at serine 126 for several reasons. First, on its own the active form of GSK-3 cannot phosphorylate serine 126 of paxillin *in vitro*. Second, expression of constitutively active GSK-3 cannot induce serine 126 phosphorylation *in vivo* (data not shown). Third, disruption of the priming site, serine 130, can inhibit serine 126 phosphorylation both *in vivo* and *in vitro.* These results suggest that paxillin is phosphorylated by an ERK/GSK-3 dual kinase mechanism, and that the major regulatory event is substrate priming for GSK-3 phosphorylation by ERK.

Why do cells use such a complicated regulatory mechanism to control GSK-3 mediated substrate phosphorylation? The requirement for the precise temporal and spatial regulation of downstream signaling may be the answer to this question. Signaling events might only be transduced under local conditions where the priming kinase is activated and where GSK-3 remains active due to the absence of inhibitory signals regulating GSK-3 serine phosphorylation. In macrophages, LPS induces PI3K/AKT activation resulting in the phosphorylation-dependent inactivation of GSK-3. This results in the nuclear accumulation of β-catenin and transcription of genes regulated by the β-catenin/Lef complex (331). In

contrast, GSK-3 mediated paxillin phosphorylation is stimulated by ERK activation via a priming mechanism upon LPS stimulation. Thus two different downstream signaling pathways controlled by LPS have opposing effects on GSK-3 signaling, one impairing β-catenin phosphorylation and the other promoting paxillin phosphorylation. Similarly, in neuronal cells, both activation and inhibition of GSK-3 regulated signaling processes are apparently required to promote neuronal cell polarity and axon extension. The dephosphorylation of two GSK-3 substrates, APC and CRMP-2, is required for axon extension and concomitantly the phosphorylation of MAP1b by GSK-3 is required (104,160,500,509). We have now shown that paxillin is phosphorylated by GSK-3 downstream of NGF stimulation and that phosphorylation of paxillin is required for efficient neurite outgrowth. These paradoxical observations can be reconciled if populations of active and inactive GSK-3 are spatially segregated within the cell.

Paxillin plays an important role in neurite outgrowth since a number of mutants act in a dominant negative fashion to retard this process. Expression of a paxillin variant lacking the LD4 motif inhibits the neurite extension of PC12 cells upon EGF stimulation (230). Expression of the paxillin p38 MAPK phosphorylation site mutant S85A also strongly inhibits the neurite outgrowth of PC12 cells following NGF stimulation (202). It has been reported that both GSK-3 and ERK activity are required for neurite outgrowth (44,368), and phosphorylation of paxillin at serine 126 and 130 via GSK-3 and ERK is also important for NGF-induced neurite extension in PC12 cells. Collectively this data suggests that multiple sites of phosphorylation on paxillin, which are regulated by different kinases, may all function in the control of neuronal cell polarity. This is an intriguing mechanism where

multiple signaling pathways may converge on the same substrate to regulate a biological response.

How ERK/GSK-3 mediated phosphorylation of paxillin modulates cytoskeleton rearrangement remains to be elucidated. Since serine 126 and 130 are in proximity to the LD2 motif, it seems likely that phosphorylation regulates binding to other proteins. However, our results and other published data suggest phosphorylation of paxillin at these sites does not appreciably alter the binding of paxillin to any proteins that are known to dock to LD2 (data not shown) (479). It is also possible that paxillin phosphorylation is involved in control of the activity of paxillin associated signaling molecules. In paxillin null cells, the associated tyrosine kinase FAK exhibits a defect in tyrosine phosphorylation, suggesting a role for paxillin in controlling its activity (169). However, null cells re-expressing wild type paxillin or the S126A/S130 mutant exhibit similar levels of FAK phosphorylation (data not shown). Perhaps paxillin phosphorylation modulates the activity of other associated enzymes including the ArfGAP, PKL/Git1, and the Rac exchange factor, Cool-1/Pix. Interestingly, phosphorylation of serine 83 of paxillin has been linked to the control of Rac activity in epithelial cells (219). Investigation of the mechanism of control of downstream signaling pathways by serine 126/130 phosphorylation of paxillin will be the focus of future investigations.

2.6 Acknowledgements

We thank Adi Dubash for laying some of the experimental groundwork for this study. Thanks to Dr. William Snider, Dr. Glenn Matsushima and Dr. Sheila Thomas for providing reagents for this project. Thanks to members of the lab, Danielle Scheswohl, Jessica Harrell and Martin Playford, for productive discussions. We are indebted to Dr. Jim Bear for the use

of his microscope facility and members of his lab, particularly Liang Cai, for advice. This work was supported by NIH grants CA90901 and HL45100 (to MDS).

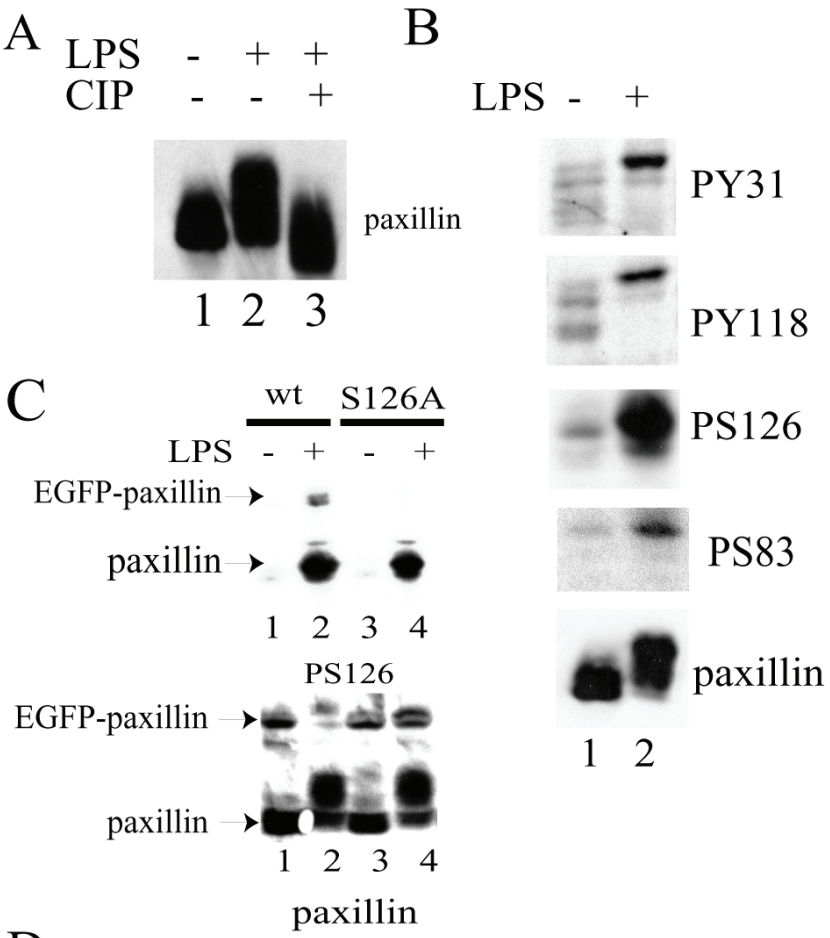

A
LPS - + +
CIP - - +
paxillin
1 2 3
B
LPS - +
PY31
PY118
PS126
PS83
paxillin
1 2
C
wt S126A
LPS - + - +
EGFP-paxillin
paxillin
1 2 3 4
PS126
EGFP-paxillin
paxillin
1 2 3 4
paxillin
D
LPS - 5' 10' 30' 60' 90' - + - +
PS126
paxillin
1 2 3 4 5 6 7 8 9 10
Primary Macrophages J774 Bac1
E
Lysate
IP
Sup
paxillin
1 2 3

Figure 2.1 LPS induces paxillin tyrosine and serine phosphorylation. (A) RAW264.7 cells were serum starved overnight (lane 1), then stimulated with 1 μg/ml LPS for 1 hr (lanes 2, 3) prior to lysis. Paxillin was immunoprecipitated from 500 μg of cell lysate. Half of the immune complex from the LPS stimulated sample was incubated with calf intestine alkaline phosphatase for half an hour (lane 3). The immune complexes were then analyzed by Western blotting. (B) Cell lysates from unstimulated (lane 1) and LPS stimulated (lane 2) RAW264.7 cells were Western blotted using phosphospecific (top 4 panels) or paxillin antibodies (bottom panel). (C) RAW264.7 cell line derivatives stably expressing EGFP-paxillin (lanes 1, 2) or EGFP-paxillinS126A (lanes 3, 4) were serum starved (lanes 1, 3) or starved and then stimulated with LPS (lanes 2, 4). Cell lysates were blotted with the PS126 (top) or paxillin antibody (bottom). The positions of the exogenous EGFP-paxillin and endogenous paxillin are indicated. (D) Primary peritoneal macrophages (lanes 1-6) were stimulated with LPS (1 μg/ml) for the indicated times prior to lysis. J774 cells (lanes 7, 8) and Bac1 cells (lanes 9, 10) were starved (lanes 7, 9) or stimulated with LPS (1 μg/ml)(lanes 8, 10) for 1 hr prior to lysis. Lysates were blotted with PS126 and paxillin antibodies. (E) Lysates from RAW 264.7 cells stimulated with LPS were analyzed. 50 μg of lysate was directly analyzed (lane 1) or 500 μg of lysate was immunoprecipitated using PS126. Ten percent of the immune complex (lane 2) and supernatant (lane 3) was analyzed by Western blotting for paxillin.

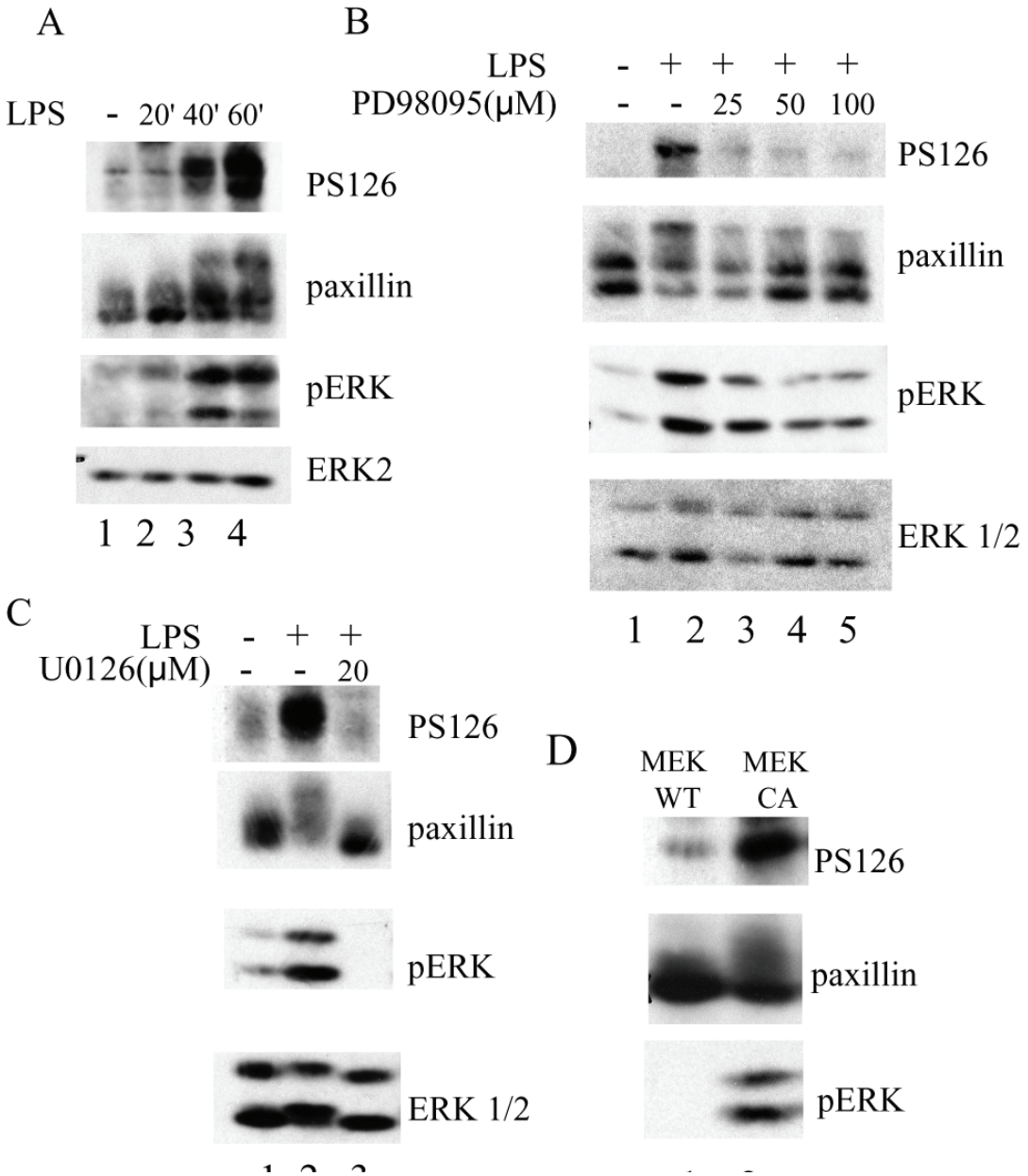


Figure 2.2 LPS induced paxillin serine 126 phosphorylation is ERK-dependent. (A) RAW264.7 cells were serum starved overnight (lane 1), and then stimulated with 1 μg/ml LPS for the indicated time (lane 2-4). Cell lysates were blotted with the PS126, paxillin, pERK and ERK2 antibodies. (B) RAW264.7 cells were serum starved overnight (lane 1), and then pretreated with the MEK inhibitor PD98095 at the dose shown for 1 hr. Cells were stimulated with LPS (1 μg/ml) for 1 hr. Cell lysates were blotted with PS126, paxillin, pERK and ERK antibodies. (C) RAW264.7 cells were treated as in B, except MEK was inhibited with 20 μM U0126. (D) Wild type or constitutively activated MEK was transiently expressed in 293 cells and lysates were blotted with the PS126, paxillin and pERK antibodies.

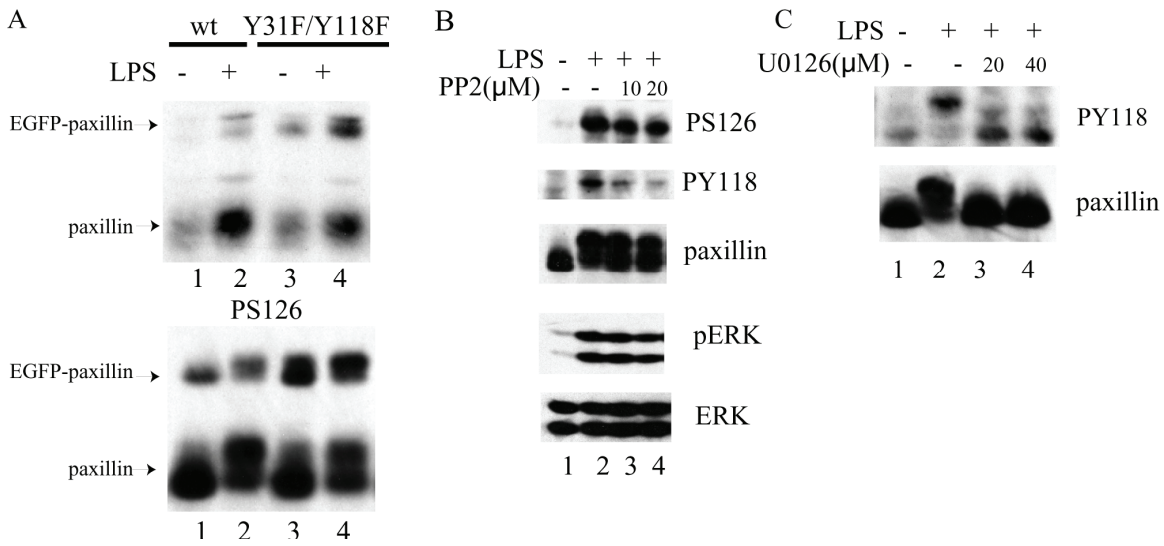


Figure 2.3 Paxillin serine and tyrosine phosphorylation are regulated by separate pathways. (A) RAW264.7 cell line derivatives transiently expressing EGFP-paxillin (lanes 1, 2) or EGFP-paxillinY31F/Y118F (lanes 3, 4) were stimulated with LPS (lanes 2, 4) and cell lysates blotted with the paxillin and PS126 antibodies. The positions of the exogenous EGFP-paxillin and endogenous paxillin are indicated. (B) RAW264.7 cells were serum starved overnight (lane 1), and then pretreated with the Src inhibitor PP2 at the doses shown for 20 min. Cells were stimulated with LPS (1 μg/ml) for 1 hr. Cell lysates were blotted with PS126, PY118, paxillin, pERK, or ERK antibodies. (C) RAW264.7 cells were serum starved overnight (lane 1), and then were pretreated with U0126 at the dose shown for 1 hr. Cells were stimulated with LPS (1 μg/ml) for 1 hr. Cell lysates were blotted with paxillin and PY118 antibodies.

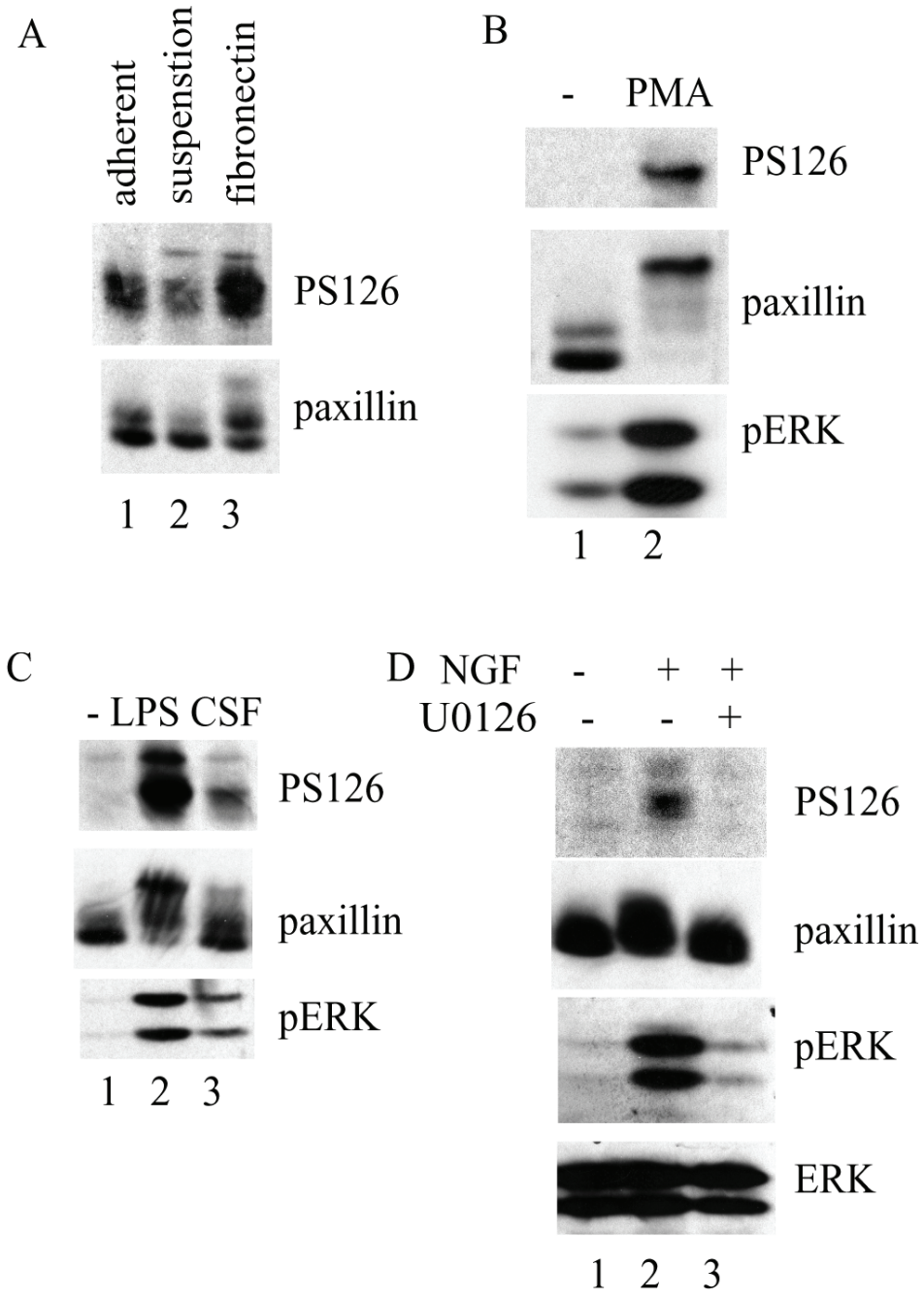


Figure 2.4 Paxillin serine 126 phosphorylation is induced by multiple stimuli. (A) RAW264.7 cells were trypsinized, incubated in suspension at 37°C for half an hour (lane 2), then replated on fibronectin coated plates (50 μg/ml) for half an hour (lane 3) at 37°C prior to lysis. Cell lysates were blotted with paxillin and PS126 antibodies. (B) and (C) RAW264.7 cells were serum starved overnight (lane 1), and then were stimulated with PMA (100 ng/ml)(lane 2 in panel B), LPS (1 μg/ml) (lane 2 in panel C) and CSF (1.32 nM) (lane 3 in panel C) for 1 hr. Cell lysates were blotted with paxillin, PS126 and pERK antibodies. (D) PC12 cells were starved in DMEM containing 1% FBS for 6 hr, treated with 20 μM U0126 for 1 hr (lane 3), and stimulated with 100 ng/ml NGF for 2 hr (lanes 2, 3). Cell lysates were blotted with PS126, paxillin and pERK antibodies.

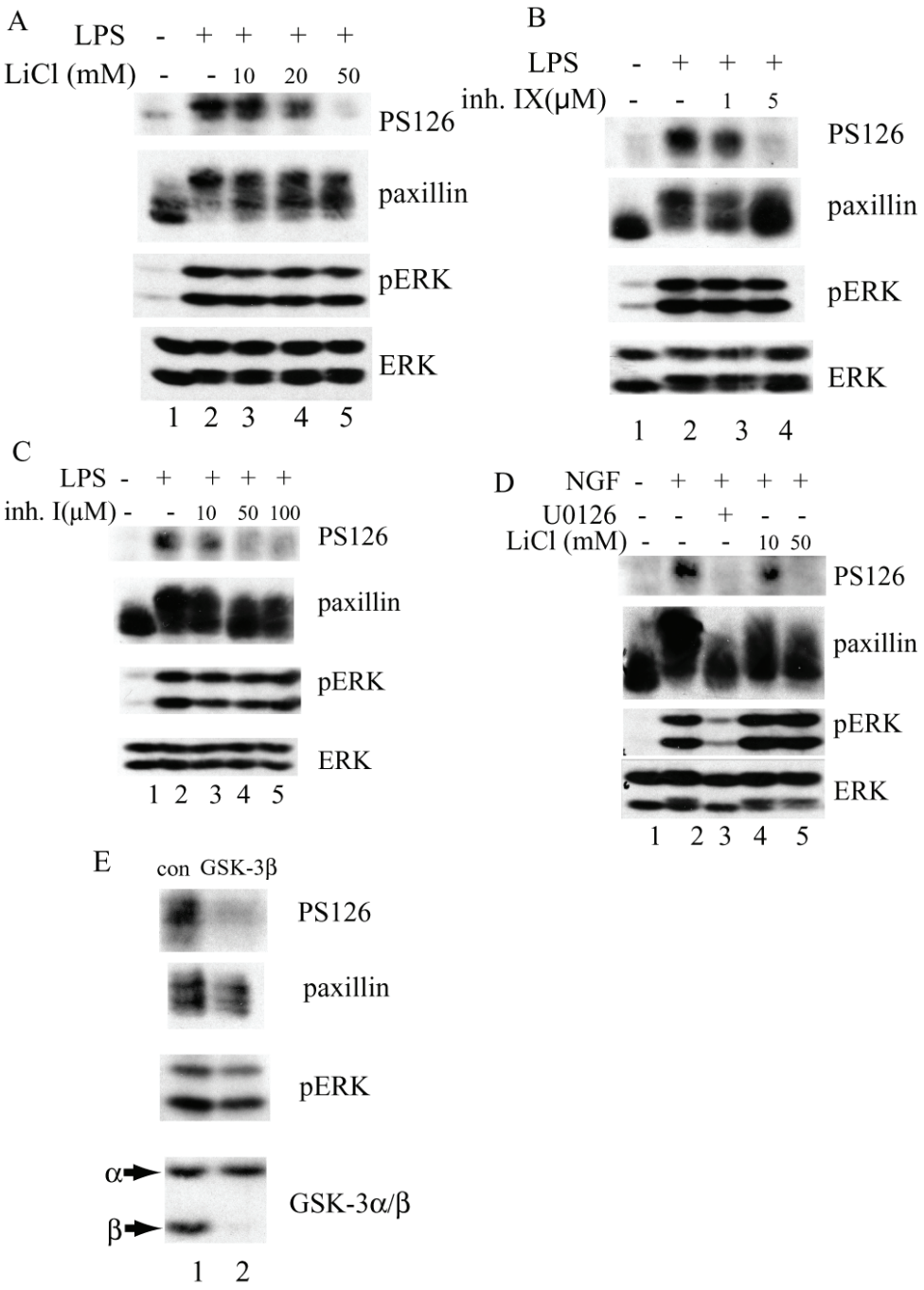


Figure 2.5 Phosphorylation of serine 126 is abolished by GSK-3 inhibitors. (A, B & C) RAW264.7 cells were serum starved overnight (lane 1), then pretreated with LiCl, GSK-3 inhibitor IX or GSK-3 inhibitor I at the dose indicated for 1 hr. Cells were stimulated with LPS (1 μg/ml) for 1 hr. Cell lysates were blotted with the indicated antibodies. (D) PC12 cells were starved in DMEM containing 1% FBS for 6hr, treated with the indicated drug for 1hr, and stimulated with 100 ng/ml NGF for 2 hr. Cell lysates were blotted with the indicated antibodies. (E) RAW264.7 cells were transfected with the control or GSK-3β siRNAs, starved and stimulated with LPS. Lysates were blotted for PS126, paxillin, pERK and GSK-3α/β.

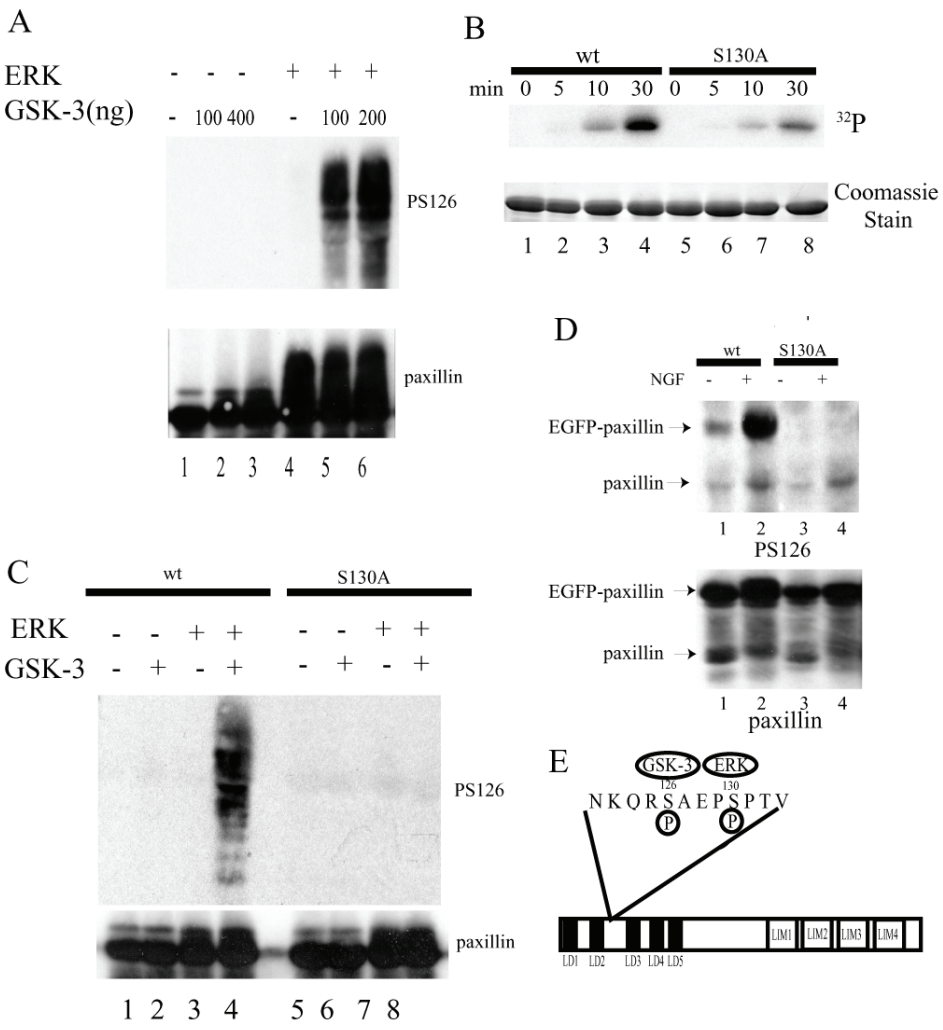


Figure 2.6 Phosphorylation of paxillin is mediated by ERK/GSK-3 dual kinase mechanism. (A) Recombinant GST-N-C3 was incubated with active GSK-3 and ERK1, alone or in combination, in 50 µl of kinase reaction buffer at 30°C for 60 min. Samples were resolved by SDS-PAGE, and immunoblotted using the PS126 or paxillin antibodies. (B) GST-N1-C1A was incubated with ERK in kinase reaction buffer containing γ[^{32}P]-ATP and the samples resolved by SDS-PAGE. The autoradiograph and Coomassie stained gel are shown. (C) The wild type GST-N-C3 or GST-N-C3 variant containing an alanine substitution for serine 130 were treated as in A. (D) PC12 cells transiently expressing EGFP-paxillin (lanes 1, 2) or EGFP-paxillinS130A (lanes 3, 4) were challenged with NGF (lanes 2, 4) and cell lysates were blotted with the paxillin and PS126 antibodies. The positions of the exogenous EGFP-paxillin and endogenous paxillin are shown. (E) A model for phosphorylation of paxillin by ERK/GSK-3 dual kinase mechanism is show.

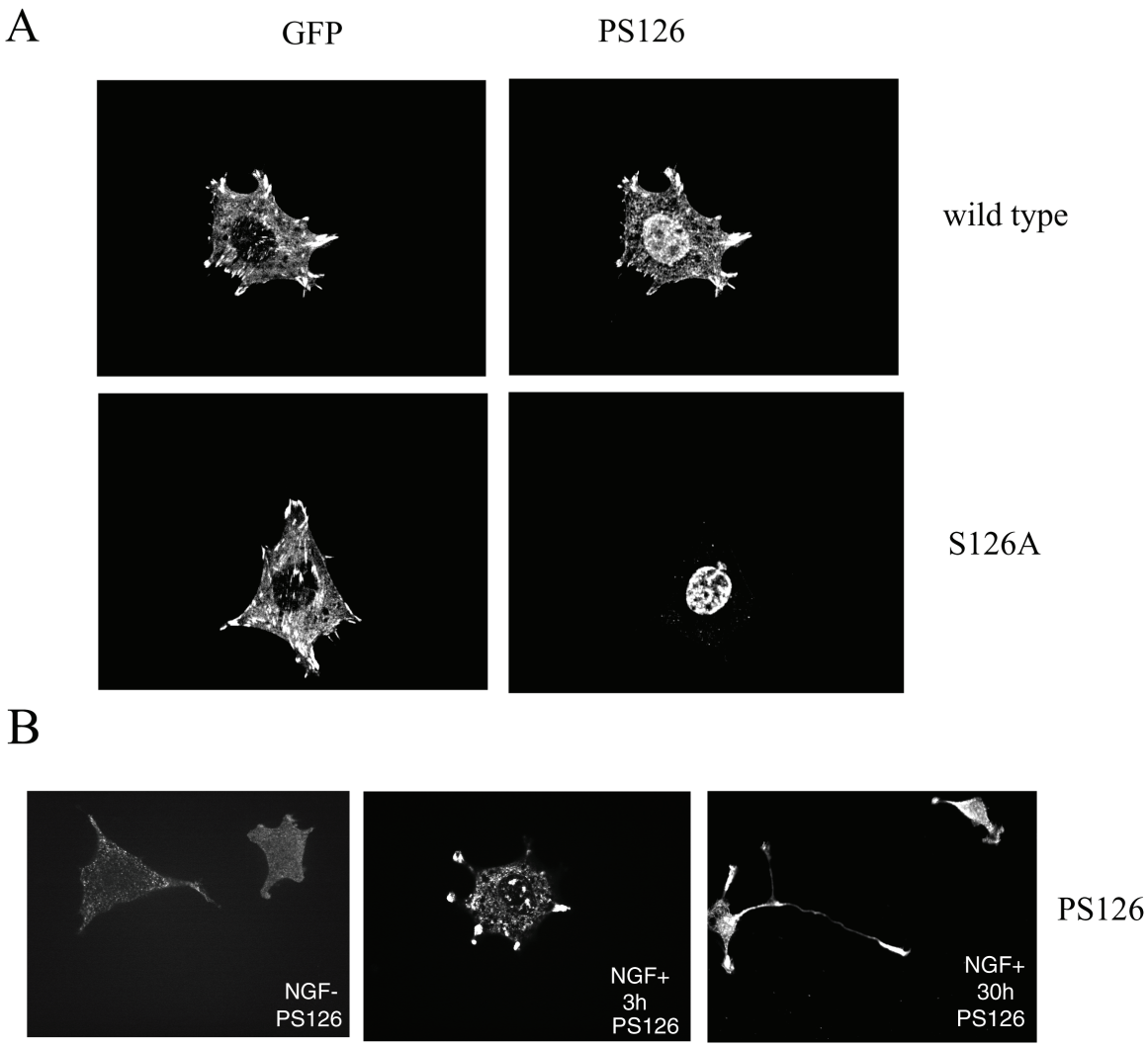


Figure 2.7 Subcellular localization of the serine 126 phosphorylated form of paxillin in fibroblasts. (A) Paxillin null fibroblasts were transiently transfected with EGFP-paxillin (top panels) or the EGFP-paxillinS126A mutant (bottom panels). Twenty-four hours later, the cells were trypsinized, held in suspension for 45 minutes and then plated onto fibronectin coated coverslips for 60 minutes in serum-free medium. Localization of the protein was visualized by fluorescent microscopy (left panels). Localization of paxillin that was phosphorylated on serine 126 was determined by immunofluorescence using the PS126 antibody (right panels). (B) PC12 cells were serum starved (left panel) and stimulated with NGF (100 ng/ml) for 3 hours (middle panel) or 30 hours (right panel). Cells were fixed and immunostained using the PS126 antibody.

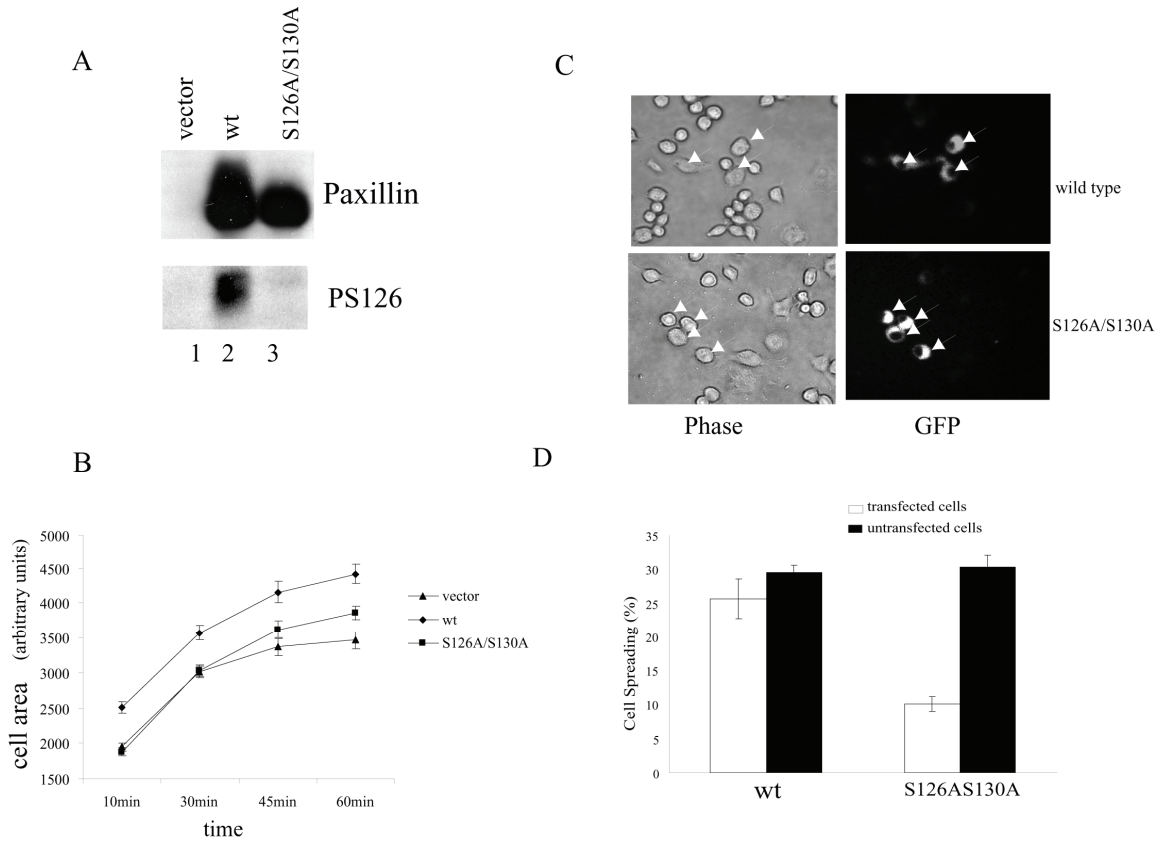
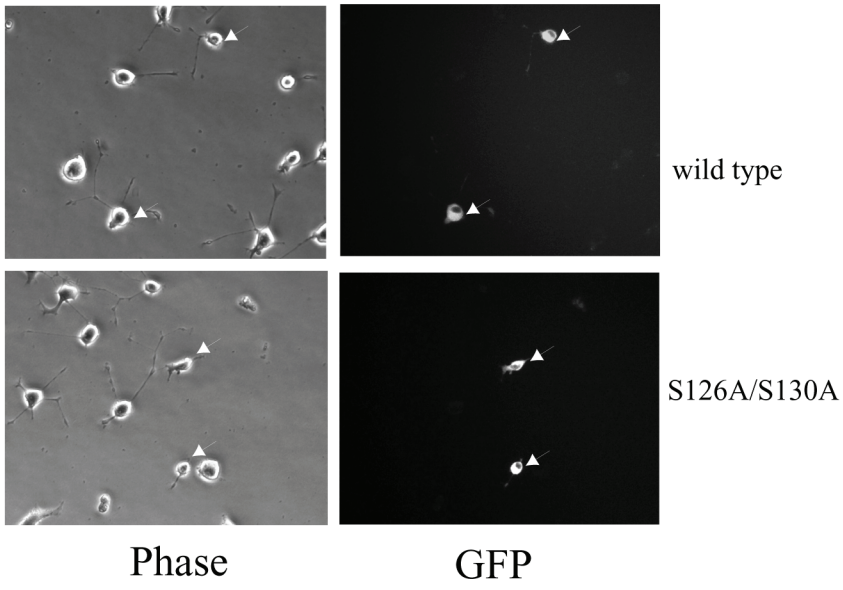


Figure 2.8 ERK/GSK-3 mediated phosphorylation of paxillin is involved in cell spreading. (A) Lysates from paxillin null cells or cells re-expressing wild type or S126A/S130A paxillin were analyzed by Western blotting for paxillin (top panel) or PS126 (bottom panel). (B) Cells were serum starved overnight and then plated on fibronectin. At various times the area of spreading cells was determined using Image J software (>50 cells per experiment) and the average from 3 experiments plotted. The data at each time point was analyzed using one-way ANOVA ($P<0.0001$ at each time) and the Dunnett post test (for wild type expressing cells $P<0.01$ at each time; for S126A/S130A $P<0.05$ only at 60 minutes). (C) RAW264.7 cells or derivatives stably expressing EGFP-paxillin or EGFP-paxillinS126A/S130A were challenged with 1 μg/ml LPS as described. Cell spreading was recorded after 3 hr of LPS stimulation. Phase contrast images (left panels) and fluorescent images (right panels) of cells transfected with EGFP-paxillin (top panels) or EGFP-paxillinS126A/S130A (bottom panels) are shown. (D) GFP negative, untransfected cells and GFP positive cells expressing exogenous paxillin were scored as spread or unspread and expressed as percentage of cells spread. Greater than 100 GFP positive cells were scored in each experiment. The data was plotted as the mean ± SE from three experiments. The P value is less than 0.05 (unpaired student t-test).

