Published in final edited form as:

Biochemistry. 2017 September 05; 56(35): 4722–4731. doi:10.1021/acs.biochem.7b00616.

Regulation of Focal Adhesion Kinase through a Direct Interaction with an Endogenous Inhibitor

Taylor J. Zak¹, Yevgenia E. Koshman², Allen M. Samarel², Seth L. Robia^{*,1}

¹Department of Cell and Molecular Physiology, Cardiovascular Research Institute, Stritch School of Medicine, Loyola University Chicago, Maywood, IL 60153

²Department of Medicine, Cardiovascular Research Institute, Stritch School of Medicine, Loyola University Chicago, Maywood, IL 60153

Abstract

Focal adhesion kinase (FAK) plays a key role in integrin and growth factor signaling pathways. FAK related non-kinase (FRNK) is an endogenous inhibitor of FAK that shares its primary structure with the C-terminal third of FAK. FAK S910 phosphorylation is known to regulate FAK protein-protein interactions, but the role of the equivalent site on FRNK (S217) is unknown. Here we determined that S217 is highly phosphorylated by ERK in cultured rat aortic smooth muscle cells. Blocking phosphorylation by mutation (S217A) greatly increased FRNK inhibitory potency, resulting in strong inhibition of FAK autophosphorylation at Y397 and induction of smooth muscle cell apoptosis. FRNK has been proposed to compete for FAK anchoring sites in focal adhesions, but we did not detect displacement of FAK by WTFRNK or superinhibitory S217A-FRNK. Instead, we found FRNK interacted directly with FAK and this interaction is markedly increased for the superinhibitory S217A-FRNK. The results suggest that FRNK limits growth and survival signaling pathways by binding directly to FAK in an inhibitory complex, and this inhibition is relieved by phosphorylation of FRNK at S217.

Keywords

focal adhesion kinase (FAK); focal adhesion kinase related non-kinase; extracellular regulated kinase (ERK); vascular smooth muscle cells; focal adhesions; protein phosphorylation; apoptosis; Fluorescence resonance energy transfer (FRET); Total internal reflection fluorescence (TIRF)

*Correspondence to: Seth L. Robia, Ph.D., Department of Cell and Molecular Physiology, Cardiovascular Research Institute, Loyola University Chicago, Stritch School of Medicine, 2160 S. First Avenue, Maywood, Illinois 60153. Telephone: 708-216-2522, Fax: 708-216-6308, srobia@luc.edu.

Author Contributions: TJZ designed the study, conducted all the experiments, analyzed the data, and wrote the paper. YEK created critical reagents and methods. AMS designed the study, assisted in the experiments, analyzed data, and wrote the paper. SLR designed the study, assisted in the experiments, analyzed data, and wrote the paper. All authors reviewed the experimental results and approved the final manuscript.

SUPPORTING INFORMATION

Western blot confirming angiotensin II activation of ERK

Western blot demonstrates that ERK inhibition also decreases FAK Serine 910 phosphorylation

Western blot demonstrating a linear increase in WT- and S217A- FRNK expression with increase in volume of adenovirus used to balance WT and mutant FRNK expression

Conflict of interest: The authors declare that they have no conflicts of interest with the contents of this article.

INTRODUCTION

Focal adhesion kinase (FAK) is a 125 kDa non-receptor protein tyrosine kinase responsible for activating many signaling pathways including those responsible for cell growth¹, survival², and migration³⁻⁵. FAK is expressed ubiquitously⁵ and its knockout is lethal during embryogenesis of the vascular system⁶. FAK is so named because of its striking localization to cellular structures called focal adhesions. These dynamic structures mechanically connect the extracellular matrix to the intracellular cytoskeleton and serve as localized domains of cell signaling⁷⁻⁹. Once anchored in the focal adhesion, FAK undergoes a variety of post-translational modifications that regulate its kinase activity and the scaffolding of FAK with other proteins in signaling complexes^{3, 5}. FAK comprises three domains (Fig. 1A): a kinase domain with tyrosine specificity, a focal adhesion targeting (FAT) domain that binds to proteins within focal adhesions and localizes FAK to the focal adhesion microenvironment, and an auto-inhibitory protein 4.1, ezrin, radixin and moesin homology (FERM) domain that prevents FAK kinase activity until FAK is activated by diverse stimuli³.

