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FRNK Regulatory Complex Formation with FAK Is Regulated by ERK Mediated Serine 217 Phosphorylation

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FRNK REGULATORY COMPLEX FORMATION WITH FAK IS REGUALTED BY ERK MEDIATED SERINE 217 PHOSPHORYLATION

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Dedicated to my wife Stacey

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LIST OF ABBREVIATIONS

A.U.	arbitrary units
Arp3	actin-related protein 3 (a component of the Arp2/3 complex)
ASAP1	ARF GTPase-activating protein containing SH3, ANK repeats, and PH domain
CER	mCerulean
Crk	CT10 regulator of kinase
DES	Drug eluting stent
EGFR	epidermal growth factor receptor
ERK	extracellular signal-regulated kinase
FAT	focal adhesion targeting domain of FAK/FRNK
FAK	focal adhesion kinase
FERM	4.1, ezrin, radixin, and moesin domain
FRNK	focal adhesion kinase related non-kinase
FRET	Fluorescence resonance energy transfer
GFP	enhanced Green Fluorescent protein
Grb2	growth factor receptor-bound protein 2
GTP	guanosine triphosphate
JNK	c-Jun N-terminal kinase
KD	Kilodalton
PCI	Percutaneous coronary intervention

P130CAS	Crk-associated substrate
PI3K	Phospatidylinositol 3-kinase
PIP2	phospho-inositol 4,5-bisphosphate
PKB	Protein kinase B
PP1	protein phosphatase-1
PYK2	Protein tyrosine kinase 2, AKA RAFTK
MAPK	Mitogen-activated protein kinases
MBD2	methyl CpG-binding protein 2
Mdm2	Mouse double minute 2 homolog
Min	Minutes
RASM	Rat aortic smooth muscle cell
RhoA	ras homolog gene family member A
S217	residue serine 217 of FRNK
S910	residue serine 910 of FAK
SH2	N-terminal Src homology 2
SOS	son of sevenless
Src	cellular src kinase (c-src), proto-oncogene tyrosine-protein kinase Src
TIRF	Total Internal Reflection Fluorescence
VEGFR3	vascular endothelial growth factor receptor-3
WT	wild type
Y397	residue tyrosine 397 of FAK

ABSTRACT

Focal adhesion kinase related non-kinase (FRNK) is an endogenous inhibitor of focal adhesion kinase (FAK) that has traditionally been used to inhibit FAK signaling in a variety of experiments and is also an important endogenous regulator of FAK signaling. More recently, FRNK has been shown to be of increasing importance in some pathologic conditions. Despite the increasing importance of FRNK, the molecular mechanism by which it functions remains unclear. In addition, FRNK contains several phosphorylation sites with unknown importance and function. Here I hypothesize that FRNK can inhibit FAK by binding directly to FAK within focal adhesions. Furthermore, I propose that serine phosphorylation at residue 217 of FRNK reduces its ability to bind to FAK, highlighting a potential regulatory mechanism for the newly described FAK-FRNK interaction. I found using co-immunoprecipitation and fluorescence resonance energy transfer that FRNK can bind directly to FAK. FRNK serine phosphorylation occurs in vitro and in vivo, and FRNK S217 is phosphorylated in an ERK-dependent fashion. Additionally, mutation of serine 217 on FRNK to alanine resulted in increased FAK binding. Cells overexpressing S217A FRNK exhibited increased rates of apoptosis as measured by flow cytometry. Taken together, I propose that FAK-FRNK complexes are an important mechanism of FRNK inhibition of FAK and that this mechanism is regulated by ERK phosphorylation of FRNK at serine 217. This information could be used to rationally develop treatments to treat diseases involving abnormal smooth muscle cell growth in response to injury, such as in-stent restenosis.

CHAPTER ONE

CELL SIGNALING AND FAK/FRNK

CELL SIGNALING.

Cell signaling is an essential component of normal cell physiology that allows cells to respond to their environment. Perturbations of normal cell signaling can lead to pathologic states. The ability of cells to respond to their environment is essential for life. Cell signaling is defined as the complex combination of signals that control the function of cells at the molecular level. These signals can originate from within the cell or outside the cell. Receptors are proteins that are typically on the cell surface that take an extracellular signal and transduce that signal into an intracellular signal. For example in the human eye the receptor rhodopsin converts the extracellular signal of light and converts it into the intracellular signal of increasing the concentration of GTP bound dissociated Gα proteins in the cell[1]. The now transduced intracellular signal, increased GTP bound Gα proteins, can then bind to and activate an enzyme called phosphodiesterase that converts cGMP to GMP[2]. The net result being a decrease in cGMP concentration which causes additional signals such as ion channel closure which ultimately signal to the brain and enable sight. This example highlights two important aspects of cell signaling cascades: amplification and second messengers.

Amplification refers to the increase in potency of the signal as it propagates downstream. In the rhodopsin example, when a single receptor is activated by light it changes conformation and catalyzes the conversion of GDP-bound Gα to GTP-bound Gα.

The receptor stays in this activating conformation long enough to convert multiple GDP-bound Gα subunits to multiple GTP-bound Gα subunits. Each individual GTP-bound Gα is able to bind to a phosphodiesterase and activate it and each phosphodiesterase is able to convert many molecules of cGMP to GMP. Thus a single activation of just one rhodopsin receptor results in a larger decrease in intracellular GMP concentration. The ability of cells to amplify signals in this manner is crucial to cell signaling.

Second messengers enable efficient and multifunctional cell signaling. In the rhodopsin example cGMP acts as a second messenger, meaning the light signal is converted into a decrease in cGMP which is a small molecule. The advantage of a small molecule second messenger is that it is able to rapidly diffuse throughout the cell, a function that transmembrane proteins cannot perform. Additionally, second messengers allow for a single signal, such as light in the rhodopsin example, to impact multiple functions. It was already mentioned that cGMP concentration can regulate ion channel opening, but cGMP can also activate protein kinase G which results in different signaling pathways being activated. Thus, second messengers allow for signals to travel and can allow signals to diverge in order to activate multiple pathways simultaneously.

PROTEIN PHOSPHORYLATION.

A major component of many cell signaling pathways is protein phosphorylation. Protein phosphorylation was discovered in 1954, with the discovery that phosphorylase b is converted to phosphorylase a by attaching the gamma-phosphate from ATP to a serine residue[3]. The enzymes responsible for phosphorylation are called kinases and the enzymes responsible for removing phosphorylation are called phosphatases. The balance of kinase activity and phosphatase activity ultimately determines the equilibrium status of a particular protein's phosphorylation. Once phosphorylated (or de-phosphorylated) a protein undergoes conformational changes that can activate or inactivate the protein's function. For example, many kinases themselves are regulated by phosphorylation and are only active after they have been phosphorylated.

A great example of these kinds of phosphorylation cascades is the mitogen activated protein kinase (MAPK) family of proteins. The substrate of several MAPKs is another MAPK, leading to a series of kinase activations which amplifies the original signal and allows for differential signaling. For example, when activated MAPKKKs phosphorylate MEK1/2 (a MAPKK), which then phosphorylates ERK1/2 (a MAPK), which then phosphorylates transcription factors and other cellular targets to exert effects on the cell [4]. However, activation of MAPKKKs will also lead to phosphorylation of MKK7 (a MAPKK) which will lead to phosphorylation of JNK (a MAPK) which will phosphorylate a different set of transcription factors. These kinase cascades are interconnected, but are differentially regulated in such a way that different stimuli can promote a preference for a subset of kinases to be preferentially activated, while still activating many of the

different kinases[4]. It is now known that protein phosphorylation occurs in many proteins, and is the most important post-translational modification in biology.

REVIEW OF THE PROTEIN FAMILY NON-RECEPTOR TYROSINE KINASES.

In order to better understand the function of focal adhesion kinase (FAK) and its endogenous inhibitor focal adhesion kinase related non-kinase (FRNK) the structure and function of the focal adhesion kinase family of non-receptor tyrosine kinases will be reviewed. Non-receptor tyrosine kinases function by adding a phosphate group to substrate proteins on tyrosine residues. The phosphorylation of these proteins can cause conformational changes that change the function of substrate proteins or create protein binding sites, ultimately effecting cell physiology such as growth, survival, and migration. Non-receptor tyrosine kinases are not transmembrane proteins like the related family of receptor tyrosine kinases. Receptor tyrosine kinases bind ligands, such as growth factors, which results in a conformational change that activates the kinase domain of the protein. In contrast, non-receptor tyrosine kinases must be activated by means other than extracellular ligand binding because they reside in the cytoplasm.

Cellular Src kinase (Src) is a well-studied non-receptor tyrosine kinase that exemplifies the way non-receptor tyrosine kinases are activated. In an unstimulated cell, Src exists in an inhibited conformation where a C-terminal phosphorylated tyrosine 527 binds to an N-terminal Src homology 2 (SH2) domain, folding the protein and decreasing the activity of the kinase domain [5]. Dephosphorylation of Src tyrosine 527 by a phosphatase abolishes SH2 binding and allows the protein to unfold, activating the kinase domain. Another method of Src activation involves the Src SH2 domain binding to a different protein's phosphorylated tyrosine, allowing the protein to remain unfolded

while the tyrosine 527 is still phosphorylated. Once in an open active conformation, Src can auto-phosphorylate a different residue, tyrosine 416, which stabilizes the active conformation. Src cannot bind extracellular ligands, but can be activated by the downstream actions of extracellular ligands. For example, epidermal growth factor receptor (EGFR) autophosphorylates intracellular tyrosine residues after binding its ligand and forming a homodimer [6]. These phosphorylated tyrosine residues can now serve as binding sites for the N terminal Src SH2 domain and prevent auto-inhibition of the Src kinase domain by binding to the C terminal phosphorylated tyrosine 527. This provides a direct mechanism for EGFR ligand binding to contribute to Src signaling pathways. Additionally, once bound to EGFR through the SH2 domain, the now active Src can phosphorylate the EGFR, further enhancing the growth signal [7]. In this manner non-receptor tyrosine kinases can respond to a much larger variety of extracellular ligands compared to their transmembrane receptor counterparts and can integrate many different mitogenic signals. The Src and EGFR example highlights the importance of protein-protein interactions in non-receptor tyrosine kinase signaling.

DISCOVERY OF FOCAL ADHESION KINASE.

FAK was discovered in 1992 by Hanks et. al. [8] who showed that the protein was phosphorylated on tyrosine residues in adherent cells, but contained little phospho-tyrosine in re-suspended cells. Their initial paper also demonstrated tyrosine kinase activity, a focal adhesion localization pattern, and the ability of growth substrates such as fibronectin to increase FAK tyrosine phosphorylation. These observations established an early link to integrin signaling. In 1993 Schaller et. al. [9] described an endogenously occurring truncated form of FAK lacking a kinase domain termed focal adhesion kinase

related non-kinase (FRNK). Initial observations of FRNK included localization to focal adhesions, providing evidence that the C-terminal portion of FAK was involved in focal adhesion localization. This C-terminal domain would later be named the focal adhesion targeting (FAT) domain. In 1995 Hanks et. al. [10] provided evidence for a link to Src kinase, in particular that Src phosphorylation of FAK tyrosine residues 576 and 577 increase FAK kinase activity. Additionally, Hanks et. al. [11] identified a v-Crk-associated tyrosine kinase substrate (p130Cas) as a FAK binding partner which utilizes SH3 domains to bind to FAK proline rich regions, which began to establish FAK as an important scaffolding protein. Also in 1995, a global FAK knockout mouse was generated that resulted in embryonic death due to cardiovascular defects[12]. Thus, in a few short years FAK was identified and determined to be essential for life and important in Src and integrin signaling pathways.

FAK AND FRNK ARE IMPORTANT REGULATORS OF CELL PHYSIOLOGY.

As reviewed below, FAK and its endogenous inhibitor FRNK are important regulators of a variety of cell signaling pathways. FAK is a non-receptor protein tyrosine kinase that plays a pivotal role in growth, migration, and anti-apoptotic pathways. FAK is encoded by the PTK2 gene and is composed of three main domains; an N-terminal band 4.1, ezrin, radixin, moesin (FERM) domain, a tyrosine kinase domain, and a focal adhesion targeting (FAT) domain (Figure 1)[13]. Alterations in FAK signaling commonly occur in cancer, which has driven most of the research on FAK signaling. FAK knockout mice die in utero, highlighting the importance of this signaling pathway[14]. While FAK is ubiquitously expressed in adult cells, FRNK expression is more tightly regulated. FRNK is encoded by the same gene as FAK, but transcription starts at an intronic promoter

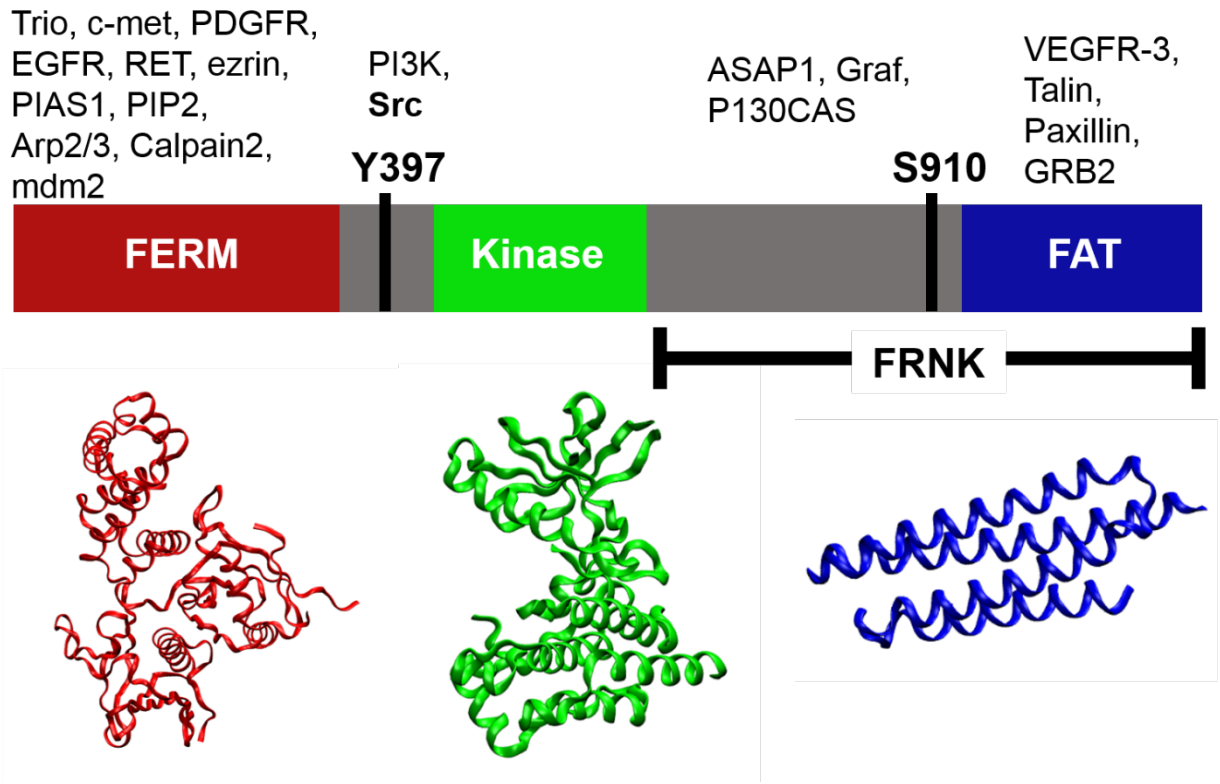

Figure 1. Diagram of the crystal structure of FAK and FRNK
The three main domains of FAK and their crystal structures. The sequence of FRNK is indicated below the schematic. The phosphorylation sites tyrosine 397 and serine 910 (217 on FRNK) are also indicated. For the FERM domain, PDB structure 2AL6 was used. For the kinase domain, PDB structure 1MP8 was used. For the FAT domain, PDB structure 1K40 was used. Some key interactions mediated by each domain are listed above their respective binding sites.

resulting in a truncated peptide[15]. FRNK is expressed only during development and in adult cells FRNK expression is limited to specific cell types and is specifically upregulated in pathologic tissues. The ability of FRNK to inhibit one of the most important signaling pathways in the cell and the specific expression pattern of FRNK in a subset of adult cells make FRNK an ideal locus to target for pharmacological intervention in several disease states.

FOCAL ADHESIONS PERFORM STRUCTURAL AND SIGNALING FUNCTIONS.

Both FAK and FRNK localize to focal adhesions, which are specialized cell structures that mechanically connect the cytoskeleton to the extracellular matrix through integrins. In order for cells to adhere to the extracellular matrix in tissues transmembrane proteins must be physically linked to the cytoskeleton. In particular, integrins are transmembrane proteins that bind to extracellular matrix components such as fibronectin[16]. By themselves, integrins would only mechanically connect the extracellular matrix to the fluid plasma membrane, which would be insufficient to transmit the forces required for cell function. In order to support the forces required for cell migration, integrins must be mechanically linked to the cytoskeleton, in particular actin stress fibers. Actin stress fibers are bundles of actin filaments of opposite orientations that are associated with crosslinking proteins as well as contractile proteins such as myosin II[17]. The cellular structure that performs the vital function of linking integrins to actin stress fibers is the focal adhesion. Focal adhesions are comprised of numerous proteins that participate in multiple protein-protein interactions. Individual proteins such as talin contain integrin and actin binding sites and directly mediate the physical connection of integrins to cytoskeleton [18]. Talin also contains a binding site for vinculin, a protein that is also capable of binding to actin. Paxillin is another focal adhesion protein that can bind to integrins and other focal adhesion proteins[19], but cannot bind directly to actin. However, paxillin can bind to vinculin. Thus, in the absence of talin an integrin to actin mechanical link could still be made through integrin-paxillin, paxillin-vinculin, and vinculin-actin protein-protein interactions. In fact, knockdown of talin results in cells that are still able to generate focal adhesions, adhere to extracellular

matrix, and migrate[20]. There over 60 proteins that localize to focal adhesions[21], many of which mediate multiple protein-protein interactions like the examples listed above. Thus, focal adhesions are protein rich structures where many protein-protein interactions among many different proteins mediate the structural connection of the extracellular matrix to the actin cytoskeleton.

In addition to focal adhesions' structural function, focal adhesions also play an important role in cell signaling. Many of the focal adhesion proteins that have a structural function also function as powerful modulators of cell signaling[22]. For example, vinculin knock out in cancer cells results in enhanced metastasis and increased resistance to apoptosis [23]. It was found that this effect was caused by altering interactions of the other focal adhesion proteins FAK and paxillin and ultimately ERK signaling [23]. Other focal adhesion proteins serve little structural function, but are powerful regulators of cell signaling. FAK is a great example of a focal adhesion protein that performs a signaling function and can alter cell growth, migration, and survival. FAK can be activated by growth factor receptors such as EGFR or integrin activation. FAK also acts on other focal adhesion proteins, altering their ability to structurally link extracellular matrix and cytoskeleton[22]. Focal adhesion signaling, and specifically FAK signaling is critical to normal cell signaling.

THE CRYSTAL STRUCTURE OF FAK PROVIDES INSIGHT INTO FAK AND FRNK FUNCTIONS.

The first crystal structure of the FAK kinase domain was described in 2002[24] around the same time as the structure for the FAK focal adhesion targeting (FAT) domain was determined to be a four helix bundle[25] (Figure 1). The third and final domain, the band 4.1, ezrin, radixin, moesin (FERM) domain, was crystalized in 2006 and

showed similar structure to other FERM domains despite a significantly different amino acid sequence[26]. The structure of FAK's three domains have been extensively studied and will be discussed individually after a brief explanation of the function of the FAK as a whole protein.

OVERVIEW OF THE STRUCTURE AND FUNCTION OF FAK.

FAK is composed of three main domains: a FERM domain, a kinase domain, and a FAT domain (Figure 1). The main function of the FERM domain is to mediate FAK dimerization and auto-inhibit the FAK kinase domain until activation. The main function of the kinase domain is to phosphorylate tyrosine residues, and is predominantly involved in auto-phosphorylation of FAK once dimerization has occurred. In particular, auto-phosphorylation of FAK tyrosine 397 results in Src binding and further signaling. Last, the most important role of the FAT domain is to bind to paxillin and other focal adhesion proteins causing FAK to be localized to focal adhesions. In order to fully describe the function of FAK each domain will be discussed individually while keeping this overview view in mind.

THE FERM DOMAIN.

The 4.1, ezrin, radixin, and moesin (FERM) domain of FAK is present in many proteins involved in linking the cytoskeleton to receptors and has a conserved sequence consisting of three lobes. The first FERM protein identified was erythrocyte band protein 4.1 (4.1) and like most FERM domains contains an actin binding domain [27]. 4.1 protein localizes and stabilizes the cytoskeleton. FERM domains consist of three lobes, F1, F2, and F3 starting with F1 at the N-terminus. The three lobes act together as a cohesive unit in order to bind to various molecules including proteins and lipids (binding partners

are indicated in Figure 1). The active FERM domain then serves as a scaffolding protein that brings together different proteins into a local environment that causes changes in protein activity or function. In the present study, the FERM domain is of great importance because I hypothesize that it mediates a FAK FRNK interaction and the FERM domain mediates most of the protein-protein interactions available to FAK but not to FRNK. Thus, the FERM domain will be discussed extensively.

The FERM domain inhibits the kinase domain. The FERM domain of focal adhesion kinase has been well studied and multiple different mechanisms of activation have been proposed. The FAK FERM domain was shown to inhibit FAK kinase activity in experiments where deleting the first 375 amino acids resulted in an increase in kinase activity [28], suggesting activation of the FERM domain requires dissociation from the kinase domain. This mechanism of FERM domain inhibition was confirmed by a crystal structure.[29] The crystal structure showed that the auto inhibited state inhibits FAK activity through multiple mechanisms including: blocking the kinase active site, prevention of Src binding to phospho-tyrosine 397, and blocking Src from phosphorylating tyrosine residues 576 and 577 on the kinase activation loop. Partial FERM domain deletion mutants confirmed this mechanism by causing increased FAK tyrosine phosphorylation and Src binding [28,30].

The FERM kinase interaction was shown to be mediated through a basic patch of amino acids on the F2 loop of the FERM domain[31]. Researchers mutated the positively charged lysine residues at position 216, 218, 222 and an arginine at position 221 to create a mutant without the basic patch dubbed KAKTLR [31]. The KAKTLR mutant was

unable to interact with purified FAK kinase domain in GST pulldown assays. Surprisingly, expression of the KAKTLR mutant FAK in cells resulted in a decrease in FAK tyrosine 397 phosphorylation, the opposite effect expected if the autoinhibition of the kinase domain by the FERM domain was prevented. This data was the first to suggest that the FERM domain can either inhibit or activate the kinase domain, and or FAK association with other kinases such as Src. The KAKTLR sequence is of particular interest because it may be the region of FAK that binds to FRNK, which will be discussed later.

Regulation of FERM domain auto-inhibition and phospholipids. The importance of FERM-kinase interaction in regulating FAK activity lead many researchers to look for a ligand that can cause FERM dissociation from the kinase domain. Purified FERM domain was shown to bind to phospho-inositol 4,5-bisphosphate (PIP2) using an anisotropy in vitro assay [32]. Experiments using the KAKTLR mutant with an additional lysine to alanine substitution showed that this basic patch was required for PIP2 binding[32]. Furthermore, they showed that the FERM domain could localize to the plasma membrane in the absence of the FAT domain and that this localization pattern was abolished with the KAKTLR mutant. Furthermore, Cai et. al. showed that adding PIP2 to an in vitro kinase assay could increase FAK tyrosine phosphorylation further implicating acidic phospholipids as a FERM domain ligand[32]. Phospatidylinositol 3-kinase (PI3K) binding to FAK was also shown to be important in mediating FAK migration signaling, further suggesting the importance of PIP2 in FERM regulation of FAK [33].

Regulation of FERM domain auto-inhibition and integrins and force. In addition to PIP2, integrin binding has been shown to cause FERM domain conformational

changes[34]. Using a FRET based biosensor, it was shown that FERM domain conformational changes occur in response to plating cells on fibronectin, consistent with integrin activation and clustering releasing FERM-kinase domain inhibition[34]. Additionally, they showed that FAK conformation was dependent on tension applied to actin stress fibers by reducing tension via myosin II phosphorylation inhibition[34]. This confirmed that the FERM domain inhibition of the kinase activity responds to force applied to focal adhesions from actin stress fibers. Furthermore, it was determined that these conformational changes occur independently of FAK tyrosine 397 phosphorylation via experiments using a tyrosine 397 to alanine mutant biosensor[32,34]. However, another group generated a similar biosensor with the fluorophore moved only 22 amino acids but reported an opposite change in FRET efficiency upon activation[32,34]. The authors of the second paper suggested this difference was not due to different distances between donor and acceptor fluorophores, but instead changes in fluorophore dipole orientation. Simply put, they believed the sensors were measuring a twisting motion instead of the usual increase in distance. Regardless, both authors concluded that FERM domain changes occurred before Y397 phosphorylation.

Regulation of FERM domain inhibition and other proteins. FERM domain protein-protein interactions have also been implicated in regulating FERM inhibition of FAK (Several interacting proteins listed in Figure 1). Protein inhibitor of activated STAT1 (PIAS1) was shown to interact with the FERM domain of FAK with multiple methods [35]. PIAS1 is a small ubiquitin-like modifier (SUMO) ligase and in cells facilitates the Sumoylation of FAK lysine 152, and increased FAK autophosphorylation at tyrosine 397

[35]. FERM domain interaction with the protein ezrin, another FERM family protein, was shown to increase FAK tyrosine 397 phosphorylation in a Src independent fashion [36]. Similarly, the protein trio was also shown to bind to the FAK FERM domain, be phosphorylated by FAK, and to also increase FAK tyrosine 397 phosphorylation [37]. The FAK FERM domain has also been shown to interact with the growth factor receptor c-met [38]. The basic patch of residues in the F2 lobe, the KAKTLRK sequence, was shown to be required for c-met interaction with FAK. Again, c-met interaction with FAK resulted in increased FAK tyrosine 397 phosphorylation and increased FAK signaling in the form of cell migration[38]. Both platelet derived growth factor receptor (PDGFR) and epidermal growth factor receptor (EGFR) are able to bind to the FERM domain, and binding was required for efficient growth signaling [39]. Rearranged during transfection (RET) is a receptor tyrosine kinase that has also been shown to bind to the FAK FERM domain [40]. RET and FAK both contribute to phosphorylation of each other, and this RET interaction is dependent on the KAKTLRK basic sequence of the FAK FERM domain [40]. Other protein-protein interactions also exist with the FERM domain, but are probably of less importance in inducing FAK tyrosine 397 phosphorylation [13]. These numerous protein-protein interactions allow the FERM domain to regulate FAK kinase activity in response to a wide variety of stimuli, establishing FAK as a convergence point of many cell signaling pathways. Furthermore, the ability of the KAKTLR sequence to mediate important protein-protein interactions could explain why FRNK binding to FAK at the KAKTLR sequence would result in FAK signaling defects.

Regulation of FERM domain inhibition and pH. In addition to protein-protein interactions and acidic phospholipids, it has been proposed that FERM auto-inhibiton of FAK can be relieved by changes to intracellular pH [41]. Lawson and Schlaepfer bring attention to the fact that upon cell adhesion, intracellular pH is increased in an integrin dependent manner[41]. Futhermore, experiments done with a paxillin based pH sensor showed that the local pH at focal adhesions was higher than in the rest of the cell, and that FAK tyrosine 397 phosphorylation is dependent on high pH, but independent of pH if the FERM domain is not present[42]. Additionally, they point out that FAK contains a histidine at position 58 in the FERM domain that is close in three dimensional space to tyrosine 397[42]. Mutation of this histidine to alanine reduced FAK tyrosine 397 phosphorylation dependence on pH, suggesting it is involved in conformational changes that alter 397 phosphorylation based on local focal adhesion proton concentration [42]. Thus, the local environment of the focal adhesion, in particular the local pH which is altered upon cell adhesion, may also serve to regulate FERM domain inhibition of FAK tyrosine 397 phosphorylation and subsequent signaling pathways.