A



B

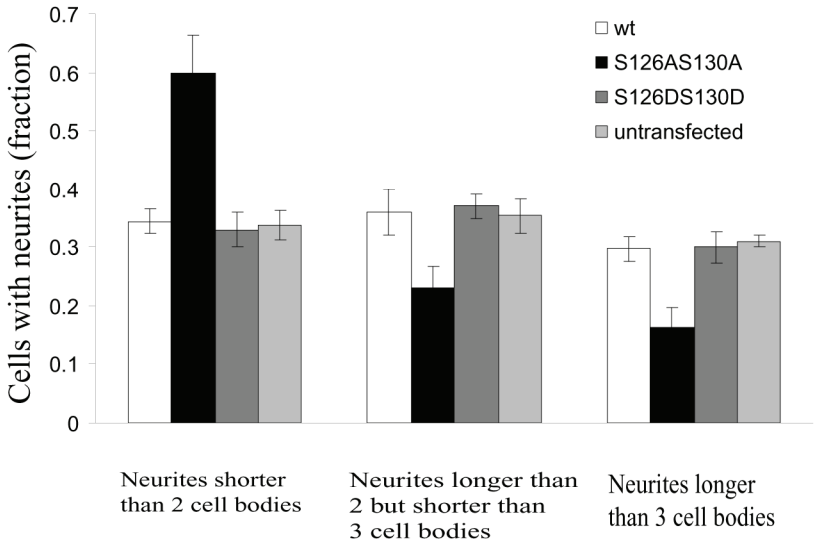


Figure 2.9 ERK/GSK-3 mediated phosphorylation of paxillin is involve in NGF induced neurite outgrowth. (A) PC12 cells transiently expressing EGFP-paxillin or EGFP-paxillinS126A/S130A were plated on collagen-coated petri dishes and treated with 100 ng/ml NGF. Neurite outgrowth was recorded after 36 hr of NGF stimulation. Phase contrast images (left panels) and fluorescent images (right panels) of cells transfected with EGFP-paxillin (top panels) or EGFP-paxillinS126A/S130A (bottom panels) are shown. (B) Quantitative analysis of neurite outgrowth from three independent experiments is shown. In each experiment, greater than 100 GFP negative, untransfected cells (light grey bars) and

GFP positive cells from the EGFP-paxillin (white bars), EGFP-paxillinS126A/S130A (black bars) and EGFP-paxillinS126D/S130D (dark grey bars) transfected populations were scored. The data was plotted as the mean ± SE from three experiments. In each group the wild type and S126A/S130A data was analyzed using one way ANOVA and Dunnett post test (for each: ANOVA, $P<0.0002$; Dunnett, $P<0.01$).

CHAPTER 3
Spatial and Temporal Regulation of FAK Activity in Living Cells

This chapter has previously been accepted under the same title as : Xinming Cai, Daniel Lietha, Derek F. Ceccarelli, Andrei V. Karginov, Zenon Rajfur, Ken Jacobson, Klaus M. Hahn, Michael J. Eck, Michael D. Schaller Mol Cell Biol. 2007 Oct 29; [Epub ahead of print]

3.1 Abstract

Focal adhesion kinase is an essential kinase that regulates developmental processes and functions in the pathology of human disease. An intramolecular autoinhibitory interaction between the FERM and catalytic domains is a major mechanism of regulation. Based upon structural studies, a FRET based FAK biosensor that discriminates between autoinhibited and active conformations of the kinase was developed. This biosensor was used to probe FAK conformational change in live cells and the mechanism of regulation. The biosensor demonstrates directly that FAK undergoes conformational change *in vivo* in response to activating stimuli. A conserved FERM domain basic patch is required for this conformational change and for interaction with a novel ligand for FAK, acidic phospholipids. Binding to PIP2-containing phospholipid vesicles activated and induced conformational change in FAK *in vitro*, and alteration of PIP2 levels *in vivo* changed the level of activation of the conformational biosensor. These findings provide direct evidence of conformational regulation of FAK in living cells and novel insight into the mechanism regulating FAK conformation.

3.2 Introduction

Focal adhesion kinase (FAK) is an essential non-receptor tyrosine kinase since FAK null mice exhibit embryonic lethality (145). In endothelial cells FAK is required for the proper development of the vasculature (26,413), and in neurons FAK regulates netrin-mediated axon outgrowth and dendrite formation (330,378). In addition to these roles in development, FAK is also implicated in the pathology of disease. FAK expression is required in cardiomyocytes to promote hypertrophy and fibrosis in response to cardiac stress and potentially plays a role in the development of heart disease (111,355). Overexpression of

FAK is observed in many types of cancer (146), and experiments using animal models have implicated FAK in tumor formation and metastasis in a number of neoplasms, including cancer of the brain, breast and skin (322,326,458).

Despite the importance of FAK in controlling multiple developmental and pathological events, the molecular mechanism of regulation remains incompletely elucidated. Like many kinases post-translation modification, particularly phosphorylation, is a major regulatory mechanism. Tyrosine 397 is the major site of auto-phosphorylation and mutation of this site abrogates the biological activity of FAK (387). This site primarily serves a scaffolding function, providing a docking site for a number of proteins containing SH2 domains, including Src and PI3K (386). The interaction with FAK occupies both the SH2 and SH3 domains of Src preventing intramolecular inhibitory interactions resulting in stabilization of Src in its active conformation (440). Complex formation also serves to direct Src to its substrates, which include FAK itself. Activated Src phosphorylates FAK on multiple sites including two tyrosine residues in the activation loop, Y576 and Y577, which function in regulating catalytic activity (48).

A number of protein-protein interactions also regulate FAK activity. FIP200 is a negative regulator of kinase activity (452). The interaction of FIP200 with FAK is complex, with multiple FIP200 binding sites in FAK (4). This complex dissociates upon cell adhesion, which is a stimulus leading to FAK activation (2). The interaction of FAK with growth factor receptors positively regulates FAK signaling. Stimulation of fibroblasts promotes FAK binding to the EGF and PDGF receptors, which is required for EGF- and PDGF-induced chemotaxis (425). Stimulation of cells with HGF promotes the direct association of FAK with the Met receptor and this interaction is required for HGF induced activation of

FAK (82). A potential ligand-binding site within the N-terminal domain of FAK, consisting of a series of basic residues, is required for maximal activation of FAK and subsequent downstream signaling following cell adhesion to fibronectin (120). This same site forms the binding site for tyrosine phosphorylated Met receptor (81), and thus this sequence is required for activation of FAK in response to diverse stimuli.

Multiple studies support a model of regulation whereby the FAK FERM domain interacts with the FAK catalytic domain to impair catalytic activity in the inactive state. Truncation of the FERM domain increases FAK tyrosine phosphorylation and/or kinase activity (69,233,400,406,447). Further, the FERM domain can interact in trans with the catalytic domain *in vivo* and inhibit its activity (95). Mutation of K38 within the FERM domain disrupts the FERM/kinase domain interaction, and when introduced into the full-length molecule activates FAK (89). The molecular details of the FERM/catalytic domain interaction were revealed by X-ray crystallography (294). In this complex, the FERM domain blocks the active site of the kinase domain inhibiting access to the ATP and substrate binding sites. Further, the activation loop lies within the cleft between the FERM and kinase domains. These studies support a model of FAK activation entailing a switch from autoinhibited to active conformation, however, the challenging task of testing this model under physiological conditions in living cells has yet to be undertaken.

In this paper, we report the development of two biosensors that can measure two important facets of FAK activation in live cells; 1) phosphorylation of Y397, which reflects a FAK scaffolding function, and 2) a conformational change associated with FAK activation. The novel conformational biosensor directly demonstrates that a conformation change in FAK occurs in living cells upon FAK activation. The biosensor was further used to probe

the spatial regulation of FAK and mechanisms involved in controlling conformation changes. Acidic phospholipids are identified as novel ligands for FAK. PIP2-containing vesicles alter FAK conformation *in vitro* and modulating PIP2 levels alters FAK conformation *in vivo*. These findings support the model that FAK is regulated via interaction with lipid ligands and provides mechanistic insight into the observation that acidic phospholipids are required for FAK activation (59,248,302,315).

3.3 Materials and Methods

Cell culture. 293T cells were maintained in DMEM F-12 medium containing 10% FBS and HeLa cells in DMEM containing 10% FBS. Cells were transfected using Lipofectamine Plus (Invitrogen) according to manufacturer's instructions. HeLa cells were starved in DMEM overnight prior to stimulation with ligands. To measure adhesion dependent activation cells were maintained adhered to substrate or incubated in suspension at 37^{o}C for 1 hour prior to lysis.

Molecular Biology. To generate CFAK the full length FAK cDNA was inserted into ECFP-C1, in frame with CFP. Citrine was generated from the EYFP-C1 vector by mutation of glutamine 69 to methionine (188). To generate citrine-dSH2, two Src SH2 domains were sequentially introduced into the citrine construct. This was achieved by PCR amplification of the cSrc SH2 domain (553-894) and insertion of two copies of the SH2 coding sequence between the XhoI and PstI sites within citrine vector. To generate FAK conformation probes, six-nucleotide insertions encoding a Not I site were introduced into different sites of CFAK. Citrine was amplified with primers containing Not I sites and the PCR product was introduced into the Not I site within CFAK. All mutations were generated using the QuickChange mutagenesis strategy. The full-length constructs were analyzed by sequencing

to verify the intended mutations and ensure that no unintended mutations were present. The expression construct for PIPKIα was the generous gift of Richard Anderson (University of Wisconson) and a YFP-SopB construct was a generous gift of Jorge Galán (Yale University). For this study, the SopB encoding sequences were amplified by PCR and subcloned into pcDNA3.1.

Fluorometric measurements of FRET. For live cell fluorometric measurements, 293 cells expressing different constructs were typsinized and suspended in PBS. The cells were analyzed using a Fluorolog SPEX 168 fluorometer or a Spectrofluorophotometer RF-1501 (Shimadzu). The cell suspension was excited at 425 nm and an emission scan was acquired from 450 to 550 nm. The spectra of different samples in a single experiment were normalized to CFP emission of a reference spectrum.

Measuring FRET by Microscopy. HeLa cells expressing biosensors were plated on fibronectin (Sigma 5 ug/ml) coated coverslips (Lucas Highland). After incubation at 37^{o}C for one hour the cells were transferred to the microscope's heated chamber (Warner Instrument Corporation TC-344B) in Ham's F-12 K medium without phenol red (Biosource), 25 mM HEPES, pH 7.4 and 5% FBS. Images were collected using a Zeiss 100X 1.3 N.A. lens, a Zeiss Axiovert 100TV microscope, a Cool Snap ES digital camera and Metamorph software (Universal Imaging). The filters and dichroic mirror setting for FRET images were 1) CFP: D436/20, D470/40; 2) FRET: D436/20, HQ535/30; and 3) YFP: HQ500/20, HQ535/30 (359). The CFP, FRET and YFP images were recorded using 2*2 binning. For ligand stimulation, transfected HeLa cells were serum starved overnight, transferred to the microscope's heated chamber and imaged, before and after ligand stimulation, in HBSS

buffer with 20 mM HEPES, pH 7.4 and 2 g/l glucose. At each time point, images were collected using a Zeiss 40X 1.3NA Plan NeoFluor lens and 4*4 binning.

For photobleaching assays, HeLa cells expressing the FAK biosensor were illuminated to excite CFP and imaged in both the CFP and FRET channels at 11-second intervals. The YFP acceptor was photo bleached by pulse illumination for 6 seconds at each 11-second interval. The mean intensity from the whole cell was measured at each time point and normalized to the zero time point at the initiation of photobleaching. For laser scanning confocal microscopy, 293 cells expressing the donor (CFAK), acceptor (citrine-dSH2) or both were analyzed. Images were recorded using a LSM 510 laser scanning confocal microscope (Zeiss) in lambda stack mode. For photo bleaching, the cells were photo bleached at 488 nm. The emission spectra following stimulation at 458 nm were recorded before and after photobleaching.

Image processing and analysis. Images were processed as previously described (67,73,258,358). CFP, FRET, and citrine images were background subtracted and registered. For the FAK conformation biosensor, the background subtracted CFP image was divided by the background subtracted FRET image to get a pixel-to-pixel CFP/FRET ratio image. The CFP images were thresholded to generate a binary mask with a value of zero outside the cell and a value of one inside the cell. The CFP/FRET ratio images were multiplied with the mask and displayed in pseudocolors scaled from the lowest to the highest signal within the cell, eliminating pixels outside the 5-95% range on the intensity histogram, to provide a more reasonable estimate of the biosensor's useful dynamic range. For the FAK auto-phosphorylation biosensor, the images were processed using the following equation,

Corrected FRET ($FRET^C$)= FRET-a*CFP-b*citrine, (487)

where a is the percentage of CFP bleed through (determined from cells expressing only CFP-FAK), b is the percentage of citrine bleed through (determined from cells expressing only expressing citrine-dSH2).

To quantify the focal adhesion and cytoplasm FRET values, two segmented images were generated using a threshold function. Two segmented images were used to generate binary masks for focal adhesions and the cytoplasm. The FRET ratio images were multiplied with the two masks to generate the ratio images for focal adhesions and cytoplasm. To determine the relationship between focal adhesion FRET value and distance to the cell margin, the focal adhesion binary mask was used to generate an individual segmented region around each focal adhesion. The segmented regions for focal adhesions were loaded to the focal adhesion CFP/FRET ratio image. The individual focal adhesion parameters, including average intensity and x,y value of each focal adhesion, were exported to Excel to generate a focal adhesion database. The cell margin was plotted based on the registered CFP image and transformed to a set of x and y values using Image J. The set of margin x and y values were imported into Excel. The minimal margin distance was measured by determining the minimal value of $\sqrt{(x_1 - x_2)^2 + (y_1 - y_2)^2}$, where x_1,y_1 are the coordinates of each focal adhesion and x_2,y_2 are the coordinates of each point at the margin of the cell.

Lipid Binding Assays. Lipid vesicles were prepared by mixing chloroform-dissolved phospholipids (Avanti Polar Lipids) in the appropriate ratios keeping the PE/PC mass ratio as 3:1, while other lipids were supplemented to the final concentration indicated. The mixture was dried using a speed vacuum for 15 min. The dried lipid mixture was suspended and sonicated in lipid binding buffer (40 mM Hepes pH 7.5, 2 mM DTT,150 mM NaCl) to a final concentration 2.5 μg/μl. For cosedimentation, 4 μg of GST fusion protein was incubated

with 250 μg of lipid vesicles on ice for 1 h. The mixtures were subsequently centrifuged at 110,000g for 1 hr at 4°C. The supernatants were collected. The pellets were suspended in 100 μl SDS buffer and boiled for 2 min to dissolve. Both the supernatants and pellets were mixed with Laemmli sample buffer and boiled for 5 min. The samples were analyzed by SDS-PAGE and Coomassie Blue staining. For fluorescence polarization the purified FERM domain (65) was incubated with BODIPY labeled phosphoinosotides with C_6-acyl chains (62). Increasing concentrations of purified protein was added to 12.5 nM fluorescent phosphoinositide in buffer containing 20 mM HEPES pH 7.0, 150 mM Nacl and 5 mM β-mercaptoethanol. Anisotropy measurements were taken at 21^{o}C using a Beacon 2000 fluorescence polarization instrument. Binding curves and dissociation constants were determined using the program Prism (GraphPad Software Inc.).

Protein purification and kinase assay. The GST-fusion proteins were induced and purified as previously described (119,316). For immune complex kinase assays, FAK and its variants were immunoprecipitated using the BC4 polyclonal antiserum or GFP antibody and the immune complexes incubated with 2 μg of GST-paxillin-N-C3 using previously described kinase reaction conditions (317). The reaction was terminated by the addition of sample buffer and phosphorylation of paxillin examined by Western blotting.

Expression of the recombinant fragment of FAK containing the FERM and catalytic domains has been described (293). For *in vitro* kinase assays using recombinant proteins, 200 ng of the recombinant FAK fragment and 1 μg Src (SH3+SH2+Kinase) were incubated with liposomes in kinase reaction buffer (100 mM Tris pH7.5, 100 mM $MgCl_2$, 50 μM ATP) for 30 min. The reaction was terminated by the addition of sample buffer and phosphorylation of recombinant FAK was examined by Western blotting.

Protein analysis. Cells were lysed in ice-cold modified radioimmunoprecipitation assay buffer. Lysates were clarified, and protein concentrations were determined using the bicinchoninic acid assay (Pierce, Rockford, IL). Immunoprecipitations were performed using a polyclonal FAK antibody (BC4) or a GFP monoclonal antibody (Roche). For Western blotting the FAK antibody (447) and phosphotyrosine antibody (4G10) were from Millipore, the PY576 antibody from Biosource and the paxillin polyclonal antibody was previously described (438).

3.4 Results

Development of a FAK autophosphorylation biosensor. Tyrosine397 is the major FAK autophosphorylation site, which is critical for FAK function and serves as a scaffolding site to recruit other proteins into complex. To visualize FAK autophosphorylation status in living cells, a genetically encoded FRET-based biosensor was developed. As a similar biosensor has been described (20), it will only be briefly introduced. CFP was fused to the FAK N-terminus to serve as the FRET donor and citrine, a variant of YFP, was fused to two Src SH2 domains to serve as the FRET acceptor (Fig. 3.1A). Since phosphorylation of tyrosine 397 regulates Src SH2 domain binding to this site, CFAK should only interact with citrine-dSH2 and produce a FRET signal when the former is phosphorylated at tyrosine 397. The interaction between CFAK and citrine-dSH2 was confirmed by co-immunoprecipitation (data not shown). Citrine-dSH2, but not citrine alone, co-immunoprecipitated with CFAK and this interaction was abolished when tyrosine 397 in CFAK was substituted with phenylalanine. Upon co-expression in 293 cells, CFAK and citrine-dSH2 co-localized peripherally near the membrane (data not shown). In contrast, CFAK-Y397F and citrine dSH2 were not co-localized. These data demonstrate that the interaction between CFAK and citrine-dSH2

depends upon phosphorylation of tyrosine 397. FRET was measured in several ways. Initially, cells were scraped from the tissue culture dish and FRET was measured in cells in suspension using a fluorometer. These measurements were made rapidly upon removing the cells from the dish (within 10 min), before exogenously expressed FAK could be inactivated and dephosphorylated. No FRET signal was detected in 293 cells expressing CFAK or citrine-dSH2 alone, whereas a strong FRET signal was detected in cells co-expressing both proteins (Fig 3.1B and Fig 3.8). Notably, no FRET signal was detected in cells co-expressing CFAK-Y397F and citrine-dSH2. Note that similar results were obtained when adherent cells were lysed and the lysates, rather than suspended cells, were analyzed (data not shown). Expression levels of the wild type and mutant biosensor were comparable (Fig 3.9A). FRET was also measured microscopically. As a further control, acceptor photobleaching was used to validate the FRET signal *in vivo*. HEK293 cells co-expressing CFAK and citrine-dSH2 were photobleached using an Argon laser (488nm). A series of fluorescent images were collected by laser scanning confocal microscopy and the average pixel intensity of the photobleached area at the CFP and FRET emission wavelengths was determined. As shown in Fig. 3.1C, photobleaching the FRET acceptor resulted in a decrease in the acceptor signal and an increase in the CFP donor signal, which was caused by the interruption of energy transfer from CFAK to citrine-dSH2.

Development of a FAK conformational biosensor. The structure of the FERM/catalytic domain complex confirms the role of the FERM domain in autoinhibition of catalytic activity and suggests that a transition from the autoinhibitory conformation to an open, active conformation is a required step for FAK activation. A FRET based FAK probe was developed to monitor such a conformational change in living cells. As shown in Fig 3.2A and

B, CFP was fused in the N-terminus of FAK and citrine was inserted into the linker between the FERM and catalytic domains to generate the CYFAK413 biosensor. As a control, citrine was inserted at the C-terminus of the catalytic domain to generate the CYFAK700 construct. The emission spectrum of these constructs following stimulation with the CFP excitation wavelength was determined using a fluorometer. Again measurements were made rapidly after taking the cells into suspension and similar results were found using lysates from adherent cells. A strong FRET peak was detected in cells expressing CYFAK413 (Fig 3.2C). In contrast a very weak FRET signal was detected in cells expressing CFAK700. Co-expression of CFAK and YFAK, in which the CFP and citrine are presented in trans, did not yield a FRET signal in this assay. In addition to the fluorometer assay, CYFAK413 was characterized by microscopy and FRET validated by acceptor photobleaching. Upon photobleaching, the citrine intensity decreased and the CFP signal increased, confirming that FRET was occurring (Fig. 3.2D). The 12% increase in CFP signal upon acceptor photobleaching is similar to that reported for the vinculin conformation biosensor (72).

To test whether the FRET signal of CYFAK413 was regulated by conformational change mutational studies were performed. The major contacts between the FERM and catalytic domains are centered around F596 of the catalytic domain, which binds in a hydrophobic pocket on the FERM domain (Fig 3.2E). Mutation of Y180 and M183, which form part of the hydrophobic pocket of the FERM domain, results in activation of FAK *in vitro* and *in vivo* (292). These activating mutations were engineered into CY413FAK and as shown in Fig. 3.2F, this mutant showed reduced FRET compared to the wild type probe. In addition to this mutant, other activating mutations that disrupt the FERM/catalytic domain interaction were also introduced into the FAK conformational probe (Fig. 3.2G). Each of

these mutants also exhibited a low FRET/CFP ratio compared with the wild type probe. The expression of these mutant biosensors was comparable to the wild type biosensor (Fig. 3.9B). These findings demonstrate that the FRET signal of this biosensor depends upon the interaction between the FERM and catalytic domains and that changes in FRET reflect changes in conformation.

Lysine 38 is a FERM domain residue that is required for the interaction between the FERM and kinase domains (88). Thus it was surprising when the crystal structure of the autoinhibited FERM/catalytic domain complex revealed that K38 did not directly contact the catalytic domain. Instead, K38 appears to interact with acidic residues within the linker extending between the FERM and catalytic domains (291). To further explore the role of K38 in FAK regulation, the K38A mutation was introduced into the CYFAK413 biosensor. Interestingly, this mutant also showed a low FRET/CFP ratio similar to that seen in CYFAK413 variants with mutations that directly disrupt the FERM/catalytic domain interaction (Fig. 3.2G). This finding supports a role for K38 in stabilizing the inactive conformation of FAK, perhaps through interactions with the linker.

The biochemical characteristics of CYFAK413 were examined to determine if incorporation of the fluorescent probes altered catalytic activity and if CYFAK413 was regulated in a manner similar to wild type FAK. The catalytic activity of CYFAK413 was tested using an *in vitro*, immune complex kinase assay. As shown in Fig. 3.3A, the kinase activity of CYFAK413 was modestly higher than wild type FAK, presumably due to the insertion of citrine in the linker, since CYFAK413 kinase activity was also higher than CFAK, which has CFP fused to the N-terminus of FAK. Notably, the activity of CYFAK413 was much lower than the activated variant CYFAK413-Y180A/M183A. To examine cell

adhesion dependent regulation, cells expressing CYFAK413 or CFAK were lysed and tyrosine phosphorylation examined following immunoprecipitation and Western blotting. Both CFAK and CYFAK413 were phosphorylated on tyrosine in adherent cells and exhibited reduced phosphotyrosine when cells were held in suspension (Fig. 3.3B). Thus, tyrosine phosphorylation of CYFAK413 was regulated by cell adhesion, similar to wild type FAK. In addition, the biochemical response of the biosensor to LPA stimulation was measured to further validate that this probe is regulated similar to wild type FAK. Following LPA stimulation, the biosensor exhibited increased tyrosine phosphorylation (Fig. 3.3C). Despite a modest increase in its catalytic activity, the fact that the biosensor was regulated similarly to wild type FAK in response to multiple stimuli suggests that CYFAK413 is a suitable biosensor for monitoring conformational changes in FAK in living cells.

Monitoring spatial regulation of FAK activity in living cells. To monitor phosphorylation of tyrosine 397 in live cells, HeLa cells transiently co-expressing CFAK and citrine-dSH2 were plated onto fibronectin coated coverslips and imaged by fluorescence microscopy. Cells were illuminated with a CFP excitation wavelength and the CFP and citrine (i.e. FRET) emissions captured. The $FRET^c$/CFP ratio image is shown in Fig 3.4A. CFAK localized prominently to focal adhesions although there was also cytoplasmic localization. The distribution of FRET indicated that phosphorylation of FAK at tyrosine 397 occurs at focal adhesions. Importantly, CFAK-397F exhibited a similar localization to CFAK, but there was no FRET signal produced in cells co-expressing CFAK-397F and citrine-dSH2. Expression of the wild type and mutant biosensors was comparable (Fig. 3.9C). The localization of FAK phosphorylated at tyrosine 397 to these sites was anticipated from studies using phosphospecific antibodies for immunofluorescence and results using a similar biosensor

from Geiger's laboratory (19,379). Strikingly, there appeared to be a heterogeneous distribution of phosphorylated FAK amongst the CFAK positive focal adhesions (Fig. 3.4A). A similar heterogeneous pattern of autophosphorylated FAK was observed by staining with a PY397 phosphospecific antibody (Fig. 3.10).

The current model of FAK regulation entails an autoinhibitory interaction between the FERM and catalytic domains and thus activation requires a conformational change relieving this inhibition. The development of the FAK conformational biosensor allows a direct test of this model in living cells. HeLa cells transiently expressing CYFAK413 were plated onto fibronectin coated coverslips and analyzed by fluorescence microscopy. Stimulation with the CFP excitation wavelength produced emission of both a CFP and a citrine signal. A representative CFP image and CFP/FRET ratio image are shown in Fig. 3.4B (note that the ratio image shows open conformation as "hot" and closed conformation as "cold"). The CFP/FRET ratio was elevated in focal adhesions, compared with the ratio seen in the cytoplasm. As a control, the activated variant CYFAK413-Y180A/M183A was analyzed in parallel. The CFP/FRET ratio was high both in focal adhesions and in the cytoplasm in cells expressing this control. Expression of the wild type and mutant biosensors was comparable (Supplemental Fig. 3.2D). This result demonstrates that the differences in CFP/FRET ratio in the CYFAK413 expressing cells were due to differences in conformation. The CFP/FRET ratio in focal adhesions and in the adjacent cytoplasm was quantified. In CYFAK413-Y180A/M183A expressing cells this ratio was the same in focal adhesions and in the cytoplasm (Fig. 3.5A). In CYFAK413 expressing cells, the CFP/FRET ratio in focal adhesions was comparable to that seen in CYFAK413-Y180A/M183A expressing cells but the cytoplasmic CFP/FRET ratio was significantly lower. These data demonstrate that FAK

exists in different conformations in different regions of the cell. While it is not surprising that FAK is active in focal adhesions, the CYFAK413 biosensor allows visualization of conformation changes in live cells, and thus provides strong support for the current model of FAK regulation.

While conformationally active FAK was found in focal adhesions, there was heterogeneity in the CFP/FRET ratio seen in focal adhesions. This did not correlate with focal adhesion size or amount of FAK in individual focal adhesions, but rather with location. FAK activity was higher in focal adhesions at the cell margin compared with internal focal adhesions and a similar pattern was observed using the autophosphorylation biosensor. This relationship was examined quantitatively by plotting the minimal distance of each focal adhesion from the cell margin versus the CFP/FRET and $FRET^c$/CFP ratio of the focal adhesion (Fig. 3.5B & C). This analysis supports the hypothesis that there is a tendency of focal adhesions near the periphery of the cell to have elevated levels of active/autophosphorylated FAK compared with focal adhesions further removed from the cell margin. Interestingly, an asymmetric FAK conformation spatial pattern was identified in many cells, i.e. FAK activation was elevated along one margin of the cell relative to its activation at the opposite margin. These findings suggest that FAK activity is regulated differentially in different focal adhesions, which may play a role in the determination of cell polarity and directional migration.

Temporal regulation of FAK conformation in living cells. To study FAK conformation changes in response to soluble ligands, HeLa cells expressing the conformational biosensor were serum starved, then stimulated with LPA. The average CFP/FRET ratio in the cell was determined over time and was found to increase following stimulation (Fig. 3.5D). The

kinetics of conformation change in FAK paralleled the kinetics of tyrosine phosphorylation of the biosensor (Fig. 3.5F). Stimulation with PDGF produced a small, transient change in the CFP/FRET ratio, while EGF stimulation did not produce a change in FRET under these conditions. The CYFAK413-Y180A/M183A constitutively active biosensor exhibited a constant CFP/FRET ratio before and after stimulation with LPA (Fig. 3.5E). These findings demonstrate that conformational changes occur in FAK in response to soluble ligands.

A FERM domain basic patch is required for conformational change. A basic patch in the F2 subdomain of the FERM domain of FAK was identified as a key motif for FAK activation *in vivo* in response to cell adhesion and stimulation of the Met receptor (80,118). This motif is on the surface of the FERM/catalytic domain complex and is close to the catalytic domain interaction site in the crystal structure. Thus, this motif is a potential site for controlling FAK conformational regulation. To determine if this basic region was required for conformational regulation of FAK, alanine substitutions for basic residues in this region were engineered into the conformational biosensor to create the K216A/K218A/R221A/K222A mutant (KAKTLRK). The wild type and mutant biosensors were transiently expressed and FRET examined by fluorometric analysis. The mutant exhibited a higher FRET/CFP ratio than the wild type biosensor suggesting that the integrity of the basic patch was required for optimally inducing the active conformation of FAK (Fig 3.6A).

Acidic phospholipids bind the basic patch within FAK FERM domain. Given the basic nature of this region, potential binding partners are likely to be acidic. Other FERM domains exhibit high affinity binding to phosphatidylinositol 4,5-bisphosphate (PIP2) via positively charged motifs (174). The structure of the FAK FERM domain precludes PIP2 binding via

the precise mechanism utilized by other FERM domains (64), however an interaction via the basic patch of the F2 subdomain remains a possibility. To examine binding to PIP2, a fluorescence polarization assay was used. Purified FERM domain was titrated into a solution of BODIPY labeled PIP2 containing C_6-acyl chains and anisotropy measured. Anisotropy was plotted against protein concentration and the dissociation constant calculated (Fig 3.6B). The KD for PIP2 was 31.52 +/- 1.7 μM and for phosphatidylinositol was >200 μM. The interaction of the FERM domain with lipids was further validated using a vesicle co-sedimentation assay. Phosphatidylethanolamine/phosphatidylcholine vesicles containing increasing amounts of PIP2 were incubated with a GST-FERM fusion protein. The vesicles were sedimented and the amount of fusion protein in the vesicle containing pellet and the supernatant was determined by SDS-PAGE and Coomassie blue staining. While the FERM domain bound poorly to PE/PC vesicles, approximately 50% of the FERM domain bound to vesicles containing 1% PIP2 and virtually all of the fusion protein associated with vesicles containing 5% and 10% PIP2 (Fig. 3.6C). To further characterize lipid binding, the interaction of the GST-FERM domain fusion protein with vesicles containing other lipids was examined. Under conditions where the protein was completely found in the pellet with PIP2 containing vesicles, GST-FERM associated weakly with PI containing vesicles (Fig. 3.6D). In contrast, the GST-FERM fusion protein bound quite well to phosphatidylserine containing vesicles. Thus, the data suggest that the interaction of GST-FERM with lipids is dictated by charge and that the structure of the head group is less important for binding *in vitro*.

The most likely lipid binding site on the FERM domain is the F2 subdomain basic patch as it is the most basic feature of the domain. To test its role in lipid binding, the

KAKTLRK mutations were engineered into the FERM domain. While the wild type GST-FERM domain associated strongly with PIP2 containing vesicles, this mutant was defective for binding (Fig. 3.6E). If the FERM domain was capable of binding lipids *in vivo* the domain might be recruited to the membrane. The FERM domain of FAK was transiently expressed in HeLa cells as a YFP fusion protein and its localization examined by confocal microscopy (Fig. 3.6G). The FERM domain partially co-localized with F-actin in ruffles and was observed at the membrane at the periphery of the cell. This pattern of localization suggests that the FERM domain is capable of associating with the membrane of the cell. Notably, KAKTLRK mutant exhibited a cytoplasmic localization and was not frequently observed in ruffles or at the membrane at the periphery of the cell (Fig. 3.6F). Expression of the wild type and mutant FERM domains was comparable (Fig. 3.9E). These data suggest that the FAK FERM domain can interact with acidic lipids *in vitro* and associate with the membrane *in vivo*, and the basic patch on the F2 subdomain is required for both of these activities.

PIP2 containing vesicles can activate FAK via conformational change. Given that the basic patch mutants are defective for signaling *in vivo* and that this region can mediate binding to PIP2 containing vesicles, it is possible that this interaction might play a role in FAK activation. This was tested using a recombinant fragment of FAK containing the FERM and catalytic domains. The fragment adopts the inactive conformation in which the Src phosphorylation sites within the activation loop of FAK are protected from phosphorylation (290). The FAK fragment was incubated with purified, active Src in lipid vesicle binding buffer. Under these conditions, Src could weakly induce FAK phosphorylation (Fig. 3.7A). Incubation of the FAK fragment with PE/PC vesicles

containing 10% PIP2 prior to phosphorylation by Src led to a dramatic increase in phosphorylation of FAK, whereas incubation with PE/PC vesicles did not (Fig. 3.7A). As a control, phosphorylation of GST fusion protein containing the activation loop peptide from FAK by Src was measured and did not change in the presence or absence of PIP2 containing vesicles. These results are consistent with the hypothesis that lipid vesicle binding leads to a conformational change in FAK exposing the activation loop for phosphorylation by Src.

Modulation of PIP2 regulates FAK conformation *in vivo*. To determine if perturbation of PIP2 levels *in vivo* could alter FAK activity, the conformational biosensor was used. To alter PIP2 levels, PIPKIα was coexpressed with the biosensor and the active state of the biosensor compared with cells expressing the catalytically inactive mutant of PIPKIα. Co-expression with PIPKIα resulted in a higher CFP/FRET ratio (indicating an increase in activated FAK) than co-expression with the catalytically inactive variant of the enzyme (Fig 3.7B). These results demonstrate that elevation of PIP2 levels in the cell promote the conversion of FAK into its active conformation. Further, the basic patch in the F2 subdomain of the FAK FERM domain is important for this effect since the KAKTLRK mutant lacking the basic patch was not responsive to altering the levels of PIP2 in the cell (Fig. 3.7B). To determine if PIP2 levels played a significant role in regulating FAK conformation under physiological conditions, SopB, an inositol polyphosphate 4-phosphatase, was co-expressed with the conformation biosensor and the effect upon the biosensor in adherent cells was examined. The conformation of the biosensor in focal adhesions was compared with its conformation in the cytoplasm by calculating the CFP/FRET ratio, where a higher ratio reflects the active conformation and a lower ratio reflects the inactive conformation (Fig. 3.7C). Expression of SopBcs, the catalytically inactive negative control, had little effect upon FAK conformation.

These cells exhibited more activated FAK in focal adhesions than the cytoplasm, similar to the results shown in Figure 3.5A (Fig. 3.7C). In contrast, SopB expression resulted in a decrease in activated FAK. In these cells the level of FAK activation in focal adhesions was only slightly higher than the level of activation in the cytoplasm. These findings demonstrate that PIP2 depletion reduces FAK activation in focal adhesions suggesting that PIP2 plays a role in regulating the conformation of FAK in cells adherent to fibronectin.

3.5 Discussion

The current model of FAK regulation evokes an intramolecular autoinhibitory interaction between the N-terminal FERM domain and central catalytic domain. Mutational and biochemical studies support this hypothesis and the crystal structure of the FERM/catalytic domain complex provides insight into the mechanism of inhibition (91,289). However, using these technologies it is not possible to probe protein conformation *in vivo*. FRET technology is a powerful tool to study protein-protein interactions in living cells. As FRET efficiency is dependent upon the distance between the two fluorophores and their relative orientation, changes in FRET correspond to changes in distance and orientation between the fluorescent probes, and in the case of a single protein fused to two fluorophores can reflect changes in conformation (304,329,357). Here, we have developed genetically encoded FRET biosensors to visualize changes in FAK conformation *in vivo*. These biosensors have provided two important pieces of evidence in support of the autoinhibitory model of FAK regulation. First, the analysis of the biosensors provides support for the crystal structure of the FERM/catalytic domain complex. CFP is encoded at the N-terminus of FAK in these biosensors and citrine was inserted at two different sites. Based upon the crystal structure, the probe insertion sites in the CYFAK413 construct are approximately 20

Å apart, and in the CYFAK700 construct the insertion sites are predicted to be 70 Å apart. As predicted from the structure, the former exhibited a high FRET signal and the latter produced a weak FRET signal. Second, using the conformational biosensor, this study provides for the first time direct evidence that a FAK conformational switch occurs *in vivo* and is associated with FAK activation. In focal adhesions the probe exhibited an open conformation, whereas probes located in the cytoplasm exhibited a closed conformation. This is consistent with the body of literature implicating FAK as a cell adhesion regulated kinase and suggests that integrin signaling contributes to the FAK conformational change. Moreover, another stimulus of FAK activation, LPA, induced a change in the wild type FAK biosensor conformation but had no effect upon a mutant biosensor that is constitutively in the open conformation. These findings demonstrate that multiple stimuli regulate the conformation of FAK and that conformational change is a general mechanism leading to FAK activation.

Spatial regulation of FAK. The FAK biosensors provide unique tools to investigate the spatial regulation of FAK. Currently, immuno-staining using FAK phosphorylation specific antibodies is used for this purpose (380), but this approach has some disadvantages. Antibody specificity is a potential problem that is often not well controlled. Further, tyrosine phosphorylation of FAK does not strictly correlate with catalytic activity (407). It is impossible to probe FAK activity in live cells using these approaches and it is currently impossible to monitor the conformation change of FAK by immunostaining.

The two biosensors report similar patterns of autophosphorylation/conformational activation. Both indicate elevated autophosphorylation/activation in peripheral focal adhesions compared with internal focal adhesions. Interestingly, this pattern is similar to the

pattern of cell traction forces in spreading cells, i.e. greatest traction forces at peripheral focal adhesions (18), which is consistent with a role for FAK in sensing mechanical stimuli. In addition, results using both biosensors indicate an asymmetry in autophosphorylation/activation of FAK in polarized cells with the highest levels in areas of cell protrusion. As FAK has been reported to regulate cell polarity (442), this asymmetric activation pattern may define the direction of polarization.

FAK and Lipid Binding. The FERM domain of FAK was shown to bind acidic phospholipids. PIP2 binding is a mechanism of regulation of ezrin, radixin and moesin, but the molecular details of the interaction differ from FAK. In radixin, the PIP2 headgroup associates with a basic pocket between the F1 and F3 subdomains of the FERM domain, whereas the corresponding site in FAK lacks the basic pocket (63,175). Although the F3 subdomain of FERM domains have a protein fold similar to PH domains, the F3 subdomain of FAK cannot bind phospholipids since it lacks the basic pocket that mediates the interaction of PH domains with phospholipids. Instead, FAK appears to bind acidic phospholipids through surface exposed basic residues at the tip of the F2 subdomain. Not surprisingly, the FERM domain of FAK binds PIP2 with lower affinity than PH domains. In contrast with the 30 μM Kd of the FERM domain, the PH domains of PLCδ and SOS-1 are reported to bind PIP2 with Kds of ~2 μM (PLCδ and SOS-1)(192,260,274). The affinity of the PH domain of spectrin ranges from 15-50 μM depending upon buffer conditions (183). Thus the affinity of the FAK FERM domain for PIP2 is in the range of lower affinity PH domain interactions. It is also notable that there is precedent for PIP2 binding to surface basic residues of proteins. For example, PIP2 associates with the tail domain of vinculin through surface basic residues rather than a discrete basic pocket (17,70).