Transcription from an alternative promoter within a FAK intron results in expression of a 41 kDa product-FAK-related non-Kinase (FRNK)¹⁰. This species is identical in amino acid sequence to the C-terminal portion of FAK, consisting of the focal adhesion targeting (FAT) domain and a proline rich region in the linker that has been implicated in signaling pathways⁴ (Fig. 1A). FRNK has been well-described as an inhibitor of FAK signaling^{2, 11-15}. Both FAK and FRNK undergo a number of post-translational modifications. In particular, serine 910 (S910) on FAK is an extracellular signal-related kinase 1/2 (ERK) phosphorylation site that regulates protein-protein interactions¹⁶. We have previously demonstrated that this site is functionally important in cardiomyocytes for regulating cell spreading and sarcomere reorganization¹⁷. There is an equivalent phosphorylation site on FRNK (S217), but little is known about the extent of FRNK phosphorylation or its functional significance. Because of the site's importance in regulating protein-protein interactions on FAK¹⁶, we hypothesized that FRNK S217 phosphorylation may modulate FRNK inhibition of growth, survival, and migration pathways. Since both FAK and FRNK contain the FAT domain that mediates anchoring in focal adhesions, FRNK has been postulated to occupy binding sites and displace FAK from the focal adhesions into the cytoplasm¹¹. This displacement mechanism has frequently been offered as an explanation of FRNK inhibitory function by us and others^{2, 12-15}. However, the degree of FAK displacement and the functional significance of this putative mechanism are unclear¹⁸⁻²¹. In the present study, we directly test the canonical displacement model and evaluate a possible alternative regulatory mechanism: direct inhibition by a physical interaction of FAK and FRNK.

FAK has previously been shown to oligomerize into functional homodimers²² and higher order oligomers²³ through FERM-FERM²², FERM-FAT²², or FAT-FAT²⁴ domain interactions. Since FRNK also possesses the FAT domain (Fig. 1A), we reasoned that FRNK may bind directly to FAK, forming a heteromeric inhibitory complex. The specific mechanism of inhibition of FAK by FRNK is of significant interest as FAK signaling pathways underlie many important physiological and pathological processes. FAK is a point of convergence of growth factor signaling, integrin signaling, and other pathways²⁵. It is

therefore not surprising that FAK dysregulation is linked to diverse cancers²⁶. In harmony with its pro-growth signaling, FAK also prevents apoptosis²⁷. The role of FRNK in disease is much less well studied, but it has been linked to pulmonary fibrosis²⁸ and neointimal hyperplasia after vascular tissue injury^{18, 29}. We and others have previously shown that FRNK is markedly upregulated after vascular injury, specifically in vascular smooth muscle cells^{18, 19, 29, 30}. This specificity makes FRNK a compelling clinical target since growth and migration of vascular smooth muscle cells, while essential for healing, can become disordered in pathological conditions. Maladaptive vascular remodeling can occur following coronary angioplasty, with restenosis (re-narrowing) of the vessel due to inappropriate growth of smooth muscle tissue at the site of injury³¹. The present study seeks to elucidate the role of FRNK in limiting smooth muscle cell growth and survival by determining the molecular mechanisms of FAK inhibition by FRNK.

MATERIALS AND METHODS

Materials and reagents.