The FERM domain as a switch instead of a kinase domain inhibitor. FERM domain protein binding is not always positively linked to tyrosine 397 phosphorylation status. For example, actin-related protein 3 (Arp3), which is part of the Arp2/3 complex, binds to the FERM domain and can induce actin polymerization which causes membrane protrusions important for cell migration [43,44]. Experiments showed that more Arp2/3 was bound to FAK when tyrosine 397 was not phosphorylated[43], the opposite effect 397 phosphorylation has on most FAK protein-protein interactions. They also noted that

Arp2/3 co-localized with FAK in small nascent adhesions and was less abundant in large mature focal adhesions[44]. This is a great example of FAK scaffolding activity and how FAK phosphorylation status can act as a switch in order to regulate protein scaffolding activity. When FAK localizes to newly forming focal adhesions it has not had time to fully activate and auto-phosphorylate at tyrosine 397. This lag time could be used by the cell to induce membrane protrusions via Arp2/3 binding. As the focal adhesion matures, 397 phosphorylation occurs and Arp2/3 is no longer able to stimulate actin polymerization. This occurs at an ideal time, because the mature focal adhesions are no longer the most distal focal adhesions, and actin polymerization from mature focal adhesions would no longer drive membrane protrusion. FAK is often described as a protein scaffold that can be activated by phosphorylation, however the Arp2/3 example highlights that some processes are inactivated by phosphorylation of tyrosine 397. Thus, it is more accurate to state that FAK is a switchable scaffold, adapting its function to its current phosphorylation state, and not simply increasing function as its tyrosine residues are phosphorylated.

FERM domain cell signaling conclusions. FAK FERM domain auto-inhibition of the FAK kinase domain phosphorylation remains unclear despite numerous studies. As described above, the FERM domain has been shown to interact with a number of molecules including acidic phospholipids, other FERM family proteins, growth factor receptors, and more. The FERM domain inhibition of FAK also responds to mechanical force and is potentially regulated by local pH. Because of the evidence surrounding all of these methods of potential FERM domain activation, it is unlikely that the FERM domain

binds to any single ligand in order to relieve its auto-inhibition of FAK. Instead multiple signals may need to combine in order to maximally enable phosphorylation of tyrosine 397. The FERM domain may function as a signal adaptor, allowing multiple different signals (protein binding, PIP2 production, pH increase, or mechanical strain) to induce FAK tyrosine 397 phosphorylation and subsequent FAK signaling. This property allows FAK to integrate many different signaling pathways into a single signaling pathway involving tyrosine 397 phosphorylation and subsequent Src binding. Additionally, it allows subsequent signaling to be tied back to the original stimulus by binding to both the stimulatory protein and Src kinase, enabling Src to in turn phosphorylate the original signal offering a potential locus of negative feedback, or a chance for signal amplification.

The FERM domain and FAK in the nucleus. In addition to regulating FAK kinase function and acting as a scaffolding domain, the FERM domain also possesses a nuclear localization sequence thought to be important in a separate FAK nuclear signaling pathway. In the present study I focus on FAK signaling within the focal adhesion, but it is important to understand that FAK has an additional function in the nucleus and that alterations in focal adhesion signaling may cause changes in FAK function in the nucleus. Expression of the FERM domain tagged to a green fluorescent protein (GFP) exhibited strong nuclear localization when examined with fluorescence microscopy [45]. Full length FAK also exhibited nuclear localization upon inhibition of nuclear export with leptomycin, and this nuclear localization was abolished by mutating positively charged residues, including but not limited to residues in the KAKTLR sequence, in the F2 lobe of

the FERM domain to alanine[45]. Expression of various FAK mutants in FAK null cells showed that the intact FERM domain was required and sufficient to decrease levels of p53 abundance [45]. Even mutation of the FAK tyrosine 397 site to alanine and rendering the kinase domain inactive did not abolish FAK mediated decreased p53 expression [45]. It was also shown with co-immunoprecipitation that FAK, specifically the F1 and F2 lobes of the FERM domain, can bind to p53. Additionally, the F3 lobe of the FERM domain was able to bind to the E3 ubiquitin ligase Mouse double minute 2 homolog (Mdm2) [45]. Additional experiments showed that FAK was able to scaffold p53 and Mdm2 leading to increased ubiquitination of p53 and subsequent p53 degradation via the proteasome [45], thus establishing a phospho-tyrosine 397 and kinase activity independent mechanism by which nuclear localized FAK can affect cell survival signaling. FAK localized to the nucleus has also been shown to interact with methyl CpG-binding protein 2 (MBD2) and decrease MBD2 binding to DNA, altering chromatin remodeling and enabling gene expression [46]. The effects of FAK in the nucleus are less understood compared to FAK function in focal adhesions. It is clear that FAK exists in a dynamic equilibrium between focal adhesions and the cytoplasm. This equilibrium may be used to alter FAK concentration in the nucleus. For example, a change in focal adhesion properties that displaces FAK to the cytoplasm would also potentially increase FAK concentration in the nucleus and alter gene expression. Thus, in the present study when comparing different FRNK mutants I assayed the ability of those FRNK mutants to displace FAK from focal adhesions in part to asses if they would have a large impact on the nuclear localization of FAK.

THE FAK KINASE DOMAIN AND FERM-KINASE LINKER.

The linker region between the FERM and kinase domains of FAK regulates Src kinase scaffolding and activation of the FAK kinase domain. The linker connects the FERM and kinase domains and consists of residues from 351 through 411 of human FAK [24,26]. The linker region contains two different Src binding sites: An SH2 binding site centered on phosphorylated tyrosine 397 and an SH3 domain 20 amino acids closer to the N-terminus that contains several proline residues. The SH2 binding domain consists of a p-YAEI sequence which matches to the Src kinase consensus sequence of p-YEEI, but is also highly dependent on adjacent negatively charged residues in the linker [47]. The adjacent SH3 domain recognition sequence does not require phosphorylation and consists of an RXXPXXP consensus sequence[26]. Together these sequences mediate Src kinase binding, but only when tyrosine 397 is phosphorylated. Thus, the phosphorylation of tyrosine 397 is the most critical step in FAK "activation" because it begins an important series of events (example cascade diagramed in Figure 2). First, it triggers Src binding via SH2 and SH3 domains. Second, Src binding to FAK relieves Src auto-inhibition[5]. The now bound and active Src phosphorylates local tyrosine residues which can create additional protein binding sites. For example, once FAK is phosphorylated at Y397, Src kinase binds and phosphorylates FAK at tyrosine 861 [48]. The newly phosphorylated tyrosine 861 now creates a binding site for the protein Crk-associated substrate (p130CAS) which binds to a proline rich region in the C-terminal third of FAK [49]. The FAK-Src-p130CAS complex is now able to phosphorylate p130CAS on fifteen YxxP motifs which then function as binding sites for proteins such as CT10

regulator of kinase (Crk), which eventually leads to increased MAPK activation [50]. Without tyrosine 397 phosphorylation, Src cannot bind and the entire scaffolding sequence is disrupted. Creation of a mouse model lacking 19 amino acids in the FERM-kinase linker, including tyrosine 397, resulted in embryonic death just four days later than total FAK knockout mice, highlighting the importance of the FAK tyrosine 397 [14]. Thus, FAK can be described as an activatable or switchable scaffold, with the main

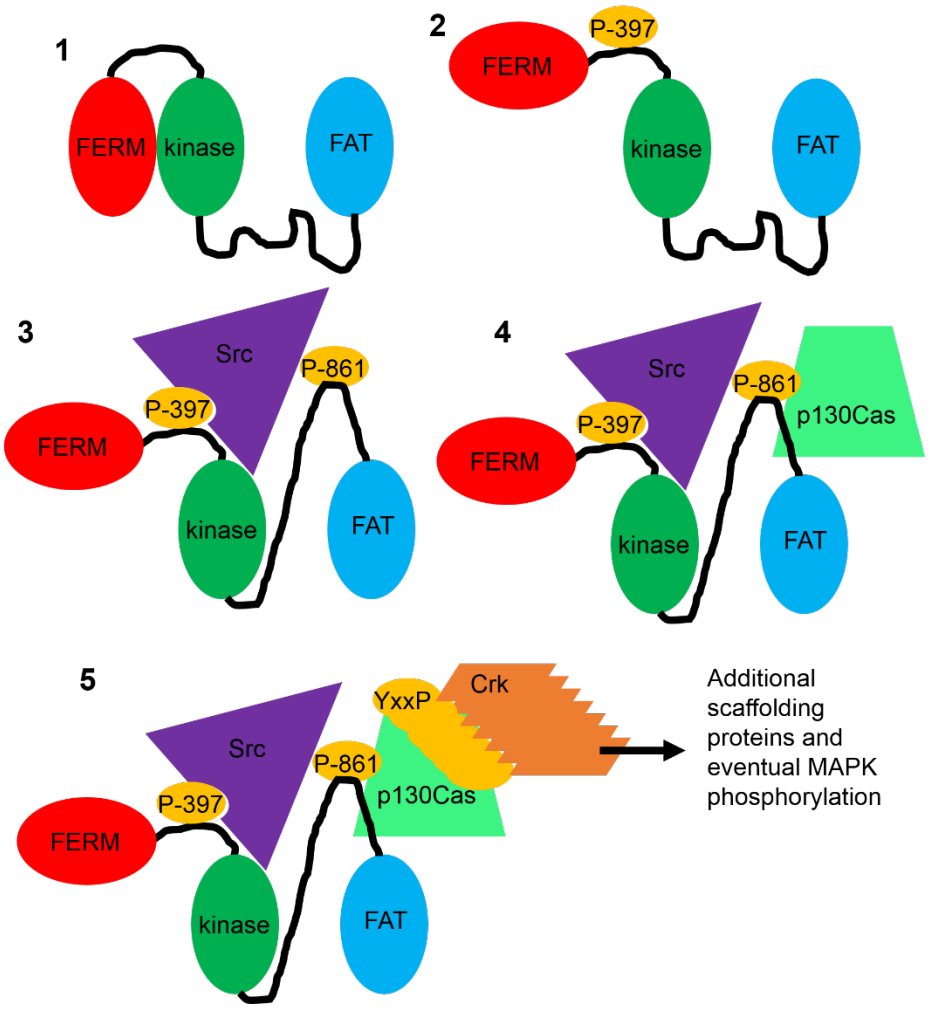

Figure 2. Diagram of FAK signaling activation
1. Inactive FAK consists of the FERM domain bound to and inhibiting the kinase domain. 2. The autoinhibition of the FERM domain is removed by an activating stimulus such as FAK dimerization or integrin signaling and the FAK kinase domain phosphorylates tyrosine 397 creating an SH2 binding site. 3. Src kinase binds to the phospho tyrosine 397 SH2 binding site and adopts and active conformation. 4. Src kinase phosphorylates residues on FAK such as tyrosine 861 which allows for p130Cas binding. 5. The newly bound p130Cas is repeatedly phosphorylated by Src on 15 YxxP repeats creating binding sites for Crk. The now bound Crk proteins serve to scaffold additional proteins and ultimately leads to MAPK phosphorylation and activation.

activating step being phosphorylation of tyrosine 397. The tyrosine 397 phosphorylation site is an excellent way to quantify how active FAK signaling is and can be probed with commercially available phospho-specific antibodies, a strategy which is used in the experiments presented in the present study.

FAK tyrosine 397 is the main substrate of the FAK kinase domain. The FAK kinase domain is essential to the function of FAK scaffolding with other proteins. The most important and well known substrate of the FAK kinase domain is the tyrosine 397 located in the linker between the FERM and kinase domains that FAK autophosphorylates when activated by cell attachment or other signals [51]. FAK that has bound Src is phosphorylated by Src on tyrosine residues 576 and 577 of a highly conserved "activation loop" within the kinase domain[10]. This mechanism of increasing kinase activity via loop phosphorylation is highly conserved between many tyrosine kinases, and is required for full activation of the FAK kinase domain [52]. Several additional substrates for the FAK kinase domain have been identified in vitro, such as paxillin, Src homology 2 domain containing transforming protein 1 (shc), p130CAS, IKB kinase α (IKKα), and others [53]. However the physiologic significance of direct FAK phosphorylation of these substrates is less clear, especially considering many of these proteins are also phosphorylated by Src which is activated by FAK kinase activation. Thus, the most important substrate for the FAK kinase domain is the tyrosine 397 of FAK which serves to activate many of the scaffolding functions of FAK.

FAK autophosphorylation is regulated by oligomerization. FAK autophosphorylation has been well studied, and recently was shown to be dependent on FAK homodimers. A very comprehensive paper published by Brami-Cherrier et al. in 2014 showed through multiple methods that FAK autophosphorylation of tyrosine 397 requires homo-dimerization of two FAK proteins 54. They showed that the dimerization of FAK is mediated by a FERM-FERM interaction centered on a tryptophan residue at

position 266 by creating a crystal structure, co-immunoprecipitation, acceptor photobleaching, chromatography, GST-pulldown, and Small angle X-ray scattering. By mutating FAK tryptophan 266 to alanine, they showed that FAK dimerization was critical to FAK autophosphorylation on tyrosine 397 through in vitro kinase assays, a forced dimerization assay, and cell culture experiments comparing phospho-tyrosine 397. In cell culture experiments, FAK dimerization was only detectable within focal adhesions. This seminal paper confirmed the auto-inhibition of the FERM-kinase domain and added an additional mechanism by which this auto-inhibition can be relieved, FAK dimerization through a FERM-FERM interaction that only occurs within the local environment of focal adhesions. The present study seeks to elaborate on the finding that FAK can dimerize and provides evidence that FRNK can also bind to FAK.

The kinase-FAT linker and FAT domain. The linker region between the kinase and FAT domains is thought to be largely unstructured and is important in mediating protein-protein interactions (Figure 1). The linker is 229 amino acids long and contains two proline rich regions as well as several phosphorylation sites [55]. The first proline rich region, located at residues 715-718, in combination with phosphorylation of tyrosine 861 creates a binding site for p130CAS, which contains an SH3 domain [49,56]. The second proline rich region, located at residues 879-882, mediates the binding of GTPase regulator associated with FAK (Graf) [57], which activates GTPase activity in ras homolog gene family member A (RhoA) and cell division control protein 42 homolog (Cdc42). RhoA and cdc42 are involved in cytoskeletal organization and cell growth. Additionally, the second proline rich region is also a binding site for ARF GTPase-activating protein

containing SH3, ANK repeats, and PH domain (ASAP1)[58]. Like P130CAS, ASAP1 is also phosphorylated by Src kinase once scaffolded to FAK [58]. ASAP1 is also a GTPase activating protein, like Graf, and functions to regulate focal adhesion development. Specifically, overexpression of ASAP1 prevents FAK and paxillin localization to focal adhesions, but does not alter vinculin localization to focal adhesions. Overexpression of a catalytically inactive ASAP1 had a less potent effect on FAK and paxillin focal adhesion localization, suggesting the GTPase activity was required to prevent FAK and paxillin localization [58]. Other proteins have been suggested to bind to the two proline rich regions in the kinase-FAT linker, such as phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) [59] and protein phosphatase-1 (PP1) [60], but the evidence of exactly how they bind to FAK is less persuasive. In conclusion, despite being unstructured the kinase-FAT linker is important in mediating several protein-protein interactions essential to FAK function as an activatable scaffold.

The kinase-FAT linker contains several phosphorylation sites. The linker between the kinase and FAT domains also contains a number of phosphorylation sites, besides the well-known tyrosine 861 site. Grigera et al. used mass spectroscopy to attempt to identify as many phosphorylation sites as possible, and noted several novel and evolutionarily conserved serine and threonine sites in the kinase-FAT linker region including T700, S722, S725, S726, S732, S766, T793, S845, S888, S891, S894, and Y899 of chicken FAK (which is 95% identical to human FAK) [55]. Some of these sites have now been established as physiologically important, such as phospho-S722 which decreases FAK kinase activity and slows cell migration [61]. The function of other sites such as S766

remain unknown, and it is possible that some of the sites with current functions may have additional functions or effects. Several of these sites with known function are located in the linker between the kinase and FAT domain and are also present on FRNK. In the present study I focus on S910, but it is possible that some of these other phosphorylation sites also play an important role in the function of FRNK.

The FAT domain mediates focal adhesion localization. The FAT domain of FAK functions to localize FAK to focal adhesions, an important aspect of its scaffolding function, and performs other functions as well. The ability of the FAT domain to localize to focal adhesions was first described in 1993 by Hildebrand et al [62]. They generated several FAK large deletion mutants and examined their localization with immunohistochemistry, narrowing the location of the FAT domain to the last 199 amino acids of FAK [62]. Additionally, by tagging proteins that normally exhibited cytoplasmic localization with the C terminal 199 fragment, they were able to induce a focal adhesion localization pattern demonstrating that the FAT domain is sufficient for focal adhesion localization [62]. Hildebrand et al. showed in 1995 that the FAK FAT domain bound to paxillin with co-immunoprecipitation and that the ability of a mutant to co-immunoprecipitate with paxillin was correlated with localization to focal adhesions [63]. This established paxillin binding as the main method of FAK localization to focal adhesions. The crystal structure revealed that the FAT domain consisted of four alpha helices which contain two hydrophobic patches on opposite sides that interact with two LD motifs located on paxillin [25,64]. This four helix shape is common among focal adhesion proteins and is shared by proteins such as vinculin, which can also bind to an LD motif

on paxillin [64,65]. Paxillin localization to focal adhesions does not require FAK or vinculin, and occurs through a separate LIM3 domain [64]. Thus, the interaction of the FAK FAT domain is the most important protein-protein interaction in causing FAK to localize to focal adhesions. However, paxillin is not required for FAK focal adhesion localization [66]. For example, a different portion of the FAT domain can interact with talin which can also interact directly with integrins [25,67]. It is likely that both paxillin and tailin interaction in combination with the rest of the protein-protein interactions of FAK work together to fully localize FAK to focal adhesions.

The FAT domain performs several signaling functions. The FAT domain is also the site of important signaling protein-protein interactions (Figure 1). After Src scaffolding to FAK phospho-tyrosine 397, Src can then phosphorylate FAK at tyrosine 925 [68]. Once phosphorylated, this residue can interact with the SH2 domain of growth factor receptor-bound protein 2 (Grb2) localizing Grb2 and its SH3 domains to the focal adhesion [68]. Once localized to focal adhesions, Grb2 can be bound by son of sevenless (SOS) through SH3 domains, which then allow SOS to interact with Ras which ultimately increases ERK activity with its GTPase function [68]. Thus, interaction of Grb2 with phospho-tyrosine 925 in the FAK FAT domain provides a mechanism by which FAK can activate a variety of kinases in the MAPK cascade, ultimately leading to cell pro-growth and migration signals. In addition to this method of MAPK casecade activation of ERK, FAK is able to form a complex directly with Calpain 2 and ERK which was shown to be important in regulating growth and migration [69]. Thus, the FAT domain is more than just a method for FAK to localize to focal adhesions and is also a powerful signaling domain.

In addition to activating ERK, the FAT domain is also able to interact with vascular endothelial growth factor receptor-3 (VEGFR-3) [70]. Co-immunoprecipitation and GST-pulldown assays confirmed the interaction was between the FAT domain and 200 amino acids from the N-terminal domain of VEGFR-3, which was first identified by phage display [70]. By overexpressing a small peptide corresponding to the VEGFR-3 sequence responsible for binding FAK, they were able to displace FAK from focal adhesions, cause cell detachment, decrease proliferation, and increase apoptosis in breast cancer cells [70]. Thus, the FAT domain can bind to multiple proteins important in cell growth and migration signaling. In the present study, it is important to consider that FRNK is also likely able to bind to some of these proteins and may exert effects beyond simple FAK inhibition.

The FAT domain has been reported to facilitate FAK oligomer formation. The FAK FAT domain has also been reported to bind to FAK domains in different ways, facilitating FAK oligomerization. In the current study, I consider this hypothesis as a potential interaction method for a FAK FRNK heterodimer. Crystal structures of FAK FAT domain were obtained in which one of the four alpha helices flipped out of the four helix bundle of the FAT domain, and occupied the space left by the same helix in another FAT four helix bundle [71]. This FAT domain dimer is sometimes referred to as a domain swapped dimer, because the H1 helix of FAK molecule A reaches over and takes the place of the H1 helix in FAK molecule B and vice versa. It is thought that the swinging out of the H1 alpha helix is important for allowing residues such as tyrosine 925 to be phosphorylated [71]. The importance of the swinging out of the H1 helix and the

formation of the domain swapped dimer has been downplayed as unimportant by some authors [72]. However, mutations that increased the ability of H1 helix to open increased FAK Y925 phosphorylation and the ability of full length FAK to bind, but paradoxically did not affect FAK Y397 phosphorylation [72]. These results are consistent with the H1 helix being an important conformational change, but not a driving force behind FAK dimerization which has a large effect on Y397 phosphorylation status and was shown to be mediated mostly through a FERM-FERM domain interaction [54]. Thus, it is possible but unlikely that a FAT-FAT interact exists. If such an interaction exists, it may be hard to detect and only occur in the local environment of proteins found within focal adhesions. Additionally, it is possible a FAT-FAT interaction exists, but serves only a minor stabilizing function in FAK dimerization.

In addition to a FAT-FAT interaction, a FAT-FERM interaction has also been reported. In the present study, a FAT FERM interaction is the leading hypothesis for the interaction of FAK and FRNK and thus the evidence for an interaction will be reviewed. In vitro small angle X-ray scattering and GST-pulldown experiments showed that the FAT domain was able to interact with a basic patch of amino acids located in the FERM domain of FAK [54]. This interaction is thought to be relatively weak compared to the FERM-FERM interaction that mediates FAK dimerization. The FERM FAT interaction has also been suggested to be important in the formation of not only FAK dimers, but also higher order FAK clusters consisting of approximately six to eight FAK proteins [13,73]. The potential for FAK to form clusters is of great importance when discussing the results of acceptor photobleaching experiments in the present study, and will be discussed further

in the results and discussion sections. The interaction of the FAT domain with the FERM domain is also enhanced by the presence of paxillin, further suggesting this interaction may be regulated by the protein dense local environment of the focal adhesion [54]. The potential ability of the FAT domain to participate in FAK dimerization is clearly important, but the exact residues responsible, and the precise regulation of this interaction are unclear. Furthermore, the effect of the kinase-FAT linker has been understudied when attempting to understand this interaction due to the experiments utilizing only the FAT domain without the linker.

FOCAL ADHESION KINASE RELATED NON-KINASE (FRNK) IS AN ENDOGENOUS INHIBITOR OF FAK.

FRNK was discovered almost immediately after the related protein FAK was identified. Within a year after FAK was discovered in 1992, FRNK was discovered to be an autonomously expressed truncated form of FAK consisting of the FAT domain and the kinase-FAT linker [9]. FRNK expression is driven by an alternative intronic promoter located in a FAK intron [15]. FRNK was originally shown to localize to focal adhesions and was phosphorylated on unknown residues when cells were plated on fibronectin [9]. In the same paper in which it was identified, FRNK was hypothesized to function as a regulator of FAK kinase activity [9] which was later described in detail by Parsons, Hildebrand, and Schaller as “The ability of p41FRNK and pmK454R, both of which are catalytically inactive, to localize to focal adhesions raises the possibility that either protein may function in dominant-negative fashion by occupying all of the potential binding sites for enzymatically active pp125FAK.”[62] Thus, in the same year it was discovered, FRNK was hypothesized to function as a dominant-negative inhibitor of FAK

which was later echoed by experiments showing FRNK overexpression reduced FAK Y397 phosphorylation, reduced phosphorylation of other focal adhesion proteins such as paxillin, and slowed the formation of focal adhesions [74]. Further in support of the dominant-negative hypothesis was the observation that overexpression of FAK in FRNK expressing cells reversed the inhibition [74]. FRNK was found to be phosphorylated on the same residues as FAK and also once phosphorylated FRNK is able to bind to the same proteins that bind to the corresponding residues on FAK, such as p130CAS after tyrosine 861 phosphorylation [56,75]. Thus, in addition to displacing FAK from focal adhesions by occupying FAT domain binding sites, FRNK is also able to bind to the proteins that scaffold to FAK. It was shown that the ability of FRNK to bind to FAK scaffolding proteins, in particular p130CAS, is essential for maximal inhibition of FAK migration signaling [76]. Thus there are at least two mechanisms of FRNK inhibition of FAK: 1. FRNK displacement of FAK from focal adhesions in a dominant-negative fashion and 2. FRNK binding to FAK binding partners such as p130CAS, preventing binding to active FAK signaling complexes.

FRNK OVEREXPRESSION CAUSES DIFFERENT EFFECTS IN DIFFERENT CELL TYPES.

The exogenous expression of FRNK has many different effects depending on the cell type it is expressed in, the concentration of FRNK, and various other factors. Original observations of FRNK in 1993 in chicken embryo cells suggested that overexpression of FRNK did not cause any abnormal phenotype [62]. However, it was shown in 1998 that overexpression of FRNK can cause cell detachment and death of breast cancer, melanoma, squamous cells carcinoma, and other cells [77-79]. Furthermore, it was shown that FRNK can induce apoptosis through a death receptor mediated pathway in human breast cancer cells [80]. It is also possible that FRNK can induce cell death simply by

causing the withdrawal of pro-growth signals usually sent through FAK-Src-ERK signaling pathways. For example, overexpression of FRNK was able to decrease ERK1/2 phosphorylation and inhibit protein synthesis in vascular smooth muscle cells [81]. It was shown in rabbit synovial fibroblasts that FRNK overexpression could induce cell death by inhibiting two different pathways: 1. An adhesion dependent FAK-Src-P130CAS complex that activates JNK and 2. An adhesion independent pathway that can be activated by serum which activates PI3K and ultimately PKB [82]. Additionally, the FAT domain was compared to FRNK in order to highlight the ability of the kinase-FAT linker, specifically the proline rich region and tyrosine 168 required to bind p130CAS, to protect against apoptosis [82]. Overexpressing the FAT domain induced apoptosis in 80% of the cells whereas overexpression of similar levels of FRNK induced apoptosis in only 30% of cells [82]. Thus FRNK may be involved in other signaling pathways besides only inhibiting FAK. The most striking example of FRNK having different effects on cell types is an experiment comparing 3Y1-v-H-Ras cancer cells (this specific cell line has overactive Ras signaling) expressing vector or FRNK. The cancer cells expressing FRNK were more than twice as invasive as the vector only control, surprisingly showing FRNK overexpression could increase migration in this specific cell type [83]. In the present study I utilize vascular smooth muscle cells in order to recapitulate as much as possible the environment of a re-stenotic artery. The differences in FAK and FRNK function in different cell types should be considered when discussing the results of any experiment involving FAK and FRNK.

FRNK AS A SIGNALING PROTEIN WITHOUT FAK.

FRNK has been implicated in FAK independent signaling. FRNK has most often been used in studies for the sole purpose of inhibiting FAK or determining the importance of the FAK C-terminal domain. More recently, studies have investigated the ability of FRNK to act as an endogenous regulator of cell signaling. For example, experiments in human umbilical vein endothelial cells (HUVECs) showed that endogenous FRNK expression can reduce IL-4 mediated cell adhesion [84]. Furthermore, knockdown of both FAK and the related protein PYK2 did not affect this FRNK signaling pathway which suggests FRNK may perform this function independent of FAK [84].

FAK AND FRNK SERINE 910 AND 217 PHOSPHORYLATION.