The F2 basic patch is required for activation of FAK in response to cell adhesion and following HGF stimulation (79,117). This sequence binds acidic phospholipids, lipid vesicles containing PIP2 alter the conformation of FAK *in vitro*, and elevating PIP2 levels in cells results in alteration of the conformation of FAK *in vivo*. Further, reduction of PIP2 levels by expression of SopB results in decreased activation of FAK in focal adhesions in cells adherent to fibronectin. These findings support the hypothesis that acidic phospholipids, including PIP2, are physiologically relevant FAK FERM domain ligands that regulate the release of autoinhibitory interactions allowing FAK to adopt its active conformation. Additional evidence from the literature demonstrates that acidic phospholipids are important for the activation of FAK. Activation of FAK by stimulation of some G-protein coupled receptors is apparently blocked by depletion of PIP2 levels (303). Interestingly, a splice variant of PIP5KIγ co-localizes with FAK at focal adhesions and by localized generation of PIP2 might regulation FAK function (107,299). Other studies have suggested that the activity of PI3 kinase is required for activation of FAK in response to a number of different stimuli (60,251,314). We hypothesize that these acidic phospholipids might regulate FAK conformation through binding to the F2 basic patch. Following HGF stimulation, this same sequence mediates binding to tyrosine phosphorylated Met to facilitate FAK activation (84). These results suggest the intriguing hypothesis that a single site on the FERM domain of FAK can associate with either acidic phospholipids or phosphopeptides to trigger activation.

3.6 Acknowledgements

We would like to thank Drs. Sue Craig and Hui Chen, who provided invaluable advice during the development and characterization of the biosensors. Thanks also to Sean

Palmer for his guidance in setting up the lipid vesicle cosedimentation assay. This project was supported by NIH grant HL45100 (M.D.S.).

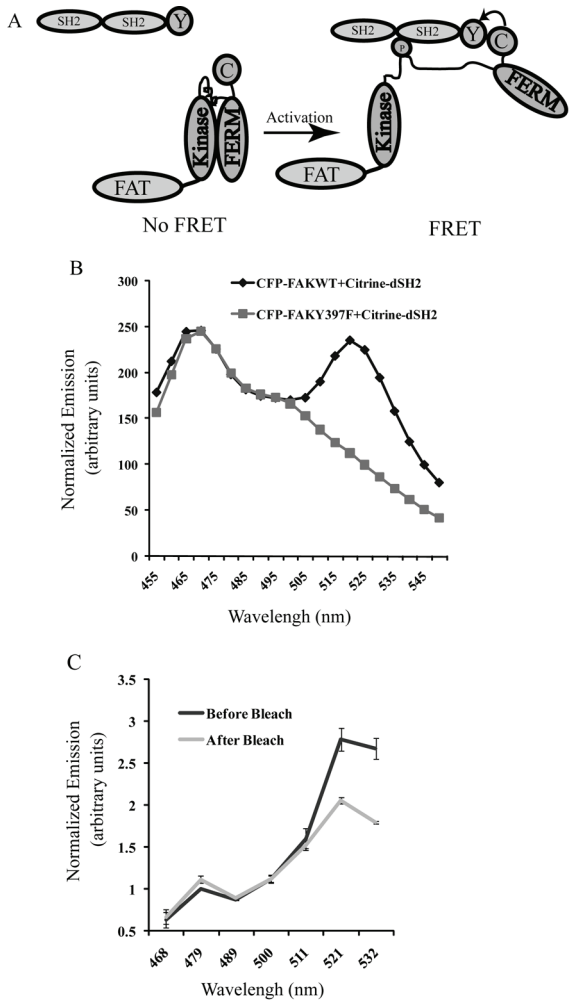


Fig. 3.1 Characterization of FAK auto-phosphorylation biosensor A) The design of the FAK auto-phosphorylation biosensor is shown. When tyrosine 397 is unphosphorylated, CFAK does not interact with citrine-dSH2 and FRET does not occur. When tyrosine 397 is phosphorylated, CFAK interacts with citrine-dSH2 and FRET occurs. (C = CFP, Y = citrine) **B)** The FAK wild-type auto-phosphorylation biosensor and the CFAK-Y397F control biosensor were expressed in HEK293 cells and analyzed by fluorometry. CFP was selectively excited at 425nm and the resulting emission spectra were normalized to the CFP emission peak. **C)** FRET in the FAK biosensor was verified by acceptor photo bleaching in living cells. HEK293 cells were photo bleached using 488 nm laser. The emission spectra produced by CFP excitation were recorded before and after photobleaching. Shown is the average of 3 experiments +/- SE.

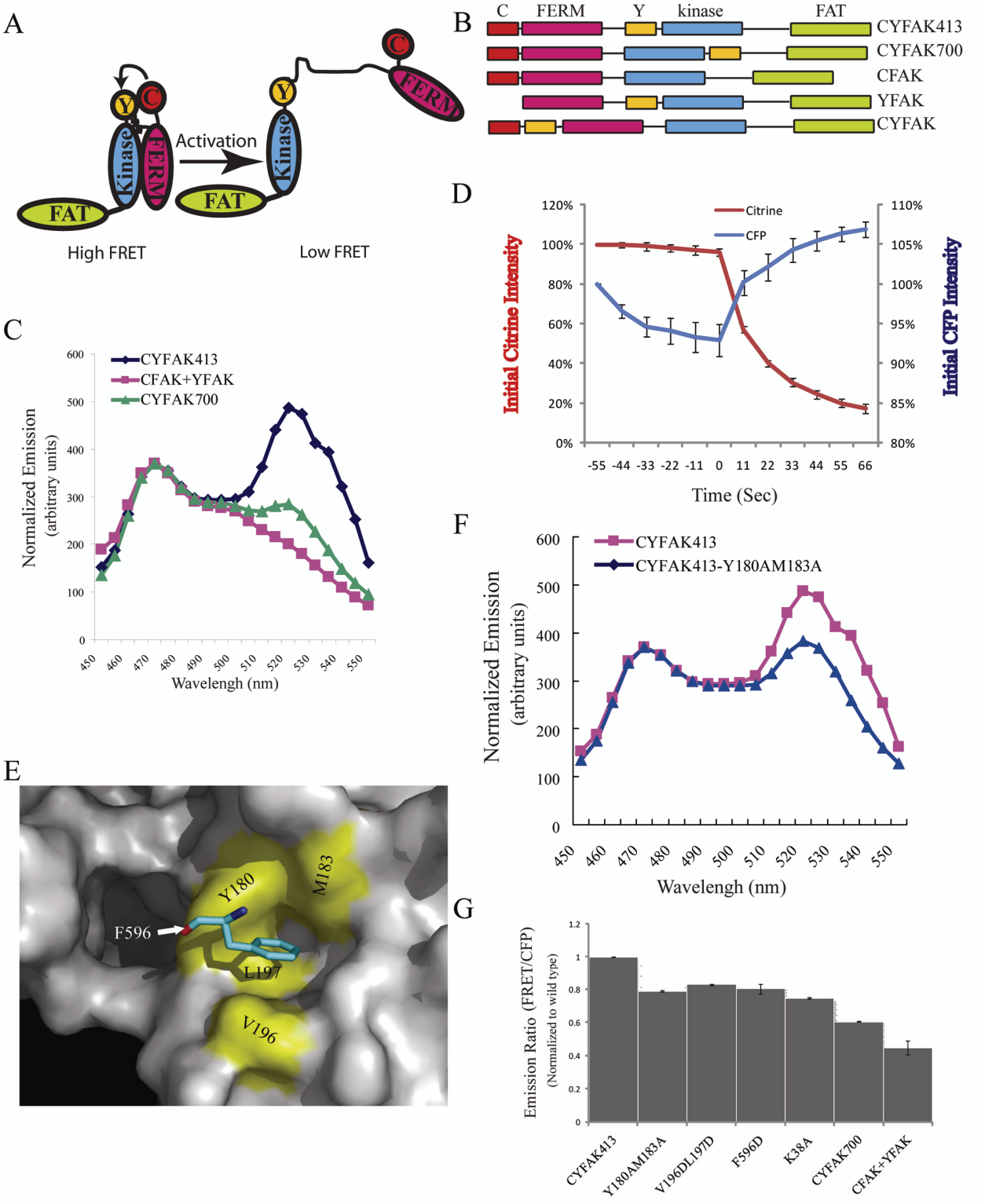


Fig. 3.2. Characterization of FAK conformation biosensor. A) The design of the FAK conformation biosensor (CYFAK413) is shown. In the inactive conformation the CFP and citrine are in proximity and FRET occurs. In the open conformation the CFP and citrine are further apart, and can rotate more freely, which will result in a reduced FRET signal. **B)** Schematic structure of the FAK biosensor and control biosensors. CFP is fused to the N-

terminus. The numbers in the construct name refer to the citrine insertion site. **C)** Normalized spectra of the FAK wild-type biosensor and control biosensors were measured by fluorometry. **D)** FRET in the FAK conformation biosensor was verified by acceptor photo bleaching in living cells. HeLa cells expressing the biosensor were imaged in both CFP and citrine channels following excitation of CFP at 11-second intervals. The acceptor (citrine) was photo bleached by pulse illumination for 6 seconds at each 11-second interval, after the zero time point. The mean intensity from whole cells was measured at each time point and normalized to the zero time point. Shown is the average from 3 experiments +/- SE. **E)** Representation of the FERM/kinase domain interface was created using Pymol and illustrates the key interaction between F596 in the kinase domain and a hydrophobic pocket in the F2 subdomain of the FERM domain (105). **F)** Normalized spectra of the FAK wild-type biosensor and a FAK biosensor with mutations designed to disrupt the FERM/kinase domain interaction are shown. **G)** Normalized FRET/CFP emission ratios of FAK biosensors containing different mutations are shown. The constructs were analyzed as in Fig 3.2C, and the FRET/CFP ratios of each were normalized to the FRET/CFP ratio of the wild type biosensor. Shown is the average of at least 3 experiments +/- SE. The results were analyzed by one-way anova ($p<0.0001$) and the Tukey's multiple comparison post test (CYFAK413 versus each construct, $p<0.001$).

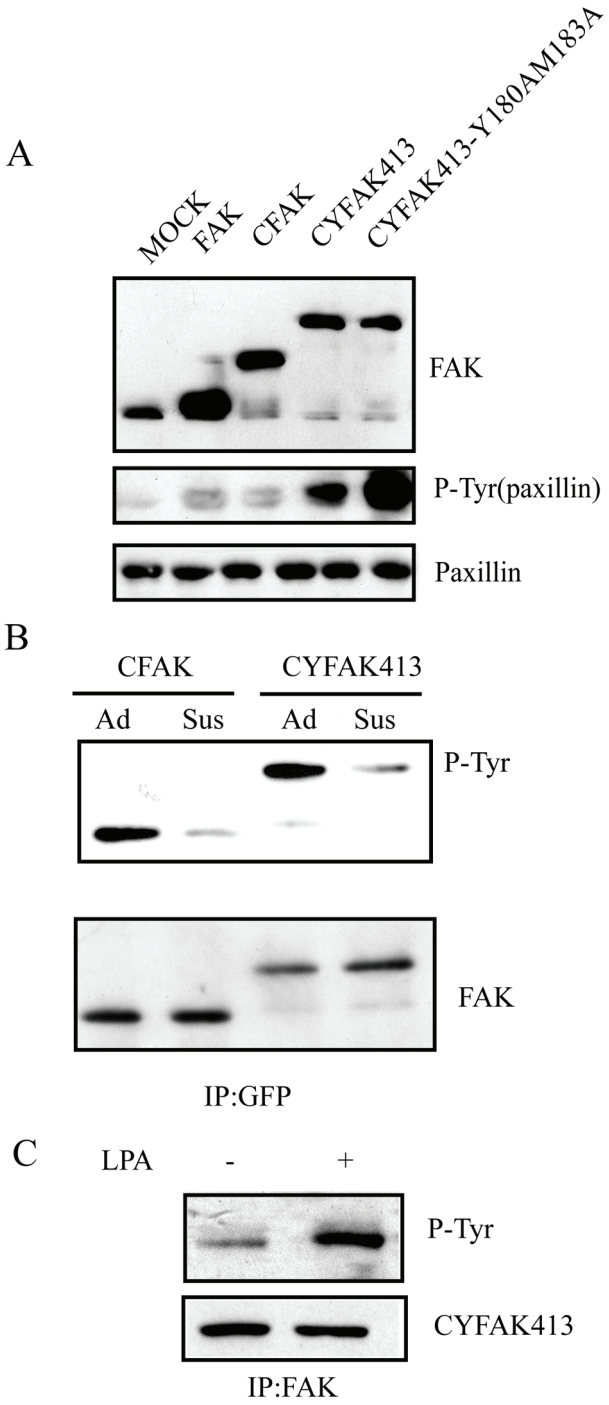


Fig. 3.3. **Biochemical characterization of the FAK conformational biosensor**. **A)** HEK293 cells expressing empty vector (Mock) or the indicated FAK constructs were lysed and immunoprecipitated using a FAK antibody. The immune complexes were incubated in an *in vitro* kinase assay utilizing recombinant GST-paxillin-N-C3 as an exogenous substrate. Phosphorylation of paxillin was detected using the 4G10 phosphotyrosine antibody. Equal amounts of substrate were verified by blotting for paxillin using a polyclonal antiserum. FAK in the immune complexes was verified by blotting for FAK. **B)** The wild type and

mutant biosensors were transiently expressed in HeLa cells and adherent cells or cells incubated in suspension at 37˚C for 1 hour were lysed. The biosensors were immunoprecipitated using a GFP antibody and the immune complexes analyzed by Western blotting for phosphotyrosine using 4G10. Equal amounts of FAK in the immune complexes were verified by blotting for FAK. **C)** HeLa cells expressing the FAK biosensor were serum starved and stimulated with LPA (200 ng/ml) for 5 minutes. The biosensor was immunoprecipitated from cell lysates and blotted with phosphotyrosine or a FAK antibody.

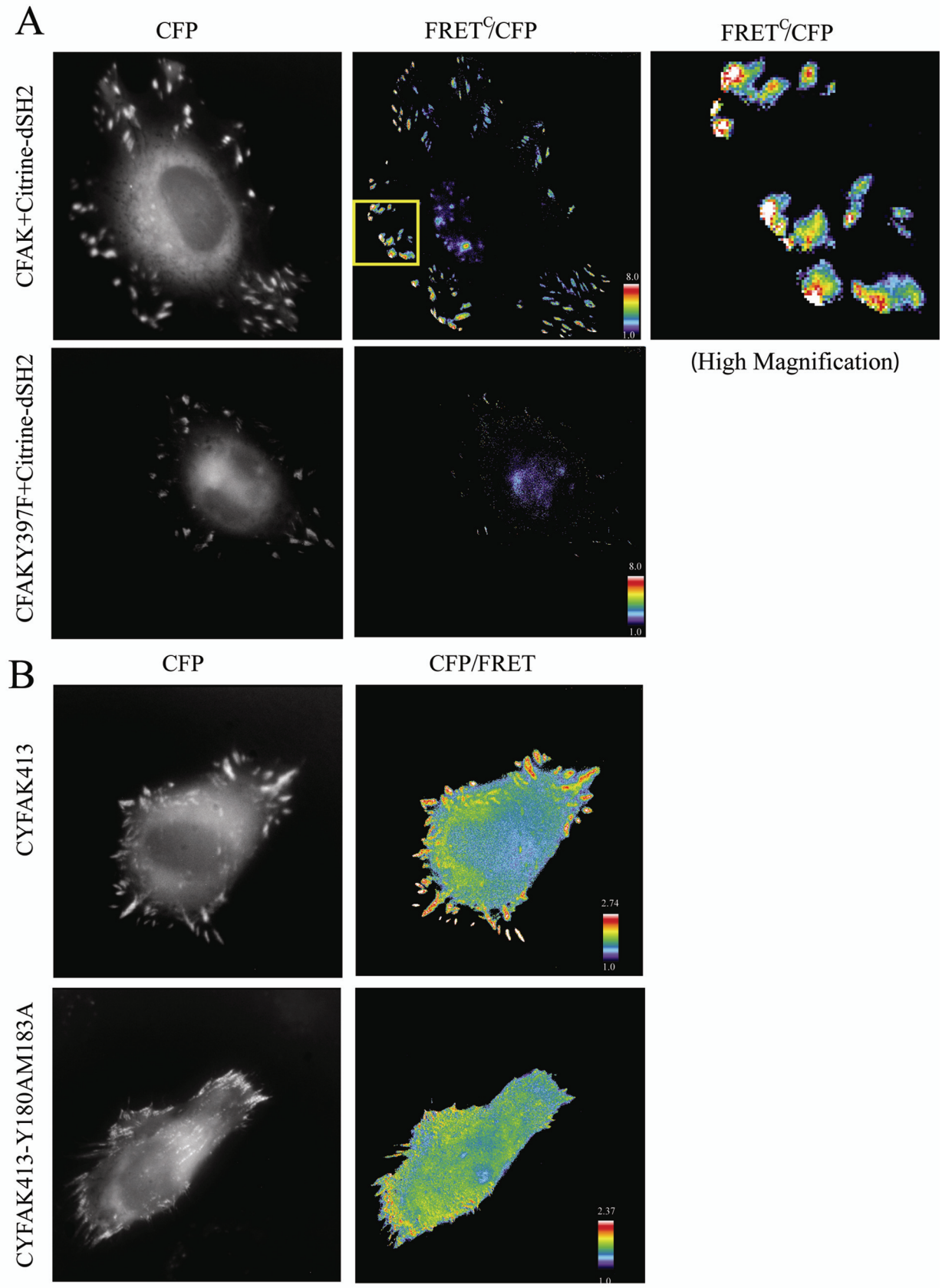

A
CFP
FRET^C/CFP
FRET^C/CFP
CFAK+Citrine-dSH2
8.0
1.0
(High Magnification)
CFAKY397F+Citrine-dSH2
8.0
1.0
B
CFP
CFP/FRET
CYFAK413
2.74
1.0
CYFAK413-Y180AM183A
2.37
1.0

Fig. 3.4. Spatial regulation of FAK activity. .**A)** HeLa cells expressing the FAK auto-phosphorylation biosensor or the 397F control were trypsinized and plated on fibronectin coated cover slips. CFP, FRET and citrine images were sequentially captured by fluorescence microscopy. CFP and $FRET^C$/CFP ratio images of the cells are shown. The ratio images are pseudocolored so that hotter colors reflect an increase in FAK auto-phosphorylation. Shown are representative images (n>5 cells). The boxed area in the CFAK + Citrine-dSH2 $FRET^c$/CFP image is shown at higher magnification to the right. **B)** HeLa cells expressing the FAK biosensor or the constitutively active mutant were trypsinized and plated on fibronectin coated cover slips. CFP, FRET and citrine images were sequentially captured by fluorescence microscopy. CFP and CFP/FRET ratio images of the cells are shown. Note that the ratio images are pseudocolored so that hotter colors reflect an increase in the open, active conformation. Representative images are shown (n>15 cells).

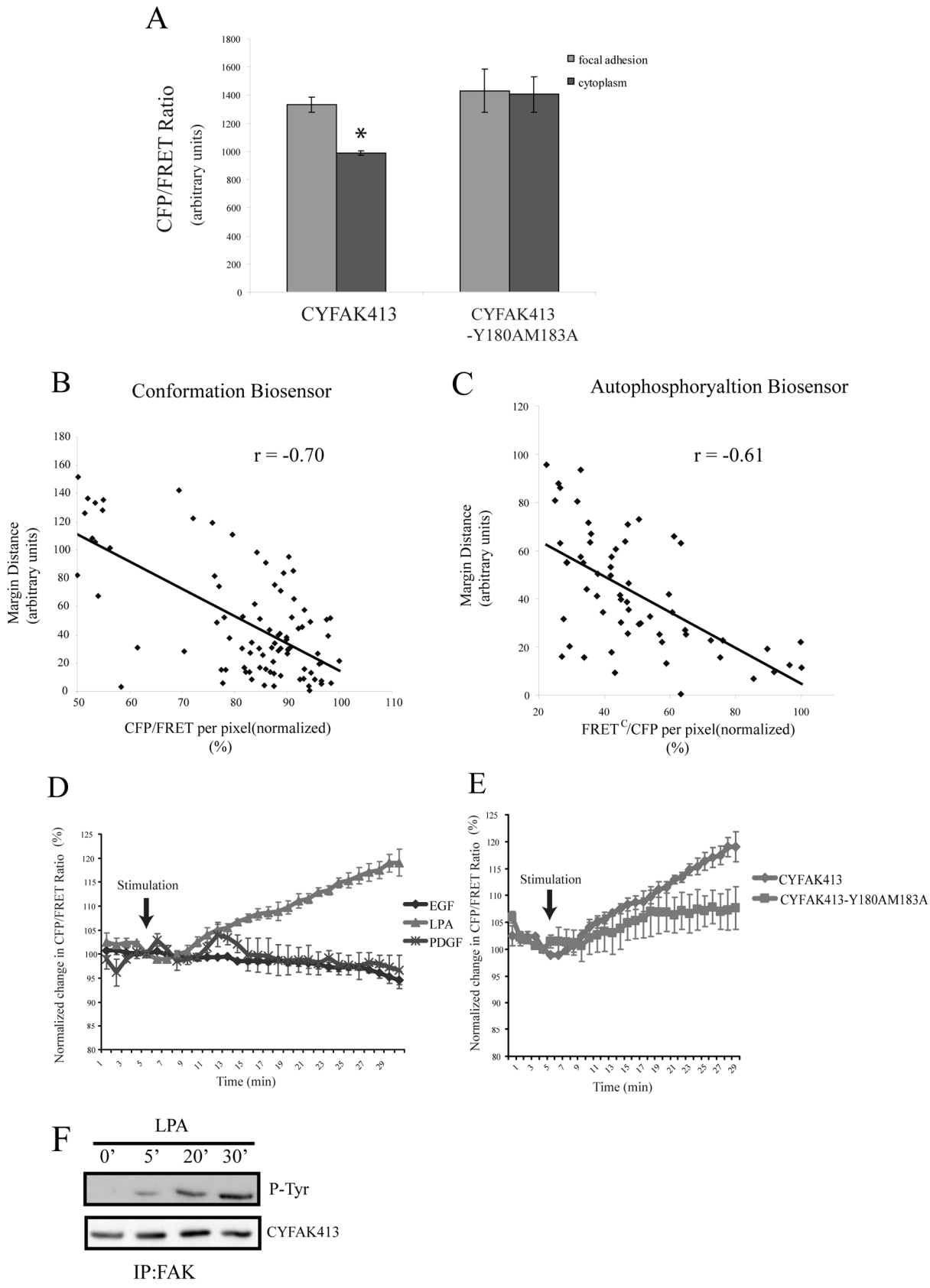

A
CFP/FRET Ratio
(arbitrary units)
focal adhesion
cytoplasm
*
CYFAK413
CYFAK413
-Y180AM183A
B
Conformation Biosensor
r = -0.70
Margin Distance
(arbitrary units)
CFP/FRET per pixel(normalized)
(%)
C
Autophosphoryaltion Biosensor
r = -0.61
Margin Distance
(arbitrary units)
FRET C/CFP per pixel(normalized)
(%)
D
Normalized change in CFP/FRET Ratio (%)
Stimulation
EGF
LPA
PDGF
Time (min)
E
Normalized change in CFP/FRET Ratio (%)
Stimulation
CYFAK413
CYFAK413-Y180AM183A
Time (min)
F
LPA
0’
5’
20’
30’
P-Tyr
CYFAK413
IP:FAK

Fig. 3.5 Spatial and temporal regulation of FAK conformation. A) The average CFP/FRET ratio of the cytoplasm or focal adhesions in HeLa cells expressing the wild type biosensor or the constitutively active variant is shown (average of n=4 cells). The focal adhesion and cytoplasmic values were analyzed using an unpaired t test (*, $p<0.0005$). **B & C)** The relationship between biosensor activity and focal adhesion location is shown. The distance of each focal adhesion from the cell margin is plotted versus the average CFP/FRET or $FRET^C$/CFP ratio, where the highest ratio has been normalized to 100 per cent. Representative data from single cells is shown (n=4). r = correlation coefficient. **D & E)** HeLa cells expressing the FAK biosensor were serum starved and simulated with LPA (200 ng/ml), EGF (50ng/ml) or PDGF (50ng/ml). CFP and FRET images were sequentially captured at 1-minute intervals. The mean CFP/FRET ratio of cells was calculated and normalized to the ratio at the time of stimulation. The change in the emission ratio of the FAK biosensor following EGF, PDGF or LPA stimulation is shown in A. The change in the emission ratio of the wild type FAK biosensor is compared with that of the constitutively active FAK biosensor mutant following LPA stimulation in B. Shown is the average response +/- SE (n=3 cells). **F)** HeLa cells expressing the biosensor were stimulated with LPA and lysed at the indicated times. The biosensor was immunoprecipitated and blotted with a phosphotyrosine antibody (top) or FAK antibody as a loading control (bottom).

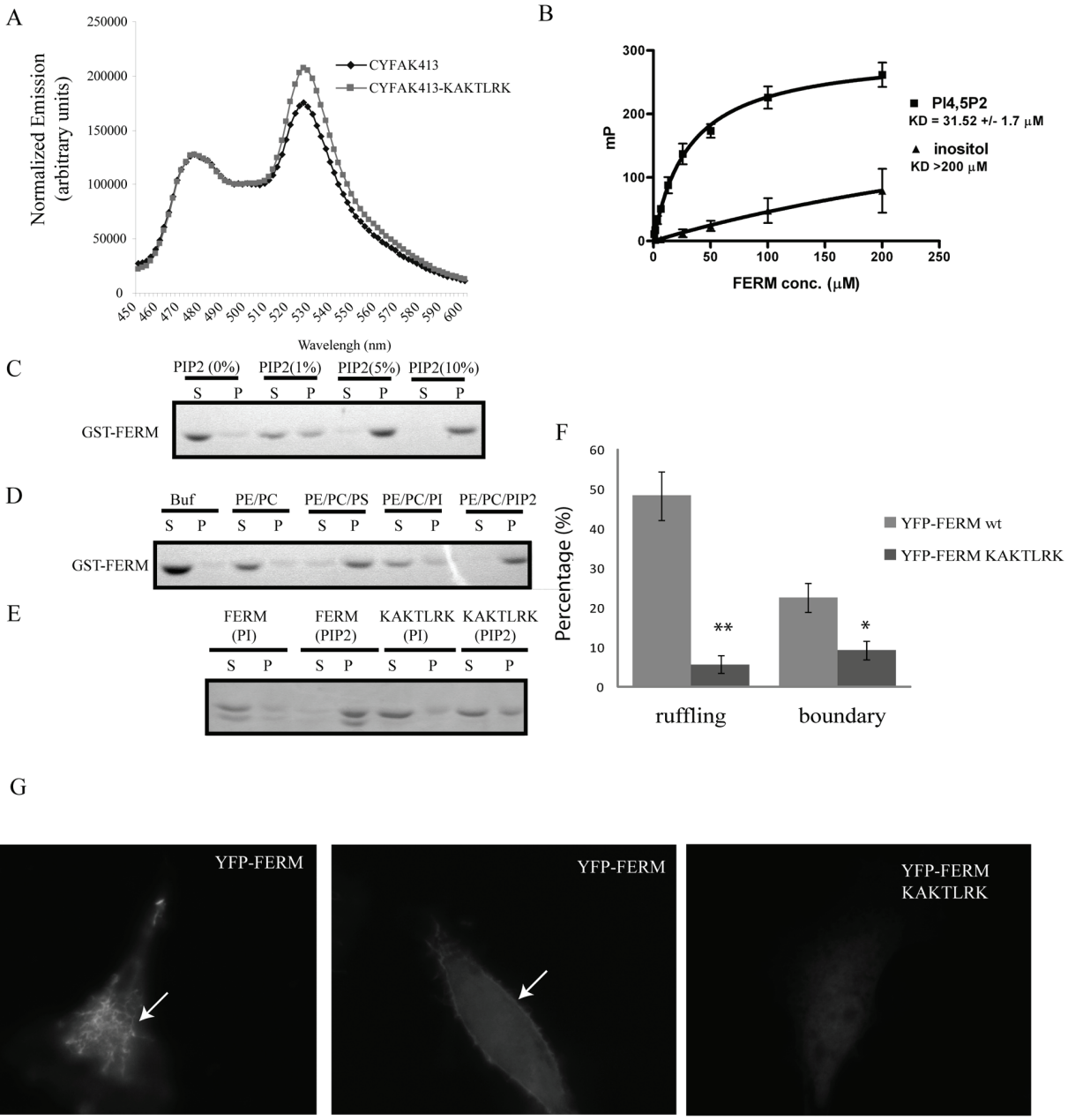


Fig. 3.6 PIP2 associates with FAK FREM domain. Acidic phospholipids bind the basic patch within FAK FERM domain. **A)** Normalized FRET/CFP emission ratios of the wild type FAK biosensor and the basic patch mutant are shown. The mutant FRET/CFP ratio was normalized to FRET/CFP ratio of the wild type biosensor. Shown is a representative experiment (n=3). **B)** The purified recombinant FERM domain was incubated with BODIPY-labeled phospholipids and binding measured by fluorescence polarization. Anisotropy (mP – millipolarization units) is plotted against FERM domain concentration. The average of 3 experiments +/-SD is shown. **C)** PE/PC vesicles containing increasing amounts of PIP2 were incubated with a GST-FERM fusion protein. The vesicles were sedimented by centrifugation and the amount of fusion protein in the vesicle containing pellet and the supernatant was determined was SDS-PAGE and Coomassie blue staining. **D)**

PE/PC vesicles containing 10% (mass ratio) of the indicated lipids were incubated with the GST-FERM fusion protein and analyzed as in A. **E)** PE/PC vesicles containing PIP2 or PI were incubated the wild type GST-FERM domain or a basic patch mutant (KAKTLRK) and analyzed as in A. **F,G)** HeLa cells expressing a YFP-FERM domain fusion protein or the basic patch mutant (KAKTLRK) were fixed, permeablized and then stained with rhodamine-phalloidin. Fixed cells were observed by laser scanning confocal microscopy. **F)** The percentage of cells containing the YFP-FERM constructs in ruffles or at the membrane at the edge of the cell were scored (average of 3 experiments +/- SD; >100 cells counted per experiment; **, $p<0.005$; *, $p<0.05$). **G)** Representative images of cells expressing the YFP-FERM domain (2 left panels) or the YFP-FERM KAKTLRK mutant (right panel) are shown. Arrows indicate YFP-FERM localization in ruffles (left panel) and at the membrane at the periphery of the cell (middle panel).

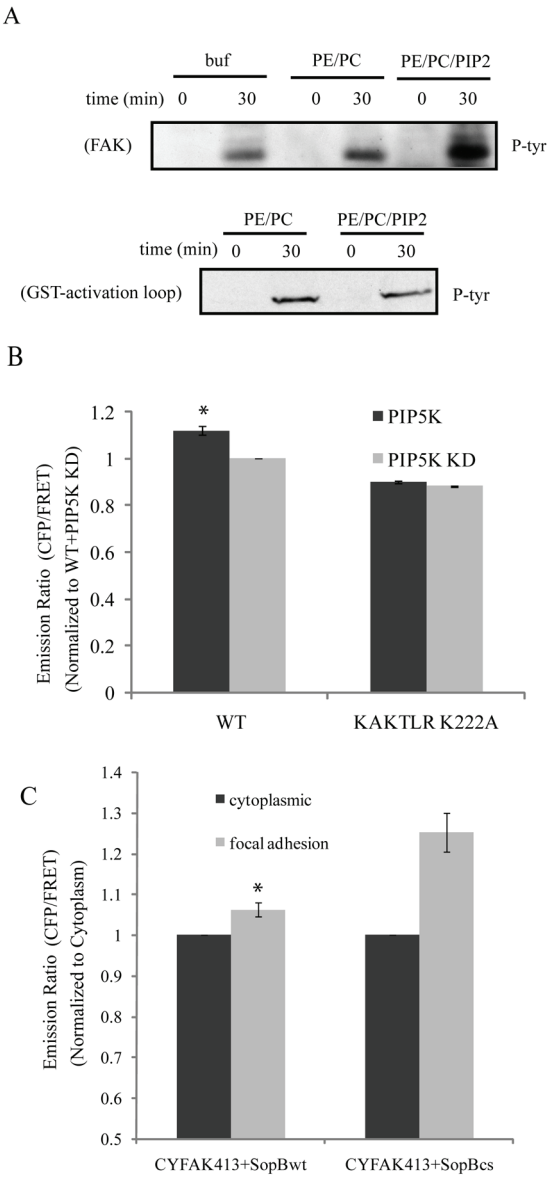


Fig. 3.7 PIP2 induces conformation change in FAK. A) A recombinant fragment of FAK containing the FERM and catalytic domains was incubated with Src (SH3+SH2+Kinase) in the presence of the indicated liposomes in kinase reaction buffer, or in buffer alone for 30 min. As a control substrate a GST fusion protein containing a peptide mimicking the activation loop of FAK was used. The reaction was terminated by the addition of sample buffer and phosphorylation of the substrates was examined by western blotting with a phosphotyrosine antibody. **B)** The wild type biosensor and the F2 basic patch mutant biosensor (KAKTLRK) were co-expressed with PIP5KIα or a catalytically defective mutant of PIP5KIα (KD). The CFP/FRET ratio in each case was determined by fluorometry. The average of 3 experiments +/- SE is shown. The emission ratios observed in the presence of wild type and catalytically inactive PIPKIα were analyzed using an unpaired t test (*, $p<0.05$). **C)** The average CFP/FRET ratio of the cytoplasm or focal adhesions in HeLa cells co-expressing the wild type biosensor and wild type SopB or the catalytically inactive mutant

SopBcs is shown (average of n=6 cells+/-SE). The focal adhesion values in SopB and SopBcs expressing cells were analyzed using an unpaired t test (*, $p<0.0025$).

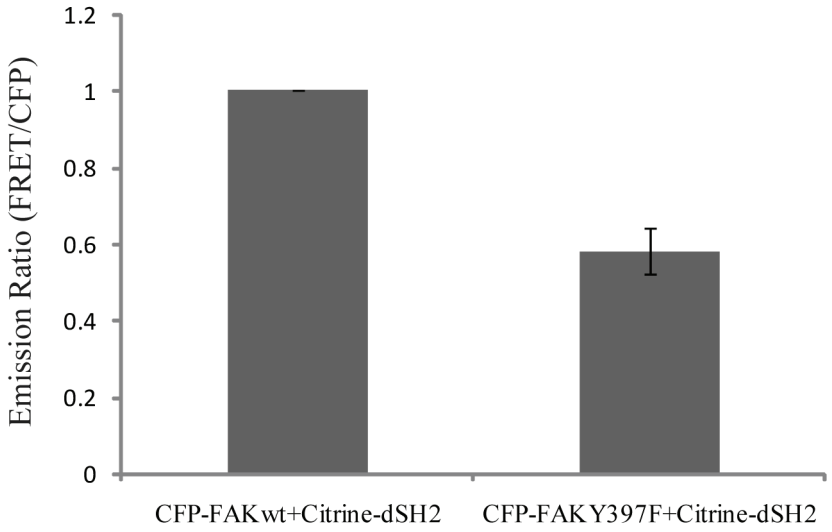


Fig 3.8 The emission ratio of FAK auto-phosphorylation biosensor. The FAK wild-type auto-phosphorylation biosensor and the CFAK-Y397F control biosensor were expressed in HEK293 cells and analyzed by fluorometry. CFP was selectively excited at 425nm and the resulting emission spectra were measured. Shown are the average FRET/CFP ratios (normalized to the wild type biosensor). The average of 3 experiments +/- SE is shown.

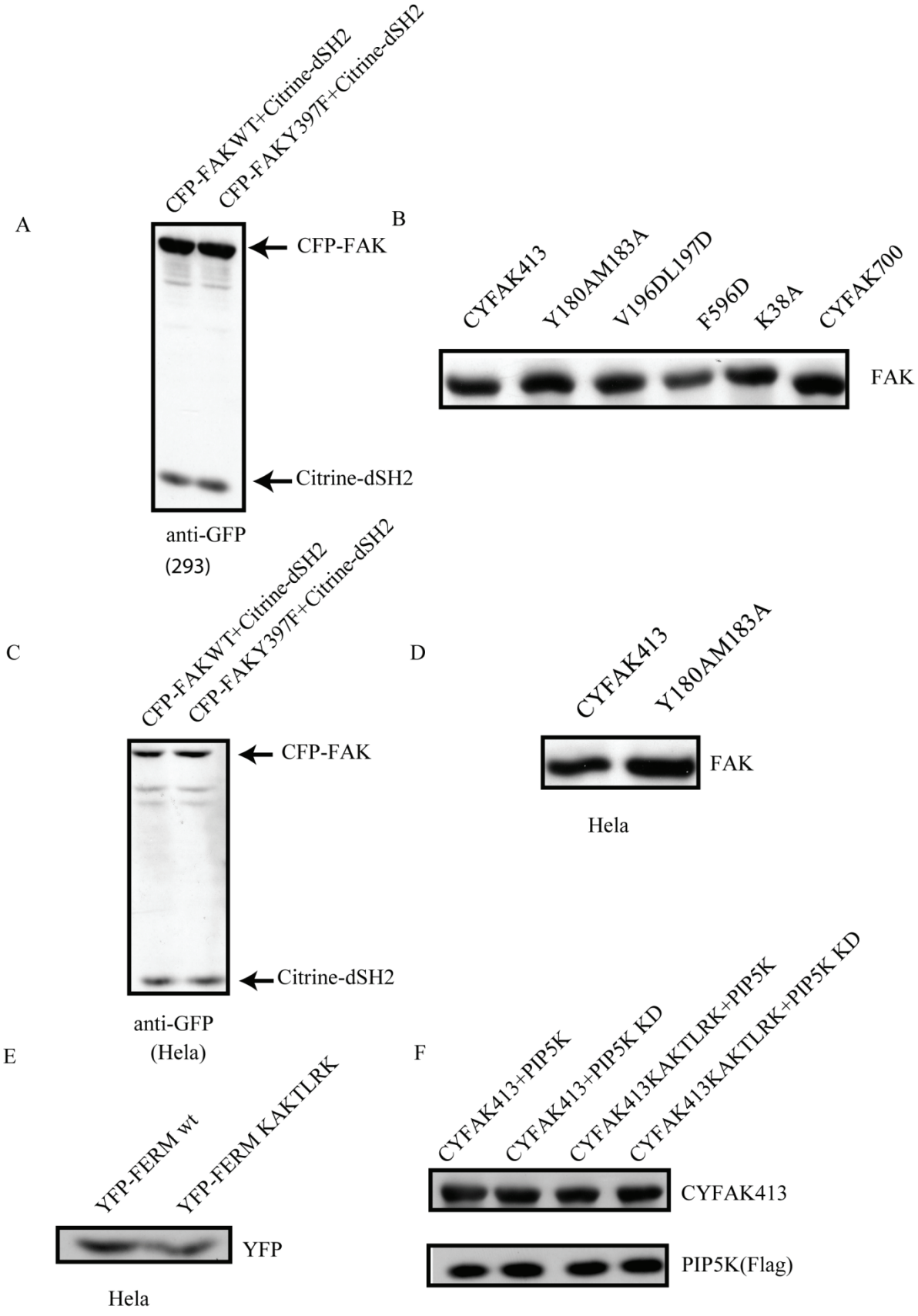

A
CFP-FAKWT+Citrine-dSH2
CFP-FAKY397F+Citrine-dSH2
CFP-FAK
Citrine-dSH2
anti-GFP
(293)
B
CYFAK413
Y180AM183A
V196DL197D
F596D
K38A
CYFAK700
FAK
C
CFP-FAKWT+Citrine-dSH2
CFP-FAKY397F+Citrine-dSH2
CFP-FAK
Citrine-dSH2
anti-GFP
(Hela)
D
CYFAK413
Y180AM183A
FAK
Hela
E
YFP-FERM wt
YFP-FERM KAKTLRK
YFP
Hela
F
CYFAK413+PIP5K
CYFAK413+PIP5K KD
CYFAK413KAKTLRK+PIP5K
CYFAK413KAKTLRK+PIP5K KD
CYFAK413
PIP5K(Flag)

Figure 3.9 The expression of FAK biosensors. A) Lysates of 293 cells expressing the wild type or Y397F mutant autophosphorylation biosensor were probed with a GFP antibody to compare expression levels. **B)** Lysates of 293 cells expressing the wild type and mutant conformation biosensors, which are analyzed in Fig. 3.2, were Western blotted with a FAK antibody to compare levels of expression. **C)** Lysates of HeLa cells expressing the wild type or Y397F mutant autophosphorylation biosensors, which are analyzed in Fig. 3.5, were blotted for GFP to compare expression levels. **D)** Lysates of HeLa cells expressing the wild type conformation biosensor and the Y180AM183A constitutively active mutant, which are analyzed in Fig. 3.5, were probed with a FAK antibody to compare levels of expression. **E)** Lysates of HeLa cells expressing the wild type FERM domain or the KAKTLRK mutant FERM domain, which are analyzed in Fig. 3.6, were Western blotted with a GFP antibody to compare expression levels. **F)** Lysates of 293 cells expressing the wild type conformation biosensor, the KAKTLRK mutant and PIPK5 kinase variants, which are analyzed in Fig. 3.7, were Western blotted for FAK to compare expression levels of the biosensor or PIP5 kinase to compare expression levels of the wild type and mutant proteins.