Tissue culture dishes were from Nunc (Naperville, IL). 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). Rabbit anti-FAK (P-Y397) phosphospecific antibody (700255) and anti-FAK (P-S910) antibody (44596G) were from Thermo-Fisher (Camarillo, CA). GFP antibody (1e4) was from Stressgen (Ann Arbor, MI). N-terminal FAK antibody (FAK-specific, BD #610087) was from Millipore (Darmstadt, Germany). A C-terminal FAK/FRNK antibody (sc-558) that recognizes both species was obtained from Santa Cruz (Dallas, TX). GAPDH antibody (NB300–221) was from Novus Biologicals (Littleton, CO). Rainbow molecular weight standards and enhanced chemiluminescence (ECL) kits were from Amersham (Arlington Heights, IL). 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), IR dye 680RD donkey anti-mouse, or Bio-Rad Goat anti Rabbit conjugated to HRP for ECL.

Cell culture.

Rat aortic smooth muscle cells (RASMs) were isolated as previously described³² and maintained in DMEM containing 10% FBS. Cells up to the 24th passage were used.

Adenoviral constructs.

Replication-defective adenoviruses (Adv) expressing GFP, GFP-wild-type (WT) FRNK, nuclear-encoded β -galactosidase (β gal), FLAG-FAK and FLAG-WT-FRNK were generated as previously described². Cerulean (Cer) WT-FRNK, mCherry-WT-FRNK, YFP-FAK, and cyan fluorescent protein (CFP) S217A-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 was determined by dilution assay in HEK293 cells grown in 96-well clusters. To ensure equal expression of WT FRNK and

S217A FRNK for comparative experiments, we determined the expression of each using viral dose protein expression analysis (Supplemental Fig. 3).

Kinase inhibitor experiments.

Equal numbers of RASMs were plated onto 10cm plates in 10% serum containing DMEM and infected with 300 MOI 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 10 μ M, PD98059 (PD98) at 30 μ M, SP600125 (SP600) at 40 μ M, Y-27632 (Y27) at 10 μ M, SB 203580 (SB203) at 10 μ M, U0126 at 10 μ M, and Angiotensin II (AT II) at 1 μ M. 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 probed with either a total FAK/FRNK C-terminal antibody or a phosphospecific 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. 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.

DAPI nuclei assay.

RASMs were counted and placed into microcentrifuge tubes where they were infected with GFP, Cer-FRNK, CFP-S217A-FRNK, or no adenovirus, using Western blot analysis to balance expression levels. Cells were then plated into chamber slides and allowed to grow for 48h. Cells were then fixed in 4% paraformaldehyde and stained with DAPI. Cells were imaged 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 nuclei.

Flow cytometry.

Cells were prepared as above and infected with equal amounts of Cer-WT-FRNK, CFPS217A-FRNK, control β gal or no adenovirus. WT- and S217A-FRNK expression were balanced with Western blot analysis. After 48h of infection cells were harvested, including attached and detached cells, placed in a 2ml centrifuge tube, 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 were 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.

FAK Y397 assay.

RASMs were plated onto 10 cm plates, infected in suspension with increasing volumes of mCherry-FRNK or CFP-S217A-FRNK, and cultured for 24 hours. For WT FRNK, the added volumes of virus were 0.2, 0.5, 1, 2, and 4 μ l. The volumes of S217A FRNK virus that yielded a comparable range of FAK Y397 phosphorylation inhibition were 2.5, 5, 10, 20, and 40 μ l. Cells were scraped into cell lysis buffer¹⁹ containing Pefabloc, vanadate, leupeptin, and aprotinin and then subjected to Western blotting using total FAK/FRNK (sc-558) and phosphospecific (700255) antibodies. GAPDH was used as a loading control. Two ECL film exposures were obtained, approximately 1 sec (short exposure) and 5 min (long exposure). The resulting densities of FRNK and FAK Y397 were plotted against each other for both the WT and mutant FRNK to compare relative potency. Only the highest molecular weight total FRNK band was used for the quantification of total FRNK. All comparisons were done on samples within the same gel.

FAK displacement.