FAK and FRNK are phosphorylated on serine residues 910 and 217 respectively. In the present study, I find a dramatic phenotype when mutating FRNK serine 217 to alanine and thus the current information available about this phosphorylation site will be discussed. FAK and FRNK serine 910 and 217 phosphorylation were first identified in 2001 and were shown to be increased during mitosis [85]. It was then discovered that Serine 910 phosphorylation was regulated independently from FAK tyrosine 397 phosphorylation through ERK [72,86]. For example phosphorylation of serine 910 can be achieved with PDGF stimulation even while the cytoskeleton and focal adhesions are disrupted with drugs such as cytochalasin D [86]. A FAK mutant with serine 910 mutated to alanine resulted in increased binding to paxillin as determined by co-immunoprecipitation and fluorescence recovery after photobleaching (FRAP) [87]. The same FAK serine 910 to alanine mutant was also shown in overactive Ras cancer cells to abolish Pin1 and PTP-PEST binding which they showed was able to decrease FAK

tyrosine 397 phosphorylation and promote metastasis [83]. The S910 residue is located near the H1 alpha helix (a helix that has been proposed to swing out of the FAT domain four helix bundle). Studies on mutants that have an increased ability to cause swinging out of the H1 helix show increased S910 phosphorylation by ERK, however, the same mutant also shows decreased paxillin binding[72]. The complex nature of focal adhesions makes the cause and effect difficult to assess in some cases. For example, the increased ability of the FAK-S910A mutant to bind to paxillin may be a downstream effect of PTP-PEST dephosphorylation of focal adhesion components that changes the local environment leading to an increased affinity of FAK for paxillin. It is also possible that the FAK S910A mutant simply has a different conformation that improves both PTP-PEST binding and paxillin binding. Regardless of the mechanism, the ability of non-phosphorylatable FAK 910 mutants to alter migration and other physiologic parameters like sarcomere organization[87] highlights the importance of this phosphorylation site. However, despite the importance of this site in FAK, no studies have been published on the significance of the corresponding site in FRNK, serine 217.

FAK IS DEGRADED BY CASPASE GENERATING FRNK-LIKE PROTEINS.

FRNK consists of the same amino acid sequence as truncated FAK and functions as an inhibitor of FAK, however FAK itself can be degraded generating proteins similar to FRNK. The presence of these FAK degradation products is of great interest because they are seen frequently on western blots in the present study when looking for FRNK, and are a potential confounding variable in experiments where FRNK-FAK interaction is measured. Experiments where caspase enzymes were activated by pro-apoptotic factors such as Apo-2L induce FAK cleavage over a 24 hour period [88]. FAK was cleaved

predominantly into 85KD and 77KD fragments and was shown to be cleaved by multiple caspase enzymes. However, the smaller cleavage products were overlooked until a year later when researchers examined the entire length of a western blot[89]. They found that FAK is able to be cleaved into the original 85 and 77 KD fragments, but also a 35 and 40 KD fragment. Furthermore, they identified the cleavage sites at residues D704 and D772 located in the kinase-FAT linker. Thus, cleavage of FAK by caspases can generate two FRNK like species: one that contains a large portion of the kinase-FAT linker and the FAT domain, and one that contains predominantly the FAT domain (but still contains S910 and is missing one of the proline rich regions). Additionally, FRNK contains both of these caspase cleavage sites, although they did not investigate the ability of FRNK to be cleaved. Thus, in response to apoptotic stimuli FAK signaling is inhibited via the destruction of full length FAK and the introduction of FRNK-like degradation products that inhibit FAK signaling with a mechanism presumed to be similar to FRNK.

PYK2: ANOTHER FAK FAMILY PROTEIN TYROSINE KINASE.

Protein tyrosine kinase 2 (PYK2) also known as related adhesion focal tyrosine kinase (RAFTK) was identified in 1995 as an additional member of the FAK family [90]. PYK2 shares the same three main domains of FAK, but is regulated by calcium and exhibits a more cytoplasmic localization compared to the focal adhesion pattern of FAK[91]. PYK2 is expressed in vascular smooth muscle cells and has been shown to be an important factor in regulating smooth muscle cell contraction [92]. However, PYK2 seems to be less important than FAK in regulating vascular smooth muscle cell invasion in response to injury, despite also being inhibited by FRNK, [93] and so I have chosen to focus on only FAK in the present study.

FAK AND FRNK IN DISEASE STATES.

Both FAK and FRNK expression are altered in various disease states, wherein they contribute to aberrant cell signaling. FAK expression has been linked to several human cancers by multiple papers shortly after its identification in 1992 [94-96]. Furthermore, cancer research indicated that integrin and FAK signaling were linked to extracellular regulated kinase (ERK) signaling [97]. By the early 2000's FAK had become a hot area of cancer research because of its involvement with many signaling pathways important in cancer that regulated not only growth and survival, but also migration and invasion of metastatic cancer cells [98]. Furthermore, FAK had been shown to be involved in cancers of many different tissues including: head and neck, thyroid, prostate, breast, colon, and brain [98]. FAK was shown to be important in primary tumor cells, but also FAK was identified as having an important role in angiogenesis [99]. The involvement of FAK in cancer would drive research to identify the molecular mechanisms of FAK function.

Because of the importance of FAK overexpression in many cancers, FAK inhibitors have been extensively studied. The first small molecule inhibitor of FAK was generated in 2007 and called PF-573,228 [100]. This molecule was designed with high throughput screening and rational design and inhibits the FAK kinase domain catalytic activity. This compound is highly effective at reducing FAK tyrosine 397 phosphorylation status and slows focal adhesion turnover. Treatment with PF-573,228 was also able to reduce cell growth and migration, but failed to induce apoptosis. Despite being the first FAK specific inhibitor, PF-573,228 remains in pre-clinical trials for cancer therapy [101]. However, 4 additional FAK inhibitors have entered phase I clinical trials. PF-562,271 was found safe in clinical trials, but was abandoned because it showed undesirable

pharmacokinetics [102]. Several other compounds are in ongoing clinical trials as of November 2016. In the present study I utilize PF-573,228 in experiments, additionally, FAK inhibitors could provide a starting point for drugs that target FRNK or be used in combination with future FRNK specific treatments.

FRNK IS A POTENTIAL DRUG TARGET IN VASCULAR INJURY AND IN THE PREVENTION OF RE-STENOSIS.

FRNK is of specific interest in vascular smooth muscle cells, especially following vascular injury and its associated neointimal formation. While FAK is expressed in most adult cells, FRNK expression is limited to developing tissues and only some subsets of adult cells. In rats, FRNK was detected mostly in the aorta and lungs and in situ hybridization assays determined that FRNK is expressed in the media of arteries, but not in the endothelial cell layer [103,104]. There have been some reports of endogenous FRNK expression in endothelial cells, but they did not use adult tissue [84]. The endothelial cell layer is responsible for hemostasis of the stented artery and current drug eluting stents prevent healing of the endotheial cell layer leading to increased in-stent thrombosis[105]. A drug eluting stent using a FRNK specific drug could allow for endothelial cell healing (because FRNK is not expressed in endothelial cells) and thus could potentially avoid the complication of stent thrombosis (and obviate the need for long term dual anti-platelet therapy).

FRNK expression follows a specific temporal pattern after vascular injury, suggesting it is performing a regulatory function. FRNK expression in arteries is relatively low before and up to 7 days following carotid artery injury, is greatly increased at 14 days after injury, and then returns to baseline levels at 21 days after injury [104]. Thus

FRNK is specifically upregulated after injury, making it a unique marker of vascular injury. FRNK knockout mice exhibited higher levels of FAK tyrosine 397 phosphorylation, increased BrdU incorporation, decreased smooth muscle cell markers, and drastic loss of smoothelin-B, a smooth muscle cell marker compared to control mice [103]. These changes suggest FRNK expression is essential for proper smooth muscle cell differentiation. Additional experiments showed that the FRNK that is upregulated 14 days after vascular injury is phosphorylated on both tyrosine 861 and 925 and that phosphorylation of FRNK at tyrosine 861 is required for inhibition of cell migration [106]. Furthermore, when the FRNK knockout mice were subjected to carotid artery injury they failed to induce smooth muscle cell markers such as smoothelin-B and alpha-smooth muscle cell actin that were upregulated by the control mice, suggesting FRNK plays an important role in vascular smooth muscle cell healing response following injury [103]. However, the FRNK knockout mice exhibited the same degree of neointima hyperplasia as control mice [103]. However, uninjured arteries in the FRNK knock out mice were identical to control mice, suggesting FRNK may only have important functions in response to injury.

As indicated above, FRNK is upregulated in vascular smooth muscle cells following mechanical injury, where the cells have switched from a quiescent and contractile phenotype towards a growing and migratory phenotype. Upregulation of FRNK during this pathologic event may be related to a phenotypic switch towards a more developmental gene program. FRNK's ability to inhibit FAK signaling and FRNK's expression which is limited to injured adult tissues makes it an attractive drug target for drugs that would benefit from targeting to pathologic tissues. It is the goal of this study

to better understand the poorly defined molecular mechanisms of FRNK in order to enable rational drug design targeting FRNK.

Potential uses for a FRNK specific drug could include coronary drug eluting stents. Coronary stents are used during percutaneous coronary intervention (PCI) to treat coronary artery disease. The procedure involves the insertion of a catheter in to the site of vessel narrowing, inflation of a balloon to expand the collapsed stent to the vessel lumen, and retraction of the catheter[107]. The resulting vessel now contains a patent lumen with the atherosclerotic plaque compressed between the stent and the vessel. Stenting is an attractive procedure because it is fast, minimally invasive, has quick recovery times, and is highly successful at returning blood flow to heart tissues[107]. However, stenting has some notable complications, including restenosis[108]. Cells injured during the stent process have the potential to exhibit aberrant growth and can grow through and around the stent leading to the formation of a neointima: an additional layer of cells made of predominantly smooth muscle cells. The development of a neointima following stenting is the currently the most common pathology by which restenosis occurs [108]. Restenosis can manifest as increasing angina or as a myocardial infarction and requires repeat stenting or bypass surgery[108]. In order to decrease rates of restenosis which range from 17-41%[108,109] for bare metal stents, drug eluting stents were developed.

Drug eluting stents are impregnated with compounds that reduce cell growth and current generation drug eluting stents are effective at reducing rates of restenosis making them the preferred stent in most cases. The addition of modern drug eluting

stents utilizing drugs such as everolimus and new polymers have reduced rates of restenosis to less than 10% [108]. However, the non-specific inhibition of cell growth caused by drug eluting stents also lead to an increase in another complication, in-stent thrombosis[105]. In stent thrombosis is the rapid formation of a blood clot in the stent and surrounding vessel that completely blocks blood flow, and can result in a myocardial infarction. In order to reduce the incidence of stent thrombosis, patients receiving a drug eluting stent are started on dual anti-platelet therapy, typically consisting of aspirin and clopidogrel for 12 months following stent placement [105,110]. The best treatment course of dual antiplatelet therapy remains a hot topic of research and debate[111-114] highlighting the importance of this aspect of drug eluting stents. Thus, current second generation drug eluting stents are effective and have acceptable rates of restenosis and in-stent thrombosis. However, stenting is currently one of the most common procedures performed in the United States[105] and further reduction of the rates of restenosis and in-stent thrombosis with new drug eluting stents should be attempted[108]. A review of in-stent restenosis published in October 2016 concluded by saying, "Restenosis represents still "the present" and not "the past" of interventional cardiology. Specifically, ISR is the most diffuse and important form of restenosis, a still open challenge also in the DES era."[108] FRNK is ideally positioned as a drug eluting stent target because of its specific expression in injured vascular smooth muscle cells, the pathologic cell type in restenosis, and is not expressed in vascular endothelial cells allowing for proper re-endothelialization of the artery theoretically preventing thrombosis. In addition, FRNK is a known potent inhibitor of both FAK signaling growth

and migration pathways which are required for neointimal formation. Thus, further investigation into the mechanism of FRNK function could lead to the development of an ideal drug eluting stent.

FRNK IN OTHER DISEASES.

In addition to coronary stenting, FRNK is has been shown to be involved in a number of other pathologies. In particular, research has shown that FRNK levels are correlated with pulmonary fibrosis in human patients[115]. Ding, Qiang et. al. showed in fibroblasts that loss of FRNK expression leads to increased growth and migration similar to that seen in vascular smooth muscles causing restenosis. They hypothesized and showed evidence that FRNK expression represses the pro-fibrotic response, and that loss of FRNK inhibition leads to pulmonary fibrosis. FRNK may be involved in many different pathologies involving non-cancerous abnormal growth and migration of cells, and therefore better understanding of its molecular mechanisms warrants further investigation in order to better understand these pathologies as well as enable rational drug design.

CHAPTER TWO

MATERIALS AND METHODS

CELL CULTURE MATERIALS AND REAGENTS.

Tissue culture dishes were purchased from from Nunc (Naperville, IL). TIRF imaging was done in Lab-Tek #1 borosilicate glass chamber slides (New York, NY). Dulbecco's Modified Eagle Medium (DMEM) was from Life Technologies (Grand Island, NY). Heat-inactivated fetal bovine serum (FBS) was from Hyclone Laboratories (Logan, UT).

ANTIBODIES AND FACS REAGENTS.

Rabbit polyclonal anti-FAK (P-Y397) phosphospecific polyclonal antibodies and anti-FAK (P-S910) were from Thermo-Fisher (Camarillo, CA). GFP antibody was from Stressgen (Ann Arbor, MI). N-terminal FAK antibody was from Millipore (Darmstadt, Germany). C-terminal FAK/FRNK antibody (used in kinase inhibitor experiments) was from Santa Cruz (Dallas, TX), or Millipore (all other experiments). GAPDH antibody was from Novus Biologicals (Littleton, CO). Anti-FLAG rabbit antibody was from Sigma (St. Louis, MO). Rainbow molecular weight standards and Enhanced Chemiluminescence (ECL) kits were from Amersham (Arlington Heights, IL). DAPI was from ThermoFisher (Camarillo, CA) and used at 300nM concentration. Annexin V conjugated to Alexfluor 488 and PI were obtained in a "Alex Fluor 488 Annexin V /dead cell apoptosis kit" (REF V13241) from Life technologies (Eugene, Oregon). All other reagents were of the highest grade commercially available and were obtained from Sigma Chemical (St. Louis,

MO). Secondary antibodies were either IR dye 800CW goat anti-rabbit (Li-Cor) or Bio-Rad Goat anti Rabbit conjugated to HRP for ECL.

KINASE INHIBITORS.

GF 109203X (GFX) at 10µM, PD98059 (PD98) at 30µM, SP600125 (SP600) at 40µM, Y-27632 (Y27) at 10µM, SB 203580 (SB203) at 10µM, U0126 at 10µM, and Angiotensin II (AT II) at 1µM. All inhibitors were purchased from calbiochem (a subsidiary of Millipore) except for AT II, which was purchased from Sigma. PD98059 (PD98) and U0126 was used to inhibit ERK1/2, GF 109203X (GFX) to inhibit PKCs, SP600125 (SP600) to inhibit JNKs, Y-27632 (Y27) to inhibit Rho kinases, and SB203580 to inhibit p38MAPK.

SDS-PAGE AND WESTERN BLOTTING SUPPLIES.

Rainbow molecular weight standards and enhanced chemiliminesence (ECL) kits were from Amersham (Arlington Heights, IL) and were used for film based detection prior to switching over to Li-Cor based infra-red antibody detection and quantification in 2016. Li-Cor Odyssey machine with secondary fluorescent secondary antibodies IRdye 680RD anti-mouse and IRdye800CW anti-rabbit instead of ECL. SDS-PAGE was performed using Hoeffer vertical gels consisting of 10% acrylamide for the separating layer and 5% acrylamide for the stacking layer. All other reagents were of the highest grade commercially available and were obtained from Sigma chemical (St. Louis, MO).

RAT CAROTID ARTERY INJURY MODEL.

The Institutional Animal Care and Use Committee of Loyola University Medical Center approved all procedures involving animals, which were handled in accordance with the American Physiological Society's "Guiding Principles in the Care and Use of Animals." The carotid arteries used in these experiments were obtained from surgeries

performed by Y. Koshman and A. Samarel. Briefly, balloon injury of the right common carotid artery was accomplished using a 2.5F double-lumen balloon catheter (Tyshak Mini Pediatric Valvuloplasty Catheter; NuMED, Inc., Hopkinton, NY), as previously described [106]. Unininjured contralateral arteries served as paired controls. Tissues were homogenized in 1ml of Tony Hunter's lysis buffer using a polytron tissue homogenizer fitted with a microgenerator. Tissue samples were used for SDS-PAGE experiments. Western blot was performed with a total FAK/FRNK antibody to an immunogen of residues 748-1053 of human FAK. Identically prepared blots were probed with a phospho-specific FAK S910 antibody to determine the relative phosphorylation of S910 on FAK and S217 on FRNK before and after injury. The carotid arteries used were obtained from surgeries performed by Y. Koshman and A. Samarel.

RAT VASCULAR SMOOTH MUSCLE CELLS.

Adult rat aortic smooth muscle cells (RASMs) were isolated by Dr. Y. Koshman as previously described and frozen at -80 until use. Once thawed, cells were maintained in DMEM containing 10% FBS. Cells up to the 22nd passage were used. A7r5 cells (a rat embryonic vascular smooth muscle cell line) were obtained from Dr. Kenneth Byron, Loyola University Chicago and were used up to the 16th passage.

ADENOVIRAL CONSTRUCTS.

Replication-defective adenoviruses (Adv) expressing GFP, GFP-wild-type (wt) FRNK, nuclear-encoded β-galactosidase (βgal), FLAG-FAK and FLAG-WT-FRNK were generated as previously described [116]. Cerulean (CER) wtFRNK, mCherry-WT-FRNK, YFP-FAK, cyan fluorescent protein (CFP) S217A-FRNK and mCherry-S217D-FRNK were generated by subcloning WT or mutant mouse constructs (generated by site-specific

mutagenesis) into the appropriate CER, CFP, YFP, or mCherry expression vectors, and Adv constructs were then generated using the AdEasy XL system. The multiplicity of viral infection (moi) was determined by dilution assay in HEK293 cells grown in 96-well clusters. Additionally, for experiments calling for equal expression of wild type and mutant FRNK constructs, a standard curve was established using western blots in order to normalize the amount of virus required to create equal levels of protein expression (Figure 3). All adenoviruses were constructed in the Samarel lab by Mr. Taehoon Kim, except for mCherry-FRNK (Jody Martin), CER-FRNK (T. Zak did made the plasmid and T. Kim the virus), YFP-FAK (T. Zak made the plasmid and T. Kim made the virus), and a re-amplification of the CFP-S910A-FRNK virus done by J. Martin.

PHOSPHO-SPECIFIC ANTIBODY FAK TYROSINE 397 ASSAY.

RASMs were plated onto 10cm plates and allowed to adhere for 24h. Cells were then infected with increasing amounts of mCherry-wtFRNK or mutant CFP-S217A-FRNK in order to establish a broad range of FRNK expression. Cells were scraped into cell lysis buffer[76] containing Pefabloc (100nM), vanadate (1mM), leupeptin (10ug/1ml), and aprotinin (10ug/1ml) and then subjected to Western blotting using total FAK and phosphospecific FAK Y397 antibodies. GAPDH was used as a loading control. The resulting densities of FRNK and FAK Y397 were plotted against each other for both the WT and mutant FRNK in order to compare relative potency. Only the highest molecular weight total FRNK band was used for the quantification of total FRNK in order to avoid quantifying differences in mutant degradation. All comparisons were done on samples within the same gel, in order to ensure accuracy.

DAPI NUCLEI STAINING AND COUNTING.

RASMs were counted and placed into 2ml micro centrifuge tubes where they were infected with equal amounts of GFP, CER-FRNK, CFP-S217A-FRNK, or no adenovirus. To ensure equal amounts of wt and S217A FRNK expression, a Western blot was used to obtain the correct ratio of each virus to ensure equal FRNK expression (Figure 3). Cells were then plated into chamber slides and allowed to grow for 48h. Cells were then fixed in 4% paraformaldehyde and stained with 10μg/ml DAPI. Cells were imaged using the same Nikon system used in acceptor photobleaching experiments and DAPI-positive nuclei counted for 3 high powered fields per well. Four separate infections for each condition were prepared on the same day, for a total of 16 wells, yielding 48 images that were hand counted for DAPI positive nuclei.

FLOW CYTOMETRY APOPTOSIS ASSAY.

Cells were prepared the same as in the DAPI nuclei assay and infected with equal amounts of CER-FRNK, CFP-S217A-FRNK, control βgal or no adenovirus. To ensure equal amounts of wt and S217A FRNK expression, a Western blot was used to obtain the correct ratio of each virus to ensure equal FRNK expression (Figure 3). After 48h of infection both attached and detached cells were placed in a 2ml centrifuge tube and

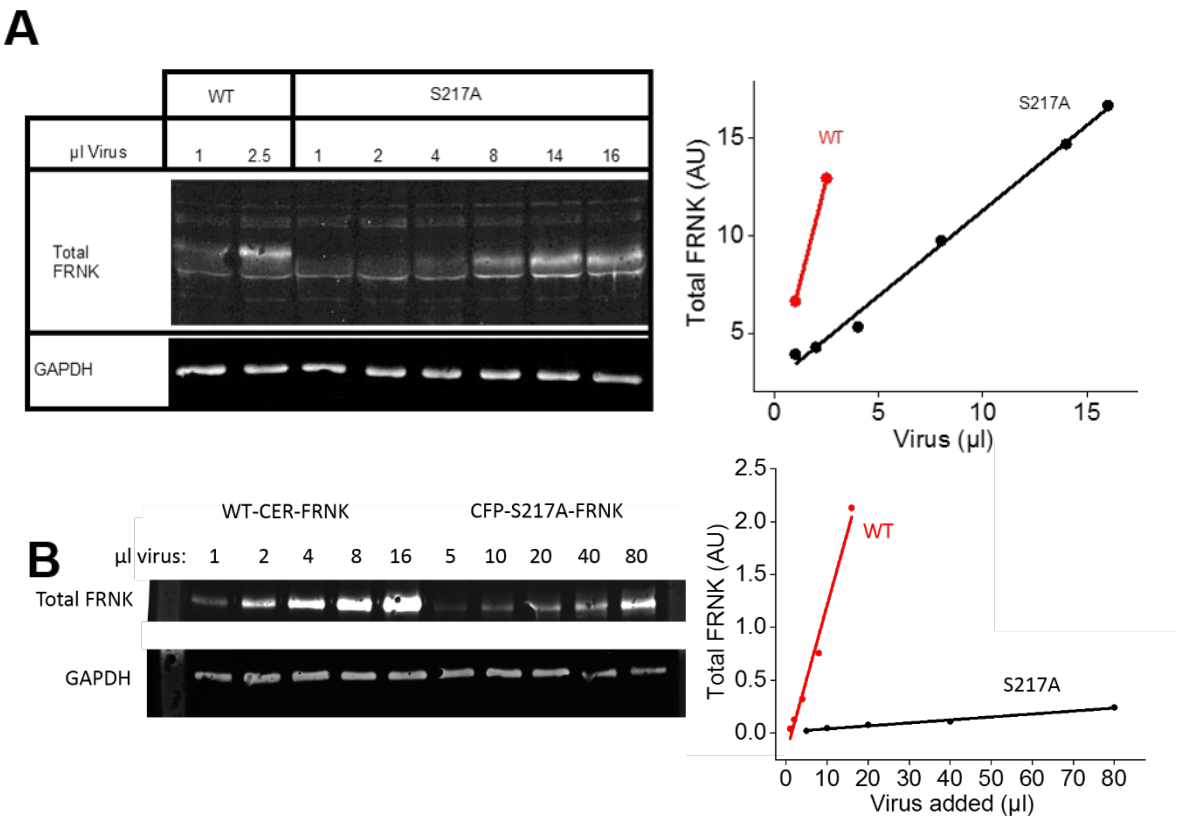

Figure 3. Adenovirus balancing by Western blot
(A) A comparison of CER-WT- and CFP-S217A-FRNK expression was performed to achieve balanced expression of WT- and S217A-FRNK. The total amount of exogenous protein increases linearly with the volume of virus added to cells. The Western blot was used to calculate the relative amount of WT- and S217A-FRNK adenovirus required for equal expression of protein. A ratio of 1:4 WT- to S217A-FRNK was used to express equal amounts of exogenous protein.
(B) A repeat of the same experiment with an additional preparation of S217A virus.

stained with annexin V conjugated to Alexfluor 488 to mark apoptotic cells and counterstained with propidium iodide (PI) as a viability marker (Thermo Fisher). Data were acquired using a BD FACSAria IIIu using BD FACSDiva v5.0.2 software. The instrument is equipped with a 488 Coherent Sapphire laser for excitation, LP mirror 502 and bandpass filter 530/30 for FITC, and LP566 and bandpass filter 585/42 for PI. Data was analyzed using FlowJo v10.1r7 software. Cell populations were gated on side and

forward scatter and then compared on the basis of annexin V and PI staining. Annexin V negative, PI negative cells were considered viable, Annexin V positive, PI positive cells were considered apoptotic, and double positive cells were considered dead. Flow cytometry analysis and sorting services were performed by the Loyola University Chicago Flow Cytometry (FACS) Core Facility.

KINASE INHIBITOR EXPERIMENTS.

Equal numbers of RASMs were plated onto 10cm plates in 10% serum containing DMEM and infected with 300moi of FLAG-WT-FRNK for 24h. Then each plate was treated with the following kinase inhibitors and agonists at the following concentrations: GF 109203X (GFX) at 10μM, PD98059 (PD98) at 30μM, SP600125 (SP600) at 40μM, Y-27632 (Y27) at 10μM, SB 203580 (SB203) at 10μM, U0126 at 10μM, and Angiotensin II (AT II) at 1μM. All kinase inhibitors were incubated with the cells for 1h and AT II was incubated for 10 min before the cells were harvested in cell lysis buffer. Cell extracts were then sonicated, centrifuged, and the protein containing supernatant fractions collected. Protein samples were then boiled for 10 min and subjected to SDS-PAGE and Western blotting. Two identical gels were prepared - one was probed with a total FAK/FRNK C-terminal antibody, and the other a phospho-specific antibody to FAK/FRNK S910/S217. An uninfected sample was run to rule out any FAK degradation products being identified by the pS910 antibody around the same molecular weight of FLAG-FRNK. For statistical analysis the experiments were repeated 3 times, with 3 separate infections and treatment with inhibitors. The individual samples were normalized to the total signal on each individual gel and the ratio of pS217 / total FRNK

was compared. The uninfected sample was not used in the statistical analysis because there was no signal.

CO-IMMUNOPRECIPITATION.

RASMs or A7r5s expressing GFP-FRNK were grown in 10 cm dishes, scraped, and sonicated in a non-denaturing buffer containing 0.5% Nonidet P-40 and 1% sodium deoxycholate. The sample was centrifuged for 20 min at 50,000xg, and the protein-containing supernatant fraction was collected. Each supernate was immunoprecipitated with 5 μg of the indicated antibodies overnight, complexes were bound to protein A/G agarose or magnetic (Dynabeads) beads for 2h at 4°C, and then washed three times with 1ml of the same non-denaturing buffer. Immune complexes were then removed from protein A/G beads by incubating with 2X SDS-PAGE sample buffer and boiling for 10 min. Eluted protein samples were subjected to SDS-PAGE and Western blotting.

TOTAL INTERNAL REFLECTION FLUORESCENCE MICROSCOPY.

Total Internal Reflection Fluorescence (TIRF) excitation is a useful method to study proteins and biological processes that are near the plasma membrane of cells. TIRF excitation is accomplished by obliquely aiming a laser light beam at a thin piece of glass (such as a cover slip) which contains different refractive indices above and below the glass [117,118]. In a microscope, the light exits the objective traveling at an angle in immersion oil, hits the glass, and then is reflected as it fails to enter the aqueous solution (cell culture media, water, etc.). In order to achieve this reflection, the incident angle must be greater than a critical angle, which is defined by Snell's law [117].

$$\Theta_c = \sin^{-1}(n_1/n_2)$$

Where Θ_c is the critical angle, n_1 is the refractive index of the sample and n_2 is the refractive index of the glass coverslip. In order to increase the angle of incidence past the critical angle in a microscope, a laser can be focused off of the center of the rear lens of the objective (Figure 4). The laser beam hits the glass/sample interface and reflects away from the coverslip creating an electromagnetic field located at the coverslip interface. This field is what is then used to excite fluorophores within the sample [117].