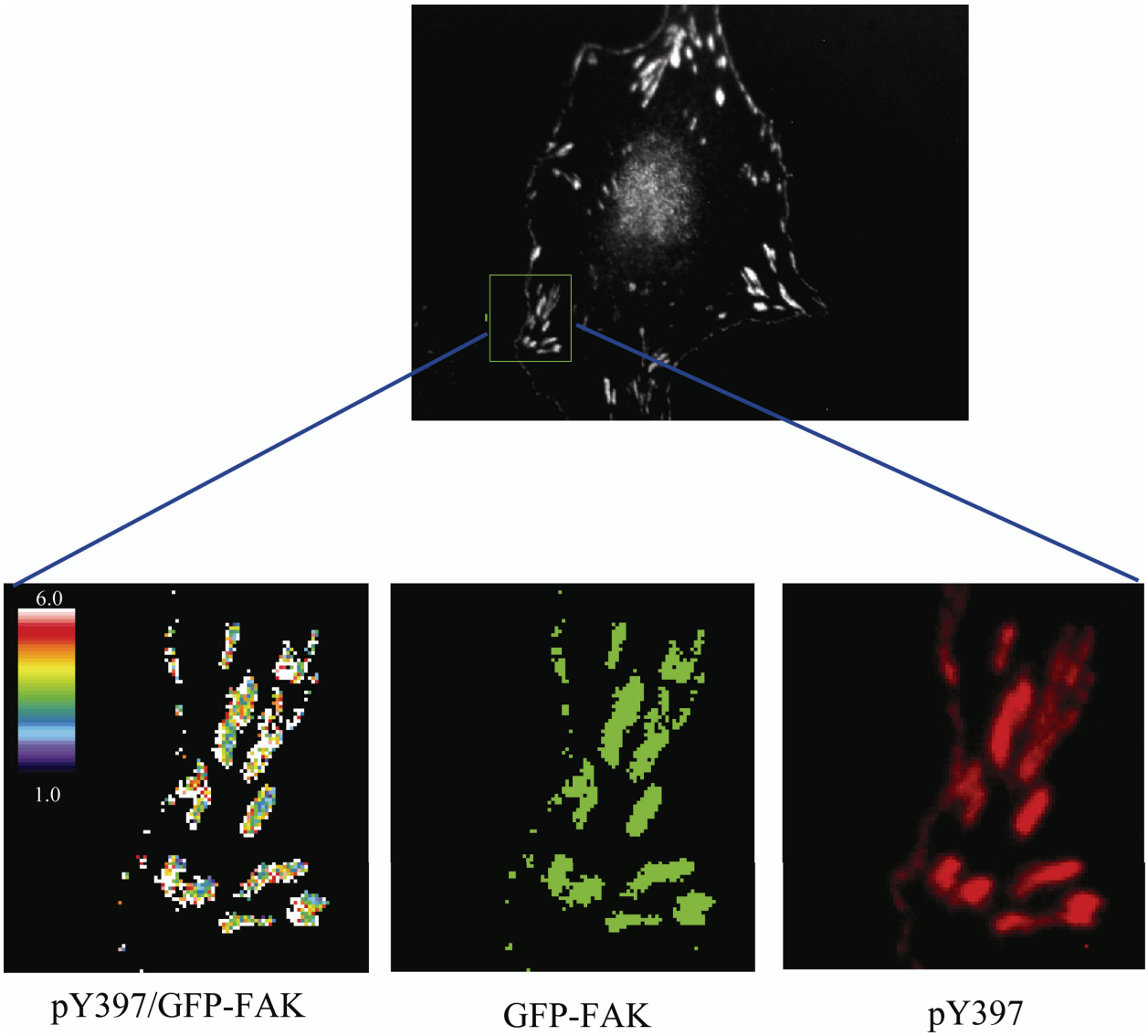


Fig 3.10 FAK auto-phosphorylation in focal adhesions. HeLa cells expressing GFP-FAK were fixed and stained with the PY397 antibody and rhodamine labeled secondary antibody. The top panel shows the PY397 staining pattern. The lower panels show a higher magnification of the boxed region. The left panel is the PY397/GFP ratio image, the middle panel is the GFP image and the right panel is the PY397 image.

CHAPTER 4
Pyk2 Regulates Fcγ Receptor Mediated Phagocytosis in Macrophage

4.1 Abstract

Pyk2 is a non-receptor tyrosine kinase expressed in a variety of tissues including cells derived from hematopoietic lineages. It regulates cell migration, survival and cell morphology. Here we report that Pyk2 is involved in regulating Fcγ receptor (FcγR) meditated phagocytosis in macrophages. Pyk2 is tyrosine phosphorylated following stimulation of the FcγR and this occurs through a Src family kinase and Syk dependent mechanism. Further, Pyk2 is enriched in phagocytotic cups during phagocytosis of IgG opsonized particles. RNAi, pharmacological and genetic approaches were used to demonstrate that Pyk2 is required for efficient FcγR mediated phagocytosis in a macrophage cell line and primary macrophages. Biochemical studies suggest that Pyk2 regulates downstream tyrosine phosphorylation events during phagocytosis, most strikingly of cytoskeletal targets. The results implicate Pyk2 as a regulator of FcγR-mediated phagocytosis and demonstrate that Pyk2 is required for optimal particle engulfment.

4.2 Introduction

Proline-rich tyrosine kinase (Pyk2) is a member of the focal adhesion kinase (FAK) family of kinases and shares a number of characteristics with FAK (15). Pyk2, like FAK, contains three major domains: an N-terminal FERM domain, a central kinase domain, and a C-terminal domain that contains binding sites for signaling molecules and the focal adhesion targeting (FAT) sequence of FAK. Pyk2 and FAK also share many binding partners. Both Pyk2 and FAK are activated by a number of stimuli, including integrin-dependent cell adhesion, growth factors and ligands for GPCRs. Pyk2 and FAK can regulate similar biological events, e.g. regulation of the cytoskeleton to control cell spreading and cell migration. However, there are a number of differences between Pyk2 and FAK, and the two

are not simply redundant. FAK is prominently localized to focal adhesions in adherent cells, whereas Pyk2 is not well localized in focal adhesions (121,398). However, in response to certain stimuli, Pyk2 can dramatically re-localize to focal adhesions (305). FAK is ubiquitously expressed in almost all tissues, while Pyk2 expression is restricted to specific tissues, like neuronal, epithelial and hematopoietic cells. Some of these tissues express both FAK and Pyk2, whereas other cells like macrophages predominantly express Pyk2 and express FAK at relatively low levels (122). Interestingly, *pyk2*$^{-/-}$ mice exhibit hematopoietic cell defects, including reduced marginal zone B cells in the spleen, defective recruitment of macrophages to sites of inflammation and altered macrophage morphology and motility(166,339).

As "professional phagocytes" an important function for macrophages is the engulfment of foreign particles. Particle engulfment can occur through multiple mechanisms, including direct engulfment of particles, e.g. bacteria, FcγR-mediated phagocytosis and complement-mediated phagocytosis (453). These are important cellular anti-microbe strategies in innate immunity that are distinguished by the receptors and signaling mechanisms controlling particle engulfment. During FcγR-mediated phagocytosis, immunoglobulin opsonized pathogens are recognized and bound by cell surface FcγR, the particles engulfed by the cell and the internalized pathogens are destroyed in the phagosome. Multiple signal pathways cooperate to orchestrate the phagocytic process, including pathways controlled by tyrosine kinases, lipid kinases and the Rho family of GTPases (432). When IgG-opsonized microbes interact with the FcγR, two members of the Src family of tyrosine kinases, Lyn and Hck, are activated and induce the tyrosine phosphorylation of the ITAM motifs in the cytoplasmic tail of the FcγR. This creates a high affinity binding site for

the SH2 domains of Syk and Syk is recruited to the tyrosine phosphorylated ITAM. Syk is responsible for triggering the activation of downstream signals, which includes the Rho family and PI3 kinase signaling pathways. Two members of the Rho family, Cdc42 and Rac, are implicated in controlling cytoskeletal events associated with particle engulfment. Cdc42 regulates the formation of the phagocytotic cup and Rac regulates closure of the phagocytic cup and particle engulfment. However, the function of RhoA in FcγR-mediated phagocytosis is still controversial due to the inconstant results from the different kind of cells (53,173). PI3K signaling is critical for the phagosome formation (433). It could be also evolved in the other stages of phagocytosis, such as pseudopod extension (101).

Since Pyk2 regulates cytoskeleton rearrangements both in macrophages and fibroblasts, and previous studies demonstrate that Pyk2 is activated in human macrophages following binding of IgG coated beads (245), it seems likely that Pyk2 may also play a role in controlling FcγR mediated phagocytosis. Our data support the hypothesis that Pyk2 is involved in macrophage signaling during phagocytosis. Pyk2 is activated upon ligand binding to the FcγR and accumulates at the phagocytotic cup during phagocytosis. Multiple strategies were employed to inhibit Pyk2 in a macrophage cell line and primary macrophages. These results demonstrate that inhibition of Pyk2 signaling impairs FcγR mediated phagocytosis. Further, these results implicate Pyk2 in the control of tyrosine phosphorylation of cytoskeletal proteins and in inducing maximal activation of PI3 kinase in response to FcγR signaling.

4.3 Methods

Cells and reagents. RAW264.7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS). HEK293T cells were

maintained in DMEM-F12 containing 10% FBS. Bone marrow–derived macrophages were isolated and cultured as described (137). Wortmannin and PP2 were purchased from Calbiochem. The small molecule Pyk2 and FAK inhibitors, GR-222 and PF-228, were from Pfizer. Phosphotyrosine (4G10) and FAK antibodies (447) were from Millipore. Phosphospecific antibodies recognizing FAK, ERK and Akt were from Invitrogen. Syk, ERK2 and general mouse IgG antibodies were from Santa Cruz and the Akt antibody was from Cell Signaling. The actin antibody was from Chemicon and the GFP antibody was from Roche.

Plasmid and siRNA oligos transfection. HEK293T cells were transfected using Lipofectamine Plus according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). Raw264.7 cells were transfected with the Amaxa Nucleofector Kit V according to the manufacturer's instructions. For siRNA assay, RAW264.7 cells were transfected with 100 nM siRNA using Transit TKO® according to the manufacturer's instructions (Mirus, Madison, WI). The FAK siRNA sequence is 5'-AAGAUUACCAAUGCCUCCAAA-3'. Pyk2 siRNA (smart pool®) is designed by Dharmacon. All oligos were synthesized by Dharmacon (Lafayette, CO).

Immunostaining and imaging. Raw264.7 cells expressing fluorescent proteins were seeded on cover slips and incubated with IgG opsonized latex beads. Cells were fixed in 3.7% formaldehyde and imaged using a Zeiss LSM510 confocal microscope. Bone marrow–derived macrophages were seeded on cover slips and incubated with IgG opsonized RBCs. Cells were fixed in 3.7% formaldehyde solution for 8 min. The fixed cells were permeablized with 0.1% Triton X-100 for 5min. Non-specific binding to the FcγR was blocked by Fc-Block®. Pyk2 was detected using Pyk2 mAb (BD Transduction Laboratories)

and a fluorescein-conjugated anti-mouse antibody (Jackson ImmunoResearch Labs) as described previously (46). The fixed cells were visualized using fluorescence microscopy.

Molecular biology. Pyk2 point mutations were created by PCR using the Quick-Change mutagenesis kit (Stratagene, La Jolla, CA). Sequence analysis was performed on each mutant to verify the intended point mutations and that no unintended mutations were present. Pyk2 truncation mutants were created by subcloning PCR amplified Pyk2 fragments into the pcDNA or Citrine vectors.

FcγR mediated phagocytosis and binding assays. To prepare IgG opsonized red blood cells (RBCs), RBCs were washed with 100ul PBS twice. The washed RBCs were incubated with rabbit-anti-sheep RBCs (1:300) for 1h at room temperature. The opsonized RBCs were washed with PBS once and diluted to $4*10^6$/ml. Raw264.7 cells were split into 24 well dishes ($1*10^5$ cells/well) in culture medium for 2h. Then the cells were serum starved for another 2h and incubated with opsonized RBCs at 37ºC for various times. The cells were washed twice with serum free medium. RBCs that were not engulfed by the macrophages were lysed by incubation in distilled water for 30 seconds. The cells were fixed in 3.7% paraformaldehyde. The percentage of phagocytic cells (containing at least one red blood cell) was determined by counting under a microscope. In some experiments, the macrophages were incubated with drugs for 1h before the assay. The phagocytosis assay using IgG opsonized latex beads was performed as previous described (321). For the binding assay, macrophages were incubated with opsonized RBCs at 4ºC for 20min. The cells were washed twice with SFM and fixed in 3.7% paraformaldehyde. The binding index was counted as the number of RBCs per cell.

Phagocytosis biochemical assay. Bone marrow cells were seeded in p35 dish and incubated at 37^{o}C for 7 days. Then differentiated macrophages were serum starved overnight and equilibrated with assay medium (DMEM, 0.1%BSA, 10mM HEPES PH7.4) on ice. The cells were incubated with the 2.4G2 monoclonal antibody (10ug/ml, BD Pharmingen) for 30min. After two washes with ice-cold assay medium, FcγR were clustered by addition of 30ug/ml rabbit anti-rat IgG (Sigma) in assay medium and incubation at 37 ºC for various times prior to lysis. For stimulation, ice cold cells were incubated with opsonized RBCs for 30 min at 4 ºC and transferred to 37 ºC for various times. The cells were washed with serum-free medium and lysed.

Cell lysis, protein analysis, and immunoprecipitation. Cells were lysed in ice-cold modified radioimmunoprecipitation assay buffer. Lysates were clarified, and protein concentrations were determined using the bicinchoninic acid assay (Pierce, Rockford, IL). For immunoprecipitations, antibodies were incubated with cell lysate at 4°C for 1 h. Immune complexes were precipitated at 4°C for 1 h with protein A-Sepharose beads (Sigma, St. Louis, MO) coated with AffiniPure rabbit anti-mouse immunoglobulin G (Jackson ImmunoResearch Labs, West Grove, PA). Immune complexes were washed twice with ice-cold lysis buffer and once with ice-cold PBS. Beads were resuspended in sample buffer and boiled to elute the proteins, and the samples were analyzed by Western blotting.

4.4 Results

FcγR mediated phagocytosis induces tyrosine phosphorylation of multiple signaling proteins. Clustering the FcγR in bone marrow derived macrophages using a specific antibody, 2.4G2, resulted in increased tyrosine phosphorylation (Fig. 4.1A). Tyrosine phosphorylation was also enhanced when cells were challenged with IgG opsonized RBCs,

which induce FcγR mediated phagocytosis (Fig. 4.1A). One evident tyrosine phosphorylated band exhibits a molecular weight similar to Pyk2 suggesting that this tyrosine kinase may be a substrate for phosphorylation following the activation of FcγR. To test this possibility Pyk2 was immunoprecipitated from these lysates and Western blotted using a phosphotyrosine antibody. The result indicates that tyrosine phosphorylation of Pyk2 was increased following FcγR clustering, suggesting that Pyk2 was activated during FcγR mediated phagocytosis (Fig. 4.1B). In addition to Pyk2, FAK was also examined. While FAK is expressed at very low levels in macrophages, other phagocytic cells, such as neutrophils, express FAK, as do a number of macrophage cell lines, such as J774 and Raw264.7 cells (133). Tyrosine phosphorylation of FAK was measured in Raw 264.7 cells following stimulation with IgG-opsonized RBC or FcγR crosslinking (Fig. 4.1C). These results demonstrate that FAK is also tyrosine phosphorylated during FcγR mediated phagocytosis. To investigate the molecular mechanism regulating Pyk2 tyrosine phosphorylation, primary macrophages were pre-treated with the pharmacological inhibitors prior to stimulation of FcγR. Tyrosine phosphorylation of Pyk2 was blocked by both PP2 (an inhibitor of the Src family kinases), piceatannol (an inhibitor of Syk) and Syk inhibitor III (Fig. 4.1D). All drugs blocked tyrosine phosphorylation of Pyk2 in response to crosslinking of FcγR. Interestingly, tyrosine phosphorylation of Pyk2 is unimpaired upon treatment with wortmanin, which is an inhibitor of PI3 kinase (Fig. 4.1D). These results suggest that phagocytosis induced Pyk2 tyrosine phosphorylation depends upon the SFK/Syk activation but not lipid signaling.

The subcellular localization of Pyk2 during phagocytosis was also investigated. Raw264.7 cells co-expressing YFP-Pyk2 and the empty pECFP vector were incubated with

IgG opsonized beads and the fluorescent probes visualized using confocal microscopy. YFP-Pyk2 accumulated in the phagocytotic cup, whereas CFP was not enriched in this region of the cell (Fig. 4.2A). The spatial distribution of endogenous Pyk2 was investigated in primary bone marrow derived macrophages. The macrophages were incubated with IgG opsonized RBCs and immunostained using a Pyk2 monoclonal antibody. Fc-Block® was used to block non-specific binding to the FcγR. Phagocytic cups were visualized by phase contrast microscopy (Fig. 4.2B). Endogenous Pyk2 was also enriched in phagocytotic cups during phagocytosis (Fig. 4.2B). A non-specific mouse IgG was used as a negative control for the Pyk2 monoclonal antibody and produced no evident immunofluorescent signal in the cells. These results demonstrate that Pyk2 is also recruited to sites of FcγR mediated phagocytosis.

The interaction of Pyk2 with tyrosine kinases implicated in the regulation of phagocytosis was tested. Both Pyk2 and FAK are known to interact with Src and other members of this family of tyrosine kinases and myeloid expressed Src family kinase members play a role in the control of FcγR mediated phagocytosis (136). To further explore these interactions, Lyn-GFP was co-expressed with Pyk2 in 293T cells and the two were found to associate by co-immunoprecipitation and Western blotting (Fig. 4.3A). Pyk2 was recovered from Lyn immune complexes and GFP-Lyn was detected in Pyk2 immune complexes. As anticipated the auto-phosphorylation site (Y402) of Pyk2, which conforms to a high affinity Src SH2 domain binding site, was required for this interaction (Fig. 4.3B). Syk is a second tyrosine kinase implicated in the regulation of FcγR mediated phagocytosis and a Pyk2/Syk complex transiently forms following TNF stimulation of neutrophils (177). The interaction of Syk with Pyk2 was therefore further investigated. GFP-Syk and Pyk2 were co-expressed in 293T cells and complexes examined by co-immunoprecipitation and

Western blotting (Fig. 4.3A). Pyk2 was detected in Syk immune complexes and Syk was found in Pyk2 immune complexes. Interestingly, the Pyk2 auto-phosphorylation site is not required for this interaction, which suggests that Lyn and Syk interact with Pyk2 via different mechanisms (Fig. 4.3B). Very similar results were obtained with FAK. Upon co-expression in 293T cells FAK associated with Lyn and Syk (Fig. 4.3C). Further the association of FAK with Lyn required the autophosphorylation site of FAK (Y397) whereas binding to Syk did not (Fig. 4.3D). The interaction with Syk was mediated by the N-terminal FERM domain of Pyk2. Whereas wild type Pyk2 co-immunoprecipitated with Syk, a Pyk2 mutant with a deletion of the N-terminal domain of Pyk2 (Pyk2ΔN) failed to associate with Syk (Fig. 4.3E). Further, when Syk was co-expressed with the FERM domain of Pyk2, expressed as a citrine-FERM domain fusion protein, Syk was co-immunoprecipitated with citrine-FERM (Fig. 4.3F). This interaction was verified using an *in vitro* pull down assay. A GST-Pyk2 FERM domain fusion protein bound Syk from cell lysates, whereas the GST control did not (Fig. 4.3G). While the interaction with FAK and Src family kinases is well established, it was important to demonstrate that endogenous Pyk2 and Syk could form a complex. The complex between the endogenous proteins was identified in Raw264.7 cells by co-immunoprecipitation and Western blotting (Fig. 4.3H).

The FcγR mediated tyrosine phosphorylation of Pyk2 and translocation of Pyk2 to phagocytic cups suggests that Pyk2 may play an important role in phagocytosis. Similarly, FAK could also play an important function in phagocytosis. This may be true in macrophage cell lines, like Raw264.7 cells, which express both tyrosine kinases. However, in primary macrophages, which express predominantly Pyk2 and very low levels of FAK, Pyk2 seems the more likely candidate (Fig. 4.4A). The function of Pyk2 in phagocytosis was

investigated using multiple strategies. First, the expression of Pyk2 and FAK in Raw264.7 cells was diminished using siRNAs. These siRNAs effectively reduced expression of the target protein (Fig. 4.4B). Two different assays were performed to evaluate phagocytosis. The first assay utilized IgG-opsonized latex beads and the second IgG-opsonized RBCs as targets for phagocytosis. Using both assays, the phagocytic activity of Raw264.7 cells was evidently impaired by siRNA knockdown of Pyk2 or FAK (Fig. 4.4C and D). Maximal impairment of phagocytosis was achieved when both Pyk2 and FAK were silenced in these cells. However, the binding index of IgG-opsonized RBCs was not changed in these cells, suggesting that Pyk2 and FAK had no effect on the ability of the FcγR to bind its ligand and were required for a subsequent step in phagocytosis. The second method to address the function of Pyk2 and FAK in phagocytosis used a pharmacological strategy. Recently, small molecule inhibitors recognizing FAK (PF-228) or both FAK and Pyk2 (GR-222) were developed (428). These were tested in Raw264.7 cells. Tyrosine phosphorylation of FAK was dramatically impaired by both drugs, whereas Pyk2 phosphorylation was inhibited by GR-222, but not by PF-228 (Fig. 4.4E). The effect of these inhibitors on FcγR mediated phagocytosis was then tested in Raw264.7 cells (Fig. 4.4F). Interestingly, phagocytosis was partially impaired by PF-228, which inhibited FAK but not Pyk2, and phagocytosis was dramatically impaired by GR-222, which targeted both kinases. Again, inhibition of FAK and Pyk2 had no effect upon binding IgG-opsonized particles. The effect of Rho kinase in FcγR mediated phagocytosis was also tested since Rho kinase is also potentially inhibited by GR-222, albeit at higher concentrations than Pyk2 and FAK. Phagocytosis was unimpaired when cells were treated with Y-27632, which is consistent with previously published data (342). Thus, the effect of GR-222 on phagocytosis was apparently due to its effects upon

Pyk2 and FAK. The third strategy used a genetic approach to investigate the function of Pyk2 in primary macrophages. The phagocytic ability of bone marrow derived macrophages isolated from wild type and *pyk2*$^{-/-}$ mice was compared. The *pyk2*$^{-/-}$ macrophages exhibited a deficiency in the phagocytosis of IgG-opsonized RBCs that was more evident at early times than at later times (Fig. 4.4G). The null macrophages exhibited binding of opsonized RBCs that was comparable to wild type macrophages (Fig. 4.4H). The results of all three experimental approaches support the hypothesis that Pyk2 functions to regulate FcγR mediated phagocytosis in macrophages. In cells also expressing FAK, e.g. Raw264.7 cells, FAK also plays a role in regulating phagocytosis.

Since Pyk2 is involved in the FcγR mediated phagocytosis, it is interesting to understand which pathways are regulated by Pyk2 in phagocytosis. The tyrosine phosphorylation pattern was compared between wild type and *pyk2*$^{-/-}$ bone-marrow derived macrophages. The increased phosphorylation of some specific bands was partially impaired (Fig. 4.5A). Several specific signaling pathways were examined based upon the role of Pyk2/FAK in regulating ERK activation, PI3K signaling and tyrosine phosphorylation of adhesion-associated proteins. ERK is activated during FcγR-mediated phagocytosis and crosslinking of FcγR in primary macrophages. The level of ERK activation in wild type and *pyk2*$^{-/-}$ macrophages was similar suggesting that Pyk2 is dispensable for FcγR-induced ERK activation (Fig. 4.5 B). PI3K signaling was monitored by measuring Akt phosphorylation. Akt phosphorylation was dramatically increased during FcγR-mediated phagocytosis and crosslinking of FcγR. There was a modest reduction in Akt phosphorylation in the null macrophages, suggesting that Pyk2 plays a minor role in regulating PI3K and Akt phosphorylation during the phagocytosis of IgG-opsonized particles (Fig. 4.5 B). Paxillin is

a Pyk2 substrate that translocates to the phagocytotic cup in FcγR mediated phagocytosis (162,243)**.** The tyrosine phosphorylation of paxillin is enhanced during receptor crosslinking and FcγR-mediated phagocytosis. The *pyk2*$^{-/-}$ macrophages exhibited a dramatic deficiency in tyrosine phosphorylation of paxillin, demonstrating an important role for Pyk2 in controlling paxillin tyrosine phosphorylation during FcγR-mediated phagocytosis in primary macrophages (Fig. 4.5B).

4.5 Discussion

Here, we report that Pyk2 is involved in FcγR-mediated phagocytosis. Pyk2 is translocated to the phagocytotic cup and becomes tyrosine phosphorylated during phagocytosis. Pyk2 interacts with two important kinases that regulate phagocytosis of IgG opsonized particles, Lyn and Syk. Pharmacological studies suggest that tyrosine phosphorylation of Pyk2 depends on the activity of those two tyrosine kinases. The activation of Pyk2 is independent of PI3K. As *pyk2*$^{-/-}$ macrophages exhibit a modest reduction in phosphorylation of Akt, Pyk2 might play a minor role in regulating PI3K signaling. These cells showed a dramatic reduction in tyrosine phosphorylation of paxillin during phagocytosis, demonstrating a key role for Pyk2 in regulating phosphorylation of cytoskeletal proteins during this process. Most importantly, cells lacking Pyk2 or treated to inhibit Pyk2 catalytic activity exhibit impaired FcγR-mediated phagocytosis. While other studies have alluded to a role in phagocytosis, this is the first report directly demonstrating that Pyk2 regulates FcγR-mediated phagocytosis.

During FcγR-mediated phagocytosis, Pyk2 activation depends on Src family kinases and Syk. The pharmacological approach does not distinguish which myeloid specific Src family kinase is responsible, but given the redundancy of the members of the family, it is

likely that both Lyn and Hck can regulate Pyk2 phosphorylation. It is well established that Src family kinases associate with the focal adhesion family kinases by SH2 domain interactions and regulate Pyk2/FAK tyrosine phosphorylation (51,110,390,405). Pyk2 is reported to associate with Syk and the related tyrosine kinase, Zap70 (178,242). Our study has extended these observations to determine one mechanism of interaction between these kinases. The N-terminal FERM domain of Pyk2/FAK mediates this interaction. The FERM domain functions as a negative regulatory element to control catalytic activity of FAK, and presumably Pyk2, and also serves a scaffolding function by docking with a number of other proteins. Phosphorylation of Pyk2 is reportedly controlled by Syk in a number of scenarios (52,449,495). There is some concern regarding the use of piceatannol as a Syk inhibitor in studies exploring Pyk2 signaling, since piceatannol is known to directly inhibit the catalytic activity of the Pyk2-related kinase, FAK (264). Our study supports these pharmacological studies by using another Syk inhibitor to document a role for Syk in controlling Pyk2 phosphorylation during phagocytosis. Genetic approaches have also been applied to define a role for Syk upstream of FAK. For example, a variant of the RBL 2H3 mast cell line lacking Syk shows a defect in Pyk2 tyrosine phosphorylation in response to FcεRI signaling (336).

Pyk2 possibly regulates phagocytosis via several different mechanisms. Pyk2 may regulate signaling via phospholipids in macrophages since the *pyk2*$^{-/-}$ macrophages exhibit a modest decrease in Akt phosphorylation during phagocytosis. However, the modest reduction indicates that Pyk2 plays a lesser role and other signaling pathways predominantly function in controlling PI3K signaling during phagocytosis. Other studies support a role for Pyk2 in the regulation of PI3K in response to other stimuli. For example, *pyk2*$^{-/-}$ macrophages exhibit defects in PI3K activation in response to integrin dependent cell

adhesion (337). The pharmacological inhibitors of Pyk2/FAK used in this study are competitive inhibitors of ATP and since they inhibit phagocytosis, the phosphorylation of downstream substrates is implicated in the regulation of phagocytosis by Pyk2. Paxillin phosphorylation is markedly defective for phosphorylation in *pyk2*$^{-/-}$ macrophages. This is potentially a mechanism of regulation of phagocytosis by Pyk2. Tyrosine phosphorylation of paxillin creates binding sites for the Crk adaptor protein (395), which in turn can recruit guanine nucleotide exchange factors for Rho family proteins into complex. One such exchange factor, the Dock180/Elmo complex, functions to promote GTP binding to Rac and was identified as a Crk binding protein (184). Interestingly, CrkII and Dock180/Elmo were recently implicated in controlling Rac activation and FcγR-mediated phagocytosis in RAW 264.7 cells (271). This signaling pathway is potentially under the control of Pyk2 in primary macrophages and will be an important area of investigation in future studies.

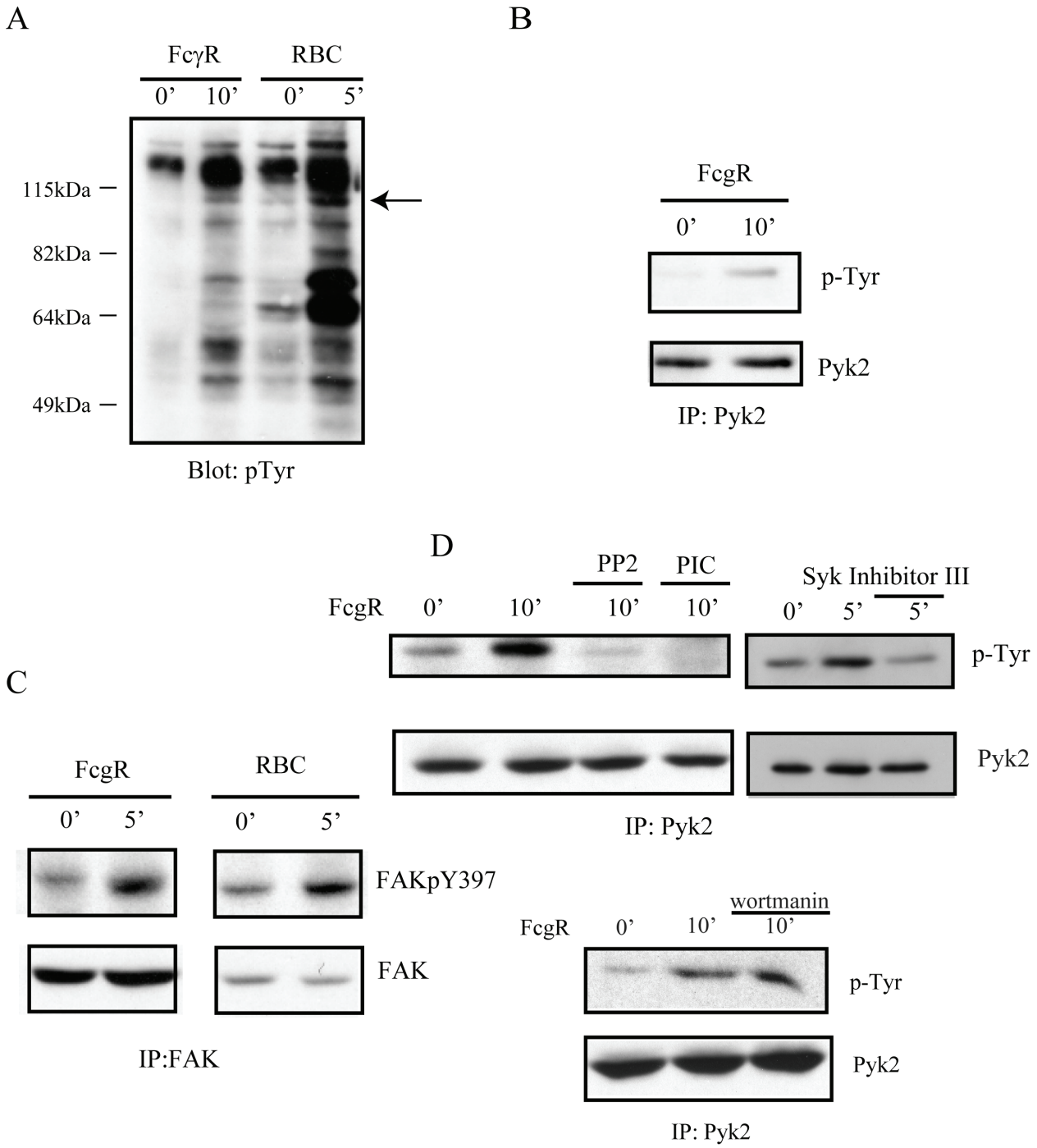


Figure 4.1 Pyk2 is tyrosine phosphorylated in a Src family kinase- and Syk-dependent manner during FcγR mediated phagocytosis. A) Bone marrow derived macrophages were stimulated by incubation with the anti- FcγR mAb 2.4G2 followed by anti–rat IgG cross-linking or by addition of IgG opsonized RBCs for the indicated times. Cells were lysed, and equivalent amounts of cellular protein were Western blotted with pTyr (4G10) antibody. A band exhibits a molecular weight similar to Pyk2 is labeled with arrow. **B)** Bone marrow derived macrophages were stimulated by incubation with the anti- FcγR mAb 2.4G2 followed by anti–rat IgG cross-linking for the indicated time. Pyk2 was immunoprecipitated using the Pyk2 mAb and Western blotted with indicated antibodies. **C)** Raw264.7 cells were

stimulated by incubating with anti- FcγR mAb (2.4G2) followed by anti–rat IgG cross-linking or IgG opsonized RBCs for the indicated times. Cells were lysed, and equivalent amounts of cellular protein were blotted with FAK and FAK pY397 antibodies. **D)** BMMs were pretreated with DMSO or the indicated inhibitors and stimulated by incubation with anti- FcγR mAb 2.4G2 followed by anti–rat IgG cross-linking for the indicated time. Pyk2 was immunoprecipitated using the Pyk2 mAb and blotted with indicated antibodies.

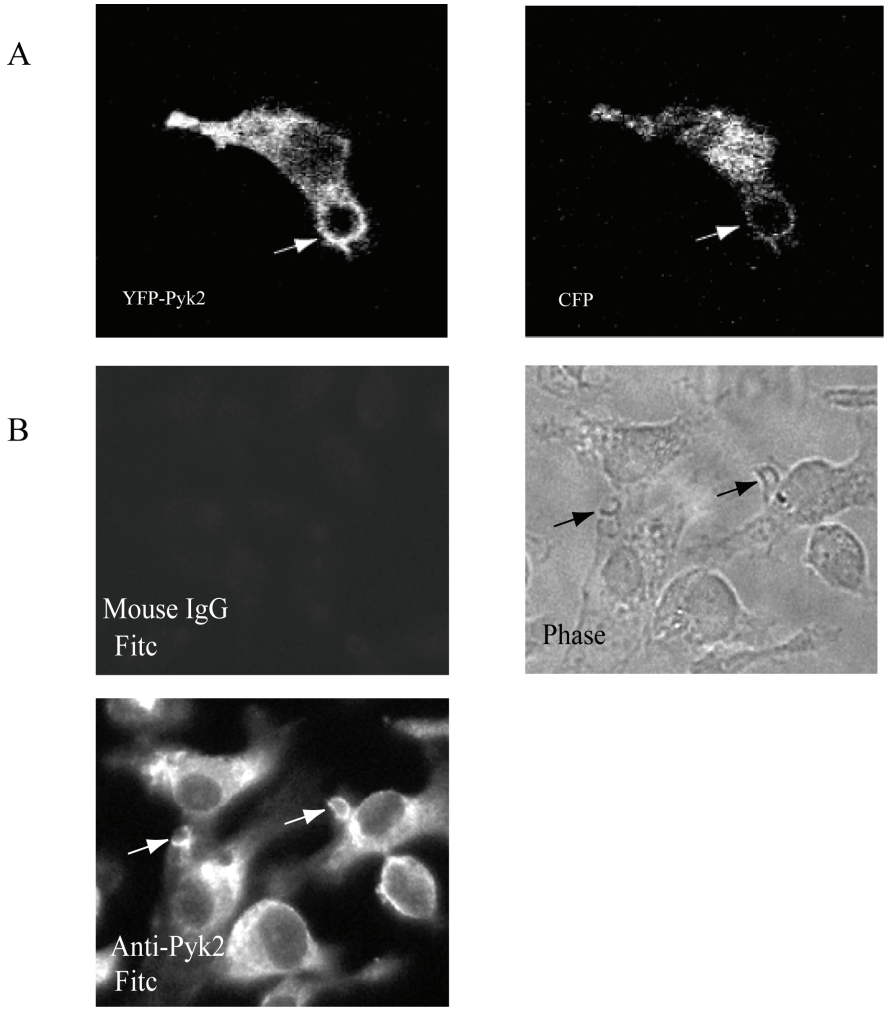


Figure 4.2 Pyk2 accumulates in phagocytotic cups during FcγR mediated phagocytosis. **A)** Raw 264.7 cells co-expressing YFP-Pyk2 and CFP were incubated with IgG opsonized latex beads for 5min. The cells were fixed and visualized by laser confocal microscopy. **B)** BMMs were stimulated with IgG opsonized RBC for 5min. Localization of Pyk2 was visualized by immunofluorescence using a Pyk2 monoclonal antibody. Mouse IgG was used as negative control.

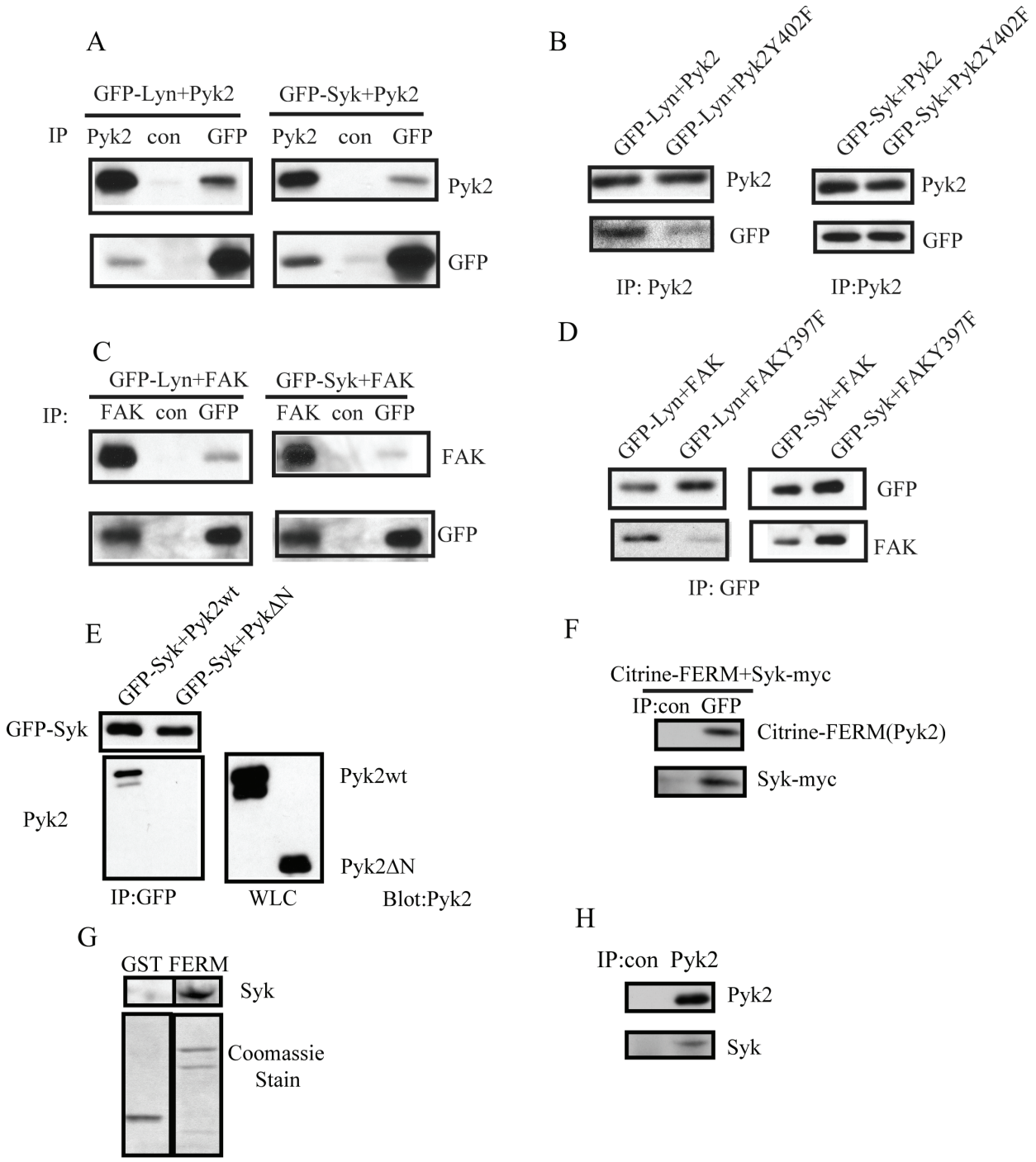


Figure 4.3 Pyk2 interacts with Lyn and Syk via different mechanisms. A-F) 293T cells expressing indicated constructs were lysed and immunoprecipitated with indicated antibodies. The immune complexes were blotted with indicated antibodies. **G)** Raw264.7 cells were lysed, precleared with GST, and then incubated with GST or GST-FERM (Pyk2). Endogenous Syk bound to the beads were detected by western blotting using Syk antibody. The equivalent amounts of GST fusion protein were visualized by Coomassie blue staining. **H)** Raw264.7 cells lysed and immunoprecipitated with a Pyk2 antibody. The immune complexes were blotted with indicated antibodies.

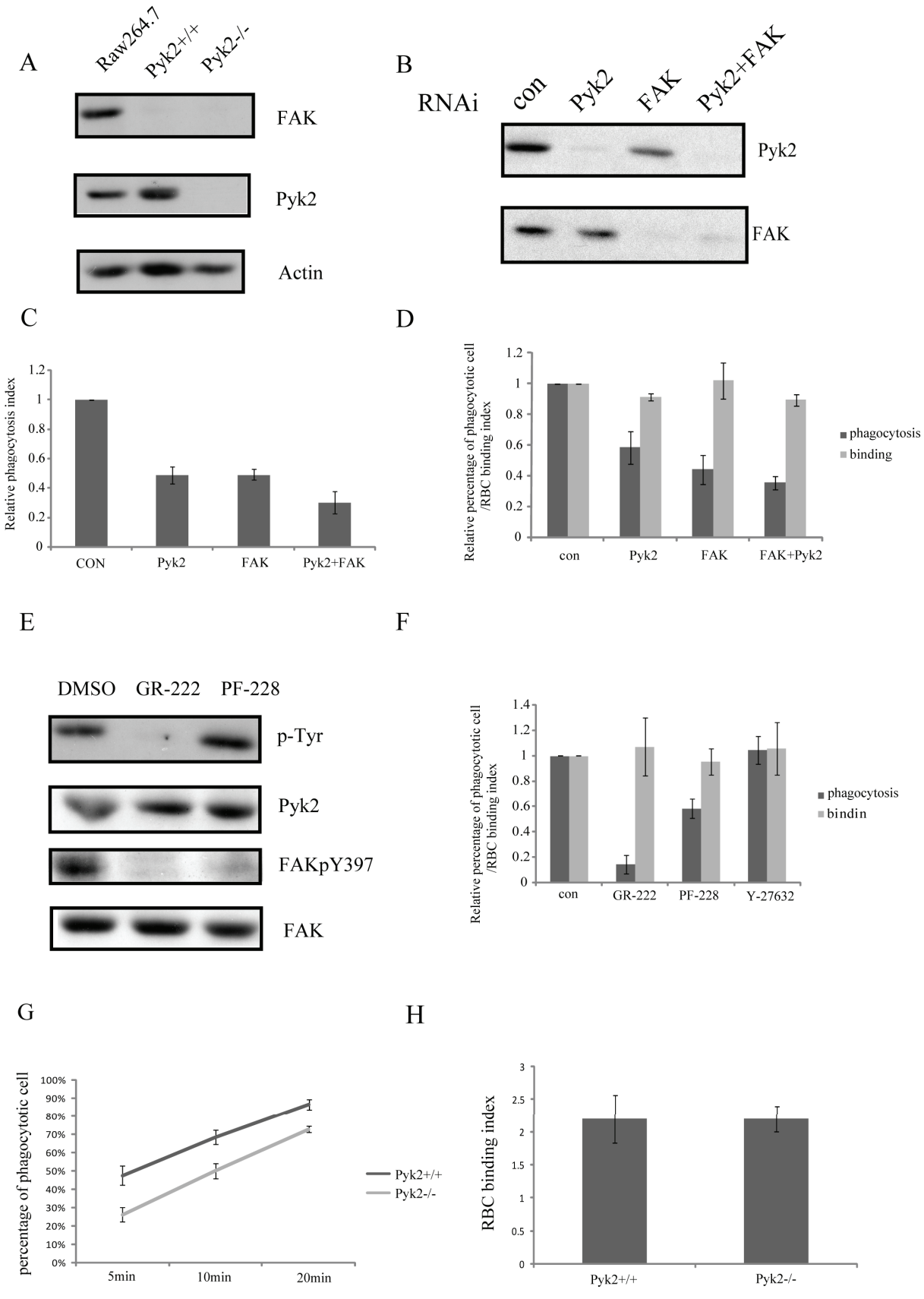

A
Raw264.7
Pyk2+/+
Pyk2-/-
FAK
Pyk2
Actin
B
RNAi
con
Pyk2
FAK
Pyk2+FAK
Pyk2
FAK
C
Relative phagocytosis index
CON
Pyk2
FAK
Pyk2+FAK
D
Relative percentage of phagocytotic cell /RBC binding index
con
Pyk2
FAK
FAK+Pyk2
phagocytosis
binding
E
DMSO
GR-222
PF-228
p-Tyr
Pyk2
FAKpY397
FAK
F
Relative percentage of phagocytotic cell /RBC binding index
con
GR-222
PF-228
Y-27632
phagocytosis
bindin
G
percentage of phagocytotic cell
5min
10min
20min
Pyk2+/+
Pyk2-/-
H
RBC binding index
Pyk2+/+
Pyk2-/-

Figure 4.4 Pyk2 and FAK regulate FcγR mediated phagocytosis. A) Equivalent amounts of indicated cell lysates were blotted with FAK, Pyk2 and Actin antibodies. **B)** Raw264.7 cells were transfected with indicated siRNA oligos. The cells were lysed after 48h and blotted with Pyk2 and FAK antibodies. **C)** Raw264.7 cells were transfected with indicated siRNA for 48h and then incubated with anti-BSA rabbit IgG opsonized latex beads for 20min. The cells were fixed and stained with rhodamine-conjugated anti-Rabbit IgG to visualize the non-internalized beads. The phagocytosis index was quantified and normalized to the control cells. Data represents means ±Standard deviation from three separate experiments. More than 100 cells were counted per experiment. The *P* value is less than 0.01 (Dunnet assay). **D)** Raw264.7 cells were transfected with indicated siRNA. The IgG opsonized RBC phagocytosis and binding assay were performed as described in methods. The percentage of phagocytosis cell and RBCs binding index were quantified and normalized to the control cells. Data represent ±Standard deviation from three separate experiments. More than 100 cells were counted per experiment. The *P* value is less than 0.01 (Dunnet assay). **E)** Raw264.7 cells were treated with DMSO, GR-222(1μM), PF-228(3 μM) for 1h and then cells were lysed and blotted with FAK and FAKpY397 antibodies. Pyk2 was immunoprecipitated using the Pyk2 mAb and blotted with Pyk2 and p-Tyr antibodies. **F)** Raw264.7 cells treated with DMSO, GR-222 (1μM), PF-228 (3 μM) ,for 1h. The phagocytosis and binding assay were performed as described in methods. The *P* value is less than 0.01 for these groups (Dunnet assay). Raw 264.7 cells were also treated with Y-27632 as a control. **G)** BMMs from wild type and Pyk2-/- mice were treated with IgG opsonized RBC. The percentage of phagocytosis cell in indicated time points was quantified. Data represent ±Standard deviation from three separate experiments. More than 200 cells were counted per experiment. **H)** The IgG opsonized RBC binding index of BMMs from wild type and Pyk2-/- mice. Data represent ±Standard deviation from three separate experiments. More than 200 cells were counted per experiment.

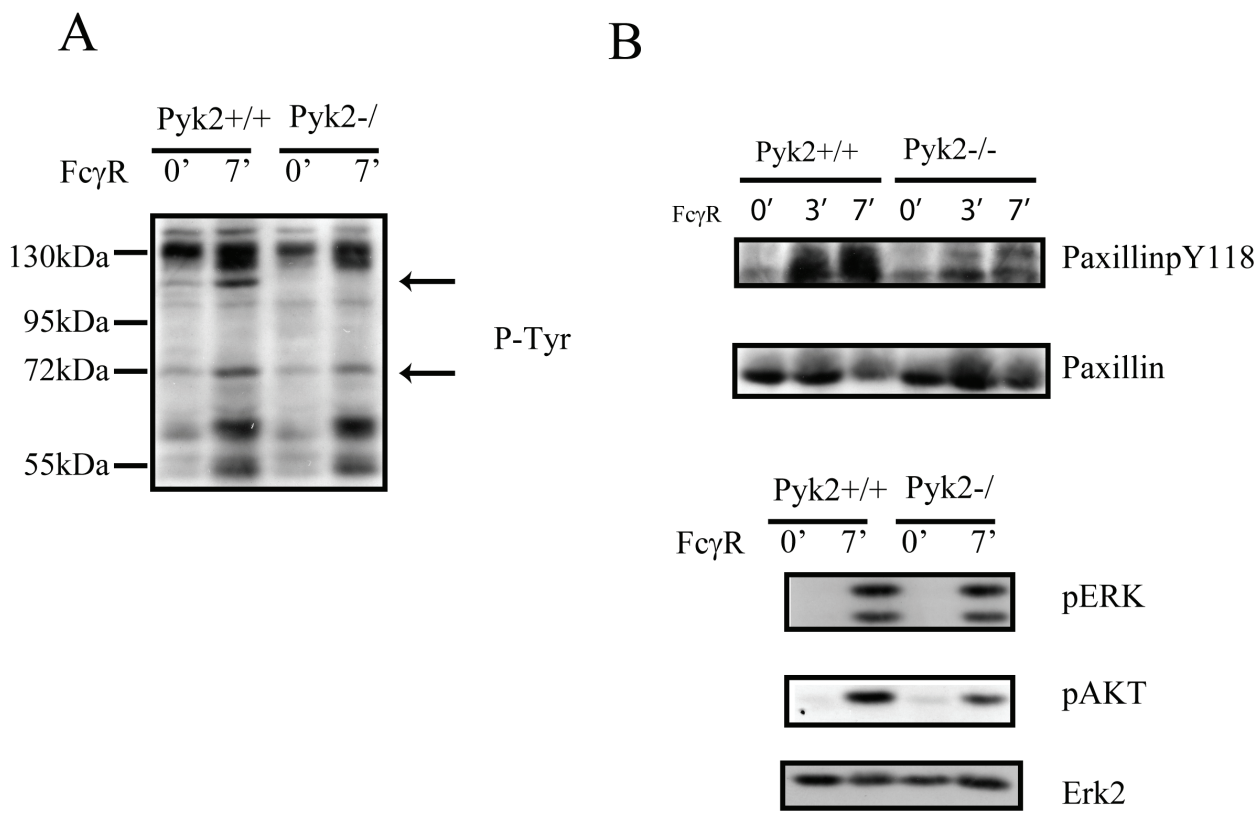


Figure.4.5 Pyk2 is required for FcγR mediated phagocytosis signaling. A-B).BMMs from wild type or Pyk2-/- mice were stimulated by incubation with anti-FcγR mAb (2.4G2) followed by anti–rat IgG cross-linking or IgG opsonized RBCs for the indicated times. Cells were lysed, and equivalent amounts of cellular protein were blotted with indicated antibodies.

CHAPTER 5
Future Directions

5.1 Future Directions For FAK Biosensor

A FAK conformational switch has proven to be an important regulatory mechanism for FAK activation. The conformational auto-inhibition model was first proposed based on biochemical studies, which was recently supported by the crystal structure of the FERM/Kinase domain in complex (87,94,288,448). Our FRET based biosensor successfully visualizes FAK conformation *in vivo* (364). The spatial and temporal regulation of FAK conformational switch was revealed in our study. However, there are a number of puzzles still waiting to be resolved.

5.1.1 Where Does FAK Change Its Conformation?

Our study suggests that FAK keeps open conformation within focal adhesions. However, the spatial regulation of FAK conformational switch in migrating cells is still unclear. A number of technical challenges make it hard to track the FRET signals in migrating cells. A long time exposure (2s per channel) of the cell to fluorescent light not only induces photo damage of the cell but also induces the photobleaching of fluorescent proteins. Moreover, the different photobleaching dynamics of CFP and Citrine might bring a systemic error into the final analysis. However, some novel technologies in microscopy may solve this problem. TIRF is a technology which illuminates a very thin layer of the specimen, e.g. 200nm. This greatly diminishes the photo bleach and phototoxity. TIRF microscopy based FRET technology has been used to track the interaction of membrane receptors (130,376). Hence, TIRF based FRET system may also benefit the study of FAK conformation during cell migration. To handle the photobleaching dynamics of different fluorescent proteins, a programmed photobleaching corrections method is used (193).

However, the photobleaching correction is a case-by-case issue. The FAK biosensor based photobleaching correction is waiting to be developed.

Keeping focal adhesions in focus is another challenge for FRET analysis of migrating cell because even a small zoom variation may introduce a deviation in FRET value. This problem might be solved by the developing auto-focus strategy (411).

In addition to the migrating cells, the spatial regulation of FAK in some specific biological processes should be investigated. For example, FAK is involved in regulating the E-cadherin mediated cell-cell junctions (our unpublished data). The N-terminal FERM domain is required for FAK cell junction targeting. Although it is hard to detect the activation of FAK in whole cell lysates, it is possible that the FAK is only activated within cell-cell junctions after the association of the FERM domain with some unknown partners. This interaction in turn promotes the conformational transition of FAK. Hence, it is important to monitor FAK conformational status within cell-cell junction. FAK has also been proposed to play critical role in neurite extension and sorting (232,284,310,373). It will be interesting to understand the spatial regulation of FAK activity within the axons.

5.1.2 When Does FAK Change Conformation?

Our study suggests that not only integrin signaling but also LPS induces a FAK conformational switch. Interestingly, PDGF was found to induce a fluctuation in FRET signal, which suggests that FAK might be transiently activated following PDGF stimulation. A number of other ligands should also be tested, including netrin, which is implicated in activating FAK via the DCC1 receptor (285,309,371). Met is another candidate that induces FAK conformational change (83). Met directly associates with FAK via its FERM domain. Interestingly, the basic residues within the FERM domain are required for Met/FAK

interaction. The FAK variant with these residues mutated exhibits a closed conformation when compared to wild type FAK (47). Hence, it is quite possible that Met might directly associate with FAK and promote its conformational switch following HGF stimulation.

5.1.3 How Does FAK Change Conformation?

The initiation of FAK activation has been a big puzzle. Based on FAK structure and FRET studies in FAK, FAK conformational transition seems to be the first step in FAK activation. However, there is still a gap between the receptors and FAK. How does the activation of receptors induce FAK conformational switch? PIP2 is proposed as a possible ligand that induces FAK conformational change. This is a reasonable explanation since PIP2 generation is observed following integrin activation (247). In addition to PIP2, PIP3 should also be tested for its potential role in FAK regulation since PIP3 is required for integrin mediated FAK activation. However, FAK conformational switch could be induced by multiple mechanisms. It is possible that FAK conformation switch is directly regulated by its interaction with some receptors. For example, the FERM domain of FAK directly associates with the integrinβ1 and β3 cytoplasmic tails (394). Moreover, the peptide which mimics the integrinβ1 cytoplasmic tail activates FAK *in vitro* (98). Thus it is possible that the FERM/integrin interaction might trigger FAK conformational switch. In addition to receptors, other FERM domain binding partners should also be investigated. Interestingly, FIP200 was proposed to inhibit the FAK activity via association with both the FERM and kinase domains (3). It is possible that the FIP200 connects the FERM and kinase domains to maintain the auto-inhibition conformation. This is also worth being investigated.

5.1.4. Why Does FAK Change Conformation?

It is interesting to know why FAK is regulated by such a complex mechanism. Here we propose a hypothesis that FAK may works as a converter to transfer a physical signal to a chemical signal. As discussed in the introduction, some physical stimuli, such as shear stress may induce the stretch of FAK, which in turn disrupts the FERM/Kinase interaction and activates FAK. This is an interesting hypothesis since FAK is an essential protein in mechanotransduction. The FAK stretch activation model will be tested both *in vivo* and *in vitro*. For *in vitro* assay, the C-terminal of purified FAK conformation biosensor will be immobilized on a surface coated with FAK C-terminus antibody. Then the N-terminus of FAK will be pulled by the magnetic beads coated with FERM domain antibody, e.g. 447. The FRET signal change will be monitored by the laser confocal microscopy. For *in vivo* assays, cells expressing biosensor will be tested in a flow shear stress assay. The FAK conformational switch will be monitored by FRET microscopy. In addition to the flow assay, laser tweezers or magnetic tweezers assay may also help us to understand the FAK conformation switch following physical stimulation (462).

5.1.5 Other Conformational Measurement Strategies

Due to the size of inserted GFP variant, the FAK conformation biosensor slightly increases its kinase activity when compared to wild type FAK. This problem might be solved by novel a dye, FlAsH, which becomes fluorescent when it binds to recombinant proteins containing a tetracysteine (TC) motif Cys-Cys-Pro-Gly-Cys-Cys (164). Due to similar excitation and emission spectrum, the FlAsH can be used to replace Citrine in FAK biosensor (194). The six amino acid insertion has little side-effects compared to the GFP variant. However, the binding specificity of FlAsH is a technical challenge for FRET assay in living cells. The labeling method needs to be improved to reduce the background noise.

Though the FRET based conformation biosensor makes it possible to visualize FAK conformation status *in vivo*, the limitations of the biosensor can never be overlooked. The complexity of observation and analysis may limit the application of FRET technology in some cases (363). An alternative method should also be developed to monitor FAK conformation. A conformation specific antibody will be a good tool to identify FAK conformation status. Such antibodies have been successfully used to identify activated integrins and stretched p130CAS (211,383). A FAK conformation specific antibody could be screened from the existing N-terminal or Kinase domain FAK antibodies. This can then be used to track FAK activation by immunoprecipitating that specific conformational FAK from cell lysates. This antibody can also be used to stain the fixed cells to visualize the spatial FAK activation pattern.

5.1.6 FRET Biosensor in a High Throughput Screen

The first generation kinase inhibitors have mostly been designed based upon the ATP binding site of the kinases. However, due to structural similarity of the various kinase ATP binding sites, it is hard to achieve a highly specific drug to target a single kinase. As mentioned in the introduction, the kinase activities of a number of kinases are regulated by distinct conformational regulatory mechanisms. The conformation based kinase inhibitor will be a promising direction for next generation drug design (287). Hence, the FRET based conformational biosensor has a great potential application for being used in high throughput screens. The target protein conformation within living cells can be read out by fluorescence microplate reader in real time. The FRET based FAK activity measurement will be a quick and simple assay, which makes it suitable for large scale screen. A number of chemically modified FRET biosensors have been used in HTS (196,334). However, the response

sensitivity is the bottleneck for the application of genetic encoded biosensors in HTS. In future, this problem might be solved by the development of new genetic coding fluorescence proteins, which might enhance the efficiency of energy transfer.

5.2 Downstream Effects of Paxillin Serine126/130 Phosphorylation

Our study suggests that the ERK/GSK-3 induced paxillin serine126/130 phosphorylation is involved in cytoskeleton reorganization (45). However, the downstream pathways regulated by paxillin serine 126/130 phosphorylation are still unknown. Interestingly, Kobayashi et al proposed that GSK-3 is required for focal adhesion turn over (254). Also GSK-3 inhibitors (LiCl and SB216763) suppress the activation of FAK and Rac. It is possible that GSK-3 regulates focal adhesion dynamics via phosphorylation of paxillin. To test this hypothesis, the focal adhesion disassembly rate of cells expressing paxillin S126A/S130A should be determined.

5.3 Downstream Effects of Pyk2 Activation in Fcγ Receptor Mediated Phagocytosis

Our study suggests that Pyk2 is required for Fcγ receptor mediated phagocytosis, possibly via tyrosine phosphorylation of paxillin and activation of PI3K. Though PI3K is well known as a critical regulator in Fcγ receptor mediated phagocytosis, the role of paxillin in phagocytosis is still unknown (12). It is possible that Pyk2 induced tyrosine phosphorylation of paxillin is required for Rac activation since tyrosine phosphorylation of paxillin is required for Rac activation in cell migration (222,361). Hence, the Pyk2/paxillin/Rac signaling should be tested in phagocytosis.

5.4 The Role of Pyk2 in Other Types of Phagocytosis

In addition, the role of Pyk2 in other types of phagocytosis should also be tested. Our preliminary study suggests that Pyk2 is not required for complement mediated phagocytosis.

However, Pyk2 is involved in Yersinia uptake in macrophage cell lines (39). So the role of Pyk2 in bacterial uptake should be tested in Pyk2 null cells. Moreover, it is interesting to understand the role of Pyk2 in apoptotic cell phagocytosis.

5.5 The Role of Pyk2 in Immune Cell Migration

Immune cell migration plays a critical role in immunological pathology. For example, leukocytes exit from circulation to the peripheral tissues during inflammation. This plays a critical role in both innate and adaptive immune responses. The defect in leukocyte integrinβ2 induces leukocyte adhesion deficiency. Patients with this deficiency suffer from recurrent bacterial infections and impaired wound healing. In addition, some diseases including multiple sclerosis are related to the cytotoxity of macrophages and TH1 cells. Restriction of the inflammation induced macrophage influx may attenuate these diseases (313).

A number of studies suggest that Pyk2 is required for cell migration of different immune cells. In leukocytes, the defects in macrophage influx were identified in Pyk2 null mice following carageenen induced acute inflammatory response (341). Our preliminary study also identified a similar defect in Pyk2 null mice following thioglycolate induced inflammatory response. Interestingly, neutrophil migration was unimpaired in above studies. The chemotaxis defect was identified in bone marrow macrophages isolated from Pyk2 null mice. Both the formation and contractility of lamellipodia is greatly impaired in these cells. Integrin induced PI3K and RhoA activation is also blocked in Pyk2 null macrophages. Lymphocytes in the Pyk2 null mice lack the marginal zone B cells, which is at least partially induced by a migration defect in B cells (168). Interestingly, a similar phenotype was identified in wild type mice which were treated with a drug to inhibit the GPCR signaling,

which suggests that Pyk2 may regulate B cell migration by regulating GPCR mediated chemotaxis. A chemotaxis defect was also identified in Pyk2 null B cells *in vitro*. No evident defects in T cell development and homing have been identified in T cells *in vivo.* However, Pyk2 is also implicated in regulating T cell migration *in vitro* (343).

As a potent regulator of cell migration, Pyk2 is essential for immune cell trafficking, especially for macrophage influx during inflammation. Understanding macrophage migration in inflammation is very important for the pathology of multiple diseases. Due to the complexity of the macrophage trafficking and the multiple effects of Pyk2 in cell migration, the role of Pyk2 in immune cell migration should be deciphered at each step of leukocyte trafficking.

Circulating leukocytes are first tethered by some receptors expressed on the surface of endothelial cells. Pyk2 is activated following integrinβ_2 activation when neutrophils are tethered by CHO cells expressing E-selectin (444). This suggests that Pyk2 may regulate the E-selectin mediated leukocyte tethering. Moreover, Pyk2 is required for integrin$\alpha_M\beta_2$ mediated neutrophil adhesion to adherent platelets (129). Little is known about the role of Pyk2 in monocyte tethering and rolling process. Pyk2 null monocytes should be tested to understand this mechanism

The tethered leukocytes are activated by chemokines, which induce leukocyte spreading and firm adhesion to endothelial cells. Pyk2 plays a critical role in this process. Pyk2 null macrophages exhibit migration defects in response to MIP-1 and SDF-1 (340). Both Ca^{2+} signaling and IP3 generation are impaired in Pyk2 null macrophages following MIP-1 stimulation. However, considering the differences between monocytes and macrophages, it will be interesting to examine Pyk2 activation in vivo.

Activated leukocytes transmigrate across the endothelial barrier to reach the site of inflammation. Pyk2 might play a critical role in both leukocytes and endothelial cells during transmigration. For endothelial cells, Pyk2 is required for ICAM-1 mediated tyrosine phosphorylation of VE-cadherin, which is required for neutrophil transmigration (9). Moreover, Pyk2 is also required for tyrosine phosphorylation of β-catenin. This is induced by the disruption of VE-cadherin mediated cell-cell adhesion (454). Pyk2 is required for Rac activation in NK cells during transmigration (154). NK cells expressing a dominant negative of Pyk2 exhibit defects in transmigration. However, we still know little about the role of Pyk2 in leukocyte transmigration. Transplantation of Pyk2 null bone marrow cells into wild type mice should be a possible strategy to exclude the effects of Pyk2 on endothelial cells. In addition to the migrating through endothelial cell junctions, the leukocytes also migrate through endothelial cells themselves (128). When circulating leukocytes are arrested, the endothelial cell forms a big protrusion cup to engulf the leukocyte. The phenotype of this cup is quite similar to the phagocytotic cup, which indicates that these two processes may share similar mechanisms. Pyk2 may also regulate the leukocyte transcellular migration.

The migration defects in Pyk2 null mice were only identified in macrophages but not neutrophils, which suggests that the Pyk2 plays distinct roles in different subsets of immune cells. One possible explanation is that FAK may complement Pyk2 function in neutrophils. Due to the absence of evident FAK expression, deletion of Pyk2 induces evident defects in macrophages (124). Interestingly, the defects in macrophage but not neutrophil migration in inflammation has been identified in transgenic mice expressing integrinα_4 Y991A, which disrupts the interaction between integrinα_4 and paxillin (132). Furthermore, the disruption of

integrinα4/paxillin interaction blocks FAK/Pyk2 activation (377). Hence integrin/paxillin/Pyk2 might work together to regulate macrophage migration.

With the development of intravital microscopy technology, it is possible to visualize leukocyte trafficking in living animals, which helps us dissect the leukocyte migration process in a real *in vivo* environment (323). Such imaging of Pyk2 null mice will helps us explain the inflammation induced macrophage influx defect among other defects.

Reference List

1. **Abassi, Y. A. and K. Vuori**. 2002. Tyrosine 221 in Crk regulates adhesion-dependent membrane localization of Crk and Rac and activation of Rac signaling. EMBO J. **21**:4571-4582.

2. **Abbi, S., H. Ueda, C. Zheng, L. A. Cooper, J. Zhao, R. Christopher, and J. L. Guan**. 2002. Regulation of focal adhesion kinase by a novel protein inhibitor FIP200. Mol.Biol.Cell **13**:3178-3191.

3. **Abbi, S., H. Ueda, C. Zheng, L. A. Cooper, J. Zhao, R. Christopher, and J. L. Guan**. 2002. Regulation of focal adhesion kinase by a novel protein inhibitor FIP200. Mol.Biol.Cell **13**:3178-3191.

4. **Abbi, S., H. Ueda, C. Zheng, L. A. Cooper, J. Zhao, R. Christopher, and J. L. Guan**. 2002. Regulation of focal adhesion kinase by a novel protein inhibitor FIP200. Mol.Biol.Cell **13**:3178-3191.

5. **Abu-Ghazaleh, R., J. Kabir, H. Jia, M. Lobo, and I. Zachary**. 2001. Src mediates stimulation by vascular endothelial growth factor of the phosphorylation of focal adhesion kinase at tyrosine 861, and migration and anti-apoptosis in endothelial cells. Biochemical Journal **360**:255-264.

6. **Akira, S. and K. Takeda**. 2004. Toll-like receptor signalling. Nat Rev Immunol **4**:499-511.

7. **Ali, M. H., P. T. Mungai, and P. T. Schumacker**. 2006. Stretch-induced phosphorylation of focal adhesion kinase in endothelial cells: role of mitochondrial oxidants. Am.J.Physiol Lung Cell Mol.Physiol **291**:L38-L45.

8. **Allen, L. A. and A. Aderem**. 1996. Molecular definition of distinct cytoskeletal structures involved in complement- and Fc receptor-mediated phagocytosis in macrophages. J.Exp.Med. **184**:627-637.

9. **Allingham, M. J., J. D. van Buul, and K. Burridge**. 2007. ICAM-1-mediated, Src- and Pyk2-dependent vascular endothelial cadherin tyrosine phosphorylation is required for leukocyte transendothelial migration. J Immunol **179**:4053-4064.

10. **Almeida, E. A., D. Ilic, Q. Han, C. R. Hauck, F. Jin, H. Kawakatsu, D. D. Schlaepfer, and C. H. Damsky**. 2000. Matrix survival signaling. From fibronectin via focal adhesion kinase to c-Jun NH_2-terminal kinase. J.Cell Biol. **149**:741-754.

11. **Almeida, E. A., D. Ilic, Q. Han, C. R. Hauck, F. Jin, H. Kawakatsu, D. D. Schlaepfer, and C. H. Damsky**. 2000. Matrix survival signaling. From fibronectin via focal adhesion kinase to c-Jun NH_2-terminal kinase. J.Cell Biol. **149**:741-754.

12. **Araki, N., M. T. Johnson, and J. A. Swanson**. 1996. A role for phosphoinositide 3-kinase in the completion of macropinocytosis and phagocytosis by macrophages. J.Cell Biol. **135**:1249-1260.

13. **Arias-Salgado, E. G., S. Lizano, S. Sarkar, J. S. Brugge, M. H. Ginsberg, and S. J. Shattil**. 2003. Src kinase activation by direct interaction with the integrin beta cytoplasmic domain. Proc.Natl.Acad.Sci.U.S.A **100**:13298-13302.

14. **Arregui, C. O., J. Balsamo, and J. Lilien**. 1998. Impaired integrin-mediated adhesion and signaling in fibroblasts expressing a dominant-negative mutant PTP1B. J.Cell Biol. **143**:861-873.

15. **Avraham, H., S. Y. Park, K. Schinkmann, and S. Avraham**. 2000. RAFTK/Pyk2-mediated cellular signalling. Cell Signal. **12**:123-133.

16. **Bagrodia, S., I. Chackalaparampil, T. E. Kmiecik, and D. Shalloway**. 1991. Altered tyrosine 527 phosphorylation and mitotic activation of p60c-src. Nature **349**:172-175.

17. **Bakolitsa, C., J. M. de Pereda, C. R. Bagshaw, D. R. Critchley, and R. C. Liddington**. 1999. Crystal structure of the vinculin tail suggests a pathway for activation. Cell **99**:603-613.

18. **Balaban, N. Q., U. S. Schwarz, D. Riveline, P. Goichberg, G. Tzur, I. Sabanay, D. Mahalu, S. Safran, A. Bershadsky, L. Addadi, and B. Geiger**. 2001. Force and focal adhesion assembly: a close relationship studied using elastic micropatterned substrates. Nat.Cell Biol. **3**:466-472.

19. **Ballestrem, C., N. Erez, J. Kirchner, Z. Kam, A. Bershadsky, and B. Geiger**. 2006. Molecular mapping of tyrosine-phosphorylated proteins in focal adhesions using fluorescence resonance energy transfer. J.Cell Sci. **119**:866-875.

20. **Ballestrem, C., N. Erez, J. Kirchner, Z. Kam, A. Bershadsky, and B. Geiger**. 2006. Molecular mapping of tyrosine-phosphorylated proteins in focal adhesions using fluorescence resonance energy transfer. J.Cell Sci. **119**:866-875.

21. **Barry, S. T. and D. R. Critchley**. 1994. The RhoA-dependent assembly of focal adhesions in Swiss 3T3 cells is associated with increased tyrosine phosphorylation and the recruitment of both pp125FAK and protein kinase C-delta to focal adhesions. J.Cell Sci. **107 (Pt 7)**:2033-2045.

22. **Bellis, S. L., J. A. Perrotta, M. S. Curtis, and C. E. Turner**. 1997. Adhesion of fibroblasts to fibronectin stimulates both serine and tyrosine phosphorylation of paxillin. Biochem.J. **325 (Pt 2)**:375-381.

23. **Bianchi, M., S. De Lucchini, O. Marin, D. L. Turner, S. K. Hanks, and E. Villa-Moruzzi**. 2005. Regulation of FAK Ser-722 phosphorylation and kinase activity by GSK3 and PP1 during cell spreading and migration. Biochem.J. **391**:359-370.

24. **Biondi, R. M. and A. R. Nebreda**. 2003. Signalling specificity of Ser/Thr protein kinases through docking-site-mediated interactions. Biochem.J. **372**:1-13.

25. **Bokoch, G. M.** 2003. Biology of the p21-activated kinases. Annu.Rev.Biochem. **72**:743-781.

26. **Braren, R., H. Hu, Y. H. Kim, H. E. Beggs, L. F. Reichardt, and R. Wang**. 2006. Endothelial FAK is essential for vascular network stability, cell survival, and lamellipodial formation. J.Cell Biol. **172**:151-162.

27. **Braren, R., H. Hu, Y. H. Kim, H. E. Beggs, L. F. Reichardt, and R. Wang**. 2006. Endothelial FAK is essential for vascular network stability, cell survival, and lamellipodial formation. J.Cell Biol. **172**:151-162.

28. **Braren, R., H. Hu, Y. H. Kim, H. E. Beggs, L. F. Reichardt, and R. Wang**. 2006. Endothelial FAK is essential for vascular network stability, cell survival, and lamellipodial formation. J.Cell Biol. **172**:151-162.

29. **Braren, R., H. Hu, Y. H. Kim, H. E. Beggs, L. F. Reichardt, and R. Wang**. 2006. Endothelial FAK is essential for vascular network stability, cell survival, and lamellipodial formation. J.Cell Biol. **172**:151-162.

30. **Braren, R., H. Hu, Y. H. Kim, H. E. Beggs, L. F. Reichardt, and R. Wang**. 2006. Endothelial FAK is essential for vascular network stability, cell survival, and lamellipodial formation. J.Cell Biol. **172**:151-162.

31. **Braren, R., H. Hu, Y. H. Kim, H. E. Beggs, L. F. Reichardt, and R. Wang**. 2006. Endothelial FAK is essential for vascular network stability, cell survival, and lamellipodial formation. J.Cell Biol. **172**:151-162.

32. **Browe, D. M. and C. M. Baumgarten**. 2003. Stretch of beta 1 integrin activates an outwardly rectifying chloride current via FAK and Src in rabbit ventricular myocytes. J.Gen.Physiol **122**:689-702.

33. **Brown, M. C., J. A. Perrotta, and C. E. Turner**. 1996. Identification of LIM3 as the principal determinant of paxillin focal adhesion localization and characterization of a novel motif on paxillin directing vinculin and focal adhesion kinase binding. J.Cell Biol. **135**:1109-1123.

34. **Brown, M. C., J. A. Perrotta, and C. E. Turner**. 1998. Serine and threonine phosphorylation of the paxillin LIM domains regulates paxillin focal adhesion localization and cell adhesion to fibronectin. Mol.Biol.Cell **9**:1803-1816.

35. **Brown, M. C. and C. E. Turner**. 1999. Characterization of paxillin LIM domain-associated serine threonine kinases: activation by angiotensin II in vascular smooth muscle cells [In Process Citation]. J.Cell Biochem. **76**:99-108.

36. **Brown, M. C. and C. E. Turner**. 2004. Paxillin: adapting to change. Physiol Rev. **84**:1315-1339.

37. **Brown, M. C. and C. E. Turner**. 2004. Paxillin: adapting to change. Physiol Rev. **84**:1315-1339.

38. **Brown, M. C. and C. E. Turner**. 2004. Paxillin: adapting to change. Physiol Rev. **84**:1315-1339.

39. **Bruce-Staskal, P. J., C. L. Weidow, J. J. Gibson, and A. H. Bouton**. 2002. Cas, Fak and Pyk2 function in diverse signaling cascades to promote Yersinia uptake. J.Cell Sci. **115**:2689-2700.

40. **Bryant, P., Q. Zheng, and K. Pumiglia**. 2006. Focal adhesion kinase controls cellular levels of p27/Kip1 and p21/Cip1 through Skp2-dependent and -independent mechanisms. Mol.Cell Biol. **26**:4201-4213.

41. **Buckbinder, L., D. T. Crawford, H. Qi, H. Z. Ke, L. M. Olson, K. R. Long, P. C. Bonnette, A. P. Baumann, J. E. Hambor, W. A. Grasser, III, L. C. Pan, T. A. Owen, M. J. Luzzio, C. A. Hulford, D. F. Gebhard, V. M. Paralkar, H. A. Simmons, J. C. Kath, W. G. Roberts, S. L. Smock, A. Guzman-Perez, T. A. Brown, and M. Li**. 2007. Proline-rich tyrosine kinase 2 regulates osteoprogenitor cells and bone formation, and offers an anabolic treatment approach for osteoporosis. Proc.Natl.Acad.Sci.U.S.A **104**:10619-10624.

42. **Burgaya, F., M. Toutant, J. M. Studler, A. Costa, M. Le Bert, M. Gelman, and J. A. Girault**. 1997. Alternatively spliced focal adhesion kinase in rat brain with increased autophosphorylation activity. J.Biol.Chem. **272**:28720-28725.

43. **Burstein, D. E., P. J. Seeley, and L. A. Greene**. 1985. Lithium ion inhibits nerve growth factor-induced neurite outgrowth and phosphorylation of nerve growth factor-modulated microtubule-associated proteins. J.Cell Biol. **101**:862-870.

44. **Burstein, D. E., P. J. Seeley, and L. A. Greene**. 1985. Lithium ion inhibits nerve growth factor-induced neurite outgrowth and phosphorylation of nerve growth factor-modulated microtubule-associated proteins. J.Cell Biol. **101**:862-870.

45. **Cai, X., M. Li, J. Vrana, and M. D. Schaller**. 2006. Glycogen synthase kinase 3-and extracellular signal-regulated kinase-dependent phosphorylation of paxillin regulates cytoskeletal rearrangement. Mol.Cell Biol. **26**:2857-2868.

46. **Cai, X., M. Li, J. Vrana, and M. D. Schaller**. 2006. Glycogen synthase kinase 3-and extracellular signal-regulated kinase-dependent phosphorylation of paxillin regulates cytoskeletal rearrangement. Mol.Cell Biol. **26**:2857-2868.

47. **Cai, X., D. Lietha, D. F. Ceccarelli, A. V. Karginov, Z. Rajfur, K. Jacobson, K. M. Hahn, M. J. Eck, and M. D. Schaller**. 2007. Spatial and Temporal Regulation of FAK Activity in Living Cells. Mol.Cell Biol.

48. **Calalb, M. B., T. R. Polte, and S. K. Hanks**. 1995. Tyrosine phosphorylation of focal adhesion kinase at sites in the catalytic domain regulates kinase activity: a role for Src family kinases. Mol.Cell Biol. **15**:954-963.

49. **Calalb, M. B., T. R. Polte, and S. K. Hanks**. 1995. Tyrosine phosphorylation of focal adhesion kinase at sites in the catalytic domain regulates kinase activity: a role for Src family kinases. Mol.Cell Biol. **15**:954-963.

50. **Calalb, M. B., T. R. Polte, and S. K. Hanks**. 1995. Tyrosine phosphorylation of focal adhesion kinase at sites in the catalytic domain regulates kinase activity: a role for Src family kinases. Mol.Cell Biol. **15**:954-963.

51. **Calalb, M. B., T. R. Polte, and S. K. Hanks**. 1995. Tyrosine phosphorylation of focal adhesion kinase at sites in the catalytic domain regulates kinase activity: a role for Src family kinases. Mol.Cell Biol. **15**:954-963.

52. **Canobbio, I., P. Lova, F. Sinigaglia, C. Balduini, and M. Torti**. 2002. Proline-rich tyrosine kinase 2 and focal adhesion kinase are involved in different phases of platelet activation by vWF. Thromb.Haemost. **87**:509-517.

53. **Caron, E. and A. Hall**. 1998. Identification of two distinct mechanisms of phagocytosis controlled by different Rho GTPases. Science **282**:1717-1721.

54. **Carragher, N. O., B. Levkau, R. Ross, and E. W. Raines**. 1999. Degraded collagen fragments promote rapid disassembly of smooth muscle focal adhesions that correlates with cleavage of pp125(FAK), paxillin, and talin. J.Cell Biol. **147**:619-630.