The electromagnetic field created by TIRF is useful for imaging. The electromagnetic field is evanescent, meaning it is non-propagating and decreases in intensity over distance. Specifically, the intensity of the field decreases as it travels perpendicular to the coverslip, i.e. as the light travels towards the sample. The decrease in intensity is exponentially related to the distance traveled and can also be further decreased by increasing the incident angle further past the critical angle [117]. The intensity of the evanescent field at any distance from the coverslip can be calculated by the equation:

$$I(z) = I(o)^{-z/d}$$

Where I(z) is the intensity at the distance z away from the coverslip, I(o) is the intensity at the coverslip, and d is a characteristic penetration depth where the intensity has

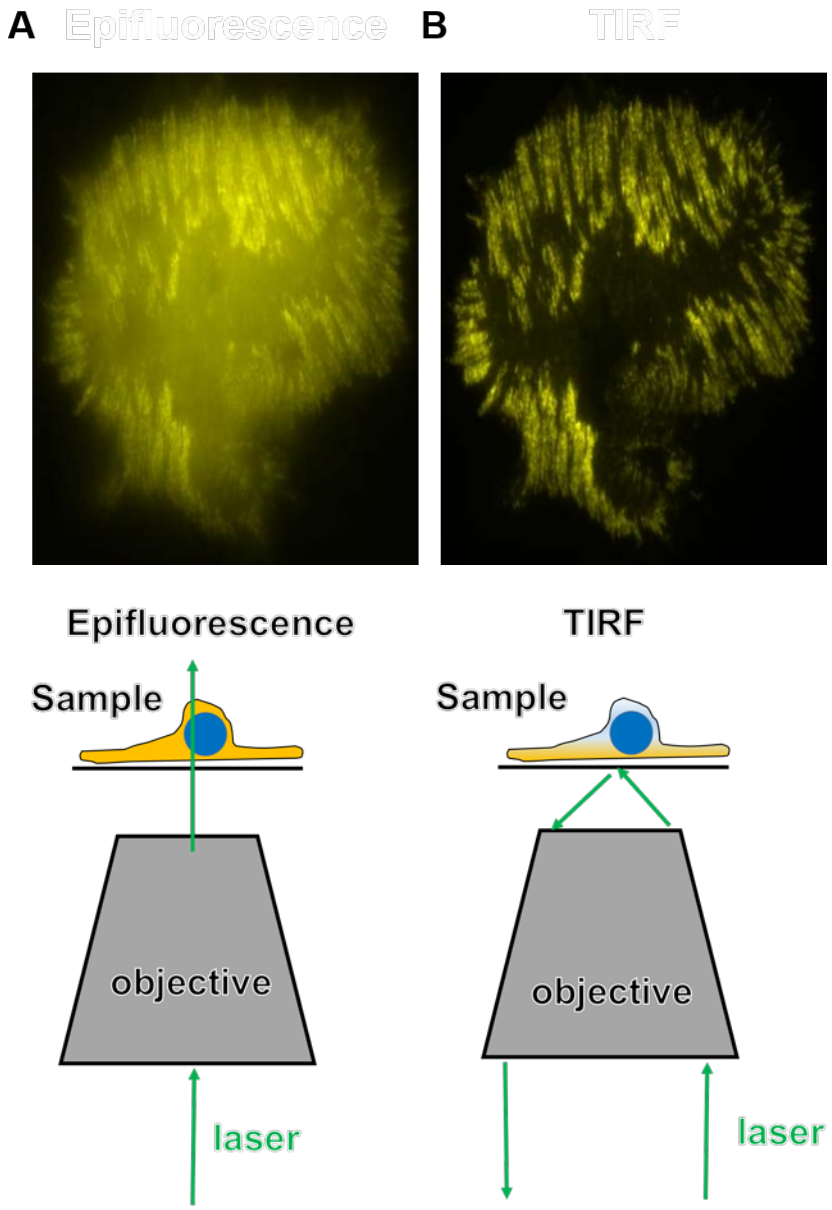

Figure 4. Diagram of TIRF Microscopy
(A) Example of a cell imaged with epifluorescence excitation expressing YFP-FAK. The cell shows localization of YFP-FAK to the cytoplasm as well as some harder to see focal adhesions.
(B) Example of a cell imaged with TIRF excitation method expressing YFP-FAK. The cell shows only focal adhesion localized YFP-FAK, which can be seen clearly now that the cytoplasmic localized proteins are no longer excited.

decreased by 37% which depends on the angle of the laser and the wavelength of

excitation light. For CFP (442 excitation wavelength) and using water and immersion oil (refractive index 1.515) the penetration depth variable can vary from 290 to 50 depending on the incident angle used. Using the lowest incident angle (the critical angle), and therefore the highest penetration depth (d)=290, the intensity of excitation 200nm away from the coverslip would be at 50% of the intensity at the coverslip and the intensity of excitation would reach 10% at 668 nm away from the coverslip. However, using the highest incident angle (limited by the numerical aperture of the objective) and therefore lowest penetration depth (d)=50, the intensity of excitation 200nm away from the coverslip would be only 2%. Thus, during an experiment the evanescent field can be rapidly manipulated and changed to select fluorophores from around 300nm to as close as 100nm, and avoids significantly exciting fluorophores further away. Thus the main advantage of TIRF is the ability to selectively excite fluorophores conjugated to proteins localized near the coverslip, such as integrins (0-20nm) and focal adhesions (20-70nm) [119]. Thus, TIRF is ideal for studying active focal adhesion proteins such as FAK and FRNK which have an active population located within focal adhesions, and an inactive population located in the cytoplasm (Figure 4).

TIRF excitation does have some drawbacks. The emission from TIRF excitation is often lower in intensity in comparison to epi-fluorescent excited samples simply because of the low total number of fluorophores being excited. Thus, sensitive cameras such as election multiplying charge coupled devices (EMCCDs), are required to efficiently image TIRF excited cells. Additionally, TIRF excitation through the objective requires an objective with a large numerical aperture (in order to angle the beam past

the critical angle), although this can be avoided by instead using an optical prism to angle the laser beam. Last, because the penetration depth of the evanescent field is dependent on the angle of incidence and the exact refractive index of the sample media, it can be difficult to obtain the exact same excitation intensity in experiments performed on different days. This makes experiments such as acceptor sensitization FRET[120] more difficult, as slight changes in intensity make obtaining valid constants to be used day to day in the 3-cube equation difficult.

FLUORESCENCE MICROSCOPY.

Fluorescence microscopy was performed with a Nikon total internal reflection fluorescence (TIRF) microscope using a 1.49 NA 100X CFI Apochromat objective and through-the-objective illumination with a 445 nm diode laser (for CFP or Cer excitation) or 514 nm Ar laser (for YFP excitation). Fluorescence emission passed through bandpass filters, 472/30 for Cer and 542/27 for YFP, and images were obtained with an emCCD camera (iXon 887).

FAK DISPLACEMENT LINE SCANS.

The fluorescence of FAK and FRNK in the focal adhesions was quantified with ImageJ using a lineout drawn radially from the cell interior toward the periphery passing through the focal adhesion of interest. Regions of the lineout sampling the focal adhesion and the cytoplasm were quantified by subtracting a background value obtained from the region of the lineout outside the cell boundary and the focal adhesion. The resulting background subtracted intensities of the cytoplasm and focal adhesion were then used to obtain a cytoplasm to focal adhesion ratio by dividing the cytoplasmic value by the focal adhesion value. The cells used for these experiment were

the same cells used in acceptor photobleaching experiments, and thus were expressing FAK and FRNK in approximately a 1 to 3 ratio.

FAK AND FRNK SUB-FOCAL ADHESION LOCALIZATION.

RASMs were infected with CER-FRNK and YFP FAK or GFP-FAK and mCherry-FRNK and imaged with TIRF excitation. Line scans were drawn from outside the cell towards the inside and the raw intensity of the fluorescence reported for each wavelength.

ACCEPTOR PHOTOBLEACHING FRET WITH TIRF EXCITATION.

RASMs were infected with CFP-S217A-FRNK or CER-WT-FRNK and YFP-WT-FAK Adv at a 1:3 ratio 24h before imaging in a two-well chamber slide. To ensure equal amounts of WT- and S217A-FRNK expression, a Western blot was used to obtain the correct ratio of each virus to ensure equal FRNK expression (Figure 3). I performed TIRF imaging of CER and YFP at 30 sec intervals to establish a baseline and then initiated progressive acceptor photobleaching [121,122], acquiring successive images of Cer and YFP with TIRF illumination alternated with 30 sec of widefield lamp illumination through a 504/12 nm bandpass filter for selective photobleaching of YFP. The objective of this protocol was to progressively photobleach YFP-FAK acceptors in the entire cell volume while selectively quantifying FRNK fluorescence intensity in the focal adhesions. This is essential because FAK localized to the cytoplasm is in rapid equilibrium with FAK localized to focal adhesions. The resulting sets of images were then analyzed in ImageJ by hand drawing regions around clusters of similar focal adhesions. All images were subjected to a constant background subtraction equal to the intensity of background regions outside the cell boundary. FRET was calculated from the pre- and post-bleach donor fluorescence intensity using the equation:

$$FRET=1-(F_{DA}/F_D)$$

Where F_{DA} = the intensity of the donor before bleaching and FD = the intensity of the donor after bleaching. To determine stoichiometry, the fluorescence of the donor was plotted against the fluorescence of the acceptor at the same time point during progressive bleaching, as shown in previous papers[121].

FLUORESCENCE LIFETIME FRET.

RASMs were infected with GFP-FAK, mCherry-FRNK, and/or FLAG-FAK at a ratio of 1:3 and then subjected to 482/18 nm TIRF illumination from a pulsed (20 MHz) supercontinuum laser (Fianium). TIRF was accomplished by directing the excitation laser beam through a Keplarian telescope and using a prism to achieve total internal reflection excitation of fluorescence in cells adhered to a coverslip optically coupled to the prism with immersion oil [117]. For those experiments, fluorescence emission was collected with a 60X Plan Apo VC water immersion objective. In all lifetime experiments, fluorescence emission was detected with an avalanche photodiode and time-correlated single photon counting (TCSPC) was performed using a Picoquant Picoharp 300 and Symphotime64 software. Donor fluorescence was collected from each cell for at least 1 min or until 500,000 photons were collected. The fluorescence decays were analyzed with a two component model (which best fit the data) and the intensity weighted average lifetime was compared to the intensity weighted average lifetime of a donor-only control. This lifetime was used to calculate FRET efficiency (E) according to the relationship E = 1- (τDA/τD). Where τDA is the lifetime of the donor in the presence of acceptor and τD is the lifetime of a donor only control. In order to obtain the average FRET for all of the samples, FRET for each GFP-FAK + mCherry-FRNK

experiment was fit and an average lifetime obtained. This lifetime was then used to calculate FRET for each cell with the average GFP-FAK only experiment lifetime of 2.617ns used for τD. These FRET values were then averaged together in order to obtain the average FRET.

ANGIOTENSIN II STIMULATION.

RASMs were serum starved overnight (for experiments performed in figures 19 and 20). Identical dishes of RASMs were stimulated with 10μM angiotensin II and then harvested at 0, 15, 30, 45, or 60 min after addition of drug. The FAK inhibitor PF 573,228 was added 1 hour prior to cell harvest without angiotensin II stimulation (P0) or after 10 min of angiotensin II stimulation P10). The harvested protein was sonicated and centrifuged and then subjected to western blot. Phospho-specific S910 antibody was used to assess FAK phosphorylation and phosphospecific ERK antibodies were used to measure ERK phosphorylation/activation. Total FAK and FRNK also examined to ensure equal loading. ERK5 and FAK tyrosine 397 were also examined using phosphospecific antibodies in a separate experiment.

STATISTICAL ANALYSIS.

Values reported here are means ± SEM. Data for multiple groups were compared by 1-way ANOVA followed by Tukey's means comparison. Data for 2 groups were compared by unpaired two tailed t-test. Data for the carotid arteries were compared by paired two tailed t-test. Differences among means were considered significant at $P<0.05$. Data were analyzed using OriginLab software (Northampton, Massachusetts)

CHAPTER THREE

RESULTS

FRNK IS PHOSPHORYLATED ON SERINE 217 IN UNINJURED AND INJURED RAT CAROTID ARTERIES.

FRNK phosphorylation has been understudied compared to FAK phosphorylation. In chapter 1 the ability of phosphorylation to alter the function of FAK and FRNK was discusses in detail. However, the function of the linker region between the kinase and FAT domains, which is present in both FAK and FRNK, contains many phosphorylation sites with both known and unknown function. Some of these phosphorylation sites, such as tyrosine 861 (discussed in section 1.10), have well defined functions and mechanisms by which they regulate cell signaling. Other sites, such as FAK serine 910 have been shown to be important in cell signaling, but the mechanism by which they function is less clear. Furthermore, most of the phosphorylation sites present on FAK have not been studied in the context of FRNK. Given the importance of FAK S910 in FAK signaling[86,87], I hypothesized it may have an important role in FRNK signaling. Furthermore, I hypothesized that the same phosphorylation site on FRNK may have different functions compared to the analogous site on FAK and that differential phosphorylation of FRNK may be involved in FRNK inhibition of FAK.

To begin my investigation, I first confirmed that FRNK is phosphorylated at serine 217 in both injured and control carotid arteries (Figure 5A). Rats were subjected to a

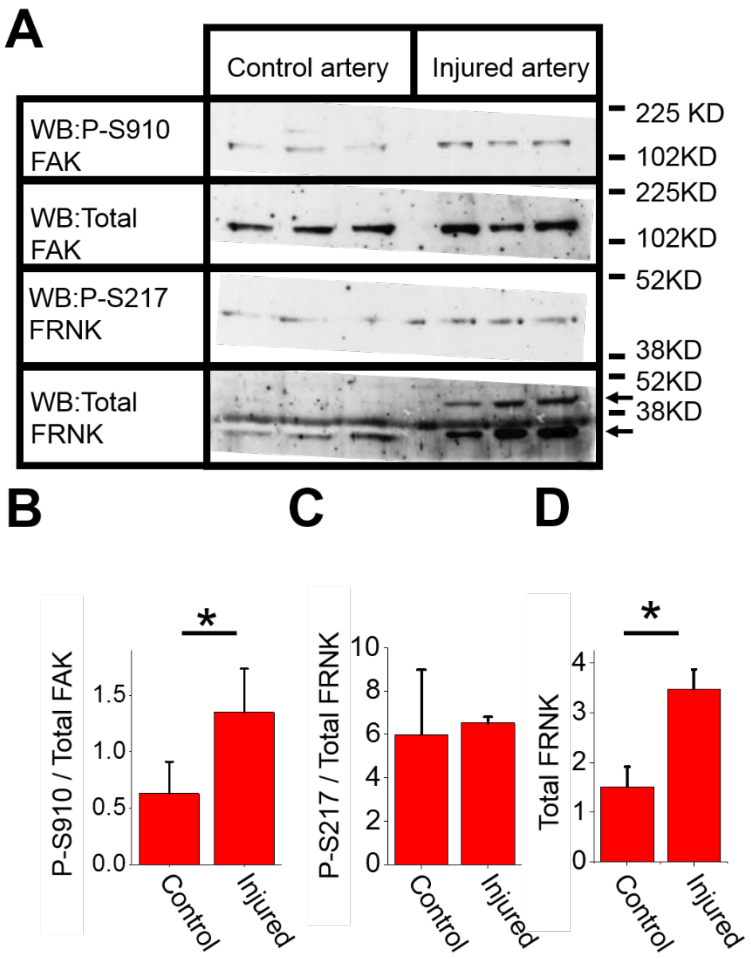

Figure 5. FAK/FRNK Serine 910/217 phosphorylation in carotid arteries
(A) Western blot comparing 50µg samples of control and injured carotid arteries. Replicate blots probed with P-S910 FAK or total FAK antibodies revealed upregulation of FAK and FRNK expression, and increased FAK phosphorylation at S910. Black arrows indicate FRNK 41 43KD doublet thought to be due to serine phosphorylation. (B,C,D) Quantification of (A) for n = 3 paired arteries.

carotid artery injury described previously[106], and then carotid arteries were homogenized two weeks after injury and 50µg of protein was subjected to western blot with phosphospecific antibodies to phospho S217. Contralateral arteries were used as uninjured controls. As expected, the total amount of FRNK increased (Figure 5D). However, the ratio of phosphorylated S217 to total FRNK did not significantly change

(Figure 5C). The ratio of FAK phospho-serine 910 to total FAK did significantly increase in response to injury (Figure 5B). Thus, FAK S910 phosphorylation appears to respond to injury. However, FRNK S217 phosphorylation did not change during injury and instead appears to be highly phosphorylated in both injured and uninjured arteries.

FRNK IS PHOSPHORYLATED ON SERINE 217 IN CULTURED RASMS OVEREXPRESSING WT FRNK.

To determine if FRNK undergoes S217 phosphorylation in cultured RASMs, I infected cells in serum containing media with an adenovirus expressing FLAG-FRNK, and 24 hours later submitted harvested protein to SDS-PAGE and Western blot analysis. As seen in Figure 6A lane 1 (DMSO), the exogenous FRNK was basally phosphorylated on S217.

FRNK S217 PHOSPHORYLATION IS DEPENDENT ON ERK1/2.

Next I wanted to determine the kinase(s) responsible for phosphorylating FRNK on serine 217. To this end, I infected RASMs maintained in serum containing media with FLAG-FRNK and used an array of kinase inhibitors to see which one(s) reduced FRNK phosphorylation at serine 217 (Figure 6 A,B,C,D). I used 30µM PD98059 (PD98) to inhibit ERK1/2, 10µM GF 109203X (GFX) to inhibit PKCs, 40µM SP600125 (SP600) to inhibit JNKs, 10µM Y-27632 (Y27) to inhibit Rho kinases, and 10µM SB203580 to inhibit p38MAPK. The only inhibitor that significantly reduced FRNK serine 217 phosphorylation was PD98, suggesting that ERK phosphorylates FRNK at serine 217. In order to confirm that ERK kinase phosphorylates FRNK at serine 217, I used another ERK inhibitor (U0126)

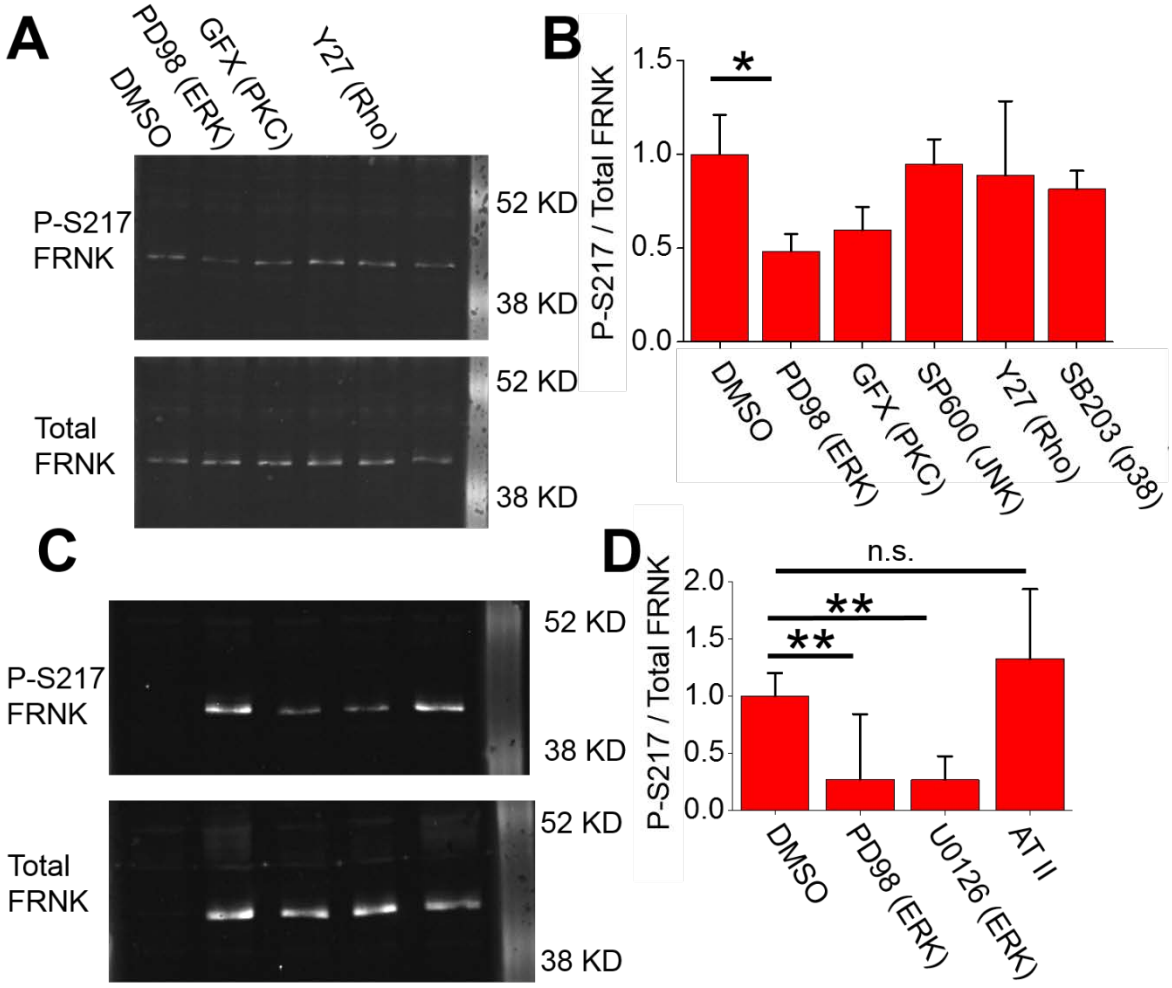

Figure 6. FRNK serine 217 is constitutively phosphorylated by ERK in RASMs
(A) Western blot of RASMs grown in serum containing media treated with various kinase inhibitors probed with P-S217 or total FRNK antibodies. Cells were treated with GF 109203X (GFX) at 10µM, PD98059 (PD98) at 30µM, SP600125 (SP600) at 40µM, Y-27632 (Y27) at 10µM, SB 203580 (SB203) at 10µM, U0126 at 10µM, and Angiotensin II (AT II) at 1µM. **(B)** Quantification of (A) for N = 3 biological replicates revealing that ERK inhibition reduced FRNK S217 phosphorylation. **(C)** Kinase inhibitors reduced FRNK S217 phosphorylation while 10min angiotensin II stimulation resulted in no significant increase in phosphorylation. **(D)** Quantification of (C) for N = 3 biological replicates show FRNK is constitutively phosphorylated at position S217 by ERK.

and also attempted to increase FRNK serine 217 phosphoryation by stimulating cells with angiotensin II (Figure 6C,D). Again, both ERK inhibitors decreased FRNK serine 217 phosphorylation. This result is not surprising considering FAK and FRNK share the same sequence containing the S910/217 site and FAK has been shown to be phosphorylated by ERK at serine 910[86,87]. Additionally, angiotensin II stimulation failed to increase 217 phosphorylation beyond the baseline control, suggesting serine 217 is relatively highly phosphorylated in RASMs stimulated with fetal bovine serum.

FRNK SERINE 217 TO ALANINE MUTANT IS SUPER-INHIBITORY WITH RESPECT TO INHIBITION OF FAK Y397 PHOSPHORYLATION.

Once FRNK serine 217 was established as a phosphorylation site, I next wanted to determine the physiological effects of phosphorylation at this site. Because initial experiments showed that FRNK was highly phosphorylated at serine 217, we generated a non-phosphorylatable mutant by changing serine to alanine. In order to use the mutant construct in fluorescence experiments, we also tagged it with a CFP fluorophore on the N terminus. A well described characteristic of FRNK is the ability of the inhibitor to reduce FAK tyrosine 397 phosphorylation upon overexpression [62]. I expressed increasing amounts of wild type and S217A mutant FRNK and examined FAK tyrosine 397 phosphorylation using a phospho-specific antibody (Figure 7A). Large amounts of wild type FRNK expression was required to achieve near complete de-phosphorylation of FAK tyrosine 397. However, relatively small amounts of S217A mutant FRNK was able to decrease FAK tyrosine 397 to undetectable levels. A direct comparison between

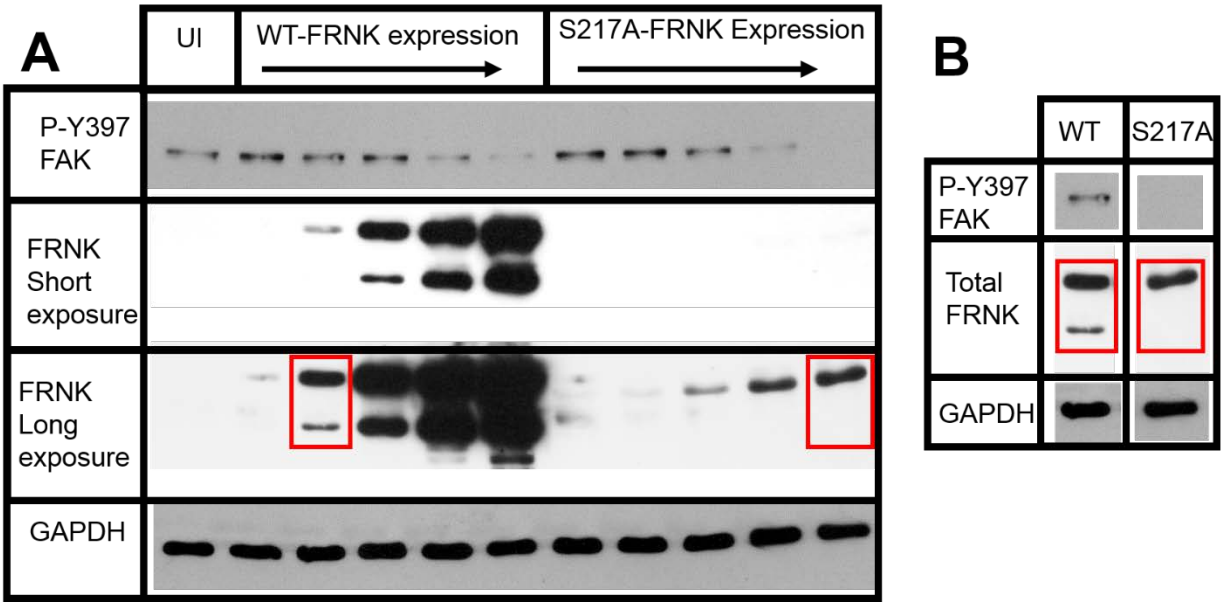

Figure 7. S217A-FRNK is a super inhibitor with respect to FAK Y397
(A) Western Blots of RASMs expressing wild type mCherry-FRNK (WT-FRNK) or mutant CFP-S217A FRNK (S217A-FRNK) were probed with FAK P-Y397 antibodies or total FRNK antibodies. Increasing expression of FRNK resulted in decreased FAK phosphorylation at activation site Y397. Red boxes identify samples with similar expression of FRNK; these lanes are reproduced at right for side-by-side comparison **(B)**. GAPDH was used as a loading control.

similar expression levels of wild type and mutant FRNK highlights the increased potency of non-phosphorylatable FRNK (Figure 7B). In this experiment, I adjusted the titer of the wtFRNK adenovirus to achieve approximately equal levels of expression of wt and S217A FRNK. As is evident from the figure, the resulting level of FAK Y397 phosphorylation was much lower in cells expressing S217A FRNK as compared to wild type. To quantify the increase in potency of the S217A mutant FRNK I plotted total FRNK vs. FAK Y397 phosphorylation for both WT and S217A FRNK (Figure 8). From this plot I fit both data sets to a line and calculated the slope for WT FRNK and S217A FRNK to be -0.134 and -1.44 respectively, yielding over a 10 fold increase in potency for S217A FRNK. Thus,

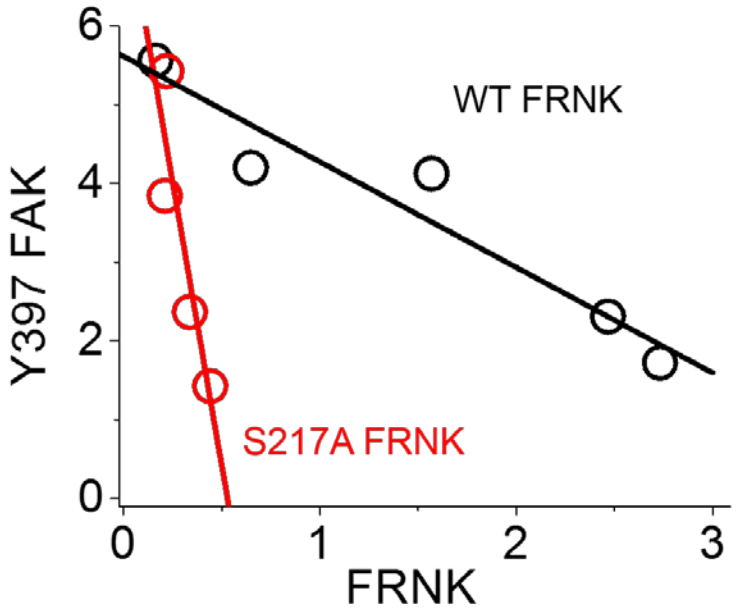

Figure 8. S217A-FRNK is 10 fold more potent with respect to Y397
The relative expression of wild type and S217A FRNK plotted vs. FAK Y397 phosphorylation shows a linear relationship between FRNK expression and Y397 phosphorylation. The slope of each line reflects the relative inhibitory potency of each construct with wild type FRNK having a slope of -0.134 and S910A FRNK having a slope of -1.44. Thus, S217A FRNK exhibits over 10fold more potent inhibition of FAK Y397 phosphorylation.

S217A FRNK is a super-inhibitory form of FRNK with respect to the ability to decrease FAK tyrosine 397 phosphorylation, a critical step in FAK activation.

FRNK SERINE 217 TO ALANINE MUTANT IS SUPER-INHIBITORY WITH RESPECT TO CELL GROWTH AND SURVIVAL.

Because S217A FRNK was a “super inhibitor” of FAK Y397 phosphorylation, I hypothesized that overexpressing it would decrease cell growth, a FAK dependent process. I expressed equal amounts of wild type FRNK or S217A FRNK alone with uninfected cells and cells infected with GFP as negative controls. Forty-eight hours after infection, RASMs were fixed with paraformaldehyde and nuclei were stained with DAPI

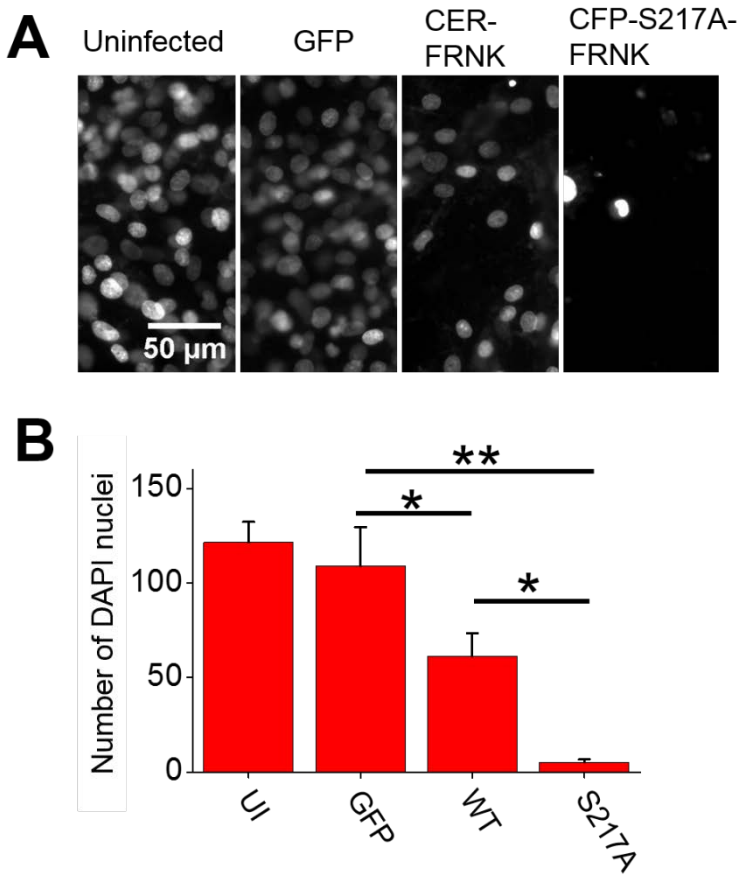

Figure 9. S217A-FRNK is a super inhibitory mutant with respect to apparent cell growth
(A) Fluorescence microscopy of cells infected with a control virus (GFP), WT-FRNK, or S217A-FRNK **(B)** Quantification of A for n=4 biological replicates demonstrated that cell growth and survival was reduced by wild type FRNK and more strongly reduced by S217A-FRNK. Data shown are mean ± SE.

and counted (Figure 9C,D). Expression of wild type FRNK reduced apparent cell growth by 45%, consistent with previous literature describing FRNK as an inhibitor of growth. Additionally, S217A FRNK reduced apparent cell growth by 95% compared to control again demonstrating an increased inhibitory potency compared to wild type. Because the apparent reduction in growth was so great, I hypothesized that mutant FRNK

expression was inducing apoptosis especially because of the ability of FAK to interact with death domain containing proteins[123]. To assess levels of apoptosis I again infected cells with wt or S217A FRNK for 48 hours. At 48 hours cells were stained with annexin V to mark apoptotic cells and counterstained with propidium iodide (PI) to mark dead cells and subjected to flow cytometry (Figure 10). Cells that were positive for annexin V and negative for PI were considered apoptotic cells. Negative controls exhibited 6% and 9% apoptotic cells, while wild type FRNK exhibited 17% apoptotic cells (a significant increase over control). Expression of S217A FRNK resulted in a large increase to 96% apoptotic cells. Thus S217A FRNK is both an inhibitor of FAK signaling and a potent inducer of apoptosis.

S217A-FRNK DOES NOT INCREASE THE DISPLACEMENT OF FAK FROM FOCAL ADHESIONS.

Once S217A-FRNK was identified as a super-inhibitory mutant, I began to investigate the mechanism by which the serine 217 phosphorylation site regulated inhibition of FAK. I initially tested to see if FRNK serine 217 dephosphorylation may increase displacement of FAK from focal adhesions in a dominant-negative fashion, the oldest and most established mechanism of FRNK inhibition of FAK [62]. To assess this

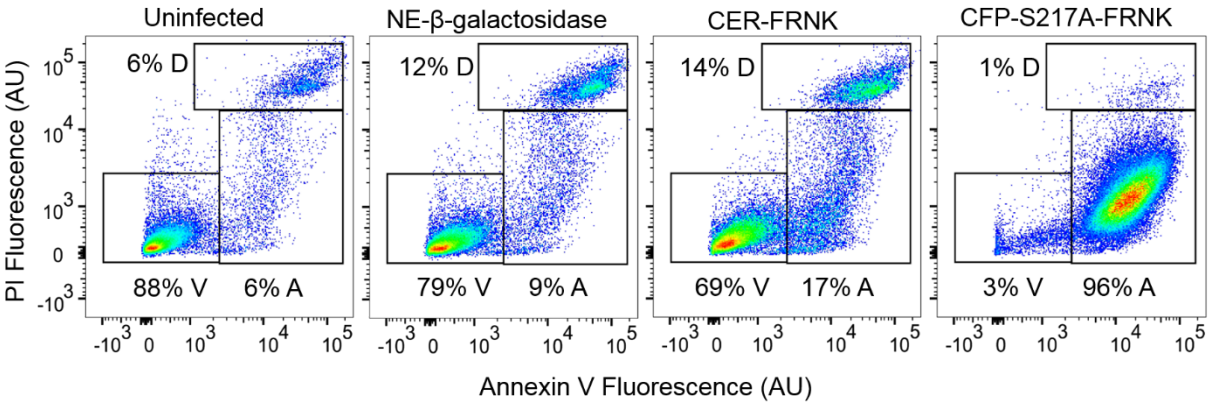

Figure 10. S217A-FRNK is a super inhibitory mutant with respect to inducing apoptosis

RASMs expressing NE-β-galactosidase control, CER-FRNK, or CFP-S217A-FRNK, were subjected to flow cytometry apoptosis analysis. Double negative cells represent viable cells (V). Annexin V positive, PI negative cells represent apoptotic cells (A), and PI positive, Annexin V negative cells represent dead cells (D). The data show that the non-phosphorylatable FRNK has increased potency for inducing apoptosis compared to wild type FRNK.

possibility, I co-expressed FAK with either wild type or S217A mutant FRNK and performed TIRF imaging to determine the intensity of FAK localized to focal adhesions (Figure 11). To quantify the relative displacement of FAK from focal adhesions, a line scan was drawn from outside the cell towards the nucleus through focal adhesions that also included some cytoplasmic fluorescence. The intensity of FAK in the cytoplasm was then divided by the intensity of FAK in the focal adhesion to obtain a ratio, and this ratio was compared between wild type FRNK and S217A FRNK expressing RASMs. The ratio for both FRNK constructs was not significantly different, suggesting that S217A FRNK does not increase the displacement of FAK from focal adhesions. Therefore, FRNK 217A super inhibition cannot be explained by an increased ability to displace FAK from focal adhesions in a dominant-negative fashion and led me to consider alternative mechanisms of inhibition.

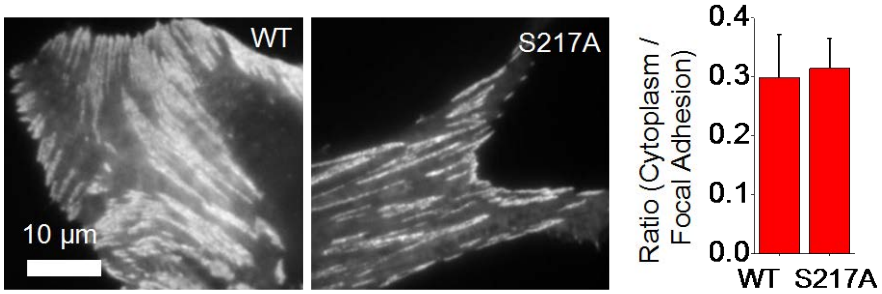

Figure 11. Expression of WT- or S217A-FRNK yields similar FAK localization to focal adhesions.
Cells expressing CER-WT- or CFP-S217A-FRNK and YFP-FAK were subjected to line scan analysis to determine the relative localization of FAK to focal adhesions. No significant differences in FAK localization were observed for N=13 cells for each condition.

S217A FRNK EXHIBITS FRET WITH FAK.

Because S217A FRNK did not alter FRNK displacement of FAK at focal adhesions, I considered the possibility that the mutation altered the ability of FRNK to interact with FAK. It has never been shown before that FRNK can interact directly with FAK, however interactions between the FAK FERM domain and isolated FAT domain (which constitutes a large portion of FRNK) have been reported in vitro [54]. Additionally, it has been proposed that the FAK FAT domain could interact with another FAT domain [71]. Both of these potential interactions could mediate a FAK-FRNK binding interaction. In addition, the S217 phosphorylation site is in close proximity to both of these potential means of FAK-FRNK interaction according to small angle X-ray scattering and crystal structure data [54,71]. Thus, I hypothesized that FRNK may bind directly to FAK and that this interaction is regulated by serine 217 phosphorylation. In order to test this hypothesis, I performed acceptor photo-bleaching FRET experiments between FAK and either wild type or S217A FRNK (Figure 12). Co-expression of fluorophore tagged FAK and FRNK showed both proteins co-localized in focal adhesions. After photo-bleaching, wild type FRNK did not

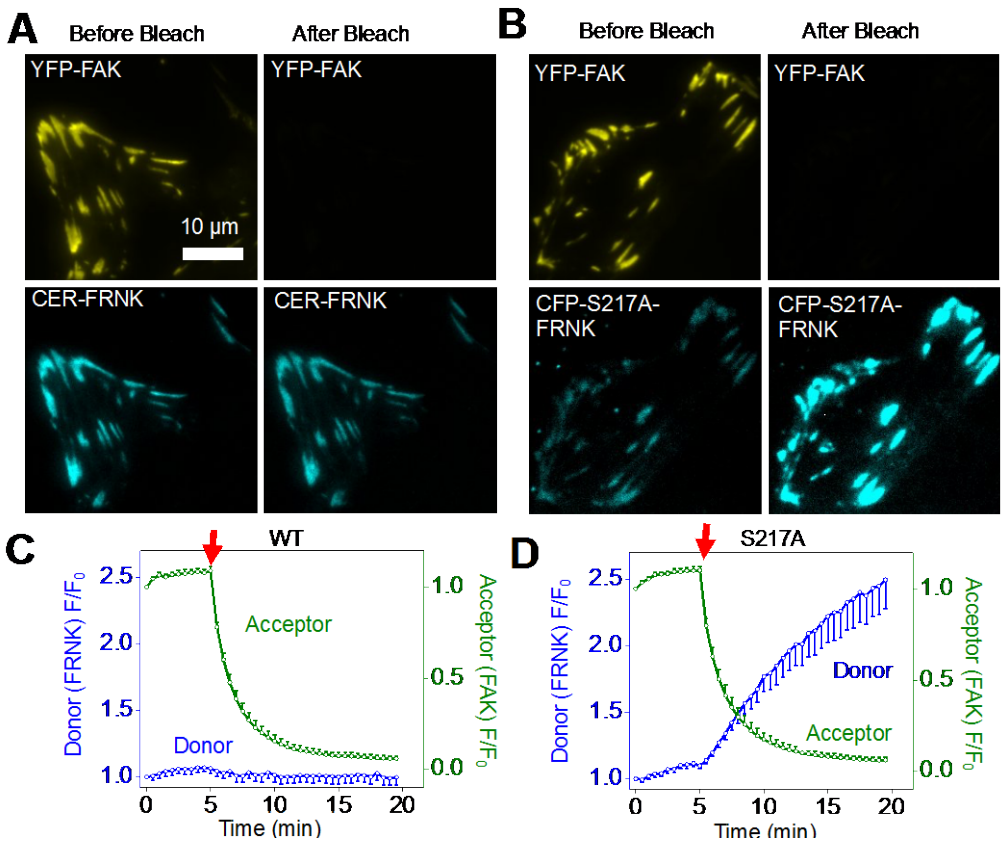

Figure 12. FAK and S217A-FRNK form oligomeric complexes.
(A, B) TIRF microscopy revealed YFP-FAK localization in focal adhesions and cytoplasm in cells co-expressing wt or S217A FRNK. Acceptor-selective photo bleaching of YFP-FAK resulted in increased fluorescence of non-phosphorylatable FRNK, but not WT-FRNK. **(C, D)** Quantification of N=12 acceptor photobleaching experiments for WT- or S217A-FRNK coexpressed with YFP-FAK. Data shown are mean ± SE. Red arrows indicate the start of photobleaching.

increase in intensity (Figure 12A, C) suggesting that there was no interaction between wild type FRNK and FAK. However, photo-bleaching caused a large increase in S217A FRNK intensity (Figure 12B,D) suggesting S217A FRNK and FAK form a dimer or oligomer. Further, it suggests S217 phosphorylation may serve to regulate the formation of this dimer or oligomer because wild type FRNK exhibited no detectable FRET with FAK using acceptor photobleaching.

CFP-S217A-FRNK exhibited an average FRET efficiency of 53.0 ± 3.3% which is higher than expected assuming a one-to-one donor-to-acceptor stoichiometry, leading me to consider the possibility of higher order oligomeric complexes. The 53% FRET efficiency calculated for S217A FRNK and FAK was higher than expected for two separate proteins forming a dimer. For example, a construct consisting of similar donor and acceptor fluorescent proteins separated by only a 5 amino acid long linker has a characteristic FRET efficiency of around 40%, lower than the observed 53% for FAK and S217A-FRNK [121,124]. Because of this high value, I considered that there might be multiple acceptors interacting with a single donor, for example multiple FAK proteins interacting with a single FRNK in an oligomeric complex. In a situation where multiple acceptors are within close proximity (close enough for FRET to occur) high FRET efficiencies like the one measured between S217A FRNK and FAK can be measured. To test this hypothesis I utilized my acceptor photobleaching data which can be used to differ between dimeric and higher order oligomeric complexes[121]. The basis of the method is shown in Figure 13A. If the interaction is dimeric, then there is only a single acceptor fluorophore quenching each donor, and bleaching of every acceptor results in the same increase in

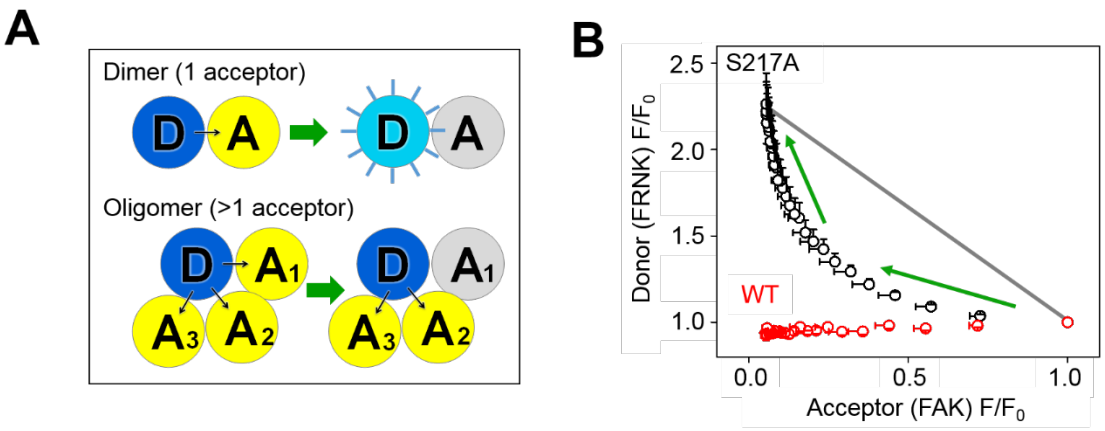

Figure 13. FAK and S217A-FRNK form oligomeric complexes.
(A) FRET (black arrows) from a donor ("D") is abolished after photobleaching of the conjugate acceptor ("A") in a dimer, resulting in increased donor brightness. For a higher order oligomer, FRET persists until all acceptors have been bleached. **(B)** The data in figure 12 plotted as donor vs. acceptor intensity revealed significant deviation from linearity for non-phosphorylatable FRNK, suggesting multiple acceptors in complex with each donor. Green line indicates the direction of time going from the bottom right to the top left of the graph. Data are mean ± SE for N=12 experiments.

donor intensity. Thus, the relationship between photo-bleaching and donor intensity would be linear. However, if there are multiple acceptors per donor, as a single acceptor is bleached the donor remains quenched by FRET with other acceptors, resulting in only small donor intensity increases during the initial photobleaching steps. However, near the end of the photobleaching process the last remaining acceptors are bleached resulting in a larger increase in donor intensity. The net result is that the relationship between bleaching and donor intensity deviates from linearity and will appear curved if

multiple acceptors are present. I plotted the donor intensity data obtained from S217A FRNK and FAK (Figure 12D) versus acceptor intensity instead of time in order to determine if there are multiple acceptors present (Figure 13B). The plot showed a high deviation from linearity, for the S217A mutant FRNK confirming that there are multiple acceptors within FRET range of each donor, suggesting that FRNK is able to interact with multiple FAK proteins in a higher order oligomer. FAK has been previously reported to form clusters consisting of six to eight FAK proteins [73]. The results obtained from my acceptor photobleaching experiments are consistent with a single molecule of FRNK interacting with a similar FAK cluster.

WILD TYPE FRNK AND FAK INTERACT.

The acceptor photo-bleaching FRET experiments used to compare wild type and S217A mutant FRNK suggested that wild type FRNK did not interact with FAK, however I hypothesized that more sensitive methods might be able to detect this interaction. I used co-immunoprecipitation experiments as a highly sensitive, but less specific method to show a FRNK FAK interaction. Cells were infected with GFP-FRNK for 24 hours. Then, an N-terminal FAK antibody was used to immunoprecipitate FAK, and co-immunoprecipitation of GFP-FRNK was probed with a GFP antibody (Figure 14). GFP-FRNK was successfully co-immunoprecipitated with FAK, suggesting wild type FRNK is able to interact with FAK. GAPDH was also examined to confirm the beads used did not non-specifically bind protein.

While the acceptor photobleaching FRET method excels at confirming protein-protein interactions and can be useful in determining the stoichiometry of protein complexes, it is not the most sensitive FRET method. In order to determine if wild type

FRNK and FAK exhibit FRET I performed lifetime fluorescence FRET experiments which can detect smaller amounts of FRET accurately. Fluorescence lifetimes are intrinsic to fluorophores and I used GFP-FAK because GFP has a mono-exponential lifetime decay conducive to FRET experiments[125]. GFP-FAK expressing RASMs were used to measure the lifetime of GFP-FAK alone (example of a single cell is shown in figure 15A, green line), and a fit of multiple decays from 11 cells yielded an average lifetime of 2.62 ± 0.03 ns. Next, cells co-expressing GFP-FAK and mCherry-FRNK were measured and yielded an average lifetime of 2.38 ± 0.03 (example decay of a single cell is shown in Figure 15A, red line). This reduction in lifetime is consistent with FRET and an average value of 8.9 ± 1% FRET efficiency was calculated for GFP-FAK and mCherry-FRNK (Figure 15B). This suggests that wild type FRNK is able to interact with FAK. To confirm the observed FRET was specific to a FAK-FRNK interaction, I performed a third experiment in which FLAG-FAK was added to compete GFP-FAK off of mCherry-FRNK. If the observed FRET was due to non-specific FRET (i.e. FAK and FRNK simply crowding in focal adhesions), then adding

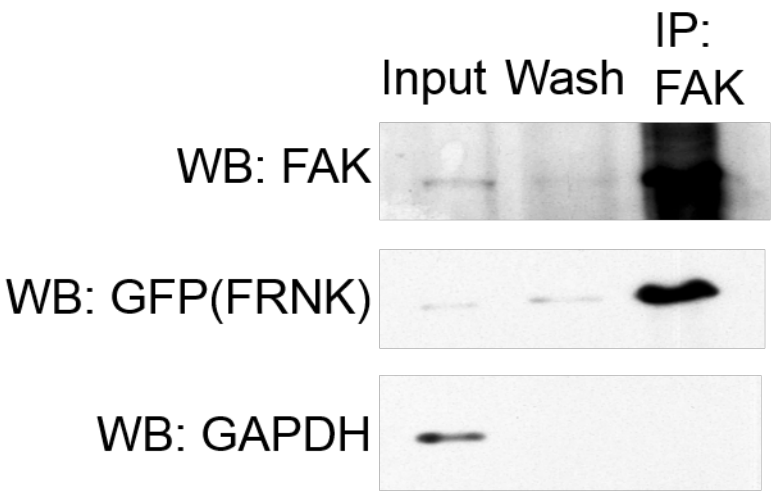

Figure 14. WT-FRNK interacts with FAK as determined by coimmunoprecipitation.
RASMs were infected with GFP-FRNK and then endogenous FAK was immunoprecipitated with an N-terminal FAK antibody. This resulted in co-immunoprecipitation of exogenously expressed GFP-FRNK. GAPDH was used to confirm washing of protein A/G beads.

additional FLAG-FAK should not reduce the apparent FRET. However, if the observed FRET was due to a specific interaction between FAK and FRNK, then the addition of flag-FAK would compete with GFP-FAK and reduce the apparent FRET. I saw a significant reduction of FRET with the addition of FLAG-FAK (Figure 15B), confirming that the observed FRET between GFP-FAK and mCherry-FRNK was due to a specific interaction.

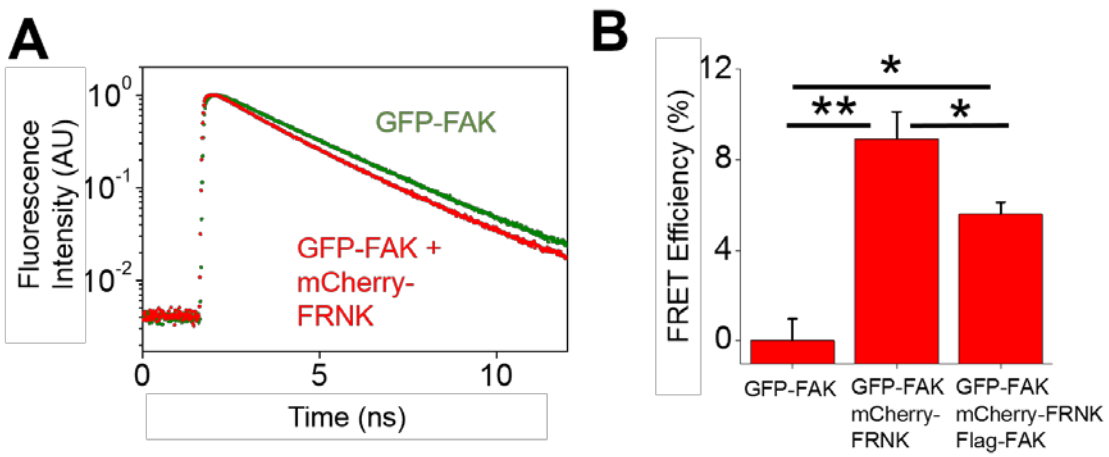

Figure 15. WT-FRNK interacts with FAK as detected by lifetime fluorescence. **(A)** An example of fluorescence lifetime measurements from two different cells: in green a GFP-FAK expressing cell and in red a cell expressing the FRET pairs GFP-FAK and mCherry-FRNK. The increased rate of decay is consistent with a small amount of FRET. **(B)** Quantification of N=11, 21, and 20 lifetime measurements for cells expressing GFP-FAK, GFP-FAK + mCherry-FRNK, and GFP-FAK + mCherry-FRNK + flag-FRNK. As a negative control, addition of flag-FRNK reduced FRET, suggesting that the FRET between FAK and FRNK was due to a specific interaction.

RECIPROCAL CO-IMMUNOPRECIPITATION EXPERIMENT.