55. **Carragher, N. O., B. Levkau, R. Ross, and E. W. Raines**. 1999. Degraded collagen fragments promote rapid disassembly of smooth muscle focal adhesions that correlates with cleavage of pp125(FAK), paxillin, and talin. J.Cell Biol. **147**:619-630.

56. **Carragher, N. O., M. A. Westhoff, V. J. Fincham, M. D. Schaller, and M. C. Frame**. 2003. A novel role for FAK as a protease-targeting adaptor protein: regulation by p42 ERK and Src. Curr.Biol. **13**:1442-1450.

57. **Carragher, N. O., M. A. Westhoff, V. J. Fincham, M. D. Schaller, and M. C. Frame**. 2003. A novel role for FAK as a protease-targeting adaptor protein: regulation by p42 ERK and Src. Curr.Biol. **13**:1442-1450.

58. **Cary, L. A., J. F. Chang, and J. L. Guan**. 1996. Stimulation of cell migration by overexpression of focal adhesion kinase and its association with Src and Fyn. J.Cell Sci. **109 (Pt 7)**:1787-1794.

59. **Casamassima, A. and E. Rozengurt**. 1998. Insulin-like growth factor I stimulates tyrosine phosphorylation of p130(Cas), focal adhesion kinase, and paxillin. Role of phosphatidylinositol 3'-kinase and formation of a p130(Cas).Crk complex. J.Biol.Chem. **273**:26149-26156.

60. **Casamassima, A. and E. Rozengurt**. 1998. Insulin-like growth factor I stimulates tyrosine phosphorylation of p130(Cas), focal adhesion kinase, and paxillin. Role of phosphatidylinositol 3'-kinase and formation of a p130(Cas).Crk complex. J.Biol.Chem. **273**:26149-26156.

61. **Cascone, I., L. Napione, F. Maniero, G. Serini, and F. Bussolino**. 2005. Stable interaction between alpha5beta1 integrin and Tie2 tyrosine kinase receptor regulates endothelial cell response to Ang-1. J.Cell Biol. **170**:993-1004.

62. **Ceccarelli, D. F., I. M. Blasutig, M. Goudreault, Z. Li, J. Ruston, T. Pawson, and F. Sicheri**. 2007. Non-canonical interaction of phosphoinositides with pleckstrin homology domains of Tiam1 and ArhGAP9. J.Biol.Chem. **282**:13864-13874.

63. **Ceccarelli, D. F., H. K. Song, F. Poy, M. D. Schaller, and M. J. Eck**. 2006. Crystal structure of the FERM domain of focal adhesion kinase. J.Biol.Chem. **281**:252-259.

64. **Ceccarelli, D. F., H. K. Song, F. Poy, M. D. Schaller, and M. J. Eck**. 2006. Crystal structure of the FERM domain of focal adhesion kinase. J.Biol.Chem. **281**:252-259.

65. **Ceccarelli, D. F., H. K. Song, F. Poy, M. D. Schaller, and M. J. Eck**. 2006. Crystal structure of the FERM domain of focal adhesion kinase. J.Biol.Chem. **281**:252-259.

66. **Ceccarelli, D. F., H. K. Song, F. Poy, M. D. Schaller, and M. J. Eck**. 2006. Crystal structure of the FERM domain of focal adhesion kinase. J.Biol.Chem. **281**:252-259.

67. **Chamberlain, C. E., V. S. Kraynov, and K. M. Hahn**. 2000. Imaging spatiotemporal dynamics of Rac activation in vivo with FLAIR. Methods Enzymol. **325**:389-400.

68. **Chan, B. P., W. M. Reichert, and G. A. Truskey**. 2004. Synergistic effect of shear stress and streptavidin-biotin on the expression of endothelial vasodilator and cytoskeleton genes. Biotechnol.Bioeng. **88**:750-758.

69. **Chan, P. Y., S. B. Kanner, G. Whitney, and A. Aruffo**. 1994. A transmembrane-anchored chimeric focal adhesion kinase is constitutively activated and phosphorylated at tyrosine residues identical to pp125FAK. J.Biol.Chem. **269**:20567-20574.

70. **Chandrasekar, I., T. E. Stradal, M. R. Holt, F. Entschladen, B. M. Jockusch, and W. H. Ziegler**. 2005. Vinculin acts as a sensor in lipid regulation of adhesion-site turnover. J.Cell Sci. **118**:1461-1472.

71. **Chen, H., D. M. Cohen, D. M. Choudhury, N. Kioka, and S. W. Craig**. 2005. Spatial distribution and functional significance of activated vinculin in living cells. J.Cell Biol. **169**:459-470.

72. **Chen, H., D. M. Cohen, D. M. Choudhury, N. Kioka, and S. W. Craig**. 2005. Spatial distribution and functional significance of activated vinculin in living cells. J.Cell Biol. **169**:459-470.

73. **Chen, H., D. M. Cohen, D. M. Choudhury, N. Kioka, and S. W. Craig**. 2005. Spatial distribution and functional significance of activated vinculin in living cells. J.Cell Biol. **169**:459-470.

74. **Chen, H. C., P. A. Appeddu, H. Isoda, and J. L. Guan**. 1996. Phosphorylation of tyrosine 397 in focal adhesion kinase is required for binding phosphatidylinositol 3-kinase. J.Biol.Chem. **271**:26329-26334.

75. **Chen, H. C., P. A. Appeddu, H. Isoda, and J. L. Guan**. 1996. Phosphorylation of tyrosine 397 in focal adhesion kinase is required for binding phosphatidylinositol 3-kinase. J.Biol.Chem. **271**:26329-26334.

76. **Chen, H. C., P. A. Appeddu, J. T. Parsons, J. D. Hildebrand, M. D. Schaller, and J. L. Guan**. 1995. Interaction of focal adhesion kinase with cytoskeletal protein talin. J.Biol.Chem. **270**:16995-16999.

77. **Chen, H. C. and J. L. Guan**. 1994. Association of focal adhesion kinase with its potential substrate phosphatidylinositol 3-kinase. Proc.Natl.Acad.Sci.U.S.A **91**:10148-10152.

78. **Chen, K., J. Huang, W. Gong, P. Iribarren, N. M. Dunlop, and J. M. Wang**. 2007. Toll-like receptors in inflammation, infection and cancer. Int.Immunopharmacol. **7**:1271-1285.

79. **Chen, S. Y. and H. C. Chen**. 2006. Direct interaction of focal adhesion kinase (FAK) with Met is required for FAK to promote hepatocyte growth factor-induced cell invasion. Mol.Cell Biol. **26**:5155-5167.

80. **Chen, S. Y. and H. C. Chen**. 2006. Direct interaction of focal adhesion kinase (FAK) with Met is required for FAK to promote hepatocyte growth factor-induced cell invasion. Mol.Cell Biol. **26**:5155-5167.

81. **Chen, S. Y. and H. C. Chen**. 2006. Direct interaction of focal adhesion kinase (FAK) with Met is required for FAK to promote hepatocyte growth factor-induced cell invasion. Mol.Cell Biol. **26**:5155-5167.

82. **Chen, S. Y. and H. C. Chen**. 2006. Direct interaction of focal adhesion kinase (FAK) with Met is required for FAK to promote hepatocyte growth factor-induced cell invasion. Mol.Cell Biol. **26**:5155-5167.

83. **Chen, S. Y. and H. C. Chen**. 2006. Direct interaction of focal adhesion kinase (FAK) with Met is required for FAK to promote hepatocyte growth factor-induced cell invasion. Mol.Cell Biol. **26**:5155-5167.

84. **Chen, S. Y. and H. C. Chen**. 2006. Direct interaction of focal adhesion kinase (FAK) with Met is required for FAK to promote hepatocyte growth factor-induced cell invasion. Mol.Cell Biol. **26**:5155-5167.

85. **Chu, B., F. Soncin, B. D. Price, M. A. Stevenson, and S. K. Calderwood**. 1996. Sequential phosphorylation by mitogen-activated protein kinase and glycogen synthase kinase 3 represses transcriptional activation by heat shock factor-1. J.Biol.Chem. **271**:30847-30857.

86. **Cicala, C., J. Arthos, A. Rubbert, S. Selig, K. Wildt, O. J. Cohen, and A. S. Fauci**. 2000. HIV-1 envelope induces activation of caspase-3 and cleavage of focal adhesion kinase in primary human CD4(+) T cells. Proc.Natl.Acad.Sci.U.S.A **97**:1178-1183.

87. **Cohen, L. A. and J. L. Guan**. 2005. Residues within the first subdomain of the FERM-like domain in focal adhesion kinase are important in its regulation. J.Biol.Chem. **280**:8197-8207.

88. **Cohen, L. A. and J. L. Guan**. 2005. Residues within the first subdomain of the FERM-like domain in focal adhesion kinase are important in its regulation. J.Biol.Chem. **280**:8197-8207.

89. **Cohen, L. A. and J. L. Guan**. 2005. Residues within the first subdomain of the FERM-like domain in focal adhesion kinase are important in its regulation. J.Biol.Chem. **280**:8197-8207.

90. **Cohen, L. A. and J. L. Guan**. 2005. Residues within the first subdomain of the FERM-like domain in focal adhesion kinase are important in its regulation. J.Biol.Chem. **280**:8197-8207.

91. **Cohen, L. A. and J. L. Guan**. 2005. Residues within the first subdomain of the FERM-like domain in focal adhesion kinase are important in its regulation. J.Biol.Chem. **280**:8197-8207.

92. **Cohen, L. A. and J. L. Guan**. 2005. Residues within the first subdomain of the FERM-like domain in focal adhesion kinase are important in its regulation. J.Biol.Chem. **280**:8197-8207.

93. **Cooley, M. A., J. M. Broome, C. Ohngemach, L. H. Romer, and M. D. Schaller**. 2000. Paxillin binding is not the sole determinant of focal adhesion localization or dominant-negative activity of focal adhesion kinase/focal adhesion kinase-related nonkinase. Mol.Biol.Cell **11**:3247-3263.

94. **Cooper, L. A., T. L. Shen, and J. L. Guan**. 2003. Regulation of focal adhesion kinase by its amino-terminal domain through an autoinhibitory interaction. Mol.Cell Biol. **23**:8030-8041.

95. **Cooper, L. A., T. L. Shen, and J. L. Guan**. 2003. Regulation of focal adhesion kinase by its amino-terminal domain through an autoinhibitory interaction. Mol.Cell Biol. **23**:8030-8041.

96. **Cooper, L. A., T. L. Shen, and J. L. Guan**. 2003. Regulation of focal adhesion kinase by its amino-terminal domain through an autoinhibitory interaction. Mol.Cell Biol. **23**:8030-8041.

97. **Cooper, L. A., T. L. Shen, and J. L. Guan**. 2003. Regulation of focal adhesion kinase by its amino-terminal domain through an autoinhibitory interaction. Mol.Cell Biol. **23**:8030-8041.

98. **Cooper, L. A., T. L. Shen, and J. L. Guan**. 2003. Regulation of focal adhesion kinase by its amino-terminal domain through an autoinhibitory interaction. Mol.Cell Biol. **23**:8030-8041.

99. **Cooper, L. A., T. L. Shen, and J. L. Guan**. 2003. Regulation of focal adhesion kinase by its amino-terminal domain through an autoinhibitory interaction. Mol.Cell Biol. **23**:8030-8041.

100. **Corsi, J. M., E. Rouer, J. A. Girault, and H. Enslen**. 2006. Organization and post-transcriptional processing of focal adhesion kinase gene. BMC.Genomics **7**:198.

101. **Cox, D., C. C. Tseng, G. Bjekic, and S. Greenberg**. 1999. A requirement for phosphatidylinositol 3-kinase in pseudopod extension. J.Biol.Chem. **274**:1240-1247.

102. **De Nichilo, M. O. and K. M. Yamada**. 1996. Integrin alpha v beta 5-dependent serine phosphorylation of paxillin in cultured human macrophages adherent to vitronectin. J.Biol.Chem. **271**:11016-11022.

103. **Deindl, S., T. A. Kadlecek, T. Brdicka, X. Cao, A. Weiss, and J. Kuriyan**. 2007. Structural basis for the inhibition of tyrosine kinase activity of ZAP-70. Cell **129**:735-746.

104. **Del Rio, J. A., C. Gonzalez-Billault, J. M. Urena, E. M. Jimenez, M. J. Barallobre, M. Pascual, L. Pujadas, S. Simo, A. La Torre, F. Wandosell, J. Avila, and E. Soriano**. 2004. MAP1B is required for Netrin 1 signaling in neuronal migration and axonal guidance. Curr.Biol. **14**:840-850.

105. **DeLano, W. L.** 2002. The PyMOL Molecular Graphics System. DeLano Scientific.

106. **Delcommenne, M., C. Tan, V. Gray, L. Rue, J. Woodgett, and S. Dedhar**. 1998. Phosphoinositide-3-OH kinase-dependent regulation of glycogen synthase kinase 3 and protein kinase B/AKT by the integrin-linked kinase. Proc.Natl.Acad.Sci.U.S.A **95**:11211-11216.

107. **Di Paolo, G., L. Pellegrini, K. Letinic, G. Cestra, R. Zoncu, S. Voronov, S. Chang, J. Guo, M. R. Wenk, and P. De Camilli**. 2002. Recruitment and regulation of phosphatidylinositol phosphate kinase type 1 gamma by the FERM domain of talin. Nature **420**:85-89.

108. **Di Paolo, G., L. Pellegrini, K. Letinic, G. Cestra, R. Zoncu, S. Voronov, S. Chang, J. Guo, M. R. Wenk, and P. De Camilli**. 2002. Recruitment and regulation of phosphatidylinositol phosphate kinase type 1 gamma by the FERM domain of talin. Nature **420**:85-89.

109. **Dikic, I., J. Schlessinger, and I. Lax**. 1994. PC12 cells overexpressing the insulin receptor undergo insulin-dependent neuronal differentiation. Curr.Biol. **4**:702-708.

110. **Dikic, I., G. Tokiwa, S. Lev, S. A. Courtneidge, and J. Schlessinger**. 1996. A role for Pyk2 and Src in linking G-protein-coupled receptors with MAP kinase activation. Nature **383**:547-550.

111. **DiMichele, L. A., J. T. Doherty, M. Rojas, H. E. Beggs, L. F. Reichardt, C. P. Mack, and J. M. Taylor**. 2006. Myocyte-restricted focal adhesion kinase deletion attenuates pressure overload-induced hypertrophy. Circ.Res. **99**:636-645.

112. **DiMichele, L. A., J. T. Doherty, M. Rojas, H. E. Beggs, L. F. Reichardt, C. P. Mack, and J. M. Taylor**. 2006. Myocyte-restricted focal adhesion kinase deletion attenuates pressure overload-induced hypertrophy. Circ.Res. **99**:636-645.

113. **DiMichele, L. A., J. T. Doherty, M. Rojas, H. E. Beggs, L. F. Reichardt, C. P. Mack, and J. M. Taylor**. 2006. Myocyte-restricted focal adhesion kinase deletion attenuates pressure overload-induced hypertrophy. Circ.Res. **99**:636-645.

114. **Doble, B. W. and J. R. Woodgett**. 2003. GSK-3: tricks of the trade for a multi-tasking kinase. J.Cell Sci. **116**:1175-1186.

115. **Doble, B. W. and J. R. Woodgett**. 2003. GSK-3: tricks of the trade for a multi-tasking kinase. J.Cell Sci. **116**:1175-1186.

116. **Doble, B. W. and J. R. Woodgett**. 2003. GSK-3: tricks of the trade for a multi-tasking kinase. J.Cell Sci. **116**:1175-1186.

117. **Dunty, J. M., V. Gabarra-Niecko, M. L. King, D. F. Ceccarelli, M. J. Eck, and M. D. Schaller**. 2004. FERM domain interaction promotes FAK signaling. Mol.Cell Biol. **24**:5353-5368.

118. **Dunty, J. M., V. Gabarra-Niecko, M. L. King, D. F. Ceccarelli, M. J. Eck, and M. D. Schaller**. 2004. FERM domain interaction promotes FAK signaling. Mol.Cell Biol. **24**:5353-5368.

119. **Dunty, J. M., V. Gabarra-Niecko, M. L. King, D. F. Ceccarelli, M. J. Eck, and M. D. Schaller**. 2004. FERM domain interaction promotes FAK signaling. Mol.Cell Biol. **24**:5353-5368.

120. **Dunty, J. M., V. Gabarra-Niecko, M. L. King, D. F. Ceccarelli, M. J. Eck, and M. D. Schaller**. 2004. FERM domain interaction promotes FAK signaling. Mol.Cell Biol. **24**:5353-5368.

121. **Dunty, J. M. and M. D. Schaller**. 2002. The amino termini of FAK family kinases regulate substrate phosphorylation, localization, and cell morphology. J.Biol.Chem. **277**:45644-45654.

122. **Duong, L. T. and G. A. Rodan**. 2000. PYK2 is an adhesion kinase in macrophages, localized in podosomes and activated by beta(2)-integrin ligation. Cell Motil.Cytoskeleton **47**:174-188.

123. **Duong, L. T. and G. A. Rodan**. 2000. PYK2 is an adhesion kinase in macrophages, localized in podosomes and activated by beta(2)-integrin ligation. Cell Motil.Cytoskeleton **47**:174-188.

124. **Duong, L. T. and G. A. Rodan**. 2000. PYK2 is an adhesion kinase in macrophages, localized in podosomes and activated by beta(2)-integrin ligation. Cell Motil.Cytoskeleton **47**:174-188.

125. **Eliceiri, B. P., X. S. Puente, J. D. Hood, D. G. Stupack, D. D. Schlaepfer, X. Z. Huang, D. Sheppard, and D. A. Cheresh**. 2002. Src-mediated coupling of focal adhesion kinase to integrin alpha(v)beta5 in vascular endothelial growth factor signaling. J.Cell Biol. **157**:149-160.

126. **Eliceiri, B. P., X. S. Puente, J. D. Hood, D. G. Stupack, D. D. Schlaepfer, X. Z. Huang, D. Sheppard, and D. A. Cheresh**. 2002. Src-mediated coupling of focal adhesion kinase to integrin alpha(v)beta5 in vascular endothelial growth factor signaling. J.Cell Biol. **157**:149-160.

127. **Embi, N., D. B. Rylatt, and P. Cohen**. 1980. Glycogen synthase kinase-3 from rabbit skeletal muscle. Separation from cyclic-AMP-dependent protein kinase and phosphorylase kinase. Eur.J.Biochem. **107**:519-527.

128. **Engelhardt, B. and H. Wolburg**. 2004. Mini-review: Transendothelial migration of leukocytes: through the front door or around the side of the house? Eur.J Immunol **34**:2955-2963.

129. **Evangelista, V., Z. Pamuklar, A. Piccoli, S. Manarini, G. Dell'elba, R. Pecce, N. Martelli, L. Federico, M. Rojas, G. Berton, C. A. Lowell, L. Totani, and S. S. Smyth**. 2007. Src family kinases mediate neutrophil adhesion to adherent platelets. Blood **109**:2461-2469.

130. **Evans, J. and D. T. Yue**. 2003. New turf for CFP/YFP FRET imaging of membrane signaling molecules. Neuron **38**:145-147.

131. **Ezratty, E. J., M. A. Partridge, and G. G. Gundersen**. 2005. Microtubule-induced focal adhesion disassembly is mediated by dynamin and focal adhesion kinase. Nat.Cell Biol. **7**:581-590.

132. **Feral, C. C., D. M. Rose, J. Han, N. Fox, G. J. Silverman, K. Kaushansky, and M. H. Ginsberg**. 2006. Blocking the alpha 4 integrin-paxillin interaction selectively impairs mononuclear leukocyte recruitment to an inflammatory site. J Clin.Invest **116**:715-723.

133. **Fernandez, R., L. A. Boxer, and S. J. Suchard**. 1997. Beta2 integrins are not required for tyrosine phosphorylation of paxillin in human neutrophils. J.Immunol. **159**:5568-5575.

134. **Fiol, C. J., A. M. Mahrenholz, Y. Wang, R. W. Roeske, and P. J. Roach**. 1987. Formation of protein kinase recognition sites by covalent modification of the substrate. Molecular mechanism for the synergistic action of casein kinase II and glycogen synthase kinase 3. J.Biol.Chem. **262**:14042-14048.

135. **Fiol, C. J., A. M. Mahrenholz, Y. Wang, R. W. Roeske, and P. J. Roach**. 1987. Formation of protein kinase recognition sites by covalent modification of the substrate. Molecular mechanism for the synergistic action of casein kinase II and glycogen synthase kinase 3. J.Biol.Chem. **262**:14042-14048.

136. **Fitzer-Attas, C. J., M. Lowry, M. T. Crowley, A. J. Finn, F. Meng, A. L. DeFranco, and C. A. Lowell**. 2000. Fcgamma receptor-mediated phagocytosis in macrophages lacking the Src family tyrosine kinases Hck, Fgr, and Lyn. J.Exp.Med. **191**:669-682.

137. **Fitzer-Attas, C. J., M. Lowry, M. T. Crowley, A. J. Finn, F. Meng, A. L. DeFranco, and C. A. Lowell**. 2000. Fcgamma receptor-mediated phagocytosis in macrophages lacking the Src family tyrosine kinases Hck, Fgr, and Lyn. J.Exp.Med. **191**:669-682.

138. **Fonseca, P. M., R. Y. Inoue, C. B. Kobarg, D. P. Crosara-Alberto, J. Kobarg, and K. G. Franchini**. 2005. Targeting to C-terminal myosin heavy chain may explain mechanotransduction involving focal adhesion kinase in cardiac myocytes. Circ.Res. **96**:73-81.

139. **Foster, S. L., D. C. Hargreaves, and R. Medzhitov**. 2007. Gene-specific control of inflammation by TLR-induced chromatin modifications. Nature **447**:972-978.

140. **Franchini, K. G., A. S. Torsoni, P. H. Soares, and M. J. Saad**. 2000. Early activation of the multicomponent signaling complex associated with focal adhesion kinase induced by pressure overload in the rat heart. Circ.Res. **87**:558-565.

141. **Frey, M. T., I. Y. Tsai, T. P. Russell, S. K. Hanks, and Y. L. Wang**. 2006. Cellular responses to substrate topography: role of myosin II and focal adhesion kinase. Biophys.J. **90**:3774-3782.

142. **Frisch, S. M., K. Vuori, E. Ruoslahti, and P. Y. Chan-Hui**. 1996. Control of adhesion-dependent cell survival by focal adhesion kinase. J.Cell Biol. **134**:793-799.

143. **Fujimoto, J., K. Sawamoto, M. Okabe, Y. Takagi, T. Tezuka, S. Yoshikawa, H. Ryo, H. Okano, and T. Yamamoto**. 1999. Cloning and characterization of Dfak56, a homolog of focal adhesion kinase, in Drosophila melanogaster. J.Biol.Chem. **274**:29196-29201.

144. **Funakoshi-Tago, M., Y. Sonoda, S. Tanaka, K. Hashimoto, K. Tago, S. Tominaga, and T. Kasahara**. 2003. Tumor necrosis factor-induced nuclear factor kappaB activation is impaired in focal adhesion kinase-deficient fibroblasts. J.Biol.Chem. **278**:29359-29365.

145. **Furuta, Y., D. Ilic, S. Kanazawa, N. Takeda, T. Yamamoto, and S. Aizawa**. 1995. Mesodermal defect in late phase of gastrulation by a targeted mutation of focal adhesion kinase, FAK. Oncogene **11**:1989-1995.

146. **Gabarra-Niecko, V., M. D. Schaller, and J. M. Dunty**. 2003. FAK regulates biological processes important for the pathogenesis of cancer. Cancer and Metastasis Reviews **22**:359-374.

147. **Garantziotis, S., J. W. Hollingsworth, A. K. Zaas, and D. A. Schwartz**. 2007. The Effect of Toll-Like Receptors and Toll-Like Receptor Genetics in Human Disease. Annu.Rev.Med.

148. **Garces, C. A., E. V. Kurenova, V. M. Golubovskaya, and W. G. Cance**. 2006. Vascular endothelial growth factor receptor-3 and focal adhesion kinase bind and suppress apoptosis in breast cancer cells. Cancer Res. **66**:1446-1454.

149. **Garcia-Alvarez, B., J. M. de Pereda, D. A. Calderwood, T. S. Ulmer, D. Critchley, I. D. Campbell, M. H. Ginsberg, and R. C. Liddington**. 2003. Structural determinants of integrin recognition by Talin. Molecular Cell **11**:49-58.

150. **Garcia-Garcia, E. and C. Rosales**. 2002. Signal transduction during Fc receptor-mediated phagocytosis. J.Leukoc.Biol. **72**:1092-1108.

151. **Gil-Henn, H., O. Destaing, N. A. Sims, K. Aoki, N. Alles, L. Neff, A. Sanjay, A. Bruzzaniti, P. De Camilli, R. Baron, and J. Schlessinger**. 2007. Defective microtubule-dependent podosome organization in osteoclasts leads to increased bone density in Pyk2(-/-) mice. J.Cell Biol. **178**:1053-1064.

152. **Gilmore, A. P. and K. Burridge**. 1996. Regulation of vinculin binding to talin and actin by phosphatidyl- inositol-4-5-bisphosphate. Nature **381**:531-535.

153. **Gilmore, A. P. and K. Burridge**. 1996. Regulation of vinculin binding to talin and actin by phosphatidyl- inositol-4-5-bisphosphate. Nature **381**:531-535.

154. **Gismondi, A., J. Jacobelli, R. Strippoli, F. Mainiero, A. Soriani, L. Cifaldi, M. Piccoli, L. Frati, and A. Santoni**. 2003. Proline-rich tyrosine kinase 2 and Rac activation by chemokine and integrin receptors controls NK cell transendothelial migration. J.Immunol. **170**:3065-3073.

155. **Goldberg, J., A. C. Nairn, and J. Kuriyan**. 1996. Structural basis for the autoinhibition of calcium/calmodulin-dependent protein kinase I. Cell **84**:875-887.

156. **Golubovskaya, V. M. and W. G. Cance**. 2007. Focal adhesion kinase and p53 signaling in cancer cells. Int.Rev.Cytol. **263**:103-153.

157. **Golubovskaya, V. M., R. Finch, and W. G. Cance**. 2005. Direct Interaction of the N-terminal Domain of Focal Adhesion Kinase with the N-terminal Transactivation Domain of p53. J.Biol.Chem. **280**:25008-25021.

158. **Golubovskaya, V. M., R. Finch, and W. G. Cance**. 2005. Direct Interaction of the N-terminal Domain of Focal Adhesion Kinase with the N-terminal Transactivation Domain of p53. J.Biol.Chem. **280**:25008-25021.

159. **Goold, R. G. and P. R. Gordon-Weeks**. 2005. The MAP kinase pathway is upstream of the activation of GSK3beta that enables it to phosphorylate MAP1B and contributes to the stimulation of axon growth. Mol.Cell Neurosci. **28**:524-534.

160. **Goold, R. G., R. Owen, and P. R. Gordon-Weeks**. 1999. Glycogen synthase kinase 3beta phosphorylation of microtubule-associated protein 1B regulates the stability of microtubules in growth cones. J.Cell Sci. **112 (Pt 19)**:3373-3384.

161. **Goold, R. G., R. Owen, and P. R. Gordon-Weeks**. 1999. Glycogen synthase kinase 3beta phosphorylation of microtubule-associated protein 1B regulates the stability of microtubules in growth cones. J.Cell Sci. **112 (Pt 19)**:3373-3384.

162. **Greenberg, S., P. Chang, and S. C. Silverstein**. 1994. Tyrosine phosphorylation of the gamma subunit of Fc gamma receptors, p72syk, and paxillin during Fc receptor-mediated phagocytosis in macrophages. J.Biol.Chem. **269**:3897-3902.

163. **Greenberg, S. and S. Grinstein**. 2002. Phagocytosis and innate immunity. Curr.Opin.Immunol. **14**:136-145.

164. **Griffin, B. A., S. R. Adams, J. Jones, and R. Y. Tsien**. 2000. Fluorescent labeling of recombinant proteins in living cells with FlAsH. Methods Enzymol. **327**:565-578.

165. **Guan, J. L. and D. Shalloway**. 1992. Regulation of focal adhesion-associated protein tyrosine kinase by both cellular adhesion and oncogenic transformation. Nature **358**:690-692.

166. **Guinamard, R., M. Okigaki, J. Schlessinger, and J. V. Ravetch**. 2000. Absence of marginal zone B cells in Pyk-2-deficient mice defines their role in the humoral response. Nat.Immunol. **1**:31-36.

167. **Guinamard, R., M. Okigaki, J. Schlessinger, and J. V. Ravetch**. 2000. Absence of marginal zone B cells in Pyk-2-deficient mice defines their role in the humoral response. Nat.Immunol. **1**:31-36.

168. **Guinamard, R., M. Okigaki, J. Schlessinger, and J. V. Ravetch**. 2000. Absence of marginal zone B cells in Pyk-2-deficient mice defines their role in the humoral response. Nat.Immunol. **1**:31-36.

169. **Hagel, M., E. L. George, A. Kim, R. Tamimi, S. L. Opitz, C. E. Turner, A. Imamoto, and S. M. Thomas**. 2002. The adaptor protein paxillin is essential for normal development in the mouse and is a critical transducer of fibronectin signaling. Mol.Cell Biol. **22**:901-915.

170. **Hagel, M., E. L. George, A. Kim, R. Tamimi, S. L. Opitz, C. E. Turner, A. Imamoto, and S. M. Thomas**. 2002. The adaptor protein paxillin is essential for normal development in the mouse and is a critical transducer of fibronectin signaling. Mol.Cell Biol. **22**:901-915.

171. **Hagel, M., E. L. George, A. Kim, R. Tamimi, S. L. Opitz, C. E. Turner, A. Imamoto, and S. M. Thomas**. 2002. The adaptor protein paxillin is essential for normal development in the mouse and is a critical transducer of fibronectin signaling. Mol.Cell Biol. **22**:901-915.

172. **Hagel, M., E. L. George, A. Kim, R. Tamimi, S. L. Opitz, C. E. Turner, A. Imamoto, and S. M. Thomas**. 2002. The adaptor protein paxillin is essential for normal development in the mouse and is a critical transducer of fibronectin signaling. Mol.Cell Biol. **22**:901-915.

173. **Hall, A. B., M. A. Gakidis, M. Glogauer, J. L. Wilsbacher, S. Gao, W. Swat, and J. S. Brugge**. 2006. Requirements for Vav guanine nucleotide exchange factors and Rho GTPases in FcgammaR- and complement-mediated phagocytosis. Immunity. **24**:305-316.

174. **Hamada, K., T. Shimizu, T. Matsui, S. Tsukita, and T. Hakoshima**. 2000. Structural basis of the membrane-targeting and unmasking mechanisms of the radixin FERM domain. EMBO J. **19**:4449-4462.

175. **Hamada, K., T. Shimizu, T. Matsui, S. Tsukita, and T. Hakoshima**. 2000. Structural basis of the membrane-targeting and unmasking mechanisms of the radixin FERM domain. EMBO J. **19**:4449-4462.

176. **Han, D. C. and J. L. Guan**. 1999. Association of focal adhesion kinase with Grb7 and its role in cell migration. J.Biol.Chem. **274**:24425-24430.

177. **Han, H., M. Fuortes, and C. Nathan**. 2003. Critical role of the carboxyl terminus of proline-rich tyrosine kinase (Pyk2) in the activation of human neutrophils by tumor necrosis factor: separation of signals for the respiratory burst and degranulation. J.Exp.Med. **197**:63-75.

178. **Han, H., M. Fuortes, and C. Nathan**. 2003. Critical role of the carboxyl terminus of proline-rich tyrosine kinase (Pyk2) in the activation of human neutrophils by tumor necrosis factor: separation of signals for the respiratory burst and degranulation. J.Exp.Med. **197**:63-75.

179. **Handsley, M. M. and D. R. Edwards**. 2005. Metalloproteinases and their inhibitors in tumor angiogenesis. Int.J.Cancer **115**:849-860.

180. **Hanger, D. P., K. Hughes, J. R. Woodgett, J. P. Brion, and B. H. Anderton**. 1992. Glycogen synthase kinase-3 induces Alzheimer's disease-like phosphorylation of tau: generation of paired helical filament epitopes and neuronal localisation of the kinase. Neurosci.Lett. **147**:58-62.

181. **Hanks, S. K., M. B. Calalb, M. C. Harper, and S. K. Patel**. 1992. Focal adhesion protein-tyrosine kinase phosphorylated in response to cell attachment to fibronectin. Proc.Natl.Acad.Sci.U.S.A **89**:8487-8491.

182. **Harder, K. W., N. P. Moller, J. W. Peacock, and F. R. Jirik**. 1998. Protein-tyrosine phosphatase alpha regulates Src family kinases and alters cell-substratum adhesion. J.Biol.Chem. **273**:31890-31900.

183. **Harlan, J. E., H. S. Yoon, P. J. Hajduk, and S. W. Fesik**. 1995. Structural characterization of the interaction between a pleckstrin homology domain and phosphatidylinositol 4,5-bisphosphate. Biochemistry **34**:9859-9864.

184. **Hasegawa, H., E. Kiyokawa, S. Tanaka, K. Nagashima, N. Gotoh, M. Shibuya, T. Kurata, and M. Matsuda**. 1996. DOCK180, a major CRK-binding protein, alters

cell morphology upon translocation to the cell membrane. Mol.Cell Biol. **16**:1770-1776.

185. **Hatada, M. H., X. Lu, E. R. Laird, J. Green, J. P. Morgenstern, M. Lou, C. S. Marr, T. B. Phillips, M. K. Ram, K. Theriault, and .** 1995. Molecular basis for interaction of the protein tyrosine kinase ZAP-70 with the T-cell receptor. Nature **377**:32-38.

186. **Hazeki, K., N. Masuda, K. Funami, N. Sukenobu, M. Matsumoto, S. Akira, K. Takeda, T. Seya, and O. Hazeki**. 2003. Toll-like receptor-mediated tyrosine phosphorylation of paxillin via MyD88-dependent and -independent pathways. Eur.J.Immunol. **33**:740-747.

187. **He, B., Y. H. Meng, and N. F. Mivechi**. 1998. Glycogen synthase kinase 3beta and extracellular signal-regulated kinase inactivate heat shock transcription factor 1 by facilitating the disappearance of transcriptionally active granules after heat shock. Mol.Cell Biol. **18**:6624-6633.

188. **Heikal, A. A., S. T. Hess, G. S. Baird, R. Y. Tsien, and W. W. Webb**. 2000. Molecular spectroscopy and dynamics of intrinsically fluorescent proteins: coral red (dsRed) and yellow (Citrine). Proc.Natl.Acad.Sci.U.S.A **97**:11996-12001.

189. **Hens, M. D. and D. W. DeSimone**. 1995. Molecular analysis and developmental expression of the focal adhesion kinase pp125FAK in Xenopus laevis. Dev.Biol. **170**:274-288.

190. **Hildebrand, J. D., M. D. Schaller, and J. T. Parsons**. 1993. Identification of sequences required for the efficient localization of the focal adhesion kinase, $pp125^{FAK}$, to cellular focal adhesions. J.Cell Biol. **123**:993-1005.

191. **Hildebrand, J. D., J. M. Taylor, and J. T. Parsons**. 1996. An SH3 domain-containing GTPase-activating protein for Rho and Cdc42 associates with focal adhesion kinase. Mol.Cell Biol. **16**:3169-3178.

192. **Hirose, K., S. Kadowaki, M. Tanabe, H. Takeshima, and M. Iino**. 1999. Spatiotemporal dynamics of inositol 1,4,5-trisphosphate that underlies complex Ca2+ mobilization patterns. Science **284**:1527-1530.

193. **Hodgson, L., P. Nalbant, F. Shen, and K. Hahn**. 2006. Imaging and photobleach correction of Mero-CBD, sensor of endogenous Cdc42 activation. Methods Enzymol. **406**:140-156.

194. **Hoffmann, C., G. Gaietta, M. Bunemann, S. R. Adams, S. Oberdorff-Maass, B. Behr, J. P. Vilardaga, R. Y. Tsien, M. H. Ellisman, and M. J. Lohse**. 2005. A FlAsH-based FRET approach to determine G protein-coupled receptor activation in living cells. Nat Methods **2**:171-176.

195. **Holinstat, M., N. Knezevic, M. Broman, A. M. Samarel, A. B. Malik, and D. Mehta**. 2006. Suppression of RhoA activity by focal adhesion kinase-induced activation of p190RhoGAP: role in regulation of endothelial permeability. J.Biol.Chem. **281**:2296-2305.

196. **Hong, C. A., E. Swearingen, R. Mallari, X. Gao, Z. Cao, A. North, S. W. Young, and S. G. Huang**. 2003. Development of a high throughput time-resolved fluorescence resonance energy transfer assay for TRAF6 ubiquitin polymerization. Assay.Drug Dev.Technol. **1**:175-180.

197. **Hood, J. D., R. Frausto, W. B. Kiosses, M. A. Schwartz, and D. A. Cheresh**. 2003. Differential alphav integrin-mediated Ras-ERK signaling during two pathways of angiogenesis. J.Cell Biol. **162**:933-943.

198. **Hood, J. D., R. Frausto, W. B. Kiosses, M. A. Schwartz, and D. A. Cheresh**. 2003. Differential alphav integrin-mediated Ras-ERK signaling during two pathways of angiogenesis. J.Cell Biol. **162**:933-943.

199. **Hood, J. D., R. Frausto, W. B. Kiosses, M. A. Schwartz, and D. A. Cheresh**. 2003. Differential alphav integrin-mediated Ras-ERK signaling during two pathways of angiogenesis. J.Cell Biol. **162**:933-943.

200. **Hsia, D. A., S. T. Lim, J. A. Bernard-Trifilo, S. K. Mitra, S. Tanaka, J. den Hertog, D. N. Streblow, D. Ilic, M. H. Ginsberg, and D. D. Schlaepfer**. 2005. Integrin alpha4beta1 promotes focal adhesion kinase-independent cell motility via alpha4 cytoplasmic domain-specific activation of c-Src. Mol.Cell Biol. **25**:9700-9712.

201. **Hu, B., M. J. Jarzynka, P. Guo, Y. Imanishi, D. D. Schlaepfer, and S. Y. Cheng**. 2006. Angiopoietin 2 induces glioma cell invasion by stimulating matrix metalloprotease 2 expression through the alphavbeta1 integrin and focal adhesion kinase signaling pathway. Cancer Res. **66**:775-783.

202. **Huang, C., C. H. Borchers, M. D. Schaller, and K. Jacobson**. 2004. Phosphorylation of paxillin by p38MAPK is involved in the neurite extension of PC-12 cells. J.Cell Biol. **164**:593-602.

203. **Huang, C., C. H. Borchers, M. D. Schaller, and K. Jacobson**. 2004. Phosphorylation of paxillin by p38MAPK is involved in the neurite extension of PC-12 cells. J.Cell Biol. **164**:593-602.

204. **Huang, C., C. H. Borchers, M. D. Schaller, and K. Jacobson**. 2004. Phosphorylation of paxillin by p38MAPK is involved in the neurite extension of PC-12 cells. J.Cell Biol. **164**:593-602.

205. **Huang, C., C. H. Borchers, M. D. Schaller, and K. Jacobson**. 2004. Phosphorylation of paxillin by p38MAPK is involved in the neurite extension of PC-12 cells. J.Cell Biol. **164**:593-602.

206. **Huang, C., C. H. Borchers, M. D. Schaller, and K. Jacobson**. 2004. Phosphorylation of paxillin by p38MAPK is involved in the neurite extension of PC-12 cells. J.Cell Biol. **164**:593-602.

207. **Huang, C., K. Jacobson, and M. D. Schaller**. 2004. MAP kinases and cell migration. J.Cell Sci. **117**:4619-4628.

208. **Huang, C., Z. Rajfur, C. Borchers, M. D. Schaller, and K. Jacobson**. 2003. JNK phosphorylates paxillin and regulates cell migration. Nature **424**:219-223.

209. **Huang, C., Z. Rajfur, C. Borchers, M. D. Schaller, and K. Jacobson**. 2003. JNK phosphorylates paxillin and regulates cell migration. Nature **424**:219-223.

210. **Huang, C., Z. Rajfur, C. Borchers, M. D. Schaller, and K. Jacobson**. 2003. JNK phosphorylates paxillin and regulates cell migration. Nature **424**:219-223.

211. **Humphries, M. J.** 2004. Monoclonal antibodies as probes of integrin priming and activation. Biochem.Soc.Trans. **32**:407-411.