Several additional co-immunoprecipitation experiments have further supported some of the hypotheses proposed. I showed that immunoprecipitation with an N terminal FAK antibody was able to co-immunoprecipitate GFP-FRNK (Figure 14). I performed the reciprocal experiment by expressing FLAG-FRNK in RASMs and using a FLAG antibody I immunoprecipitated FRNK and checked for the co-immunoprecipitation of endogenous FAK (Figure 16). In addition to wild type FLAG-FRNK, I also performed the experiment with a mutant FRNK, FLAG-Y168F-FRNK, which I believed would not affect FAK FRNK interaction. Both experiments resulted in immunoprecipitation of FRNK, although the total FRNK immunoprecipitated was lower than expected as there was less

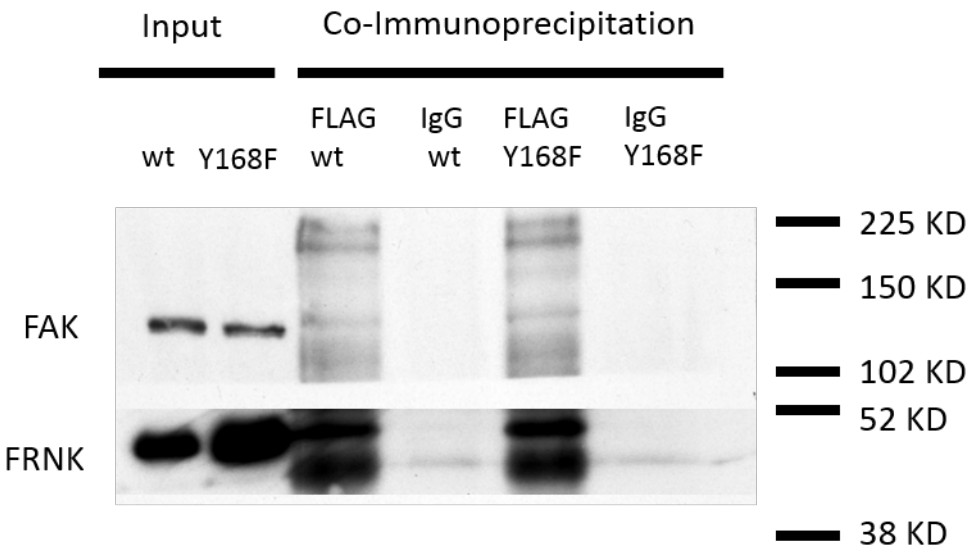

Figure 16. FLAG antibody also co-immunoprecipitates FAK and FRNK. RASMs infected with Flag-FRNK or Flag-FRNKY168F were lysed in CO-IP buffer and incubated with Flag antibodies or a non-specific IgG control. FRNK was weakly immunoprecipitated based on the intensity of the Co-IP bands relative to the input. FAK was co-immunoprecipitated with FRNK. Identical samples were incubated with non-specific IgG as a negative control, which failed to co-ip FAK and FRNK.

FRNK attached to the protein A/G beads than was present in the input. Most importantly, FAK was co-immunoprecipitated providing further evidence for a FAK FRNK interaction. As a negative control protein samples were also incubated with a non-specific IgG antibody, which failed to immunoprecipitate both FAK and FRNK.

CO-IMMUNOPRECIPITATIONS COMPARING WILD TYPE, S217D, AND S217A-FRNK.

In addition to the acceptor photobleaching data suggesting S217A FRNK has increased ability to bind FAK, co-immunoprecipitation experiments also support this hypothesis. I expressed FLAG-FRNK (42 KD), CFP-S217A-FRNK (72 KD), and mCherry-S217D-FRNK (72 KD) in RASMs and lysed the cells with non-denaturing co-

immunoprecipitation buffer. Because this experiment was performed before developing the western blot technique used to balance FRNK expression among FRNK mutants (Figure 3), the S217A FRNK was expressed at greater than 10 fold less concentration than WT (Figure 17, input lanes 1 and 2). GAPDH was used to ensure equal total protein loading. Despite vastly decreased expression of S217A-FRNK, approximately equal amounts of wild type and S217A-FRNK were co-immunoprecipitated (Figure 17). (The band present at 52 KD between the fluorophore tagged FRNK and flag-FRNK is the FAK N-terminal antibody heavy chain and should not be included in intensity assessment of the co-ip.) Thus, despite 10 fold less protein on input, S217A-FRNK was equal in abundance on co-ip to wild type FRNK suggesting that S217A-FRNK may have an increased affinity for FAK. Additionally, it is unlikely that the differences in appearance on the western blot are due to antibody recognition of S217A-FRNK, because both the input and co-ip were probed with the same FRNK antibody. S217D-FRNK expression level was similar to flag-FRNK, and also co-immunoprecipitated similarly to flag-FRNK. However, the expression of S217D-FRNK appears to have decreased the fraction of FAK that was immunoprecipitated by the N-terminal antibody; the significance of this observation is unclear.

ANGIOTENSIN II STIMULATION INCREASES ERK ACTIVITY, BUT NOT S910 PHOSPHORYLATION.

I showed that FRNK S217 is phosphorylated by ERK using kinase inhibitor experiments, and others have shown that the corresponding residue on FAK, S910, is

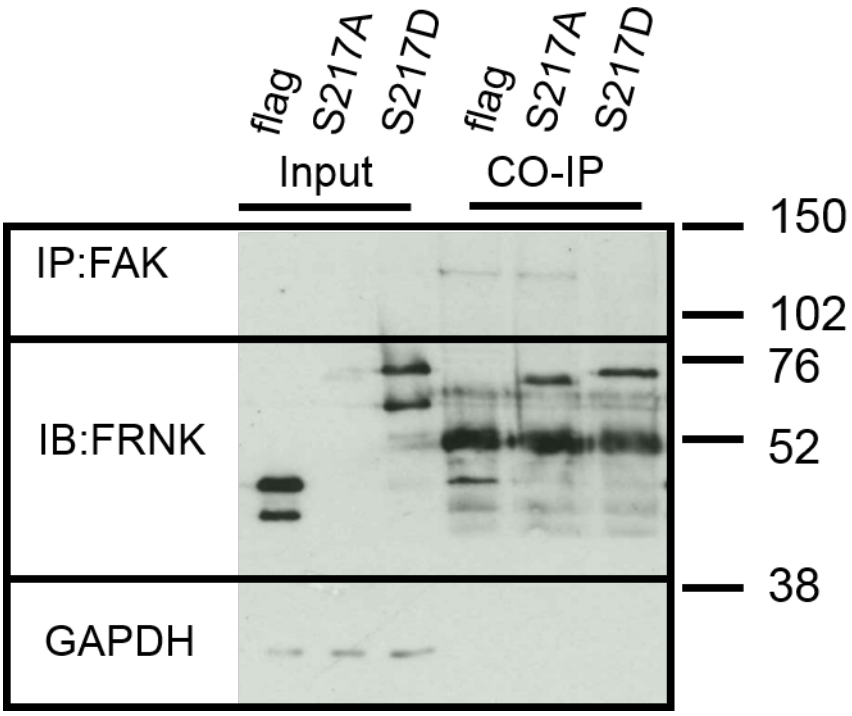

Figure 17. N-terminal FAK antibody co-immunoprecipitates wild type, S217A, and S217D FRNK.
The expression of exogenous wild type and S217D FRNK was relatively equal, however S217A FRNK expression was very low and can be seen on the input. Despite unequal expression, all three FAK variants were equally co-immunoprecipitated (flag-FRNK is detected at 42 kDa. S217A and S217D are detected at 74kDa) with a FAK N-terminal antibody suggesting S217A FRNK may have increased FAK binding. The 52DK band is the heavy chain from the immunoprecipitating antibody.

also phosphorylated by ERK [86,87]. In order to assess the effect of ERK signaling on FAK S910 phosphorylation, I serum starved cells for 24 hours and used 10µM angiotensin II to increase ERK activity. The effect angiotensin II on endogenous FAK S910 phosphorylation was then determined using phosphospecific antibodies. I examined ERK activation and FAK S910 phoshporylation at 15, 30, 45, 60, and 120 min after 10 µM angiotensin II stimulation (Figure 18). I found that ERK was maximally phosphorylated only 15 min following agonist addition, however I did not notice any FAK S910

phosphorylation changes. Because the increase in ERK phosphorylation occurred rapidly, I performed additional experiments on a shorter time scale. I harvested cells after 5, 10 and 15 min of angiotensin II stimulation in order to see if there were phosphorylation changes earlier than 15 minutes (Figure 19). I concluded that in RASMs ERK is maximally stimulated by angiotensin II at 10 to 15 min post drug addition.

In parallel with the above experiments, I also examined the effects of FAK kinase domain inhibition and angiotensin II stimulation. RASMs were incubated for one hour with a FAK kinase domain inhibitor (PF 573,228)[100] and then stimulated with 10μM angiotensin II for 10 min (Figure 18 and 19). I observed a drastic reduction in ERK phosphorylation both before and after angiotensin II demonstrating the importance of FAK in activating ERK signaling. Again, FAK S910 phosphorylation remained unchanged.

Surprisingly, I did not see any changes to FAK S910 phosphorylation in response to angiotensin II stimulation or PF 573,228 inhibition both of which drastically altered

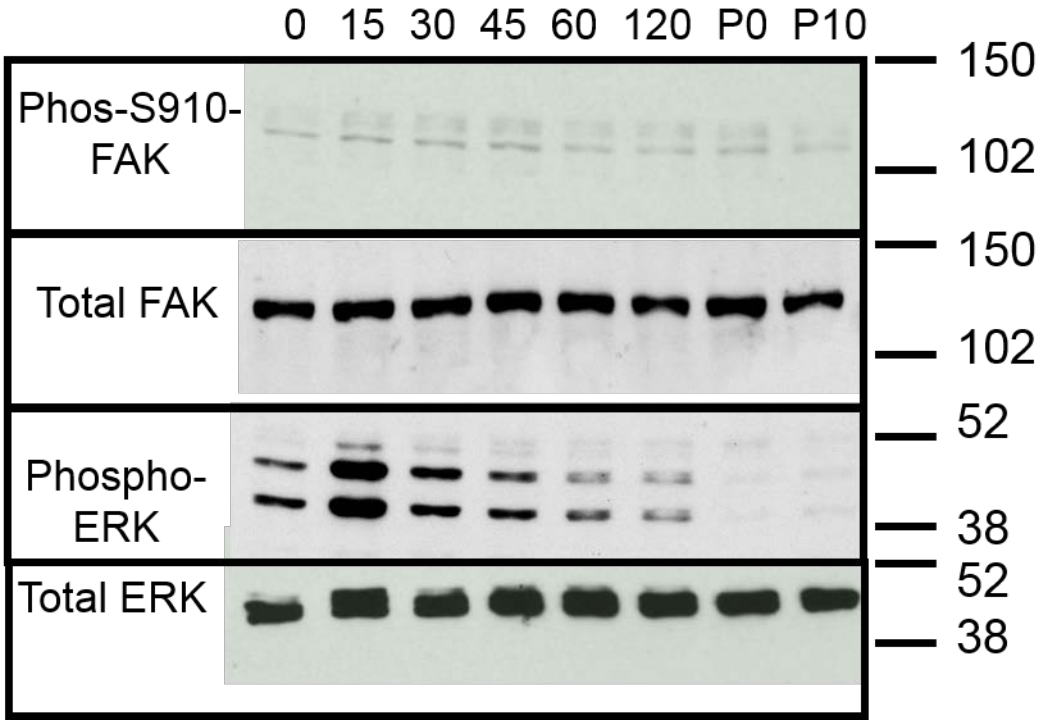

Figure 18. Angiotensin II stimulation phosphorylates ERK and FAK inhibition prevents ERK phosphorylation.
10µM angiotensin II stimulation of serum starved RASMs for 0,15, 30, 45, and 60 min activates ERK but does not increase FAK S910 phosphorylation. Incubation with FAK inhibitor PF 573,228 for 1 hour with (P10) or without (P0) angiotensin stimulation abolished ERK phosphorylation completely.

ERK activation. I expected to see an increase in FAK S910 phosphorylation with increased ERK activity based on previous studies[86,87]. It is unclear why I did not observe an increase in FAK phosphorylation. Perhaps other signaling caused by angiotensin resulted in a simultaneous increase in phosphatase activity or perhaps other changes to the focal adhesion environment balanced out the increase in ERK activity. Another possibility is that the stiffness of the cell culture conditions result in serine 910 being maximally phosphorylated, even in serum starved cells. Thus, adding angiotensin II fails to increase phosphorylation. Applying this hypothesis to the PF 573,228 results, it is possible that there are simply very few phosphatases to remove the phosphate or that 1

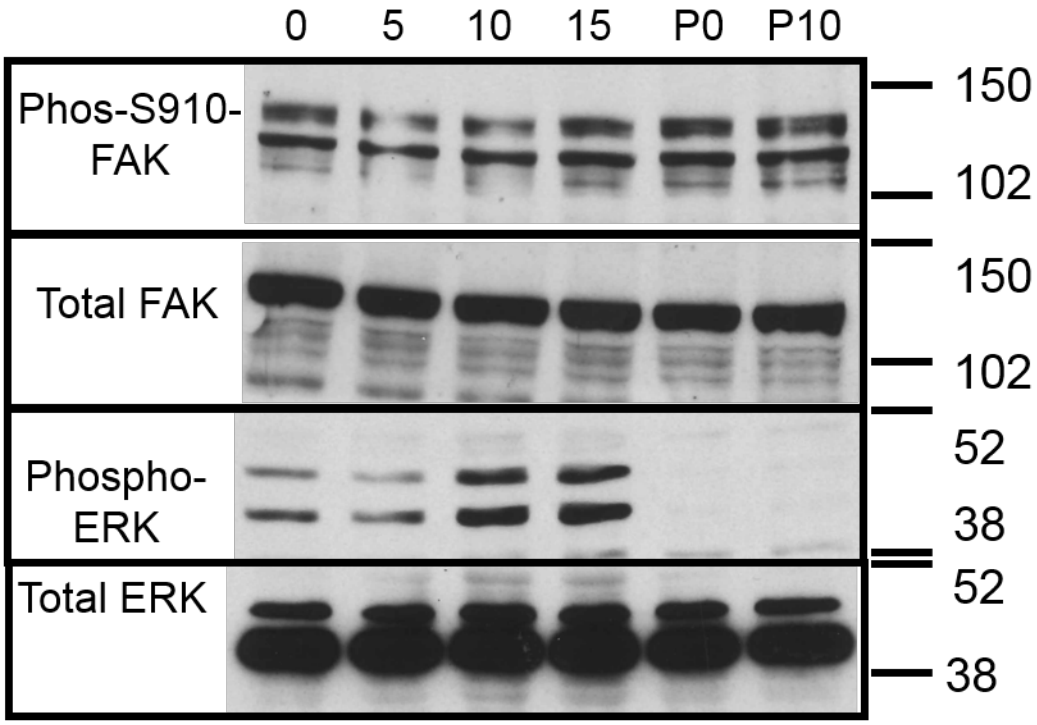

Figure 19. Angiotensin II stimulation phosphorylates ERK and FAK inhibition prevents ERK phosphorylation.
10μM angiotensin II stimulation of serum starved RASMs for 0, 5, 10, and 15 min activates ERK but does not increase FAK S910 phosphorylation. Incubation with PF 573,228 for 1 hour (P0) and with 10 min angiotensin stimulation (P10) abolished ERK phosphorylation completely.

hour of inhibition is not sufficient time for the decrease in ERK activity to effect phosphorylation levels. It is also possible that this discrepancy is due to RASM specific cell type differences compared to cardiomyocytes and cancer cells (cell type differences are discussed in section 1.12.1). Future studies are needed to fully understand the kinetics of FAK serine 910 phosphorylation in response to ERK.

ERK5 has also been proposed to phosphorylate FAK at S910, and I examined ERK5 activation following angiotensin II stimulation [87]. ERK5 behaves exactly the same as ERK1/2, increasing in activity after 15 min of angiotensin II stimulation and becoming completely inactive following PF 573,228 incubation (Figure 20). Additionally, I confirmed that PF 573,228 was able to decrease FAK Y397 phosphorylation as previously

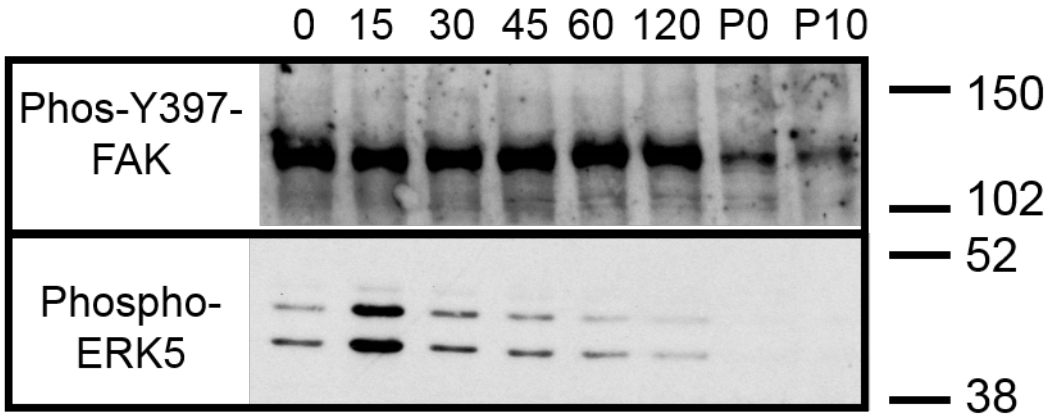

Figure 20. ERK5 behaves similarly to ERK1/2.
ERK 5 is also activated by angiotensin II stimulation and is inhibited by PF 573,228. FAK Y397 phosphorylation is not significantly altered by angiotensin II stimulation, but is drastically changed by PF 573,228.

described[100], however the angiotensin II stimulation did not have a significant effect on FAK Y397 phosphorylation (Figure 20). This may explain in part the lack of FAK S910 phosphorylation increases in the preceding experiments by showing that in RASMs FAK signaling is mostly upstream of ERK phosphorylation and shows little effects with respect to Y397 phosphorylation upon angiotensin II stimulation.

CONFIRMATION OF S217 PHOSPHOSPECIFIC ANTIBODY AND S217D MUTANT OBSERVATIONS.

We utilized S910 phosphospecific antibodies for many assays involving both FAK and FRNK. In order to confirm the specificity of this established commercially available antibody I expressed mCherry-wild type, CFP-S217A, and mCherry-S217D FRNK in cells and subjected them to western blotting with phosphospecific S910 (217) antibody and a total FAK/FRNK antibody (Figure 21). The wild type FRNK showed high amounts of phospho 217 on both the full length and assumed degradation products. As expected, the S217A FRNK was not detected by the antibody. The S217D FRNK was barely

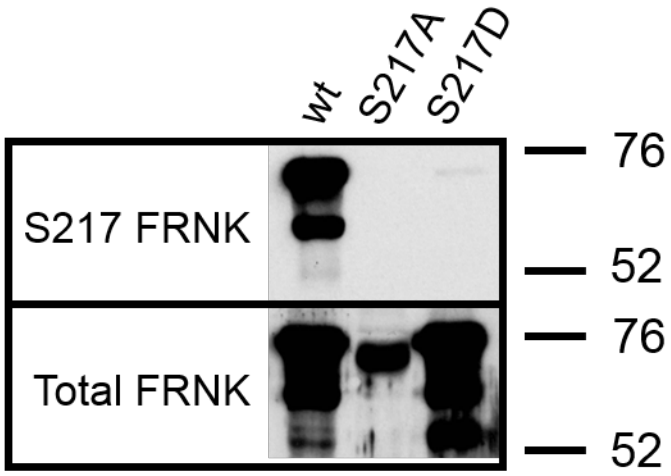

Figure 21. Phosphospecific antibodies weakly recognize S217D and do not recognize S217A FRNK.
Wild type FRNK is highly phosphorylated as detected by a S910 (217) phosphospecific antibody. S217A was not detected and S217D was barely detectable.

detectable on western blot. The phosphospecific antibody binding to a phosphomimetic does not perfectly reflect the ability of the S217D mutant to bind to focal adhesion proteins. However, it is an example of a protein-protein interaction which suggests the S217D-FRNK may act as a poor phosphomimetic because other phosphomimetic serine mutants are able to be recognized by phosphospecific antibodies[126].

S217D FRNK INHIBITS FAK TYROSINE 397 PHOSPHORYLATION MORE THAN WILD TYPE FRNK.

Because the S217A mutant FRNK exhibited such a dramatic phenotype, I wanted to assess the ability of S217D mutant FRNK to inhibit FAK. In order to compare the ability of S217D FRNK to inhibit FAK tyrosine 397, I used phosphospecific antibodies to FAK tyrosine 397 and expressed increasing amounts of either wild type or S217D FRNK (Figure 22). Both wild type and S217D mutant FRNK were able to decrease FAK tyrosine 397 phosphorylation. In order to compare the potency of the S217D mutant with wild

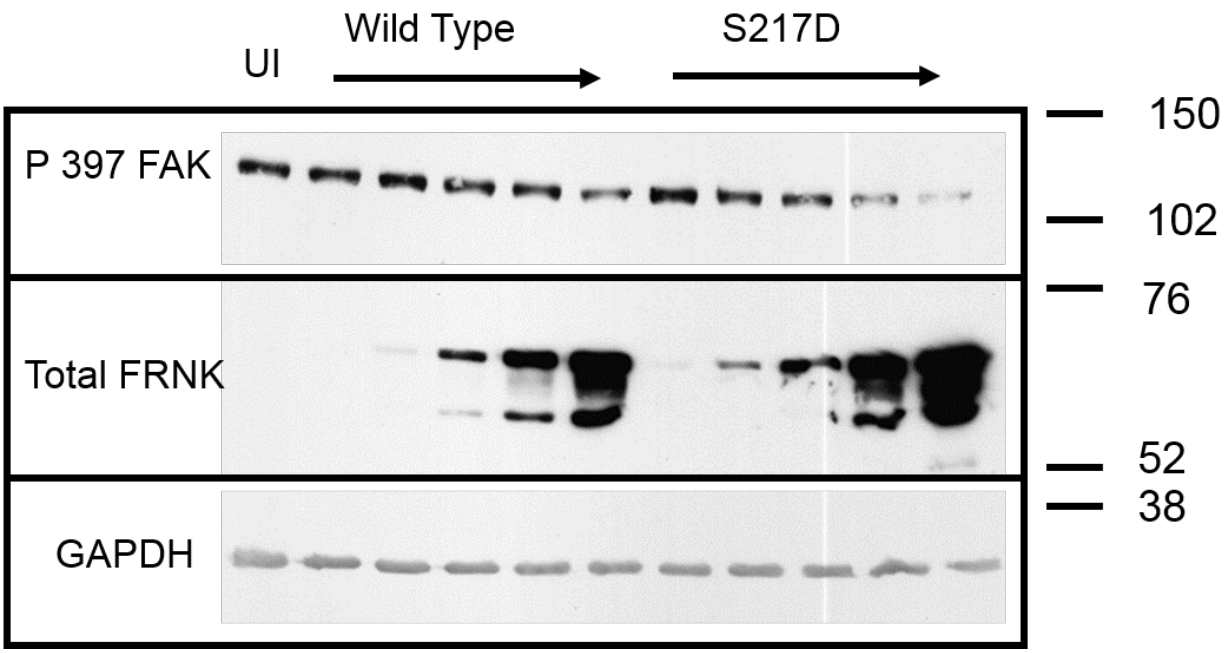

Figure 22. S217D FRNK inhibits FAK Y397 phosphorylation.
S217D FRNK is a slightly more potent inhibitor of FAK Y397 phosphorylation. Cells infected with increasing amounts of wild type FRNK or S217D mutant FRNK were subjected to western blot with phosphospecific antibodies to FAK tyrosine 397. UI was a uninfected control.

type FRNK, a plot of the FRNK concentration vs. FAK Y397 phosphorylation wasgenerated (Figure 23). An analysis of inhibition as a function of FRNK expression revealed the slope of the wild type FRNK to be -0.09 (AU), while the slope of the S217D mutant FRNK was -0.18. This suggests that the S217D FRNK is approximately twice as potent as wild type in respect to inhibiting FAK Y397 phosphorylation. Thus, S217D FRNK exhibited increased inhibition compared to wild type, but was not as prominent as the S217A mutant which exhibited over 10 fold increased inhibition compared to wild type. It is possible that the S217D mutant, which was created in an attempt to mimic phosphorylation, does not recapitulate the same function as phosphorylated S217

FRNK. Instead, the S217D FRNK may reflect an intermediate phosphorylation state between non-phosphorylatable S217A and the highly phosphorylated wild type FRNK. Thus, I hypothesize that S217D FRNK exhibits an intermediate ability to inhibit FAK with respect to FAK Y397 phosphorylation and should not be used to mimic phosphorylated FRNK.

FAK AND FRNK EXHIBIT DIFFERENTIAL SUB FOCAL ADHESION LOCALIZATION.

Experiments in which I express fluorescent labeled FAK and FRNK under TIRF excitation revealed differential sub-focal adhesion localization of the two proteins. While FAK and FRNK both localize to focal adhesions, I observed a consistent differential sub-focal adhesion localization (the distribution of the two proteins within a single focal adhesion). In Figure 24A a RASM expressing CER-FRNK and YFP-FAK is shown with an inset zoomed in picture of a focal adhesion exhibiting differential sub-focal adhesion

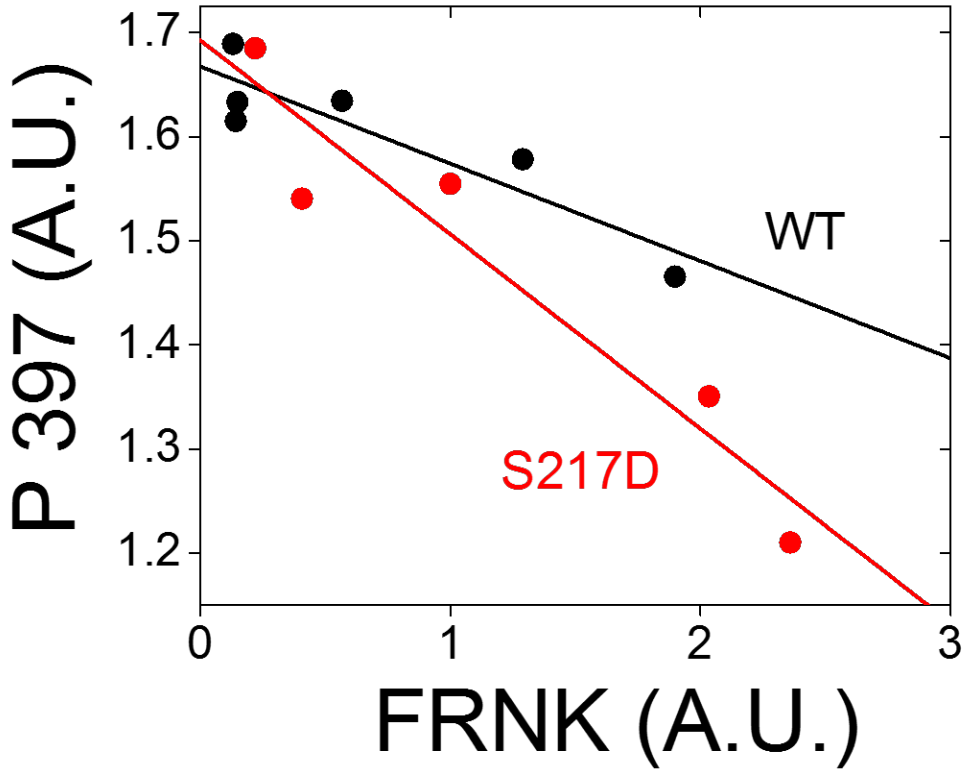

Figure 23. S217D exhibits similar potency compared to wild type FRNK.
Plot of data in Figure 11. S217D has a slope twice the value of wild type FRNK, suggesting it is slightly more potent than wild type FRNK with respect to FAK Y397 phosphorylation inhibition.

localization. Drawing a linescan through this focal adhesion and plotting the relative intensity of both FAK and FRNK reveals clear differential localization for the two proteins, with FRNK preferring the proximal side of the focal adhesion (Figure 24B). This observation was surprising because FAK and FRNK focal adhesion localization is mediated mostly by the FAT domain, which is identical in sequence in both FAK and FRNK[62]. It is possible that this differential localization is due to differential phosphorylation of focal adhesion proteins such as paxillin[127] in combination with FERM

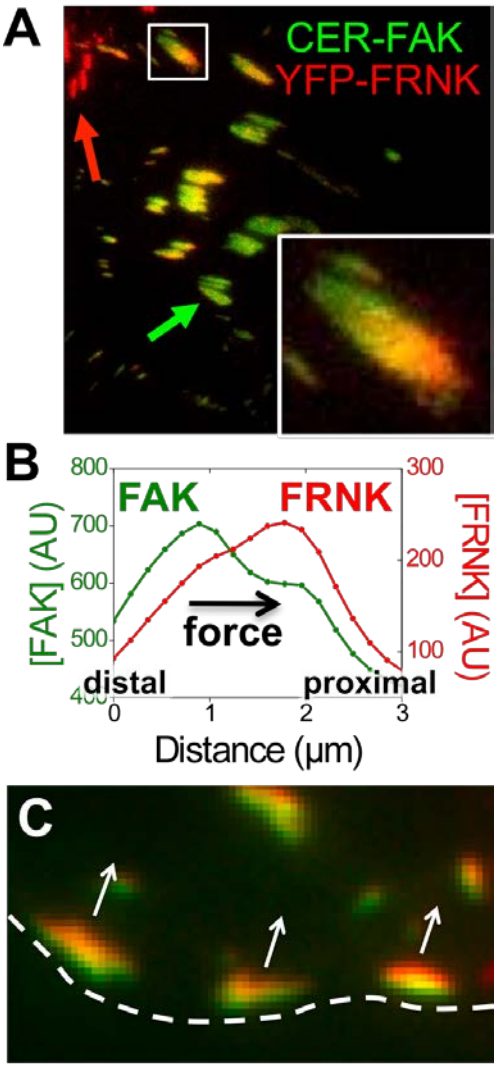

Figure 24. FAK and FRNK sub-focal adhesion localization.
(A) A cell expressing CER-FAK and YFP-FRNK imaged with TIRF excitation. **(B)** A line scan of the focal adhesion in A showing the distribution of FAK and FRNK. **(C)** A broad set of focal adhesions displaying the differential localization along the longitudinal axis of the focal adhesion, suggesting a "purse string" like mechanism of force being applied to the focal adhesions.

domain protein-protein interactions present only in FAK. An alternative hypothesis is that the focal adhesion is under different amounts of stress as the actin fiber travels up and away from the plasma membrane. In support of the theory that this differential localization is based on force, broad focal adhesions have been observed which have

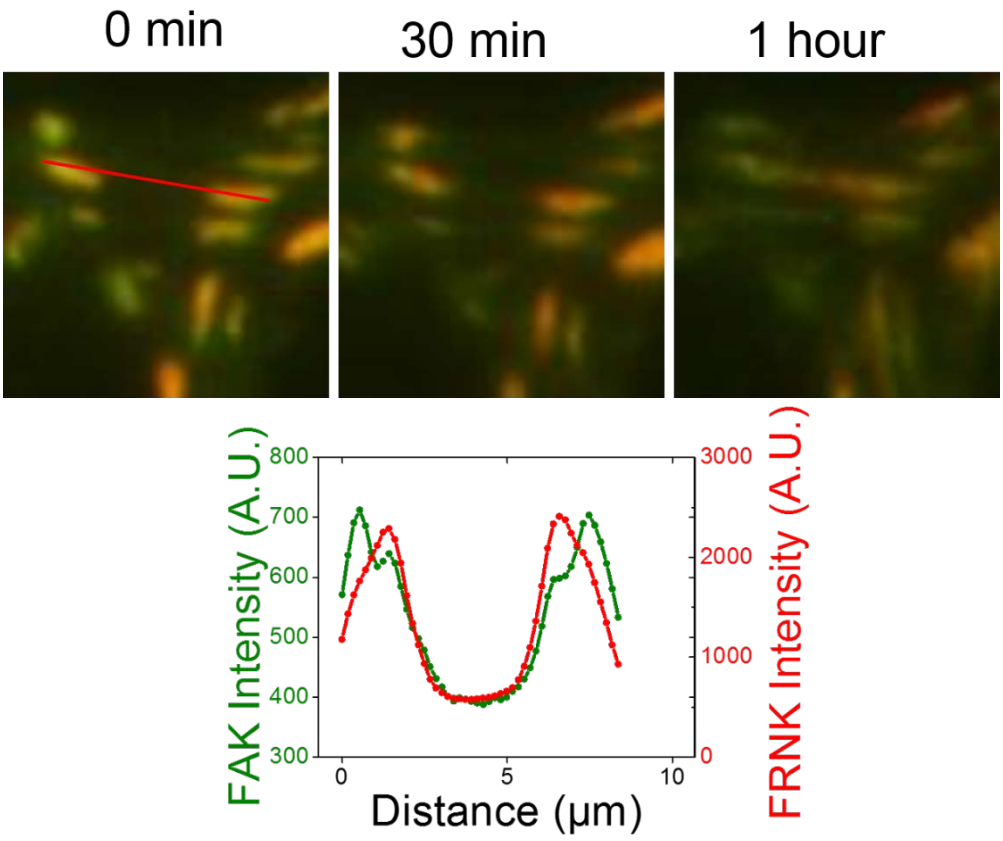

Figure 25. FRNK localization is not always proximal.
Three images from a movie of a cell expressing GFP-FAK and mCherry-FRNK. In the first from focal adhesions close to each other are shown with a line scan drawn through them. At 30 min striking polarization between FAK and FRNK can be seen. At 1 hour the focal adhesions are beginning to disassemble as they are pulled towards each other.

this differential localization on a lengthwise axis (Figure 24C). The dotted line here represents the plasma membrane (determined from phase contrast). I hypothesize that the cortical actin network that attaches focal adhesions circumferentially and is pulled tight, analogous to a purse string, applying force to the broad side of the focal adhesion and creating this type of localization pattern. Thus, within focal adhesions FRNK may respond to force differently than FAK.

In my observations, FRNK always preferentially localizes to the actin side of the focal adhesion. I further examined FAK and FRNK sub-focal adhesion localization by taking a movie of cells with TIRF excitation. In a time-lapse experiment I observed two focal adhesions near each be pulled towards each other, suggesting they were attached via actin stress fiber (Figure 25 red line indicates stress fiber location and line scan). The stark differential localization of FAK and FRNK was again observed. However, the localization of FRNK was not towards the proximal side of the focal adhesion, but was instead towards the side of the focal adhesion connected to the actin stress fiber (proximal on one focal adhesion and distal on the other with respect to the center of the cell). Thus, I hypothesize that the differential localization of FAK and FRNK is based on the force applied to focal adhesions from actin stress fibers.

CHAPTER FOUR

DISCUSSION

FAK AND FRNK FORM OLIGOMERS.

One of the central findings in the present study is that FAK and FRNK can bind directly to one another within focal adhesions. FAK oligomerization and intermolecular phosphorylation was hypothesized early after the discovery of FAK because of the similarities between FAK and receptor tyrosine kinases which dimerize and perform intermolecular phosphorylation[8]. Since then, the hypothesis that FAK can perform intra- and/or inter- molecular autophosphorylation has been tested and data has shown that FAK can perform both types of phosphorylation at the Y397 residue [29,39,128]. However, the relative contributions of intra- and inter- molecular autophosphorylation, and therefore the importance of FAK dimerization, remained less clear.

FRNK-FAK INHIBITORY COMPLEX FORMATION.

More recently it has been shown that formation of a specific FAK dimer, mediated by a FERM FERM interaction, regulates FAK Y397 phosphorylation and signaling function[54]. This paper provided strong evidence with a variety of techniques that FAK forms dimers only within the local environment of focal adhesions and suggested that inter-molecular phosphorylation of FAK at Y397 was the more important method of FAK activation. My results show that FRNK is similarly able to bind directly to

FAK within focal adhesions and raises interesting possibilities. For instance, if FRNK binds directly to FAK, does it inhibit FAK dimer formation? If so then FRNK would decrease Y397 phosphorylation by the prevention of FAK Y397 intermolecular phosphorylation. However, it is also possible that FRNK instead binds to existing FAK dimers and causes a conformational change resulting in decreased Y397 phosphorylation without disrupting the FAK-FAK association. Indeed, in my acceptor photobleaching experiments I found that a mutant form of FRNK was able to form higher order oligomeric complexes with a single FRNK in complex with multiple FAK proteins. This result suggests that FRNK may not disrupt FAK dimerization, but instead may cause other changes that result in inhibition of FAK dimerization and FAK activation.

POTENTIAL FERM-FAT INTERACTION.

My co-immunoprecipitation and FRET experiments showed that FRNK can form complexes with FAK, but did not elucidate that exact regions of FAK and FRNK that mediate this interaction. Previously, several different regions of FAK have been proposed to mediate FAK dimerization. The most convincing of these proposed loci is the FERM-FERM interaction discussed in the preceding paragraph[54]. However, a FERM-FERM interaction cannot explain the novel observed FAK-FRNK interaction because FRNK lacks the FERM domain. Thus, I considered other proposed loci of FAK-FAK interactions as a starting point for potential regions mediating the FAK-FRNK interaction observed in the present study. The same study that highlighted a FERM-FERM interaction also proposed a less important FAT-FERM interaction existed that could stabilize the more important FERM-FERM interaction[54]. Their proposed FAT FERM

interaction was supported by in vitro data such as GST-pulldowns of the FAT domain (not full length FRNK) with the FERM domain and some small angle X-ray scattering data showing a FAT sized shape in close proximity to the FERM domain. Interestingly, by mutating the FERM domain KAKTLR patch (discussed at length in section 1.9) they saw a loss of FERM-FAT GST-pulldown suggesting the FERM-FAT interaction may involve the KAKTLR sequence. This possibility is consistent with the FRNK-FAK interaction occurring simultaneously with the FAK-FAK dimer as the KAKTLR patch is on a different lobe than the residues mediating the FAK-FAK dimer[54]. Furthermore, if the hypothesis proposed by Brami-Cherrier et al. that the FAT-FERM interaction helps stabilize FAK dimers is correct, then the expression of FRNK would disrupt, but not abolish FAK dimers. The disruption of FAK dimers would allow for FRNK binding to FAK to decrease Y397 phosphorylation (by inhibiting inter-molecular phosphorylation) while at the same time still allow for FRNK to exhibit FRET with multiple FAK species. Thus, a FRNK-FAK interaction mediated by FRNK binding to the KAKTLR FERM domain sequence is consistent with both the data in the present study and current reports of FAK dimerization. Last, mutation of the KAKTLR sequence has been reported to cause many significant changes in FAK function, which could be due to altering the FRNK-FAK interaction. Additional FRNK-FAK acceptor photobleaching FRET experiments using FAK KAKTLR mutants should be performed to test this hypothesis.

POTENTIAL FAT-FAT INTERACTION.

In addition to the FAT-FERM hypothesis of FRNK-FAK interaction, it is possible that FRNK interacts with FAK through a FAT-FAT domain interaction. Studies on full length FAK and the isolated FAT domain have proposed that the FAT domain alone can

form a dimer (discussed in section 1.11.4) [71]. This hypothesis consists of the four helix bundle of the FAT domain opening into a 3 helix bundle with the remaining helix reaching out an interacting with another open 3 helix FAT domain. This hypothesis has decent evidence behind it including the creation of two mutants that are more likely to be in open and closed states[72]. These mutants showed that by increasing the ability of the helix to separate from the four helix bundle, FAK phosphorylation is increased at residues Y925, Y861, and S910. However, the same mutation that resulted in increased phosphorylation of these key residues also resulted in a decreased focal adhesion localization pattern. It is possible that FAK dimers (mediated by a FERM-FERM interaction) could bind to FRNK via a FAT-FAT interaction. Such a system would be consistent with my data that a single FRNK binds to multiple FAK proteins. However, the swinging out of the helix decreases FRNK binding to paxillin and potentially decreases focal adhesion localization. In experiments with FRNK binding to FAK I do not see a difference in focal adhesion localization suggesting this may not be the mechanism by which FRNK binds to FAK, however this hypothesis remains a possibility. Future acceptor photobleaching FRET experiments utilizing the open and closed mutants[72] could be used to test the possibility of a FAT-FAT interaction between FAK and FRNK.

THE RELATIVE IMPORTANCE OF FAK-FRNK INTERACTIONS AND FRNK S217 PHOSPHORYLATION.

We investigated FRNK S217 (corresponding to FAK S910) phosphorylation with multiple methods. First, because FRNK S217 phoshporylation had never been investigated (previously only FAK phosphorylation was studied), I found that FRNK was phosphorylated at S217 in my basal smooth muscle cell culture conditions and in both

uninjured and injured rat carotid arteries. Additionally, because FAK has been reported to be phosphorylated by ERK at postion S910 [86,87,129], I confirmed that FRNK is also phosphorylated by ERK by using ERK inhibitors. I attempted in increase FRNK and FAK S910 phosphorylation by stimulating ERK with angiotensin II, but did not observe an increase in either in contrast to previous reports with FAK[87]. However, RASMs were utilized in the experiments in the current work while others used cardiomyocytes and cancer cell lines. There could be cell type specific differences in the signaling pathways of angiotensin II responsible for the observed FAK S910 discrepancy (see section 1.12.1). Regardless of FAK and FRNK S910/217 phosphorylation in other cell types, my observations lead me to conclude that FRNK is highly phosphorylated at S217 by ERK in RASMs.

THE S217D MUTANT MAY BE A POOR PHOSPHOMIMETIC.

Next I wanted to determine the effects of FRNK S217 phosphorylation on the relative potency of FRNK. In order to do this I generated both a phospho-mimetic and non-phosphorylatable S217 FRNK mutants. The S217D FRNK mutant exhibited potency similar to wild type FRNK. However, S217A FRNK exhibited a marked ability to potently inhibit FAK Y397, cell growth, and an ability to induce apoptosis. Thus I have termed S217A FRNK a super inhibitor of FAK signaling. The intermediate effects of the S217D FRNK could be explained if an alanine to aspartic acid mutation is a poor phosphomimetic. The endogenous FRNK is highly phosphorylated at S217 and an aspartic acid residue is not as negatively charged as a phosphate group. In support of this theory, a S910 phosphospecific antibody poorly recognized the S217D mutant. Regardless, my analysis will focus on the S217A mutant due to the more prominent

effect and the certainty of the effect of the serine to alanine substitution (non-phosphorylatable).

S217A INHIBITION OCCURS VIA REGULATORY COMPLEX FORMATION.

Despite the powerful effect of the S217A mutation on cell growth, FAK Y397 phosphorylation, and apoptosis, S217A FRNK expression had no detectable effect on FAK localization to focal adhesions. This result was surprising due to the currently held belief that FRNK inhibition of FAK is in large part due to the ability of FRNK to displace FAK from focal adhesions[62,74]. Thus, S217A FRNK must exert its inhibitory effects via a mechanism other than displacing FAK from focal adhesions. I tested the ability of S217A FRNK to bind directly to FAK and found that it had a remarkably higher ability to bind to FAK compared to wild type FRNK. Thus, I have observed a correlation between FRNK-FAK complex formation and the inhibition of FAK signaling. Based on this correlation I hypothesize that a large portion of FRNK's inhibitory potential comes from the ability to bind directly to FAK and form a FRNK-FAK regulatory complex. This regulatory complex results in inhibited FAK signaling. A model of this new mechanism of inhibition is shown in Figure 26. These inhibitory regulatory complexes are disrupted by ERK phosphorylation of FRNK residue S217 which results in more active FAK signaling. The now active FAK signaling then further activates ERK, resulting in a positive feedback loop by which more FRNK is phosphorylated at S217, more FRNK-FAK complexes are disrupted, and even more FAK is active and signaling downstream. This negative

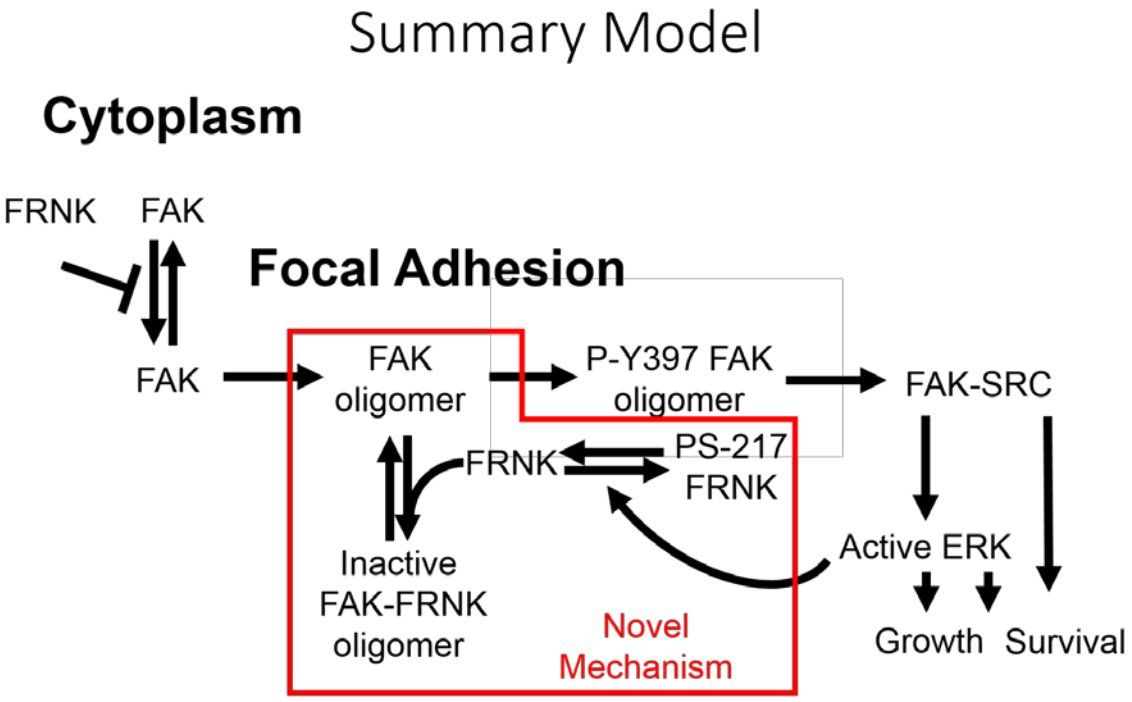

Figure 26. Summary model of FRNK regulatory complex inhibition
Classical FRNK inhibition of FAK occurs by preventing FAK translocation from the cytoplasm to the focal adhesion. The mechanism of FRNK inhibition of FAK described in the present work is highlighted in red. Under normal circumstances, FAK oligomerizes, autophosphorylates at Y397 and binds to SRC resulting in downstream activation of ERK, growth, and survival. If FRNK is present, FRNK can bind directly to FAK within focal adhesions and inhibit FAK Y397 phosphorylation and subsequent signaling. The FRNK-FAK regulatory complex (oligomer) is inhibited by ERK phosphorylation of FRNK at S217.

feedback loop could serve as a way to end FRNK inhibition of FAK in cells that have finished healing or if never activated could serve to push cells that never healed properly towards apoptosis.

OTHER POSSIBLE MECHANISMS OF S217 PHOSPHORYLATION INHIBITION.

There are other mechanisms which could explain the effect of the S217A mutation on FAK signaling. For example, S217A mutation could increase Pin1 and PTP-PEST binding (as reported previously for FAK[83]) and the resulting scaffolded PTP-PEST

could de-phosphorylate a nearby FAK Y397. In addition to this hypothesis, S217A FRNK could compete for FAK binding partners within the focal adhesion. For example, the FRNK Y168F mutant does not increase FAK displacement from focal adhesions, but instead localizes to focal adhesions where it competes with FAK for p130CAS. The net result is a decreased ability for p130CAS to scaffold to FAK and be fully phosphorylated on its YxxP repeats and a reduction in downstream signaling[76]. It is possible that S217A FRNK similarly competes with FAK for Pin1 or an unidentified protein and exerts its inhibitory effect through this mechanism. All of these mechanisms are possible, but none of them require the observed FRNK complex formation with FAK. It is possible that FRNK complex formation with FAK is simply a coincidence, but this possibility is unlikely considering this complex appears to be tightly regulated by a conserved ERK phosphorylation site. Future studies on the ability of FRNK to bind to Pin1, and the ability of FRNK to inhibit FAK in Pin1 knockout cells, could test this potential mechanism.

UTILIZING THIS INFORMATION TO TREAT IN-STENT RESTENOSIS.

I have identified a novel protein-protein interaction between FAK and FRNK in vascular smooth muscle cells (the pathologic cell type in restenosis) which results in the inhibition of growth and the induction of apoptosis. Furthermore, this interaction appears highly dependent on FRNK S217 being de-phosphorylated. There are several ways in which this information can be utilized to potentially treat for in-stent restenosis, and some of these possibilities will now be discussed individually.

Small molecule inhibition of FAK through FRNK-FAK complexes. I have hypothesized that ERK is responsible for regulating the FRNK-FAK inhibitory complex through S217 phosphorylation and this information suggests that small molecule ERK inhibitors could

be used to treat in-stent restenosis. In fact, PD98059 has been used to treat the same rat carotid injury model of restenosis used in the present study and successfully attenuated neointimal hyperplasia[130]. The authors concluded this response was due to ERK downstream suppression of transcription factors. However, given my new model of FRNK inhibition of FAK, which is highly dependent on ERK activity, it is possible that some of the observed attenuated hyperplasia was due to increased FRNK-FAK complex formation. In order to further target the FAK pathway, small molecule FAK inhibitors (which are readily available and currently in human clinical trials, see section 1.16) could be combined with ERK inhibitors such as the aforementioned PD98059. Combination of these two drugs could allow for low doses of each to be used. By using a low dose of each drug, ERK and FAK inhibition could be minimized in cells that do not contain FRNK (such as uninjured vascular smooth muscle and endothelial cells). The preferential targeting of vascular smooth muscle cells could allow for proper healing of the endothelial lining and improved hemostasis. However, the cells that do upregulate FRNK would receive potent synergistic inhibition of FAK signaling due to direct FAK kinase inhibition and increased FRNK-FAK inhibitory complex formation due to decreased FRNK S217 phosphorylation from decreased ERK activity. This strategy of dual small molecule inhibition could easily be tested using the already established carotid artery injury model of restenosis (section 2.5) [106]. If found to potently attenuate neointimal hyperplasia, then endothelial cell layer healing could be easily assessed using immunohistochemistry. Furthermore, since both of these compounds are small

molecules the creation of a drug eluting stent using these compounds would be straightforward and thus could be easily transitioned into human clinical trials.

Virus mediated expression of S217A-FRNK in injured smooth muscle cells. In the present study I have identified a potent super inhibitory form of FRNK, S217A-FRNK. I have shown that this mutant has over 10 fold the ability to inhibit FAK Y397 phosphorylation compared to wild type FRNK. Additionally, this mutant is a potent inducer of apoptosis. One strategy to treat neo-intimal hyperplasia would be to express S217A-FRNK in injured vascular smooth muscle cells by using adenovirus at the time of stent placement. In fact, treatment of restenosis via a FRNK expressing adenovirus has been proposed and patented[131]. However, using S217A-FRNK instead of wild type FRNK may provide more potent inhibition of neointimal hyperplasia given its super-inhibitory qualities. This approach is the most straightforward way to utilize the information discovered in the present study, but has several problems. First, current drug eluting stents are already reasonably effective and have fairly low side effects. The use of an adenovirus to express FRNK could cause several side effects and poses a significant risk to patients. Furthermore, an adenovirus could express FRNK in endothelial cells potentially preventing the proper healing of the endothelial cell lining of the artery. On the other hand, I have demonstrated that FRNK inhibition is dose dependent and the exogenously expressed S217A-FRNK in combination with the endogenously upregulated FRNK in vascular smooth muscle cells would result in a more powerful effect in smooth muscle cells. The use of S217A-FRNK in inhibiting neointimal hyperplasia could be easily

tested using the aforementioned carotid artery injury model (section 2.5) [106], with addition of the S217A-FRNK adenovirus at the time of injury.

Future methods of increasing protein-protein interactions could be used. Currently most drugs used to treat patients are small molecules and about 1/3 of these drugs target G-protein coupled receptors[132]. However, more recently basic science has advanced to the point that humanized monoclonal antibodies can now be used to specifically target proteins that are exposed to the blood stream[133]. Monoclonal antibodies have been used to treat many diseases including many different cancers and autoimmune diseases. In the future, scientists may develop treatments that can specifically target protein-protein interactions inside the cell. This technology is currently being worked on, with one possibility being intrabodies, which are antibodies designed to be expressed intracellularly [134,135]. While out of reach with current technology, it is possible that in the future the novel FRNK-FAK interaction could be targeted directly with something like an intrabody. Because of the expression of FRNK only in vascular smooth muscle cells, an intrabody that stabilized the FRNK-FAK interaction could have no effect on uninjured smooth muscle cells and endothelial cells while at the same time potently inhibiting injured vascular smooth muscle cells and thus prevent restenosis

REFERENCE LIST

1. Hofmann KP, Scheerer P, Hildebrand PW, et al. A G protein-coupled receptor at work: the rhodopsin model. *Trends in biochemical sciences.* Nov 2009;34(11):540-552.

2. Arshavsky VY, Lamb TD, Pugh EN, Jr. G proteins and phototransduction. *Annual review of physiology.* 2002;64:153-187.

3. Cohen P. The origins of protein phosphorylation. *Nature cell biology.* May 2002;4(5):E127-130.

4. Wang J, Xia Y. Assessing developmental roles of MKK4 and MKK7 in vitro. *Communicative & integrative biology.* Jul 01 2012;5(4):319-324.

5. Okada M. Regulation of the SRC family kinases by Csk. *International journal of biological sciences.* 2012;8(10):1385-1397.

6. Lemmon MA, Schlessinger J, Ferguson KM. The EGFR family: not so prototypical receptor tyrosine kinases. *Cold Spring Harbor perspectives in biology.* Apr 01 2014;6(4):a020768.

7. Biscardi JS, Maa MC, Tice DA, Cox ME, Leu TH, Parsons SJ. c-Src-mediated phosphorylation of the epidermal growth factor receptor on Tyr845 and Tyr1101 is associated with modulation of receptor function. *The Journal of biological chemistry.* Mar 19 1999;274(12):8335-8343.

8. Hanks SK, Calalb MB, Harper MC, Patel SK. Focal adhesion protein-tyrosine kinase phosphorylated in response to cell attachment to fibronectin. *Proceedings of the National Academy of Sciences of the United States of America.* Sep 15 1992;89(18):8487-8491.

9. Schaller MD, Borgman CA, Parsons JT. Autonomous expression of a noncatalytic domain of the focal adhesion-associated protein tyrosine kinase pp125FAK. *Molecular and cellular biology.* Feb 1993;13(2):785-791.

10. Calalb MB, Polte TR, Hanks SK. Tyrosine phosphorylation of focal adhesion kinase at sites in the catalytic domain regulates kinase activity: a role for Src family kinases. *Molecular and cellular biology.* Feb 1995;15(2):954-963.

11. Polte TR, Hanks SK. Interaction between focal adhesion kinase and Crk-associated tyrosine kinase substrate p130Cas. *Proceedings of the National Academy of Sciences of the United States of America.* Nov 7 1995;92(23):10678-10682.

12. Ilic D, Furuta Y, Kanazawa S, et al. Reduced cell motility and enhanced focal adhesion contact formation in cells from FAK-deficient mice. *Nature.* Oct 12 1995;377(6549):539-544.

13. Walkiewicz KW, Girault JA, Arold ST. How to awaken your nanomachines: Site-specific activation of focal adhesion kinases through ligand interactions. *Progress in biophysics and molecular biology.* Oct 2015;119(1):60-71.

14. Corsi JM, Houbron C, Billuart P, et al. Autophosphorylation-independent and -dependent functions of focal adhesion kinase during development. *The Journal of biological chemistry.* Dec 11 2009;284(50):34769-34776.

15. Hayasaka H, Martin KH, Hershey ED, Parsons JT. Disruption of FRNK expression by gene targeting of the intronic promoter within the focal adhesion kinase gene. *Journal of cellular biochemistry.* Nov 1 2007;102(4):947-954.

16. Lowell CA, Mayadas TN. Overview: studying integrins in vivo. *Methods in molecular biology.* 2012;757:369-397.

17. Kassianidou E, Kumar S. A biomechanical perspective on stress fiber structure and function. *Biochimica et biophysica acta.* Nov 2015;1853(11 Pt B):3065-3074.

18. Wehrle-Haller B. Structure and function of focal adhesions. *Current opinion in cell biology.* Feb 2012;24(1):116-124.