212. **Hunger-Glaser, I., R. S. Fan, E. Perez-Salazar, and E. Rozengurt**. 2004. PDGF and FGF induce focal adhesion kinase (FAK) phosphorylation at Ser-910: dissociation from Tyr-397 phosphorylation and requirement for ERK activation. J.Cell Physiol **200**:213-222.

213. **Hunger-Glaser, I., R. S. Fan, E. Perez-Salazar, and E. Rozengurt**. 2004. PDGF and FGF induce focal adhesion kinase (FAK) phosphorylation at Ser-910: dissociation from Tyr-397 phosphorylation and requirement for ERK activation. J.Cell Physiol **200**:213-222.

214. **Ilic, D., E. A. Almeida, D. D. Schlaepfer, P. Dazin, S. Aizawa, and C. H. Damsky**. 1998. Extracellular matrix survival signals transduced by focal adhesion kinase suppress p53-mediated apoptosis. J.Cell Biol. **143**:547-560.

215. **Ilic, D., Y. Furuta, S. Kanazawa, N. Takeda, K. Sobue, N. Nakatsuji, S. Nomura, J. Fujimoto, M. Okada, and T. Yamamoto**. 1995. Reduced cell motility and enhanced focal adhesion contact formation in cells from FAK-deficient mice. Nature **377**:539-544.

216. **Ilic, D., Y. Furuta, S. Kanazawa, N. Takeda, K. Sobue, N. Nakatsuji, S. Nomura, J. Fujimoto, M. Okada, and T. Yamamoto**. 1995. Reduced cell motility and enhanced focal adhesion contact formation in cells from FAK-deficient mice. Nature **377**:539-544.

217. **Ilic, D., B. Kovacic, S. McDonagh, F. Jin, C. Baumbusch, D. G. Gardner, and C. H. Damsky**. 2003. Focal adhesion kinase is required for blood vessel morphogenesis. Circ.Res. **92**:300-307.

218. **Insall, R. H. and O. D. Weiner**. 2001. PIP3, PIP2, and cell movement--similar messages, different meanings? Dev.Cell **1**:743-747.

219. **Ishibe, S., D. Joly, Z. X. Liu, and L. G. Cantley**. 2004. Paxillin Serves as an ERK-Regulated Scaffold for Coordinating FAK and Rac Activation in Epithelial Morphogenesis. Mol.Cell **16**:257-267.

220. **Ishibe, S., D. Joly, Z. X. Liu, and L. G. Cantley**. 2004. Paxillin Serves as an ERK-Regulated Scaffold for Coordinating FAK and Rac Activation in Epithelial Morphogenesis. Mol.Cell **16**:257-267.

221. **Ishibe, S., D. Joly, Z. X. Liu, and L. G. Cantley**. 2004. Paxillin Serves as an ERK-Regulated Scaffold for Coordinating FAK and Rac Activation in Epithelial Morphogenesis. Mol.Cell **16**:257-267.

222. **Ishibe, S., D. Joly, Z. X. Liu, and L. G. Cantley**. 2004. Paxillin Serves as an ERK-Regulated Scaffold for Coordinating FAK and Rac Activation in Epithelial Morphogenesis. Mol.Cell **16**:257-267.

223. **Ishibe, S., D. Joly, Z. X. Liu, and L. G. Cantley**. 2004. Paxillin Serves as an ERK-Regulated Scaffold for Coordinating FAK and Rac Activation in Epithelial Morphogenesis. Mol.Cell **16**:257-267.

224. **Ishibe, S., D. Joly, Z. X. Liu, and L. G. Cantley**. 2004. Paxillin Serves as an ERK-Regulated Scaffold for Coordinating FAK and Rac Activation in Epithelial Morphogenesis. Mol.Cell **16**:257-267.

225. **Ishibe, S., D. Joly, X. Zhu, and L. G. Cantley**. 2003. Phosphorylation-dependent paxillin-ERK association mediates hepatocyte growth factor-stimulated epithelial morphogenesis. Mol.Cell **12**:1275-1285.

226. **Ishibe, S., D. Joly, X. Zhu, and L. G. Cantley**. 2003. Phosphorylation-dependent paxillin-ERK association mediates hepatocyte growth factor-stimulated epithelial morphogenesis. Mol.Cell **12**:1275-1285.

227. **Ishibe, S., D. Joly, X. Zhu, and L. G. Cantley**. 2003. Phosphorylation-dependent paxillin-ERK association mediates hepatocyte growth factor-stimulated epithelial morphogenesis. Mol.Cell **12**:1275-1285.

228. **Ishibe, S., D. Joly, X. Zhu, and L. G. Cantley**. 2003. Phosphorylation-dependent paxillin-ERK association mediates hepatocyte growth factor-stimulated epithelial morphogenesis. Mol.Cell **12**:1275-1285.

229. **Ishida, T., T. E. Peterson, N. L. Kovach, and B. C. Berk**. 1996. MAP kinase activation by flow in endothelial cells. Role of beta 1 integrins and tyrosine kinases. Circ.Res. **79**:310-316.

230. **Ivankovic-Dikic, I., E. Gronroos, A. Blaukat, B. U. Barth, and I. Dikic**. 2000. Pyk2 and FAK regulate neurite outgrowth induced by growth factors and integrins. Nat.Cell Biol. **2**:574-581.

231. **Ivankovic-Dikic, I., E. Gronroos, A. Blaukat, B. U. Barth, and I. Dikic**. 2000. Pyk2 and FAK regulate neurite outgrowth induced by growth factors and integrins. Nat.Cell Biol. **2**:574-581.

232. **Ivankovic-Dikic, I., E. Gronroos, A. Blaukat, B. U. Barth, and I. Dikic**. 2000. Pyk2 and FAK regulate neurite outgrowth induced by growth factors and integrins. Nat.Cell Biol. **2**:574-581.

233. **Jacamo, R. O. and E. Rozengurt**. 2005. A truncated FAK lacking the FERM domain displays high catalytic activity but retains responsiveness to adhesion-mediated signals. Biochem.Biophys.Res.Commun. **334**:1299-1304.

234. Janeway, Travers, Walport, and shlomchik. immunobiology. 2007. Garland Scinece. Ref Type: Generic

235. **Jiang, X., J. Sinnett-Smith, and E. Rozengurt**. 2007. Differential FAK phosphorylation at Ser-910, Ser-843 and Tyr-397 induced by angiotensin II, LPA and EGF in intestinal epithelial cells. Cell Signal. **19**:1000-1010.

236. **Johnson, R. P. and S. W. Craig**. 1995. F-actin binding site masked by the intramolecular association of vinculin head and tail domains. Nature **373**:261-264.

237. **Jones, G. and G. Stewart**. 2004. Nuclear import of N-terminal FAK by activation of the FcepsilonRI receptor in RBL-2H3 cells. Biochem.Biophys.Res.Commun. **314**:39-45.

238. **Jope, R. S. and G. V. Johnson**. 2004. The glamour and gloom of glycogen synthase kinase-3. Trends Biochem.Sci. **29**:95-102.

239. **Kagan, J. C. and R. Medzhitov**. 2006. Phosphoinositide-mediated adaptor recruitment controls Toll-like receptor signaling. Cell **125**:943-955.

240. **Kain, K. H. and R. L. Klemke**. 2001. Inhibition of cell migration by Abl family tyrosine kinases through uncoupling of Crk-CAS complexes. J.Biol.Chem. **276**:16185-16192.

241. **Kaplan, K. B., K. B. Bibbins, J. R. Swedlow, M. Arnaud, D. O. Morgan, and H. E. Varmus**. 1994. Association of the amino-terminal half of c-Src with focal adhesions alters their properties and is regulated by phosphorylation of tyrosine 527. EMBO J. **13**:4745-4756.

242. **Katagiri, T., T. Takahashi, T. Sasaki, S. Nakamura, and S. Hattori**. 2000. Protein-tyrosine kinase Pyk2 is involved in interleukin-2 production by Jurkat T cells via its tyrosine 402. J.Biol.Chem. **275**:19645-19652.

243. **Kedzierska, K., N. J. Vardaxis, A. Jaworowski, and S. M. Crowe**. 2001. FcgammaR-mediated phagocytosis by human macrophages involves Hck, Syk, and Pyk2 and is augmented by GM-CSF. J.Leukoc.Biol. **70**:322-328.

244. **Kedzierska, K., N. J. Vardaxis, A. Jaworowski, and S. M. Crowe**. 2001. FcgammaR-mediated phagocytosis by human macrophages involves Hck, Syk, and Pyk2 and is augmented by GM-CSF. J.Leukoc.Biol. **70**:322-328.

245. **Kedzierska, K., N. J. Vardaxis, A. Jaworowski, and S. M. Crowe**. 2001. FcgammaR-mediated phagocytosis by human macrophages involves Hck, Syk, and Pyk2 and is augmented by GM-CSF. J.Leukoc.Biol. **70**:322-328.

246. **Kim, C., N. H. Xuong, and S. S. Taylor**. 2005. Crystal structure of a complex between the catalytic and regulatory (RIalpha) subunits of PKA. Science **307**:690-696.

247. **King, W. G., M. D. Mattaliano, T. O. Chan, P. N. Tsichlis, and J. S. Brugge**. 1997. Phosphatidylinositol 3-kinase is required for integrin-stimulated AKT and Raf-1/mitogen-activated protein kinase pathway activation. Mol.Cell Biol. **17**:4406-4418.

248. **King, W. G., M. D. Mattaliano, T. O. Chan, P. N. Tsichlis, and J. S. Brugge**. 1997. Phosphatidylinositol 3-kinase is required for integrin-stimulated AKT and Raf-1/mitogen-activated protein kinase pathway activation. Mol.Cell Biol. **17**:4406-4418.

249. **King, W. G., M. D. Mattaliano, T. O. Chan, P. N. Tsichlis, and J. S. Brugge**. 1997. Phosphatidylinositol 3-kinase is required for integrin-stimulated AKT and Raf-1/mitogen-activated protein kinase pathway activation. Mol.Cell Biol. **17**:4406-4418.

250. **King, W. G., M. D. Mattaliano, T. O. Chan, P. N. Tsichlis, and J. S. Brugge**. 1997. Phosphatidylinositol 3-kinase is required for integrin-stimulated AKT and Raf-1/mitogen-activated protein kinase pathway activation. Mol.Cell Biol. **17**:4406-4418.

251. **King, W. G., M. D. Mattaliano, T. O. Chan, P. N. Tsichlis, and J. S. Brugge**. 1997. Phosphatidylinositol 3-kinase is required for integrin-stimulated AKT and Raf-1/mitogen-activated protein kinase pathway activation. Mol.Cell Biol. **17**:4406-4418.

252. **King, W. G., M. D. Mattaliano, T. O. Chan, P. N. Tsichlis, and J. S. Brugge**. 1997. Phosphatidylinositol 3-kinase is required for integrin-stimulated AKT and Raf-1/mitogen-activated protein kinase pathway activation. Mol.Cell Biol. **17**:4406-4418.

253. **Klinghoffer, R. A., C. Sachsenmaier, J. A. Cooper, and P. Soriano**. 1999. Src family kinases are required for integrin but not PDGFR signal transduction. EMBO J. **18**:2459-2471.

254. **Kobayashi, T., S. Hino, N. Oue, T. Asahara, M. Zollo, W. Yasui, and A. Kikuchi**. 2006. Glycogen synthase kinase 3 and h-prune regulate cell migration by modulating focal adhesions. Mol.Cell Biol. **26**:898-911.

255. **Koivisto, L., K. Alavian, L. Hakkinen, S. Pelech, C. A. McCulloch, and H. Larjava**. 2003. Glycogen synthase kinase-3 regulates formation of long lamellipodia in human keratinocytes. J.Cell Sci. **116**:3749-3760.

256. **Kornberg, L. J., L. C. Shaw, P. E. Spoerri, S. Caballero, and M. B. Grant**. 2004. Focal adhesion kinase overexpression induces enhanced pathological retinal angiogenesis. Invest Ophthalmol.Vis.Sci. **45**:4463-4469.

257. **Koshida, R., P. Rocic, S. Saito, T. Kiyooka, C. Zhang, and W. M. Chilian**. 2005. Role of focal adhesion kinase in flow-induced dilation of coronary arterioles. Arterioscler.Thromb.Vasc.Biol. **25**:2548-2553.

258. **Kraynov, V. S., C. Chamberlain, G. M. Bokoch, M. A. Schwartz, S. Slabaugh, and K. M. Hahn**. 2000. Localized Rac activation dynamics visualized in living cells. Science **290**:333-337.

259. **Ku, H. and K. E. Meier**. 2000. Phosphorylation of paxillin via the ERK mitogen-activated protein kinase cascade in EL4 thymoma cells. J.Biol.Chem. **275**:11333-11340.

260. **Kubiseski, T. J., Y. M. Chook, W. E. Parris, M. Rozakis-Adcock, and T. Pawson**. 1997. High affinity binding of the pleckstrin homology domain of mSos1 to phosphatidylinositol (4,5)-bisphosphate. J.Biol.Chem. **272**:1799-1804.

261. **Kurokawa, K., N. Mochizuki, Y. Ohba, H. Mizuno, A. Miyawaki, and M. Matsuda**. 2001. A pair of fluorescent resonance energy transfer-based probes for tyrosine phosphorylation of the CrkII adaptor protein in vivo. J.Biol.Chem. **276**:31305-31310.

262. **Lal, H., S. K. Verma, M. Smith, R. S. Guleria, G. Lu, D. M. Foster, and D. E. Dostal**. 2007. Stretch-induced MAP kinase activation in cardiac myocytes: Differential regulation through beta(1)-integrin and focal adhesion kinase. J.Mol.Cell Cardiol.

263. **Lal, H., S. K. Verma, M. Smith, R. S. Guleria, G. Lu, D. M. Foster, and D. E. Dostal**. 2007. Stretch-induced MAP kinase activation in cardiac myocytes: Differential regulation through beta(1)-integrin and focal adhesion kinase. J.Mol.Cell Cardiol.

264. **Law, D. A., L. Nannizzi-Alaimo, K. Ministri, P. E. Hughes, J. Forsyth, M. Turner, S. J. Shattil, M. H. Ginsberg, V. L. Tybulewicz, and D. R. Phillips**. 1999. Genetic and pharmacological analyses of Syk function in alphaIIbbeta3 signaling in platelets. Blood **93**:2645-2652.

265. **Le Boeuf, F., F. Houle, and J. Huot**. 2004. Regulation of vascular endothelial growth factor receptor 2-mediated phosphorylation of focal adhesion kinase by heat shock protein 90 and Src kinase activities. J.Biol.Chem. **279**:39175-39185.

266. **Le Boeuf, F., F. Houle, M. Sussman, and J. Huot**. 2006. Phosphorylation of focal adhesion kinase (FAK) on Ser732 is induced by rho-dependent kinase and is essential for proline-rich tyrosine kinase-2-mediated phosphorylation of FAK on Tyr407 in response to vascular endothelial growth factor. Mol.Biol.Cell **17**:3508-3520.

267. **Le Boeuf, F., F. Houle, M. Sussman, and J. Huot**. 2006. Phosphorylation of focal adhesion kinase (FAK) on Ser732 is induced by rho-dependent kinase and is essential for proline-rich tyrosine kinase-2-mediated phosphorylation of FAK on Tyr407 in response to vascular endothelial growth factor. Mol.Biol.Cell **17**:3508-3520.

268. **Le Boeuf, F., F. Houle, M. Sussman, and J. Huot**. 2006. Phosphorylation of focal adhesion kinase (FAK) on Ser732 is induced by rho-dependent kinase and is essential for proline-rich tyrosine kinase-2-mediated phosphorylation of FAK on Tyr407 in response to vascular endothelial growth factor. Mol.Biol.Cell **17**:3508-3520.

269. **Leaphart, C. L., J. Cavallo, S. C. Gribar, S. Cetin, J. Li, M. F. Branca, T. D. Dubowski, C. P. Sodhi, and D. J. Hackam**. 2007. A Critical Role for TLR4 in the Pathogenesis of Necrotizing Enterocolitis by Modulating Intestinal Injury and Repair. J Immunol **179**:4808-4820.

270. **Lee, M. S. and Y. J. Kim**. 2007. Signaling pathways downstream of pattern-recognition receptors and their cross talk. Annu.Rev.Biochem. **76**:447-480.

271. **Lee, W. L., G. Cosio, K. Ireton, and S. Grinstein**. 2007. Role of CrkII in Fcgamma receptor-mediated phagocytosis. J.Biol.Chem. **282**:11135-11143.

272. **Lehoux, S., B. Esposito, R. Merval, and A. Tedgui**. 2005. Differential regulation of vascular focal adhesion kinase by steady stretch and pulsatility. Circulation **111**:643-649.

273. **Lehoux, S., B. Esposito, R. Merval, and A. Tedgui**. 2005. Differential regulation of vascular focal adhesion kinase by steady stretch and pulsatility. Circulation **111**:643-649.

274. **Lemmon, M. A., K. M. Ferguson, R. O'Brien, P. B. Sigler, and J. Schlessinger**. 1995. Specific and high-affinity binding of inositol phosphates to an isolated pleckstrin homology domain. Proc.Natl.Acad.Sci.U.S.A **92**:10472-10476.

275. **Leucht, P., J. B. Kim, J. A. Currey, J. Brunski, and J. A. Helms**. 2007. FAK-Mediated Mechanotransduction in Skeletal Regeneration. PLoS.ONE. **2**:e390.

276. **Lewis, J. M., R. Baskaran, S. Taagepera, M. A. Schwartz, and J. Y. Wang**. 1996. Integrin regulation of c-Abl tyrosine kinase activity and cytoplasmic-nuclear transport. Proc.Natl.Acad.Sci.U.S.A **93**:15174-15179.

277. **Lewis, J. M., D. A. Cheresh, and M. A. Schwartz**. 1996. Protein kinase C regulates alpha v beta 5-dependent cytoskeletal associations and focal adhesion kinase phosphorylation. J Cell Biol. **134**:1323-1332.

278. **Li, S., P. Butler, Y. Wang, Y. Hu, D. C. Han, S. Usami, J. L. Guan, and S. Chien**. 2002. The role of the dynamics of focal adhesion kinase in the mechanotaxis of endothelial cells. Proc.Natl.Acad.Sci.U.S.A **99**:3546-3551.

279. **Li, S., P. Butler, Y. Wang, Y. Hu, D. C. Han, S. Usami, J. L. Guan, and S. Chien**. 2002. The role of the dynamics of focal adhesion kinase in the mechanotaxis of endothelial cells. Proc.Natl.Acad.Sci.U.S.A **99**:3546-3551.

280. **Li, S., J. L. Guan, and S. Chien**. 2005. Biochemistry and biomechanics of cell motility. Annu.Rev.Biomed.Eng **7**:105-150.

281. **Li, S., M. Kim, Y. L. Hu, S. Jalali, D. D. Schlaepfer, T. Hunter, S. Chien, and J. Y. Shyy**. 1997. Fluid shear stress activation of focal adhesion kinase. Linking to mitogen-activated protein kinases. J.Biol.Chem. **272**:30455-30462.

282. **Li, S., M. Kim, Y. L. Hu, S. Jalali, D. D. Schlaepfer, T. Hunter, S. Chien, and J. Y. Shyy**. 1997. Fluid shear stress activation of focal adhesion kinase. Linking to mitogen-activated protein kinases. J.Biol.Chem. **272**:30455-30462.

283. **Li, W., J. Lee, H. G. Vikis, S. H. Lee, G. Liu, J. Aurandt, T. L. Shen, E. R. Fearon, J. L. Guan, M. Han, Y. Rao, K. Hong, and K. L. Guan**. 2004. Activation of FAK and Src are receptor-proximal events required for netrin signaling. Nat.Neurosci. **7**:1213-1221.

284. **Li, W., J. Lee, H. G. Vikis, S. H. Lee, G. Liu, J. Aurandt, T. L. Shen, E. R. Fearon, J. L. Guan, M. Han, Y. Rao, K. Hong, and K. L. Guan**. 2004. Activation of FAK and Src are receptor-proximal events required for netrin signaling. Nat.Neurosci. **7**:1213-1221.

285. **Li, W., J. Lee, H. G. Vikis, S. H. Lee, G. Liu, J. Aurandt, T. L. Shen, E. R. Fearon, J. L. Guan, M. Han, Y. Rao, K. Hong, and K. L. Guan**. 2004. Activation of FAK and Src are receptor-proximal events required for netrin signaling. Nat.Neurosci. **7**:1213-1221.

286. **Li, Y. S., J. H. Haga, and S. Chien**. 2005. Molecular basis of the effects of shear stress on vascular endothelial cells. J.Biomech. **38**:1949-1971.

287. **Liao, J. J. and R. C. Andrews**. 2007. Targeting protein multiple conformations: a structure-based strategy for kinase drug design. Curr.Top.Med.Chem. **7**:1394-1407.

288. **Lietha, D., X. Cai, D. F. Ceccarelli, Y. Li, M. D. Schaller, and M. J. Eck**. 2007. Structural basis for the autoinhibition of focal adhesion kinase. Cell **129**:1177-1187.

289. **Lietha, D., X. Cai, D. F. Ceccarelli, Y. Li, M. D. Schaller, and M. J. Eck**. 2007. Structural basis for the autoinhibition of focal adhesion kinase. Cell **129**:1177-1187.

290. **Lietha, D., X. Cai, D. F. Ceccarelli, Y. Li, M. D. Schaller, and M. J. Eck**. 2007. Structural basis for the autoinhibition of focal adhesion kinase. Cell **129**:1177-1187.

291. **Lietha, D., X. Cai, D. F. Ceccarelli, Y. Li, M. D. Schaller, and M. J. Eck**. 2007. Structural basis for the autoinhibition of focal adhesion kinase. Cell **129**:1177-1187.

292. **Lietha, D., X. Cai, D. F. Ceccarelli, Y. Li, M. D. Schaller, and M. J. Eck**. 2007. Structural basis for the autoinhibition of focal adhesion kinase. Cell **129**:1177-1187.

293. **Lietha, D., X. Cai, D. F. Ceccarelli, Y. Li, M. D. Schaller, and M. J. Eck**. 2007. Structural basis for the autoinhibition of focal adhesion kinase. Cell **129**:1177-1187.

294. **Lietha, D., X. Cai, D. F. Ceccarelli, Y. Li, M. D. Schaller, and M. J. Eck**. 2007. Structural basis for the autoinhibition of focal adhesion kinase. Cell **129**:1177-1187.

295. **Lietha, D., X. Cai, D. F. Ceccarelli, Y. Li, M. D. Schaller, and M. J. Eck**. 2007. Structural basis for the autoinhibition of focal adhesion kinase. Cell **129**:1177-1187.

296. **Lietha, D., X. Cai, D. F. Ceccarelli, Y. Li, M. D. Schaller, and M. J. Eck**. 2007. Structural basis for the autoinhibition of focal adhesion kinase. Cell **129**:1177-1187.

297. **Lim, Y., H. Park, J. Jeon, I. Han, J. Kim, E. H. Jho, and E. S. Oh**. 2007. Focal adhesion kinase is negatively regulated by phosphorylation at tyrosine 407. J.Biol.Chem. **282**:10398-10404.

298. **Lim, Y., H. Park, J. Jeon, I. Han, J. Kim, E. H. Jho, and E. S. Oh**. 2007. Focal adhesion kinase is negatively regulated by phosphorylation at tyrosine 407. J.Biol.Chem. **282**:10398-10404.

299. **Ling, K., R. L. Doughman, A. J. Firestone, M. W. Bunce, and R. A. Anderson**. 2002. Type I gamma phosphatidylinositol phosphate kinase targets and regulates focal adhesions. Nature **420**:89-93.

300. **Ling, K., R. L. Doughman, A. J. Firestone, M. W. Bunce, and R. A. Anderson**. 2002. Type I gamma phosphatidylinositol phosphate kinase targets and regulates focal adhesions. Nature **420**:89-93.

301. **Ling, K., N. J. Schill, M. P. Wagoner, Y. Sun, and R. A. Anderson**. 2006. Movin' on up: the role of PtdIns(4,5)P(2) in cell migration. Trends Cell Biol. **16**:276-284.

302. **Linseman, D. A., S. D. Sorensen, and S. K. Fisher**. 1999. Attenuation of focal adhesion kinase signaling following depletion of agonist-sensitive pools of phosphatidylinositol 4,5-bisphosphate. J.Neurochem. **73**:1933-1944.

303. **Linseman, D. A., S. D. Sorensen, and S. K. Fisher**. 1999. Attenuation of focal adhesion kinase signaling following depletion of agonist-sensitive pools of phosphatidylinositol 4,5-bisphosphate. J.Neurochem. **73**:1933-1944.

304. **Lippincott-Schwartz, J., E. Snapp, and A. Kenworthy**. 2001. Studying protein dynamics in living cells. Nat.Rev.Mol.Cell Biol. **2**:444-456.

305. **Litvak, V., D. Tian, Y. D. Shaul, and S. Lev**. 2000. Targeting of PYK2 to focal adhesions as a cellular mechanism for convergence between integrins and G protein-coupled receptor signaling cascades. J.Biol.Chem. **275**:32736-32746.

306. **Liu, C., Y. Li, M. Semenov, C. Han, G. H. Baeg, Y. Tan, Z. Zhang, X. Lin, and X. He**. 2002. Control of beta-catenin phosphorylation/degradation by a dual-kinase mechanism. Cell **108**:837-847.

307. **Liu, F., M. A. Sells, and J. Chernoff**. 1998. Protein tyrosine phosphatase 1B negatively regulates integrin signaling. Curr.Biol. **8**:173-176.

308. **Liu, G., H. Beggs, C. Jurgensen, H. T. Park, H. Tang, J. Gorski, K. R. Jones, L. F. Reichardt, J. Wu, and Y. Rao**. 2004. Netrin requires focal adhesion kinase and Src family kinases for axon outgrowth and attraction. Nat.Neurosci. **7**:1222-1232.

309. **Liu, G., H. Beggs, C. Jurgensen, H. T. Park, H. Tang, J. Gorski, K. R. Jones, L. F. Reichardt, J. Wu, and Y. Rao**. 2004. Netrin requires focal adhesion kinase and Src family kinases for axon outgrowth and attraction. Nat.Neurosci. **7**:1222-1232.

310. **Liu, G., H. Beggs, C. Jurgensen, H. T. Park, H. Tang, J. Gorski, K. R. Jones, L. F. Reichardt, J. Wu, and Y. Rao**. 2004. Netrin requires focal adhesion kinase and Src family kinases for axon outgrowth and attraction. Nat.Neurosci. **7**:1222-1232.

311. **Liu, Y., J. C. Loijens, K. H. Martin, A. V. Karginov, and J. T. Parsons**. 2002. The association of ASAP1, an ADP ribosylation factor-GTPase activating protein, with focal adhesion kinase contributes to the process of focal adhesion assembly. Mol.Biol.Cell **13**:2147-2156.

312. **Lorenz, M., H. Yamaguchi, Y. Wang, R. H. Singer, and J. Condeelis**. 2004. Imaging sites of N-wasp activity in lamellipodia and invadopodia of carcinoma cells. Curr.Biol. **14**:697-703.

313. **Luster, A. D., R. Alon, and U. H. von Andrian**. 2005. Immune cell migration in inflammation: present and future therapeutic targets. Nat Immunol **6**:1182-1190.

314. **Lymn, J. S., S. J. Rao, G. F. Clunn, K. L. Gallagher, C. O'Neil, N. T. Thompson, and A. D. Hughes**. 1999. Phosphatidylinositol 3-kinase and focal adhesion kinase are early signals in the growth factor-like responses to thrombospondin-1 seen in human vascular smooth muscle. Arterioscler.Thromb.Vasc.Biol. **19**:2133-2140.

315. **Lymn, J. S., S. J. Rao, G. F. Clunn, K. L. Gallagher, C. O'Neil, N. T. Thompson, and A. D. Hughes**. 1999. Phosphatidylinositol 3-kinase and focal adhesion kinase are early signals in the growth factor-like responses to thrombospondin-1 seen in human vascular smooth muscle. Arterioscler.Thromb.Vasc.Biol. **19**:2133-2140.

316. **Lyons, P. D., J. M. Dunty, E. M. Schaefer, and M. D. Schaller**. 2001. Inhibition of the Catalytic Activity of Cell Adhesion Kinase beta by Protein-tyrosine Phosphatase-PEST-mediated Dephosphorylation. J.Biol.Chem. **276**:24422-24431.

317. **Lyons, P. D., J. M. Dunty, E. M. Schaefer, and M. D. Schaller**. 2001. Inhibition of the Catalytic Activity of Cell Adhesion Kinase beta by Protein-tyrosine Phosphatase-PEST-mediated Dephosphorylation. J.Biol.Chem. **276**:24422-24431.

318. **Manes, S., E. Mira, C. Gomez-Mouton, Z. J. Zhao, R. A. Lacalle, and A. Martinez**. 1999. Concerted activity of tyrosine phosphatase SHP-2 and focal adhesion kinase in regulation of cell motility. Mol.Cell Biol. **19**:3125-3135.

319. **Mansour, H., P. P. de Tombe, A. M. Samarel, and B. Russell**. 2004. Restoration of resting sarcomere length after uniaxial static strain is regulated by protein kinase Cepsilon and focal adhesion kinase. Circ.Res. **94**:642-649.

320. **Masson-Gadais, B., F. Houle, J. Laferriere, and J. Huot**. 2003. Integrin alphavbeta3, requirement for VEGFR2-mediated activation of SAPK2/p38 and for Hsp90-dependent phosphorylation of focal adhesion kinase in endothelial cells activated by VEGF. Cell Stress.Chaperones. **8**:37-52.

321. **May, R. C., E. Caron, A. Hall, and L. M. Machesky**. 2000. Involvement of the Arp2/3 complex in phagocytosis mediated by FcgammaR or CR3. Nat.Cell Biol. **2**:246-248.

322. **McLean, G. W., N. H. Komiyama, B. Serrels, H. Asano, L. Reynolds, F. Conti, K. Hodivala-Dilke, D. Metzger, P. Chambon, S. G. Grant, and M. C. Frame**. 2004. Specific deletion of focal adhesion kinase suppresses tumor formation and blocks malignant progression. Genes Dev. **18**:2998-3003.

323. **Mempel, T. R., M. L. Scimone, J. R. Mora, and U. H. von Andrian**. 2004. In vivo imaging of leukocyte trafficking in blood vessels and tissues. Curr.Opin.Immunol **16**:406-417.

324. **Miki, H., K. Miura, and T. Takenawa**. 1996. N-WASP, a novel actin-depolymerizing protein, regulates the cortical cytoskeletal rearrangement in a PIP2-dependent manner downstream of tyrosine kinases. EMBO J. **15**:5326-5335.

325. **Mitra, S. K., D. A. Hanson, and D. D. Schlaepfer**. 2005. Focal adhesion kinase: in command and control of cell motility. Nat.Rev.Mol.Cell Biol. **6**:56-68.

326. **Mitra, S. K., S. T. Lim, A. Chi, and D. D. Schlaepfer**. 2006. Intrinsic focal adhesion kinase activity controls orthotopic breast carcinoma metastasis via the regulation of urokinase plasminogen activator expression in a syngeneic tumor model. Oncogene **25**:4429-4440.

327. **Mitra, S. K., D. Mikolon, J. E. Molina, D. A. Hsia, D. A. Hanson, A. Chi, S. T. Lim, J. A. Bernard-Trifilo, D. Ilic, D. G. Stupack, D. A. Cheresh, and D. D. Schlaepfer**. 2006. Intrinsic FAK activity and Y925 phosphorylation facilitate an angiogenic switch in tumors. Oncogene **25**:5969-5984.

328. **Mitra, S. K., D. Mikolon, J. E. Molina, D. A. Hsia, D. A. Hanson, A. Chi, S. T. Lim, J. A. Bernard-Trifilo, D. Ilic, D. G. Stupack, D. A. Cheresh, and D. D. Schlaepfer**. 2006. Intrinsic FAK activity and Y925 phosphorylation facilitate an angiogenic switch in tumors. Oncogene **25**:5969-5984.

329. **Miyawaki, A.** 2003. Visualization of the spatial and temporal dynamics of intracellular signaling. Dev.Cell **4**:295-305.

330. **Moeller, M. L., Y. Shi, L. F. Reichardt, and I. M. Ethell**. 2006. EphB receptors regulate dendritic spine morphogenesis through the recruitment/phosphorylation of focal adhesion kinase and RhoA activation. J.Biol.Chem. **281**:1587-1598.

331. **Monick, M. M., A. B. Carter, P. K. Robeff, D. M. Flaherty, M. W. Peterson, and G. W. Hunninghake**. 2001. Lipopolysaccharide activates Akt in human alveolar macrophages resulting in nuclear accumulation and transcriptional activity of beta-catenin. J.Immunol. **166**:4713-4720.

332. **Nadruz, W., Jr., M. A. Corat, T. M. Marin, G. A. Guimaraes Pereira, and K. G. Franchini**. 2005. Focal adhesion kinase mediates MEF2 and c-Jun activation by stretch: role in the activation of the cardiac hypertrophic genetic program. Cardiovasc.Res. **68**:87-97.

333. **Nagy, T., H. Wei, T. L. Shen, X. Peng, C. C. Liang, B. Gan, and J. L. Guan**. 2007. Mammary Epithelial-specific Deletion of the Focal Adhesion Kinase Gene Leads to Severe Lobulo-Alveolar Hypoplasia and Secretory Immaturity of the Murine Mammary Gland. J.Biol.Chem. **282**:31766-31776.

334. **Nakayama, G. R., P. Bingham, D. Tan, and K. A. Maegley**. 2006. A fluorescence polarization assay for screening inhibitors against the ribonuclease H activity of HIV-1 reverse transcriptase. Anal.Biochem. **351**:260-265.

335. **Nayal, A., D. J. Webb, C. M. Brown, E. M. Schaefer, M. Vicente-Manzanares, and A. R. Horwitz**. 2006. Paxillin phosphorylation at Ser273 localizes a GIT1-PIX-PAK complex and regulates adhesion and protrusion dynamics. J.Cell Biol. **173**:587-589.

336. **Okazaki, H., J. Zhang, M. M. Hamawy, and R. P. Siraganian**. 1997. Activation of protein-tyrosine kinase Pyk2 is downstream of Syk in FcepsilonRI signaling. J.Biol.Chem. **272**:32443-32447.

337. **Okigaki, M., C. Davis, M. Falasca, S. Harroch, D. P. Felsenfeld, M. P. Sheetz, and J. Schlessinger**. 2003. Pyk2 regulates multiple signaling events crucial for macrophage morphology and migration. Proc.Natl.Acad.Sci.U.S.A **100**:10740-10745.

338. **Okigaki, M., C. Davis, M. Falasca, S. Harroch, D. P. Felsenfeld, M. P. Sheetz, and J. Schlessinger**. 2003. Pyk2 regulates multiple signaling events crucial for macrophage morphology and migration. Proc.Natl.Acad.Sci.U.S.A **100**:10740-10745.

339. **Okigaki, M., C. Davis, M. Falasca, S. Harroch, D. P. Felsenfeld, M. P. Sheetz, and J. Schlessinger**. 2003. Pyk2 regulates multiple signaling events crucial for macrophage morphology and migration. Proc.Natl.Acad.Sci.U.S.A **100**:10740-10745.

340. **Okigaki, M., C. Davis, M. Falasca, S. Harroch, D. P. Felsenfeld, M. P. Sheetz, and J. Schlessinger**. 2003. Pyk2 regulates multiple signaling events crucial for macrophage morphology and migration. Proc.Natl.Acad.Sci.U.S.A **100**:10740-10745.

341. **Okigaki, M., C. Davis, M. Falasca, S. Harroch, D. P. Felsenfeld, M. P. Sheetz, and J. Schlessinger**. 2003. Pyk2 regulates multiple signaling events crucial for macrophage morphology and migration. Proc.Natl.Acad.Sci.U.S.A **100**:10740-10745.

342. **Olazabal, I. M., E. Caron, R. C. May, K. Schilling, D. A. Knecht, and L. M. Machesky**. 2002. Rho-kinase and myosin-II control phagocytic cup formation during CR, but not FcgammaR, phagocytosis. Curr.Biol. **12**:1413-1418.

343. **Ostergaard, H. L. and T. L. Lysechko**. 2005. Focal adhesion kinase-related protein tyrosine kinase Pyk2 in T-cell activation and function. Immunol Res. **31**:267-282.

344. **Owen, J. D., P. J. Ruest, D. W. Fry, and S. K. Hanks**. 1999. Induced focal adhesion kinase (FAK) expression in FAK-null cells enhances cell spreading and migration requiring both. Mol.Cell Biol. **19**:4806-4818.

345. **Owen, J. D., P. J. Ruest, D. W. Fry, and S. K. Hanks**. 1999. Induced focal adhesion kinase (FAK) expression in FAK-null cells enhances cell spreading and migration requiring both. Mol.Cell Biol. **19**:4806-4818.

346. **Owen, J. D., P. J. Ruest, D. W. Fry, and S. K. Hanks**. 1999. Induced focal adhesion kinase (FAK) expression in FAK-null cells enhances cell spreading and migration requiring both. Mol.Cell Biol. **19**:4806-4818.

347. **Palazzo, A. F., C. H. Eng, D. D. Schlaepfer, E. E. Marcantonio, and G. G. Gundersen**. 2004. Localized stabilization of microtubules by integrin- and FAK-facilitated Rho signaling. Science **303**:836-839.

348. **Palmer, R. H., L. I. Fessler, P. T. Edeen, S. J. Madigan, M. McKeown, and T. Hunter**. 1999. DFak56 is a novel Drosophila melanogaster focal adhesion kinase. J.Biol.Chem. **274**:35621-35629.

349. **Parsons, J. T.** 2003. Focal adhesion kinase: the first ten years. J.Cell Sci. **116**:1409-1416.

350. **Patel, S., B. Doble, and J. R. Woodgett**. 2004. Glycogen synthase kinase-3 in insulin and Wnt signalling: a double-edged sword? Biochem.Soc.Trans. **32**:803-808.

351. **Patel, S., B. Doble, and J. R. Woodgett**. 2004. Glycogen synthase kinase-3 in insulin and Wnt signalling: a double-edged sword? Biochem.Soc.Trans. **32**:803-808.

352. **Paul, R., Z. G. Zhang, B. P. Eliceiri, Q. Jiang, A. D. Boccia, R. L. Zhang, M. Chopp, and D. A. Cheresh**. 2001. Src deficiency or blockade of Src activity in mice provides cerebral protection following stroke. Nat.Med. **7**:222-227.

353. **Peng, X., M. S. Kraus, H. Wei, T. L. Shen, R. Pariaut, A. Alcaraz, G. Ji, L. Cheng, Q. Yang, M. I. Kotlikoff, J. Chen, K. Chien, H. Gu, and J. L. Guan**. 2006. Inactivation of focal adhesion kinase in cardiomyocytes promotes eccentric cardiac hypertrophy and fibrosis in mice. J.Clin.Invest **116**:217-227.

354. **Peng, X., M. S. Kraus, H. Wei, T. L. Shen, R. Pariaut, A. Alcaraz, G. Ji, L. Cheng, Q. Yang, M. I. Kotlikoff, J. Chen, K. Chien, H. Gu, and J. L. Guan**. 2006. Inactivation of focal adhesion kinase in cardiomyocytes promotes eccentric cardiac hypertrophy and fibrosis in mice. J.Clin.Invest **116**:217-227.

355. **Peng, X., M. S. Kraus, H. Wei, T. L. Shen, R. Pariaut, A. Alcaraz, G. Ji, L. Cheng, Q. Yang, M. I. Kotlikoff, J. Chen, K. Chien, H. Gu, and J. L. Guan**. 2006. Inactivation of focal adhesion kinase in cardiomyocytes promotes eccentric cardiac hypertrophy and fibrosis in mice. J.Clin.Invest **116**:217-227.

356. **Peng, X., H. Ueda, H. Zhou, T. Stokol, T. L. Shen, A. Alcaraz, T. Nagy, J. D. Vassalli, and J. L. Guan**. 2004. Overexpression of focal adhesion kinase in vascular endothelial cells promotes angiogenesis in transgenic mice. Cardiovasc.Res. **64**:421-430.

357. **Pertz, O. and K. M. Hahn**. 2004. Designing biosensors for Rho family proteins--deciphering the dynamics of Rho family GTPase activation in living cells. J.Cell Sci. **117**:1313-1318.

358. **Pertz, O., L. Hodgson, R. L. Klemke, and K. M. Hahn**. 2006. Spatiotemporal dynamics of RhoA activity in migrating cells. Nature **440**:1069-1072.

359. **Pertz, O., L. Hodgson, R. L. Klemke, and K. M. Hahn**. 2006. Spatiotemporal dynamics of RhoA activity in migrating cells. Nature **440**:1069-1072.

360. **Petit, V., B. Boyer, D. Lentz, C. E. Turner, J. P. Thiery, and A. M. Valles**. 2000. Phosphorylation of tyrosine residues 31 and 118 on paxillin regulates cell migration through an association with CRK in NBT-II cells. J.Cell Biol. **148**:957-970.

361. **Petit, V., B. Boyer, D. Lentz, C. E. Turner, J. P. Thiery, and A. M. Valles**. 2000. Phosphorylation of tyrosine residues 31 and 118 on paxillin regulates cell migration through an association with CRK in NBT-II cells. J.Cell Biol. **148**:957-970.

362. **Petit, V., B. Boyer, D. Lentz, C. E. Turner, J. P. Thiery, and A. M. Valles**. 2000. Phosphorylation of tyrosine residues 31 and 118 on paxillin regulates cell migration through an association with CRK in NBT-II cells. J.Cell Biol. **148**:957-970.

363. **Piston, D. W. and G. J. Kremers**. 2007. Fluorescent protein FRET: the good, the bad and the ugly. Trends Biochem.Sci. **32**:407-414.

364. **Piston, D. W. and G. J. Kremers**. 2007. Fluorescent protein FRET: the good, the bad and the ugly. Trends Biochem.Sci. **32**:407-414.

365. **Polte, T. R. and S. K. Hanks**. 1995. Interaction between focal adhesion kinase and Crk-associated tyrosine kinase substrate p130Cas. Proc.Natl.Acad.Sci.U.S.A **92**:10678-10682.

366. **Qi, J. H. and L. Claesson-Welsh**. 2001. VEGF-induced activation of phosphoinositide 3-kinase is dependent on focal adhesion kinase. Exp.Cell Res. **263**:173-182.

367. **Qui, M. S. and S. H. Green**. 1992. PC12 cell neuronal differentiation is associated with prolonged p21ras activity and consequent prolonged ERK activity. Neuron **9**:705-717.

368. **Qui, M. S. and S. H. Green**. 1992. PC12 cell neuronal differentiation is associated with prolonged p21ras activity and consequent prolonged ERK activity. Neuron **9**:705-717.

369. **Reiske, H. R., S. C. Kao, L. A. Cary, J. L. Guan, J. F. Lai, and H. C. Chen**. 1999. Requirement of phosphatidylinositol 3-kinase in focal adhesion kinase- promoted cell migration. J.Biol.Chem. **274**:12361-12366.

370. **Reiske, H. R., S. C. Kao, L. A. Cary, J. L. Guan, J. F. Lai, and H. C. Chen**. 1999. Requirement of phosphatidylinositol 3-kinase in focal adhesion kinase- promoted cell migration. J.Biol.Chem. **274**:12361-12366.

371. **Ren, X. R., G. L. Ming, Y. Xie, Y. Hong, D. M. Sun, Z. Q. Zhao, Z. Feng, Q. Wang, S. Shim, Z. F. Chen, H. J. Song, L. Mei, and W. C. Xiong**. 2004. Focal adhesion kinase in netrin-1 signaling. Nat.Neurosci. **7**:1204-1212.

372. **Ren, X. R., G. L. Ming, Y. Xie, Y. Hong, D. M. Sun, Z. Q. Zhao, Z. Feng, Q. Wang, S. Shim, Z. F. Chen, H. J. Song, L. Mei, and W. C. Xiong**. 2004. Focal adhesion kinase in netrin-1 signaling. Nat.Neurosci. **7**:1204-1212.

373. **Ren, X. R., G. L. Ming, Y. Xie, Y. Hong, D. M. Sun, Z. Q. Zhao, Z. Feng, Q. Wang, S. Shim, Z. F. Chen, H. J. Song, L. Mei, and W. C. Xiong**. 2004. Focal adhesion kinase in netrin-1 signaling. Nat.Neurosci. **7**:1204-1212.

374. **Richardson, A. and T. Parsons**. 1996. A mechanism for regulation of the adhesion-associated proteintyrosine kinase pp125FAK [published erratum appears in Nature 1996 Jun 27;381(6585):810]. Nature **380**:538-540.

375. **Rico, B., H. E. Beggs, D. Schahin-Reed, N. Kimes, A. Schmidt, and L. F. Reichardt**. 2004. Control of axonal branching and synapse formation by focal adhesion kinase. Nat.Neurosci. **7**:1059-1069.

376. **Riven, I., S. Iwanir, and E. Reuveny**. 2006. GIRK channel activation involves a local rearrangement of a preformed G protein channel complex. Neuron **51**:561-573.

377. **Rose, D. M., S. Liu, D. G. Woodside, J. Han, D. D. Schlaepfer, and M. H. Ginsberg**. 2003. Paxillin binding to the alpha 4 integrin subunit stimulates LFA-1 (integrin alpha L beta 2)-dependent T cell migration by augmenting the activation of focal adhesion kinase/proline-rich tyrosine kinase-2. J Immunol **170**:5912-5918.

378. **Round, J. and E. Stein**. 2007. Netrin signaling leading to directed growth cone steering. Curr.Opin.Neurobiol. **17**:15-21.

379. **Ruest, P. J., S. Roy, E. Shi, R. L. Mernaugh, and S. K. Hanks**. 2000. Phosphospecific antibodies reveal focal adhesion kinase activation loop phosphorylation in nascent and mature focal adhesions and requirement for the autophosphorylation site. Cell Growth Differ. **11**:41-48.

380. **Ruest, P. J., S. Roy, E. Shi, R. L. Mernaugh, and S. K. Hanks**. 2000. Phosphospecific antibodies reveal focal adhesion kinase activation loop phosphorylation in nascent and mature focal adhesions and requirement for the autophosphorylation site. Cell Growth Differ. **11**:41-48.

381. **Sakai, T., S. Li, D. Docheva, C. Grashoff, K. Sakai, G. Kostka, A. Braun, A. Pfeifer, P. D. Yurchenco, and R. Fassler**. 2003. Integrin-linked kinase (ILK) is required for polarizing the epiblast, cell adhesion, and controlling actin accumulation. Genes Dev. **17**:926-940.

382. **Sakai, T., S. Li, D. Docheva, C. Grashoff, K. Sakai, G. Kostka, A. Braun, A. Pfeifer, P. D. Yurchenco, and R. Fassler**. 2003. Integrin-linked kinase (ILK) is required for polarizing the epiblast, cell adhesion, and controlling actin accumulation. Genes Dev. **17**:926-940.

383. **Sawada, Y., M. Tamada, B. J. Dubin-Thaler, O. Cherniavskaya, R. Sakai, S. Tanaka, and M. P. Sheetz**. 2006. Force sensing by mechanical extension of the Src family kinase substrate p130Cas. Cell **127**:1015-1026.

384. **Sawada, Y., M. Tamada, B. J. Dubin-Thaler, O. Cherniavskaya, R. Sakai, S. Tanaka, and M. P. Sheetz**. 2006. Force sensing by mechanical extension of the Src family kinase substrate p130Cas. Cell **127**:1015-1026.

385. **Sawada, Y., M. Tamada, B. J. Dubin-Thaler, O. Cherniavskaya, R. Sakai, S. Tanaka, and M. P. Sheetz**. 2006. Force sensing by mechanical extension of the Src family kinase substrate p130Cas. Cell **127**:1015-1026.

386. **Schaller, M. D.** 2001. Biochemical signals and biological responses elicited by the focal adhesion kinase. Biochim.Biophys.Acta **1540**:1-21.

387. **Schaller, M. D.** 2001. Biochemical signals and biological responses elicited by the focal adhesion kinase. Biochim.Biophys.Acta **1540**:1-21.

388. **Schaller, M. D.** 2001. Paxillin: a focal adhesion-associated adaptor protein. Oncogene **20**:6459-6472.

389. **Schaller, M. D., C. A. Borgman, B. S. Cobb, R. R. Vines, A. B. Reynolds, and J. T. Parsons**. 1992. pp125^{FAK} a structurally distinctive protein-tyrosine kinase associated with focal adhesions. Proc.Natl.Acad.Sci.U.S.A **89**:5192-5196.

390. **Schaller, M. D., J. D. Hildebrand, J. D. Shannon, J. W. Fox, R. R. Vines, and J. T. Parsons**. 1994. Autophosphorylation of the focal adhesion kinase, pp125FAK, directs SH2- dependent binding of pp60src. Mol.Cell Biol. **14**:1680-1688.

391. **Schaller, M. D., J. D. Hildebrand, J. D. Shannon, J. W. Fox, R. R. Vines, and J. T. Parsons**. 1994. Autophosphorylation of the focal adhesion kinase, pp125FAK, directs SH2- dependent binding of pp60src. Mol.Cell Biol. **14**:1680-1688.

392. **Schaller, M. D., J. D. Hildebrand, J. D. Shannon, J. W. Fox, R. R. Vines, and J. T. Parsons**. 1994. Autophosphorylation of the focal adhesion kinase, pp125FAK, directs SH2- dependent binding of pp60src. Mol.Cell Biol. **14**:1680-1688.

393. **Schaller, M. D., J. D. Hildebrand, J. D. Shannon, J. W. Fox, R. R. Vines, and J. T. Parsons**. 1994. Autophosphorylation of the focal adhesion kinase, pp125FAK, directs SH2- dependent binding of pp60src. Mol.Cell Biol. **14**:1680-1688.

394. **Schaller, M. D., C. A. Otey, J. D. Hildebrand, and J. T. Parsons**. 1995. Focal adhesion kinase and paxillin bind to peptides mimicking beta integrin cytoplasmic domains. J.Cell Biol. **130**:1181-1187.

395. **Schaller, M. D. and J. T. Parsons**. 1995. pp125FAK-dependent tyrosine phosphorylation of paxillin creates a high- affinity binding site for Crk. Mol.Cell Biol. **15**:2635-2645.

396. **Schaller, M. D. and J. T. Parsons**. 1995. pp125FAK-dependent tyrosine phosphorylation of paxillin creates a high- affinity binding site for Crk. Mol.Cell Biol. **15**:2635-2645.

397. **Schaller, M. D. and J. T. Parsons**. 1995. pp125FAK-dependent tyrosine phosphorylation of paxillin creates a high- affinity binding site for Crk. Mol.Cell Biol. **15**:2635-2645.

398. **Schaller, M. D. and T. Sasaki**. 1997. Differential signaling by the focal adhesion kinase and cell adhesion kinase beta. J.Biol.Chem. **272**:25319-25325.

399. **Schaller, M. D. and T. Sasaki**. 1997. Differential signaling by the focal adhesion kinase and cell adhesion kinase beta. J.Biol.Chem. **272**:25319-25325.

400. **Schlaepfer, D. D., M. A. Broome, and T. Hunter**. 1997. Fibronectin-stimulated signaling from a focal adhesion kinase-c-Src complex: involvement of the Grb2, p130cas, and Nck adaptor proteins. Mol.Cell Biol. **17**:1702-1713.

401. **Schlaepfer, D. D., S. K. Hanks, T. Hunter, and G. P. van der**. 1994. Integrin-mediated signal transduction linked to Ras pathway by GRB2 binding to focal adhesion kinase. Nature **372**:786-791.

402. **Schlaepfer, D. D., S. K. Hanks, T. Hunter, and G. P. van der**. 1994. Integrin-mediated signal transduction linked to Ras pathway by GRB2 binding to focal adhesion kinase. Nature **372**:786-791.

403. **Schlaepfer, D. D., S. K. Hanks, T. Hunter, and G. P. van der**. 1994. Integrin-mediated signal transduction linked to Ras pathway by GRB2 binding to focal adhesion kinase. Nature **372**:786-791.

404. **Schlaepfer, D. D., S. Hou, S. T. Lim, A. Tomar, H. Yu, Y. Lim, D. A. Hanson, S. A. Uryu, J. Molina, and S. K. Mitra**. 2007. Tumor necrosis factor-alpha stimulates focal adhesion kinase activity required for mitogen-activated kinase-associated interleukin 6 expression. J Biol.Chem. **282**:17450-17459.

405. **Schlaepfer, D. D. and T. Hunter**. 1996. Evidence for in vivo phosphorylation of the Grb2 SH2-domain binding site on focal adhesion kinase by Src-family protein-tyrosine kinases [published erratum appears in Mol Cell Biol 1996 Dec;16(12):7182-4]. Mol.Cell Biol. **16**:5623-5633.

406. **Schlaepfer, D. D. and T. Hunter**. 1996. Evidence for in vivo phosphorylation of the Grb2 SH2-domain binding site on focal adhesion kinase by Src-family protein-tyrosine kinases [published erratum appears in Mol Cell Biol 1996 Dec;16(12):7182-4]. Mol.Cell Biol. **16**:5623-5633.

407. **Schlaepfer, D. D., K. C. Jones, and T. Hunter**. 1998. Multiple Grb2-mediated integrin-stimulated signaling pathways to ERK2/mitogen-activated protein kinase: summation of both c-Src- and focal adhesion kinase-initiated tyrosine phosphorylation events. Mol.Cell Biol. **18**:2571-2585.

408. **Schober, M., S. Raghavan, M. Nikolova, L. Polak, H. A. Pasolli, H. E. Beggs, L. F. Reichardt, and E. Fuchs**. 2007. Focal adhesion kinase modulates tension signaling to control actin and focal adhesion dynamics. J.Cell Biol. **176**:667-680.

409. **Serrels, B., A. Serrels, V. G. Brunton, M. Holt, G. W. McLean, C. H. Gray, G. E. Jones, and M. C. Frame**. 2007. Focal adhesion kinase controls actin assembly via a FERM-mediated interaction with the Arp2/3 complex. Nat.Cell Biol. **9**:1046-1056.

410. **Serrels, B., A. Serrels, V. G. Brunton, M. Holt, G. W. McLean, C. H. Gray, G. E. Jones, and M. C. Frame**. 2007. Focal adhesion kinase controls actin assembly via a FERM-mediated interaction with the Arp2/3 complex. Nat.Cell Biol. **9**:1046-1056.

411. **Shen, F., L. Hodgson, and K. Hahn**. 2006. Digital autofocus methods for automated microscopy. Methods Enzymol. **414**:620-632.

412. **Shen, T. L., A. Y. Park, A. Alcaraz, X. Peng, I. Jang, P. Koni, R. A. Flavell, H. Gu, and J. L. Guan**. 2005. Conditional knockout of focal adhesion kinase in endothelial cells reveals its role in angiogenesis and vascular development in late embryogenesis. J.Cell Biol. **169**:941-952.

413. **Shen, T. L., A. Y. Park, A. Alcaraz, X. Peng, I. Jang, P. Koni, R. A. Flavell, H. Gu, and J. L. Guan**. 2005. Conditional knockout of focal adhesion kinase in endothelial cells reveals its role in angiogenesis and vascular development in late embryogenesis. J.Cell Biol. **169**:941-952.

414. **Shen, T. L., A. Y. Park, A. Alcaraz, X. Peng, I. Jang, P. Koni, R. A. Flavell, H. Gu, and J. L. Guan**. 2005. Conditional knockout of focal adhesion kinase in endothelial cells reveals its role in angiogenesis and vascular development in late embryogenesis. J.Cell Biol. **169**:941-952.

415. **Shen, T. L., A. Y. Park, A. Alcaraz, X. Peng, I. Jang, P. Koni, R. A. Flavell, H. Gu, and J. L. Guan**. 2005. Conditional knockout of focal adhesion kinase in endothelial cells reveals its role in angiogenesis and vascular development in late embryogenesis. J.Cell Biol. **169**:941-952.

416. **Shen, T. L., A. Y. Park, A. Alcaraz, X. Peng, I. Jang, P. Koni, R. A. Flavell, H. Gu, and J. L. Guan**. 2005. Conditional knockout of focal adhesion kinase in endothelial cells reveals its role in angiogenesis and vascular development in late embryogenesis. J.Cell Biol. **169**:941-952.

417. **Shen, T. L., A. Y. Park, A. Alcaraz, X. Peng, I. Jang, P. Koni, R. A. Flavell, H. Gu, and J. L. Guan**. 2005. Conditional knockout of focal adhesion kinase in endothelial cells reveals its role in angiogenesis and vascular development in late embryogenesis. J.Cell Biol. **169**:941-952.

418. **Shen, Y., G. Schneider, J. F. Cloutier, A. Veillette, and M. D. Schaller**. 1998. Direct association of protein-tyrosine phosphatase PTP-PEST with paxillin. J.Biol.Chem. **273**:6474-6481.

419. **Sheta, E. A., M. A. Harding, M. R. Conaway, and D. Theodorescu**. 2000. Focal adhesion kinase, Rap1, and transcriptional induction of vascular endothelial growth factor. J.Natl.Cancer Inst. **92**:1065-1073.

420. **Shi, L., R. Kishore, M. R. McMullen, and L. E. Nagy**. 2002. Lipopolysaccharide stimulation of ERK1/2 increases TNF-alpha production via Egr-1. Am.J.Physiol Cell Physiol **282**:C1205-C1211.

421. **Shibuya, M. and L. Claesson-Welsh**. 2006. Signal transduction by VEGF receptors in regulation of angiogenesis and lymphangiogenesis. Exp.Cell Res. **312**:549-560.

422. **Shikata, Y., A. Rios, K. Kawkitinarong, N. DePaola, J. G. Garcia, and K. G. Birukov**. 2005. Differential effects of shear stress and cyclic stretch on focal adhesion remodeling, site-specific FAK phosphorylation, and small GTPases in human lung endothelial cells. Exp.Cell Res. **304**:40-49.

423. **Shyy, J. Y. and S. Chien**. 2002. Role of integrins in endothelial mechanosensing of shear stress. Circ.Res. **91**:769-775.

424. **Sicheri, F., I. Moarefi, and J. Kuriyan**. 1997. Crystal structure of the Src family tyrosine kinase Hck. Nature **385**:602-609.

425. **Sieg, D. J., C. R. Hauck, D. Ilic, C. K. Klingbeil, E. Schaefer, C. H. Damsky, and D. D. Schlaepfer**. 2000. FAK integrates growth-factor and integrin signals to promote cell migration. Nat.Cell Biol. **2**:249-256.

426. **Sieg, D. J., C. R. Hauck, D. Ilic, C. K. Klingbeil, E. Schaefer, C. H. Damsky, and D. D. Schlaepfer**. 2000. FAK integrates growth-factor and integrin signals to promote cell migration. Nat.Cell Biol. **2**:249-256.

427. **Sieg, D. J., D. Ilic, K. C. Jones, C. H. Damsky, T. Hunter, and D. D. Schlaepfer**. 1998. Pyk2 and Src-family protein-tyrosine kinases compensate for the loss of FAK in fibronectin-stimulated signaling events but Pyk2 does not fully function to enhance FAK- cell migration. EMBO J. **17**:5933-5947.

428. **Slack-Davis, J. K., K. H. Martin, R. W. Tilghman, M. Iwanicki, E. J. Ung, C. Autry, M. J. Luzzio, B. Cooper, J. C. Kath, W. G. Roberts, and J. T. Parsons**. 2007. Cellular characterization of a novel focal adhesion kinase inhibitor. J.Biol.Chem. **282**:14845-14852.

429. **Sonoda, Y., Y. Matsumoto, M. Funakoshi, D. Yamamoto, S. K. Hanks, and T. Kasahara**. 2000. Anti-apoptotic role of focal adhesion kinase (FAK). Induction of inhibitor-of-apoptosis proteins and apoptosis suppression by the overexpression of FAK in a human leukemic cell line, HL-60. J.Biol.Chem. **275**:16309-16315.

430. **Stewart, A., C. Ham, and I. Zachary**. 2002. The focal adhesion kinase amino-terminal domain localises to nuclei and intercellular junctions in HEK 293 and MDCK cells independently of tyrosine 397 and the carboxy-terminal domain. Biochem.Biophys.Res.Commun. **299**:62-73.

431. **Swanson, J. A. and A. D. Hoppe**. 2004. The coordination of signaling during Fc receptor-mediated phagocytosis. J.Leukoc.Biol. **76**:1093-1103.

432. **Swanson, J. A. and A. D. Hoppe**. 2004. The coordination of signaling during Fc receptor-mediated phagocytosis. J.Leukoc.Biol. **76**:1093-1103.

433. **Swanson, J. A., M. T. Johnson, K. Beningo, P. Post, M. Mooseker, and N. Araki**. 1999. A contractile activity that closes phagosomes in macrophages. J.Cell Sci. **112 (Pt 3)**:307-316.

434. **Tachibana, K., T. Sato, N. D'Avirro, and C. Morimoto**. 1995. Direct association of $pp125^{FAK}$ with paxillin, the focal adhesion- targeting mechanism of pp125FAK. J.Exp.Med. **182**:1089-1099.

435. **Tamada, M., M. P. Sheetz, and Y. Sawada**. 2004. Activation of a signaling cascade by cytoskeleton stretch. Dev.Cell **7**:709-718.

436. **Tamura, M., J. Gu, K. Matsumoto, S. Aota, R. Parsons, and K. M. Yamada**. 1998. Inhibition of cell migration, spreading, and focal adhesions by tumor suppressor PTEN. Science **280**:1614-1617.

437. **Thomas, J. W., M. A. Cooley, J. M. Broome, R. Salgia, J. D. Griffin, C. R. Lombardo, and M. D. Schaller**. 1999. The role of focal adhesion kinase binding in the regulation of tyrosine phosphorylation of paxillin. J.Biol.Chem. **274**:36684-36692.

438. **Thomas, J. W., M. A. Cooley, J. M. Broome, R. Salgia, J. D. Griffin, C. R. Lombardo, and M. D. Schaller**. 1999. The role of focal adhesion kinase binding in the regulation of tyrosine phosphorylation of paxillin. J.Biol.Chem. **274**:36684-36692.

439. **Thomas, J. W., B. Ellis, R. J. Boerner, W. B. Knight, G. C. White, and M. D. Schaller**. 1998. SH2- and SH3-mediated interactions between focal adhesion kinase and Src. J.Biol.Chem. **273**:577-583.

440. **Thomas, J. W., B. Ellis, R. J. Boerner, W. B. Knight, G. C. White, and M. D. Schaller**. 1998. SH2- and SH3-mediated interactions between focal adhesion kinase and Src. J.Biol.Chem. **273**:577-583.

441. **Tilghman, R. W., J. K. Slack-Davis, N. Sergina, K. H. Martin, M. Iwanicki, E. D. Hershey, H. E. Beggs, L. F. Reichardt, and J. T. Parsons**. 2005. Focal adhesion kinase is required for the spatial organization of the leading edge in migrating cells. J.Cell Sci. **118**:2613-2623.

442. **Tilghman, R. W., J. K. Slack-Davis, N. Sergina, K. H. Martin, M. Iwanicki, E. D. Hershey, H. E. Beggs, L. F. Reichardt, and J. T. Parsons**. 2005. Focal adhesion kinase is required for the spatial organization of the leading edge in migrating cells. J.Cell Sci. **118**:2613-2623.

443. **Torsoni, A. S., T. M. Marin, L. A. Velloso, and K. G. Franchini**. 2005. RhoA/ROCK signaling is critical to FAK activation by cyclic stretch in cardiac myocytes. Am.J.Physiol Heart Circ.Physiol **289**:H1488-H1496.

444. **Totani, L., A. Piccoli, S. Manarini, L. Federico, R. Pecce, N. Martelli, C. Cerletti, P. Piccardoni, C. A. Lowell, S. S. Smyth, G. Berton, and V. Evangelista**. 2006. Src-family kinases mediate an outside-in signal necessary for beta2 integrins to achieve full activation and sustain firm adhesion of polymorphonuclear leucocytes tethered on E-selectin. Biochem.J **396**:89-98.

445. **Toutant, M., A. Costa, J. M. Studler, G. Kadare, M. Carnaud, and J. A. Girault**. 2002. Alternative Splicing Controls the Mechanisms of FAK Autophosphorylation. Mol.Cell Biol. **22**:7731-7743.

446. **Toutant, M., A. Costa, J. M. Studler, G. Kadare, M. Carnaud, and J. A. Girault**. 2002. Alternative Splicing Controls the Mechanisms of FAK Autophosphorylation. Mol.Cell Biol. **22**:7731-7743.

447. **Toutant, M., A. Costa, J. M. Studler, G. Kadare, M. Carnaud, and J. A. Girault**. 2002. Alternative Splicing Controls the Mechanisms of FAK Autophosphorylation. Mol.Cell Biol. **22**:7731-7743.

448. **Toutant, M., A. Costa, J. M. Studler, G. Kadare, M. Carnaud, and J. A. Girault**. 2002. Alternative Splicing Controls the Mechanisms of FAK Autophosphorylation. Mol.Cell Biol. **22**:7731-7743.

449. **Tsuchida, M., S. J. Knechtle, and M. M. Hamawy**. 1999. CD28 ligation induces tyrosine phosphorylation of Pyk2 but not Fak in Jurkat T cells. J.Biol.Chem. **274**:6735-6740.

450. **Tsutsumi, R., A. Takahashi, T. Azuma, H. Higashi, and M. Hatakeyama**. 2006. Focal adhesion kinase is a substrate and downstream effector of SHP-2 complexed with Helicobacter pylori CagA. Mol.Cell Biol. **26**:261-276.

451. **Turner, C. E. and J. T. Miller**. 1994. Primary sequence of paxillin contains putative SH2 and SH3 domain binding motifs and multiple LIM domains: identification of a vinculin and pp125Fak-binding region. J.Cell Sci. **107 (Pt 6)**:1583-1591.

452. **Ueda, H., S. Abbi, C. Zheng, and J. L. Guan**. 2000. Suppression of Pyk2 kinase and cellular activities by FIP200. J.Cell Biol. **149**:423-430.

453. **Underhill, D. M. and A. Ozinsky**. 2002. Phagocytosis of microbes: complexity in action. Annu.Rev.Immunol. **20**:825-852.

454. **van Buul, J. D., E. C. Anthony, M. Fernandez-Borja, K. Burridge, and P. L. Hordijk**. 2005. Proline-rich tyrosine kinase 2 (Pyk2) mediates vascular endothelial-cadherin-based cell-cell adhesion by regulating beta-catenin tyrosine phosphorylation. J.Biol.Chem. **280**:21129-21136.

455. **Van Etten, R. A., P. K. Jackson, D. Baltimore, M. C. Sanders, P. T. Matsudaira, and P. A. Janmey**. 1994. The COOH terminus of the c-Abl tyrosine kinase contains distinct F- and G-actin binding domains with bundling activity. J.Cell Biol. **124**:325-340.

456. **Velling, T., S. Nilsson, A. Stefansson, and S. Johansson**. 2004. beta1-Integrins induce phosphorylation of Akt on serine 473 independently of focal adhesion kinase and Src family kinases. EMBO Rep. **5**:901-905.

457. **Vuori, K., H. Hirai, S. Aizawa, and E. Ruoslahti**. 1996. Introduction of p130cas signaling complex formation upon integrin- mediated cell adhesion: a role for Src family kinases. Mol.Cell Biol. **16**:2606-2613.

458. **Wang, D., J. R. Grammer, C. S. Cobbs, J. E. Stewart, Jr., Z. Liu, R. Rhoden, T. P. Hecker, Q. Ding, and C. L. Gladson**. 2000. p125 focal adhesion kinase promotes malignant astrocytoma cell proliferation in vivo. J.Cell Sci. **113 Pt 23**:4221-4230.

459. **Wang, H. B., M. Dembo, S. K. Hanks, and Y. Wang**. 2001. Focal adhesion kinase is involved in mechanosensing during fibroblast migration. Proc.Natl.Acad.Sci.U.S.A **98**:11295-11300.

460. **Wang, H. B., M. Dembo, S. K. Hanks, and Y. Wang**. 2001. Focal adhesion kinase is involved in mechanosensing during fibroblast migration. Proc.Natl.Acad.Sci.U.S.A **98**:11295-11300.

461. **Wang, J. Y., F. Ledley, S. Goff, R. Lee, Y. Groner, and D. Baltimore**. 1984. The mouse c-abl locus: molecular cloning and characterization. Cell **36**:349-356.

462. **Wang, Y., E. L. Botvinick, Y. Zhao, M. W. Berns, S. Usami, R. Y. Tsien, and S. Chien**. 2005. Visualizing the mechanical activation of Src. Nature **434**:1040-1045.

463. **Wang, Y., E. L. Botvinick, Y. Zhao, M. W. Berns, S. Usami, R. Y. Tsien, and S. Chien**. 2005. Visualizing the mechanical activation of Src. Nature **434**:1040-1045.

464. **Ward, M. E., J. Y. Wu, and Y. Rao**. 2004. Visualization of spatially and temporally regulated N-WASP activity during cytoskeletal reorganization in living cells. Proc.Natl.Acad.Sci.U.S.A **101**:970-974.

465. **Webb, D. J., K. Donais, L. A. Whitmore, S. M. Thomas, C. E. Turner, J. T. Parsons, and A. F. Horwitz**. 2004. FAK-Src signalling through paxillin, ERK and MLCK regulates adhesion disassembly. Nat.Cell Biol. **6**:154-161.

466. **Webb, D. J., K. Donais, L. A. Whitmore, S. M. Thomas, C. E. Turner, J. T. Parsons, and A. F. Horwitz**. 2004. FAK-Src signalling through paxillin, ERK and MLCK regulates adhesion disassembly. Nat.Cell Biol. **6**:154-161.

467. **Webb, D. J., K. Donais, L. A. Whitmore, S. M. Thomas, C. E. Turner, J. T. Parsons, and A. F. Horwitz**. 2004. FAK-Src signalling through paxillin, ERK and MLCK regulates adhesion disassembly. Nat.Cell Biol. **6**:154-161.

468. **Webb, D. J., K. Donais, L. A. Whitmore, S. M. Thomas, C. E. Turner, J. T. Parsons, and A. F. Horwitz**. 2004. FAK-Src signalling through paxillin, ERK and MLCK regulates adhesion disassembly. Nat.Cell Biol. **6**:154-161.

469. **Webb, D. J., K. Donais, L. A. Whitmore, S. M. Thomas, C. E. Turner, J. T. Parsons, and A. F. Horwitz**. 2004. FAK-Src signalling through paxillin, ERK and MLCK regulates adhesion disassembly. Nat.Cell Biol. **6**:154-161.

470. **Werth, D. K., J. E. Niedel, and I. Pastan**. 1983. Vinculin, a cytoskeletal substrate of protein kinase C. J.Biol.Chem. **258**:11423-11426.

471. **West, A. P., A. A. Koblansky, and S. Ghosh**. 2006. Recognition and Signaling by Toll-Like Receptors. Annual Review of Cell and Developmental Biology **22**:409-437.

472. **Williams, L. M. and A. J. Ridley**. 2000. Lipopolysaccharide induces actin reorganization and tyrosine phosphorylation of Pyk2 and paxillin in monocytes and macrophages. J.Immunol. **164**:2028-2036.

473. **Williams, L. M. and A. J. Ridley**. 2000. Lipopolysaccharide induces actin reorganization and tyrosine phosphorylation of Pyk2 and paxillin in monocytes and macrophages. J.Immunol. **164**:2028-2036.

474. **Williams, L. M. and A. J. Ridley**. 2000. Lipopolysaccharide induces actin reorganization and tyrosine phosphorylation of Pyk2 and paxillin in monocytes and macrophages. J.Immunol. **164**:2028-2036.

475. **Woodring, P. J., T. Hunter, and J. Y. Wang**. 2003. Regulation of F-actin-dependent processes by the Abl family of tyrosine kinases. J.Cell Sci. **116**:2613-2626.

476. **Woodring, P. J., E. D. Litwack, D. D. O'Leary, G. R. Lucero, J. Y. Wang, and T. Hunter**. 2002. Modulation of the F-actin cytoskeleton by c-Abl tyrosine kinase in cell spreading and neurite extension. J.Cell Biol. **156**:879-892.

477. **Woodrow, M. A., D. Woods, H. M. Cherwinski, D. Stokoe, and M. McMahon**. 2003. Ras-induced serine phosphorylation of the focal adhesion protein paxillin is mediated by the Raf-->MEK-->ERK pathway. Exp.Cell Res. **287**:325-338.

478. **Woodrow, M. A., D. Woods, H. M. Cherwinski, D. Stokoe, and M. McMahon**. 2003. Ras-induced serine phosphorylation of the focal adhesion protein paxillin is mediated by the Raf-->MEK-->ERK pathway. Exp.Cell Res. **287**:325-338.

479. **Woodrow, M. A., D. Woods, H. M. Cherwinski, D. Stokoe, and M. McMahon**. 2003. Ras-induced serine phosphorylation of the focal adhesion protein paxillin is mediated by the Raf-->MEK-->ERK pathway. Exp.Cell Res. **287**:325-338.

480. **Woodrow, M. A., D. Woods, H. M. Cherwinski, D. Stokoe, and M. McMahon**. 2003. Ras-induced serine phosphorylation of the focal adhesion protein paxillin is mediated by the Raf-->MEK-->ERK pathway. Exp.Cell Res. **287**:325-338.

481. **Woodrow, M. A., D. Woods, H. M. Cherwinski, D. Stokoe, and M. McMahon**. 2003. Ras-induced serine phosphorylation of the focal adhesion protein paxillin is mediated by the Raf-->MEK-->ERK pathway. Exp.Cell Res. **287**:325-338.

482. **Woodrow, M. A., D. Woods, H. M. Cherwinski, D. Stokoe, and M. McMahon**. 2003. Ras-induced serine phosphorylation of the focal adhesion protein paxillin is mediated by the Raf-->MEK-->ERK pathway. Exp.Cell Res. **287**:325-338.

483. **Woodrow, M. A., D. Woods, H. M. Cherwinski, D. Stokoe, and M. McMahon**. 2003. Ras-induced serine phosphorylation of the focal adhesion protein paxillin is mediated by the Raf-->MEK-->ERK pathway. Exp.Cell Res. **287**:325-338.

484. **Wu, C.** 2004. The PINCH-ILK-parvin complexes: assembly, functions and regulation. Biochim.Biophys.Acta **1692**:55-62.

485. **Wu, X., B. Gan, Y. Yoo, and J. L. Guan**. 2005. FAK-mediated src phosphorylation of endophilin A2 inhibits endocytosis of MT1-MMP and promotes ECM degradation. Dev.Cell **9**:185-196.

486. **Wu, X., S. Suetsugu, L. A. Cooper, T. Takenawa, and J. L. Guan**. 2004. Focal adhesion kinase regulation of N-WASP subcellular localization and function. J.Biol.Chem. **279**:9565-9576.

487. **Xia, Z. and Y. Liu**. 2001. Reliable and global measurement of fluorescence resonance energy transfer using fluorescence microscopes. Biophys.J. **81**:2395-2402.

488. **Xie, Z., K. Sanada, B. A. Samuels, H. Shih, and L. H. Tsai**. 2003. Serine 732 phosphorylation of FAK by Cdk5 is important for microtubule organization, nuclear movement, and neuronal migration. Cell **114**:469-482.

489. **Xie, Z., K. Sanada, B. A. Samuels, H. Shih, and L. H. Tsai**. 2003. Serine 732 phosphorylation of FAK by Cdk5 is important for microtubule organization, nuclear movement, and neuronal migration. Cell **114**:469-482.

490. **Xie, Z., K. Sanada, B. A. Samuels, H. Shih, and L. H. Tsai**. 2003. Serine 732 phosphorylation of FAK by Cdk5 is important for microtubule organization, nuclear movement, and neuronal migration. Cell **114**:469-482.

491. **Xiong, W. and J. T. Parsons**. 1997. Induction of apoptosis after expression of PYK2, a tyrosine kinase structurally related to focal adhesion kinase. J.Cell Biol. **139**:529-539.

492. **Xu, W., S. C. Harrison, and M. J. Eck**. 1997. Three-dimensional structure of the tyrosine kinase c-Src. Nature **385**:595-602.

493. **Yamada, K., K. G. Green, A. M. Samarel, and J. E. Saffitz**. 2005. Distinct pathways regulate expression of cardiac electrical and mechanical junction proteins in response to stretch. Circ.Res. **97**:346-353.

494. **Yamauchi, J., Y. Miyamoto, A. Sanbe, and A. Tanoue**. 2006. JNK phosphorylation of paxillin, acting through the Rac1 and Cdc42 signaling cascade, mediates neurite extension in N1E-115 cells. Exp.Cell Res. **312**:2954-2961.

495. **Yan, S. R. and M. J. Novak**. 1999. Beta2 integrin-dependent phosphorylation of protein-tyrosine kinase Pyk2 stimulated by tumor necrosis factor alpha and fMLP in human neutrophils adherent to fibrinogen. FEBS Lett. **451**:33-38.

496. **Yano, H., Y. Mazaki, K. Kurokawa, S. K. Hanks, M. Matsuda, and H. Sabe**. 2004. Roles played by a subset of integrin-signaling molecules in cadherin based cell-cell adhesion. J.Cell Biol. **166**:283-295.

497. **Yano, H., H. Uchida, T. Iwasaki, M. Mukai, H. Akedo, K. Nakamura, S. Hashimoto, and H. Sabe**. 2000. Paxillin alpha and crk-associated substrate exert opposing effects on cell migration and contact inhibition of growth through tyrosine phosphorylation [In Process Citation]. Proc.Natl.Acad.Sci.U.S.A **97**:9076-9081.

498. **Yano, Y., J. Geibel, and B. E. Sumpio**. 1996. Tyrosine phosphorylation of pp125FAK and paxillin in aortic endothelial cells induced by mechanical strain. Am.J.Physiol **271**:C635-C649.

499. **Yoshimura, T., Y. Kawano, N. Arimura, S. Kawabata, A. Kikuchi, and K. Kaibuchi**. 2005. GSK-3beta regulates phosphorylation of CRMP-2 and neuronal polarity. Cell **120**:137-149.

500. **Yoshimura, T., Y. Kawano, N. Arimura, S. Kawabata, A. Kikuchi, and K. Kaibuchi**. 2005. GSK-3beta regulates phosphorylation of CRMP-2 and neuronal polarity. Cell **120**:137-149.

501. **Yu, D. H., C. K. Qu, O. Henegariu, X. Lu, and G. S. Feng**. 1998. Protein-tyrosine phosphatase Shp-2 regulates cell spreading, migration, and focal adhesion. J.Biol.Chem. **273**:21125-21131.

502. **Zeisel, M. B., V. A. Druet, J. Sibilia, J. P. Klein, V. Quesniaux, and D. Wachsmann**. 2005. Cross talk between MyD88 and focal adhesion kinase pathways. J.Immunol. **174**:7393-7397.

503. **Zeisel, M. B., V. A. Druet, J. Sibilia, J. P. Klein, V. Quesniaux, and D. Wachsmann**. 2005. Cross talk between MyD88 and focal adhesion kinase pathways. J.Immunol. **174**:7393-7397.

504. **Zeng, L., X. Si, W. P. Yu, H. T. Le, K. P. Ng, R. M. Teng, K. Ryan, D. Z. Wang, S. Ponniah, and C. J. Pallen**. 2003. PTP alpha regulates integrin-stimulated FAK autophosphorylation and cytoskeletal rearrangement in cell spreading and migration. J.Cell Biol. **160**:137-146.

505. **Zhai, J., H. Lin, Z. Nie, J. Wu, R. Canete-Soler, W. W. Schlaepfer, and D. D. Schlaepfer**. 2003. Direct interaction of focal adhesion kinase with p190RhoGEF. J.Biol.Chem. **278**:24865-24873.

506. **Zhang, X., C. V. Wright, and S. K. Hanks**. 1995. Cloning of a Xenopus laevis cDNA encoding focal adhesion kinase (FAK) and expression during early development. Gene **160**:219-222.

507. **Zhao, J. H., H. Reiske, and J. L. Guan**. 1998. Regulation of the cell cycle by focal adhesion kinase. J.Cell Biol. **143**:1997-2008.

508. **Zhou, F. Q. and W. D. Snider**. 2005. Cell biology. GSK-3beta and microtubule assembly in axons. Science **308**:211-214.

509. **Zhou, F. Q., J. Zhou, S. Dedhar, Y. H. Wu, and W. D. Snider**. 2004. NGF-induced axon growth is mediated by localized inactivation of GSK-3beta and functions of the microtubule plus end binding protein APC. Neuron **42**:897-912.

510. **Ziegler, W. H., U. Tigges, A. Zieseniss, and B. M. Jockusch**. 2002. A lipid-regulated docking site on vinculin for protein kinase C. J.Biol.Chem. **277**:7396-7404.