19. Schaller MD. Paxillin: a focal adhesion-associated adaptor protein. *Oncogene.* Oct 01 2001;20(44):6459-6472.

20. Zhang X, Jiang G, Cai Y, Monkley SJ, Critchley DR, Sheetz MP. Talin depletion reveals independence of initial cell spreading from integrin activation and traction. *Nature cell biology.* Sep 2008;10(9):1062-1068.

21. Wu C. Focal adhesion: a focal point in current cell biology and molecular medicine. *Cell adhesion & migration.* Jan-Mar 2007;1(1):13-18.

22. Eke I, Cordes N. Focal adhesion signaling and therapy resistance in cancer. *Seminars in cancer biology.* Apr 2015;31:65-75.

23. Subauste MC, Pertz O, Adamson ED, Turner CE, Junger S, Hahn KM. Vinculin modulation of paxillin-FAK interactions regulates ERK to control survival and motility. *The Journal of cell biology.* May 10 2004;165(3):371-381.

24. Nowakowski J, Cronin CN, McRee DE, et al. Structures of the cancer-related Aurora-A, FAK, and EphA2 protein kinases from nanovolume crystallography. *Structure.* Dec 2002;10(12):1659-1667.

25. Hayashi I, Vuori K, Liddington RC. The focal adhesion targeting (FAT) region of focal adhesion kinase is a four-helix bundle that binds paxillin. *Nature structural biology.* Feb 2002;9(2):101-106.

26. Ceccarelli DF, Song HK, Poy F, Schaller MD, Eck MJ. Crystal structure of the FERM domain of focal adhesion kinase. *The Journal of biological chemistry.* Jan 6 2006;281(1):252-259.

27. Bosanquet DC, Ye L, Harding KG, Jiang WG. FERM family proteins and their importance in cellular movements and wound healing (review). *International journal of molecular medicine.* Jul 2014;34(1):3-12.

28. Cooper LA, Shen TL, Guan JL. Regulation of focal adhesion kinase by its amino-terminal domain through an autoinhibitory interaction. *Molecular and cellular biology.* Nov 2003;23(22):8030-8041.

29. Lietha D, Cai X, Ceccarelli DF, Li Y, Schaller MD, Eck MJ. Structural basis for the autoinhibition of focal adhesion kinase. *Cell.* Jun 15 2007;129(6):1177-1187.

30. Chan PY, Kanner SB, Whitney G, Aruffo A. A transmembrane-anchored chimeric focal adhesion kinase is constitutively activated and phosphorylated at tyrosine residues identical to pp125FAK. *The Journal of biological chemistry.* Aug 12 1994;269(32):20567-20574.

31. Dunty JM, Gabarra-Niecko V, King ML, Ceccarelli DF, Eck MJ, Schaller MD. FERM domain interaction promotes FAK signaling. *Molecular and cellular biology.* Jun 2004;24(12):5353-5368.

32. Cai X, Lietha D, Ceccarelli DF, et al. Spatial and temporal regulation of focal adhesion kinase activity in living cells. *Molecular and cellular biology.* Jan 2008;28(1):201-214.

33. Reiske HR, Kao SC, Cary LA, Guan JL, Lai JF, Chen HC. Requirement of phosphatidylinositol 3-kinase in focal adhesion kinase-promoted cell migration. *The Journal of biological chemistry.* Apr 30 1999;274(18):12361-12366.

34. Papusheva E, Mello de Queiroz F, Dalous J, et al. Dynamic conformational changes in the FERM domain of FAK are involved in focal-adhesion behavior during cell spreading and motility. *Journal of cell science.* Mar 1 2009;122(Pt 5):656-666.

35. Kadare G, Toutant M, Formstecher E, et al. PIAS1-mediated sumoylation of focal adhesion kinase activates its autophosphorylation. *The Journal of biological chemistry.* Nov 28 2003;278(48):47434-47440.

36. Poullet P, Gautreau A, Kadare G, Girault JA, Louvard D, Arpin M. Ezrin interacts with focal adhesion kinase and induces its activation independently of cell-matrix adhesion. *The Journal of biological chemistry.* Oct 5 2001;276(40):37686-37691.

37. Debant A, Serra-Pages C, Seipel K, et al. The multidomain protein Trio binds the LAR transmembrane tyrosine phosphatase, contains a protein kinase domain, and has separate rac-specific and rho-specific guanine nucleotide exchange factor domains. *Proceedings of the National Academy of Sciences of the United States of America.* May 28 1996;93(11):5466-5471.

38. Chen SY, Chen HC. Direct interaction of focal adhesion kinase (FAK) with Met is required for FAK to promote hepatocyte growth factor-induced cell invasion. *Molecular and cellular biology.* Jul 2006;26(13):5155-5167.

39. Sieg DJ, Hauck CR, Ilic D, et al. FAK integrates growth-factor and integrin signals to promote cell migration. *Nature cell biology.* May 2000;2(5):249-256.

40. Plaza-Menacho I, Morandi A, Mologni L, et al. Focal adhesion kinase (FAK) binds RET kinase via its FERM domain, priming a direct and reciprocal RET-FAK transactivation mechanism. *The Journal of biological chemistry.* May 13 2011;286(19):17292-17302.

41. Lawson C, Schlaepfer DD. pHocal adhesion kinase regulation is on a FERM foundation. *The Journal of cell biology.* Sep 16 2013;202(6):833-836.

42. Choi CH, Webb BA, Chimenti MS, Jacobson MP, Barber DL. pH sensing by FAK-His58 regulates focal adhesion remodeling. *The Journal of cell biology.* Sep 16 2013;202(6):849-859.

43. Serrels B, Serrels A, Brunton VG, et al. Focal adhesion kinase controls actin assembly via a FERM-mediated interaction with the Arp2/3 complex. *Nature cell biology.* Sep 2007;9(9):1046-1056.

44. Swaminathan V, Fischer RS, Waterman CM. The FAK-Arp2/3 interaction promotes leading edge advance and haptosensing by coupling nascent adhesions to lamellipodia actin. *Molecular biology of the cell.* Apr 1 2016;27(7):1085-1100.

45. Lim ST, Chen XL, Lim Y, et al. Nuclear FAK promotes cell proliferation and survival through FERM-enhanced p53 degradation. *Molecular cell.* Jan 18 2008;29(1):9-22.

46. Luo SW, Zhang C, Zhang B, et al. Regulation of heterochromatin remodelling and myogenin expression during muscle differentiation by FAK interaction with MBD2. *The EMBO journal.* Sep 2 2009;28(17):2568-2582.

47. Lindfors HE, Drijfhout JW, Ubbink M. The Src SH2 domain interacts dynamically with the focal adhesion kinase binding site as demonstrated by paramagnetic NMR spectroscopy. *IUBMB life.* Jun 2012;64(6):538-544.

48. Choma DP, Milano V, Pumiglia KM, DiPersio CM. Integrin alpha3beta1-dependent activation of FAK/Src regulates Rac1-mediated keratinocyte polarization on laminin-5. *The Journal of investigative dermatology.* Jan 2007;127(1):31-40.

49. Lim Y, Han I, Jeon J, Park H, Bahk YY, Oh ES. Phosphorylation of focal adhesion kinase at tyrosine 861 is crucial for Ras transformation of fibroblasts. *The Journal of biological chemistry.* Jul 9 2004;279(28):29060-29065.

50. Sawada Y, Tamada M, Dubin-Thaler BJ, et al. Force sensing by mechanical extension of the Src family kinase substrate p130Cas. *Cell.* Dec 1 2006;127(5):1015-1026.

51. Schaller MD, Hildebrand JD, Shannon JD, Fox JW, Vines RR, Parsons JT. Autophosphorylation of the focal adhesion kinase, pp125FAK, directs SH2-dependent binding of pp60src. *Molecular and cellular biology.* Mar 1994;14(3):1680-1688.

52. Hunter T. Protein modification: phosphorylation on tyrosine residues. *Current opinion in cell biology.* Dec 1989;1(6):1168-1181.

53. Dwyer SF, Gao L, Gelman IH. Identification of novel focal adhesion kinase substrates: role for FAK in NFkappaB signaling. *International journal of biological sciences.* 2015;11(4):404-410.

54. Brami-Cherrier K, Gervasi N, Arsenieva D, et al. FAK dimerization controls its kinase-dependent functions at focal adhesions. *The EMBO journal.* Feb 18;33(4):356-370.

55. Grigera PR, Jeffery ED, Martin KH, Shabanowitz J, Hunt DF, Parsons JT. FAK phosphorylation sites mapped by mass spectrometry. *Journal of cell science.* Nov 1 2005;118(Pt 21):4931-4935.

56. Harte MT, Hildebrand JD, Burnham MR, Bouton AH, Parsons JT. p130Cas, a substrate associated with v-Src and v-Crk, localizes to focal adhesions and binds to focal adhesion kinase. *The Journal of biological chemistry.* Jun 7 1996;271(23):13649-13655.

57. Hildebrand JD, Taylor JM, Parsons JT. An SH3 domain-containing GTPase-activating protein for Rho and Cdc42 associates with focal adhesion kinase. *Molecular and cellular biology.* Jun 1996;16(6):3169-3178.

58. Liu Y, Loijens JC, Martin KH, Karginov AV, Parsons JT. The association of ASAP1, an ADP ribosylation factor-GTPase activating protein, with focal adhesion kinase contributes to the process of focal adhesion assembly. *Molecular biology of the cell.* Jun 2002;13(6):2147-2156.

59. Guinebault C, Payrastre B, Racaud-Sultan C, et al. Integrin-dependent translocation of phosphoinositide 3-kinase to the cytoskeleton of thrombin-activated platelets involves specific interactions of p85 alpha with actin filaments and focal adhesion kinase. *The Journal of cell biology.* May 1995;129(3):831-842.

60. Fresu M, Bianchi M, Parsons JT, Villa-Moruzzi E. Cell-cycle-dependent association of protein phosphatase 1 and focal adhesion kinase. *The Biochemical journal.* Sep 1 2001;358(Pt 2):407-414.

61. Bianchi M, De Lucchini S, Marin O, Turner DL, Hanks SK, Villa-Moruzzi E. Regulation of FAK Ser-722 phosphorylation and kinase activity by GSK3 and PP1 during cell spreading and migration. *The Biochemical journal.* Oct 15 2005;391(Pt 2):359-370.

62. Hildebrand JD, Schaller MD, Parsons JT. Identification of sequences required for the efficient localization of the focal adhesion kinase, pp125FAK, to cellular focal adhesions. *The Journal of cell biology.* Nov 1993;123(4):993-1005.

63. Hildebrand JD, Schaller MD, Parsons JT. Paxillin, a tyrosine phosphorylated focal adhesion-associated protein binds to the carboxyl terminal domain of focal adhesion kinase. *Molecular biology of the cell.* Jun 1995;6(6):637-647.

64. Brown MC, Perrotta JA, Turner CE. 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. *The Journal of cell biology.* Nov 1996;135(4):1109-1123.

65. Bakolitsa C, de Pereda JM, Bagshaw CR, Critchley DR, Liddington RC. Crystal structure of the vinculin tail suggests a pathway for activation. *Cell.* Dec 10 1999;99(6):603-613.

66. Cooley MA, Broome JM, Ohngemach C, Romer LH, Schaller MD. Paxillin binding is not the sole determinant of focal adhesion localization or dominant-negative activity of focal adhesion kinase/focal adhesion kinase-related nonkinase. *Molecular biology of the cell.* Sep 2000;11(9):3247-3263.

67. Chen HC, Appeddu PA, Parsons JT, Hildebrand JD, Schaller MD, Guan JL. Interaction of focal adhesion kinase with cytoskeletal protein talin. *The Journal of biological chemistry.* Jul 14 1995;270(28):16995-16999.

68. Schlaepfer DD, Hunter T. Evidence for in vivo phosphorylation of the Grb2 SH2-domain binding site on focal adhesion kinase by Src-family protein-tyrosine kinases. *Molecular and cellular biology.* Oct 1996;16(10):5623-5633.

69. Carragher NO, Westhoff MA, Fincham VJ, Schaller MD, Frame MC. A novel role for FAK as a protease-targeting adaptor protein: regulation by p42 ERK and Src. *Current biology : CB.* Aug 19 2003;13(16):1442-1450.

70. Garces CA, Kurenova EV, Golubovskaya VM, Cance WG. Vascular endothelial growth factor receptor-3 and focal adhesion kinase bind and suppress apoptosis in breast cancer cells. *Cancer research.* Feb 1 2006;66(3):1446-1454.

71. Hoellerer MK, Noble ME, Labesse G, Campbell ID, Werner JM, Arold ST. Molecular recognition of paxillin LD motifs by the focal adhesion targeting domain. *Structure.* Oct 2003;11(10):1207-1217.

72. Kadare G, Gervasi N, Brami-Cherrier K, et al. Conformational dynamics of the focal adhesion targeting domain control specific functions of focal adhesion kinase in cells. *The Journal of biological chemistry.* Jan 2 2015;290(1):478-491.

73. Goni GM, Epifano C, Boskovic J, et al. Phosphatidylinositol 4,5-bisphosphate triggers activation of focal adhesion kinase by inducing clustering and conformational changes. *Proceedings of the National Academy of Sciences of the United States of America.* Aug 5 2014;111(31):E3177-3186.

74. Richardson A, Parsons T. A mechanism for regulation of the adhesion-associated proteintyrosine kinase pp125FAK. *Nature.* Apr 11 1996;380(6574):538-540.

75. Schlaepfer DD, Mitra SK, Ilic D. Control of motile and invasive cell phenotypes by focal adhesion kinase. *Biochimica et biophysica acta.* Jul 5 2004;1692(2-3):77-102.

76. Koshman YE, Chu M, Engman SJ, et al. Focal adhesion kinase-related nonkinase inhibits vascular smooth muscle cell invasion by focal adhesion targeting, tyrosine 168 phosphorylation, and competition for p130(Cas) binding. *Arteriosclerosis, thrombosis, and vascular biology.* Nov 2011;31(11):2432-2440.

77. Xu LH, Yang X, Craven RJ, Cance WG. The COOH-terminal domain of the focal adhesion kinase induces loss of adhesion and cell death in human tumor cells. *Cell growth & differentiation : the molecular biology journal of the American Association for Cancer Research.* Dec 1998;9(12):999-1005.

78. Xu LH, Yang X, Bradham CA, et al. The focal adhesion kinase suppresses transformation-associated, anchorage-independent apoptosis in human breast cancer cells. Involvement of death receptor-related signaling pathways. *The Journal of biological chemistry.* Sep 29 2000;275(39):30597-30604.

79. Kornberg L. Ad-fRNK and Ad-p53 cooperate to augment drug-induced death of a transformed cell line. *Anticancer research.* Jul-Aug 2006;26(4B):3025-3031.

80. Golubovskaya V, Beviglia L, Xu LH, Earp HS, 3rd, Craven R, Cance W. Dual inhibition of focal adhesion kinase and epidermal growth factor receptor pathways cooperatively induces death receptor-mediated apoptosis in human breast cancer cells. *The Journal of biological chemistry.* Oct 11 2002;277(41):38978-38987.

81. Govindarajan G, Eble DM, Lucchesi PA, Samarel AM. Focal adhesion kinase is involved in angiotensin II-mediated protein synthesis in cultured vascular smooth muscle cells. *Circulation research.* Oct 13 2000;87(8):710-716.

82. Almeida EA, Ilic D, Han Q, et al. Matrix survival signaling: from fibronectin via focal adhesion kinase to c-Jun NH(2)-terminal kinase. *The Journal of cell biology.* May 1 2000;149(3):741-754.

83. Zheng Y, Xia Y, Hawke D, et al. FAK phosphorylation by ERK primes ras-induced tyrosine dephosphorylation of FAK mediated by PIN1 and PTP-PEST. *Molecular cell.* Jul 10 2009;35(1):11-25.

84. Sharma R, Colarusso P, Zhang H, Stevens KM, Patel KD. FRNK negatively regulates IL-4-mediated inflammation. *Journal of cell science.* Feb 15 2015;128(4):695-705.

85. Ma A, Richardson A, Schaefer EM, Parsons JT. Serine phosphorylation of focal adhesion kinase in interphase and mitosis: a possible role in modulating binding to p130(Cas). *Molecular biology of the cell.* Jan 2001;12(1):1-12.

86. Hunger-Glaser I, Fan RS, Perez-Salazar E, Rozengurt E. PDGF and FGF induce focal adhesion kinase (FAK) phosphorylation at Ser-910: dissociation from Tyr-397 phosphorylation and requirement for ERK activation. *Journal of cellular physiology.* Aug 2004;200(2):213-222.

87. Chu M, Iyengar R, Koshman YE, et al. Serine-910 phosphorylation of focal adhesion kinase is critical for sarcomere reorganization in cardiomyocyte hypertrophy. *Cardiovascular research.* Dec 1 2011;92(3):409-419.

88. Wen LP, Fahrni JA, Troie S, Guan JL, Orth K, Rosen GD. Cleavage of focal adhesion kinase by caspases during apoptosis. *The Journal of biological chemistry.* Oct 10 1997;272(41):26056-26061.

89. Gervais FG, Thornberry NA, Ruffolo SC, Nicholson DW, Roy S. Caspases cleave focal adhesion kinase during apoptosis to generate a FRNK-like polypeptide. *The Journal of biological chemistry.* Jul 03 1998;273(27):17102-17108.

90. Avraham S, London R, Fu Y, et al. Identification and characterization of a novel related adhesion focal tyrosine kinase (RAFTK) from megakaryocytes and brain. *The Journal of biological chemistry.* Nov 17 1995;270(46):27742-27751.

91. Zheng C, Xing Z, Bian ZC, et al. Differential regulation of Pyk2 and focal adhesion kinase (FAK). The C-terminal domain of FAK confers response to cell adhesion. *The Journal of biological chemistry.* Jan 23 1998;273(4):2384-2389.

92. Mills RD, Mita M, Walsh MP. A role for the Ca(2+)-dependent tyrosine kinase Pyk2 in tonic depolarization-induced vascular smooth muscle contraction. *Journal of muscle research and cell motility.* Dec 2015;36(6):479-489.

93. Koshman YE, Chu M, Engman SJ, et al. Focal adhesion kinase-related nonkinase inhibits vascular smooth muscle cell invasion by focal adhesion targeting, tyrosine 168 phosphorylation, and competition for p130(Cas) binding. *Arteriosclerosis, thrombosis, and vascular biology.* Nov;31(11):2432-2440.

94. Owens LV, Xu L, Craven RJ, et al. Overexpression of the focal adhesion kinase (p125FAK) in invasive human tumors. *Cancer research.* Jul 1 1995;55(13):2752-2755.

95. Akasaka T, van Leeuwen RL, Yoshinaga IG, Mihm MC, Jr., Byers HR. Focal adhesion kinase (p125FAK) expression correlates with motility of human melanoma cell lines. *The Journal of investigative dermatology.* Jul 1995;105(1):104-108.

96. Matsumoto K, Matsumoto K, Nakamura T, Kramer RH. Hepatocyte growth factor/scatter factor induces tyrosine phosphorylation of focal adhesion kinase (p125FAK) and promotes migration and invasion by oral squamous cell carcinoma cells. *The Journal of biological chemistry.* Dec 16 1994;269(50):31807-31813.

97. Juliano RL. Integrin signals and tumor growth control. *Princess Takamatsu symposia.* 1994;24:118-124.

98. Hecker TP, Gladson CL. Focal adhesion kinase in cancer. *Frontiers in bioscience : a journal and virtual library.* May 01 2003;8:s705-714.

99. Kim I, Kim HG, Moon SO, et al. Angiopoietin-1 induces endothelial cell sprouting through the activation of focal adhesion kinase and plasmin secretion. *Circulation research.* May 12 2000;86(9):952-959.

100. Slack-Davis JK, Martin KH, Tilghman RW, et al. Cellular characterization of a novel focal adhesion kinase inhibitor. *The Journal of biological chemistry.* May 18 2007;282(20):14845-14852.

101. Yoon H, Dehart JP, Murphy JM, Lim ST. Understanding the roles of FAK in cancer: inhibitors, genetic models, and new insights. *The journal of histochemistry and cytochemistry : official journal of the Histochemistry Society.* Feb 2015;63(2):114-128.

102. Infante JR, Camidge DR, Mileshkin LR, et al. Safety, pharmacokinetic, and pharmacodynamic phase I dose-escalation trial of PF-00562271, an inhibitor of focal adhesion kinase, in advanced solid tumors. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology.* May 1 2012;30(13):1527-1533.

103. Sayers RL, Sundberg-Smith LJ, Rojas M, et al. FRNK expression promotes smooth muscle cell maturation during vascular development and after vascular injury. *Arteriosclerosis, thrombosis, and vascular biology.* Dec 2008;28(12):2115-2122.

104. Taylor JM, Mack CP, Nolan K, Regan CP, Owens GK, Parsons JT. Selective expression of an endogenous inhibitor of FAK regulates proliferation and migration of vascular smooth muscle cells. *Molecular and cellular biology.* Mar 2001;21(5):1565-1572.

105. Claessen BE, Henriques JP, Jaffer FA, Mehran R, Piek JJ, Dangas GD. Stent thrombosis: a clinical perspective. *JACC. Cardiovascular interventions.* Oct 2014;7(10):1081-1092.

106. Koshman YE, Engman SJ, Kim T, Iyengar R, Henderson KK, Samarel AM. Role of FRNK tyrosine phosphorylation in vascular smooth muscle spreading and migration. *Cardiovascular research.* Feb 1;85(3):571-581.

107. Ghanie A. Indication for percutaneous coronary angioplasty. *Acta medica Indonesiana.* Jul 2009;41(3):159-165.

108. Buccheri D, Piraino D, Andolina G, Cortese B. Understanding and managing in-stent restenosis: a review of clinical data, from pathogenesis to treatment. *Journal of thoracic disease.* Oct 2016;8(10):E1150-E1162.

109. Kirtane AJ, Gupta A, Iyengar S, et al. Safety and efficacy of drug-eluting and bare metal stents: comprehensive meta-analysis of randomized trials and observational studies. *Circulation.* Jun 30 2009;119(25):3198-3206.

110. Holmes DR, Jr. Drug-eluting coronary-artery stents. *The New England journal of medicine.* Apr 18 2013;368(16):1558.

111. Gargiulo G, Moschovitis A, Windecker S, Valgimigli M. Developing drugs for use before, during and soon after percutaneous coronary intervention. *Expert opinion on pharmacotherapy.* 2016;17(6):803-818.

112. Majithia A, Bhatt DL. Optimal Duration of Dual Antiplatelet Therapy After Percutaneous Coronary Intervention. *Interventional cardiology clinics.* Jan 2017;6(1):25-37.

113. Kwok CS, Bulluck H, Ryding AD, Loke YK. Benefits and harms of extending the duration of dual antiplatelet therapy after percutaneous coronary intervention with drug-eluting stents: a meta-analysis. *TheScientificWorldJournal.* 2014;2014:794078.

114. Agarwal SK. Single antiplatelet therapy after percutaneous coronary intervention in patients allergic to aspirin. *Cardiovascular revascularization medicine : including molecular interventions.* Jul-Aug 2014;15(5):308-310.

115. Ding Q, Cai GQ, Hu M, et al. FAK-related nonkinase is a multifunctional negative regulator of pulmonary fibrosis. *The American journal of pathology.* May 2013;182(5):1572-1584.

116. Heidkamp MC, Bayer AL, Kalina JA, Eble DM, Samarel AM. GFP-FRNK disrupts focal adhesions and induces anoikis in neonatal rat ventricular myocytes. *Circulation research.* Jun 28 2002;90(12):1282-1289.

117. Mattheyses AL, Simon SM, Rappoport JZ. Imaging with total internal reflection fluorescence microscopy for the cell biologist. *Journal of cell science.* Nov 1 2010;123(Pt 21):3621-3628.

118. Axelrod D. Cell-substrate contacts illuminated by total internal reflection fluorescence. *The Journal of cell biology.* Apr 1981;89(1):141-145.

119. Kanchanawong P, Shtengel G, Pasapera AM, et al. Nanoscale architecture of integrin-based cell adhesions. *Nature.* Nov 25 2010;468(7323):580-584.

120. Chen H, Puhl HL, 3rd, Koushik SV, Vogel SS, Ikeda SR. Measurement of FRET efficiency and ratio of donor to acceptor concentration in living cells. *Biophysical journal.* Sep 1 2006;91(5):L39-41.

121. Kelly EM, Hou Z, Bossuyt J, Bers DM, Robia SL. Phospholamban oligomerization, quaternary structure, and sarco(endo)plasmic reticulum calcium ATPase binding measured by fluorescence resonance energy transfer in living cells. *The Journal of biological chemistry.* May 2 2008;283(18):12202-12211.

122. Blackwell DJ, Zak TJ, Robia SL. Cardiac Calcium ATPase Dimerization Measured by Cross-Linking and Fluorescence Energy Transfer. *Biophysical journal.* Sep 20 2016;111(6):1192-1202.

123. Kurenova E, Xu LH, Yang X, et al. Focal adhesion kinase suppresses apoptosis by binding to the death domain of receptor-interacting protein. *Molecular and cellular biology.* May 2004;24(10):4361-4371.

124. Koushik SV, Chen H, Thaler C, Puhl HL, 3rd, Vogel SS. Cerulean, Venus, and VenusY67C FRET reference standards. *Biophysical journal.* Dec 15 2006;91(12):L99-L101.

125. Duncan RR. Fluorescence lifetime imaging microscopy (FLIM) to quantify protein-protein interactions inside cells. *Biochemical Society transactions.* Nov 2006;34(Pt 5):679-682.

126. Shu Y, Liu X, Yang Y, Takahashi M, Gillis KD. Phosphorylation of SNAP-25 at Ser187 mediates enhancement of exocytosis by a phorbol ester in INS-1 cells. *The Journal of neuroscience : the official journal of the Society for Neuroscience.* Jan 02 2008;28(1):21-30.

127. Zaidel-Bar R, Milo R, Kam Z, Geiger B. A paxillin tyrosine phosphorylation switch regulates the assembly and form of cell-matrix adhesions. *Journal of cell science.* Jan 01 2007;120(Pt 1):137-148.

128. Toutant M, Costa A, Studler JM, Kadare G, Carnaud M, Girault JA. Alternative splicing controls the mechanisms of FAK autophosphorylation. *Molecular and cellular biology.* Nov 2002;22(22):7731-7743.

129. Hunger-Glaser I, Salazar EP, Sinnett-Smith J, Rozengurt E. Bombesin, lysophosphatidic acid, and epidermal growth factor rapidly stimulate focal adhesion kinase phosphorylation at Ser-910: requirement for ERK activation. *The Journal of biological chemistry.* Jun 20 2003;278(25):22631-22643.

130. Fahrenholz M, Real R, Kuken A, Saxena A, Orzechowski HD. Single low-dose administration of pharmacological inhibitor of mitogen-activated ERK kinase to the adventitia of the injured rat carotid artery suppresses neointima formation and inhibits nuclear ERK signaling. *European journal of pharmacology.* Sep 01 2009;617(1-3):90-96.

131. Klocke R, Hasib L, Nikol S. Recently patented applications of homologous cellular and extracellular agents as therapeutics or targets for the prevention of restenosis post-angioplasty. *Recent patents on cardiovascular drug discovery.* Jan 2006;1(1):57-66.

132. Miao Y, McCammon JA. G-protein coupled receptors: advances in simulation and drug discovery. *Current opinion in structural biology.* Dec 2016;41:83-89.

133. Geskin LJ. Monoclonal Antibodies. *Dermatologic clinics.* Oct 2015;33(4):777-786.

134. Chames P, Van Regenmortel M, Weiss E, Baty D. Therapeutic antibodies: successes, limitations and hopes for the future. *British journal of pharmacology.* May 2009;157(2):220-233.

135. Marschall AL, Dubel S. Antibodies inside of a cell can change its outside: Can intrabodies provide a new therapeutic paradigm? *Computational and structural biotechnology journal.* 2016;14:304-308.