RASMs plated at 50% confluency in DMEM containing 10% FBS with no added agonists/inhibitors were infected with a 3:1 ratio of Cer-WT-FRNK or CFP-S217A-FRNK to YFP-FAK for 24 hours and subjected to TIRF microscopy. Lineouts were applied across the short axes of focal adhesions. The lineout plots (fluorescence intensity vs. position) were fit with a

Gaussian function of the form $y = y_0 + \frac{Ae^{-\frac{4\ln(2)(x-x_c)^2}{w^2}}}{w\sqrt{\frac{\pi}{4\ln(2)}}}$ where y_0 is the base, x_c is the

center, w is the full width half maximum, and A is the amplitude of the peak. The amplitude was taken as an index of fluorescently labeled FAK or FRNK in the focal adhesion. These values were compared to determine whether there was a negative correlation between FAK focal adhesion localization and FRNK focal adhesion localization to evaluate whether the two proteins compete for binding in focal adhesions.

Co-immunoprecipitation.

RASMs 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 immunoprecipitated with 5 μ g of a FAK antibody that recognizes only the FAK kinase domain (BD biosciences #610087) (which recognizes FAK but not FRNK) overnight at 4 °C. Then complexes were bound to protein A/G beads for 2h at 4 °C, and 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. Isolated proteins were subjected to SDS-PAGE and Western blotting. Blots were probed with a different antibody (Santa Cruz sc-558) that recognizes both the immunoprecipitated FAK and co-immunoprecipitated FRNK.

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). 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, subtracting a background value obtained from the region of the lineout outside the cell boundary.

TIRF-mode acceptor photo bleaching FRET.

RASMs were infected with CFP-S217A-FRNK or Cer-WTFRNK and YFP-WT-FAK Adv at a 1:3 ratio (balanced by Western blot analysis) 24h before imaging in a two-well chamber slide. We performed TIRF imaging of Cer and YFP at 30 sec intervals to establish a baseline and then initiated progressive acceptor photobleaching³³, acquiring successive images of Cer and YFP with TIRF illumination interleaved 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. The resulting sets of images were then analyzed in ImageJ by hand drawing regions around clusters of 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 F_D = 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 bleaching³³.

Statistical analysis.

Values reported here are means $\pm$ 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. Differences among means were considered significant at $P < 0.05$. Data were analyzed using OriginLab software (Northampton, Massachusetts).

RESULTS

FRNK S217 is highly phosphorylated by ERK in RASMs.

We have previously shown that both FAK and FRNK are upregulated in injured smooth muscle. Because of our interest in FRNK as a protein upregulated during artery injury, we conducted the experiments using a primary cell line of rat aortic smooth muscle cells (RASMs)³². To determine the kinase(s) responsible for FRNK S217 phosphorylation in vascular smooth muscle cells, we treated RASMs with selected kinase inhibitors and examined FRNK S217 phosphorylation by Western blotting utilizing phospho-specific antibodies (Fig. 1B). We used PD98059 (PD98) 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 p38^{MAPK}, comparing to DMSO vehicle. PD98 was the only

inhibitor that significantly reduced FRNK S217 phosphorylation compared to untreated cells (Fig. 1C). We have previously determined that PKC is upstream from ERK in phosphorylation of FAK 910 in cardiac myocytes¹⁷, however in the present study the effect of PKC inhibition by GFX did not achieve statistical significance with an ANOVA followed by a Tukey's means comparison. We also compared the effect of PD98 inhibition with that of an additional ERK inhibitor, U0126, and observed a similar decrease in FRNK S217 phosphorylation (Fig. 1D,E). The data support the conclusion that ERK is the kinase responsible for FRNK S217 phosphorylation. Interestingly, 10 min angiotensin II (ATII) stimulation did not increase FRNK S217 phosphorylation above basal levels, suggesting that FRNK was already highly phosphorylated at the S217 site under these cell culture conditions (Fig. 1E). We confirmed angiotensin II treatment activated ERK in our cell culture model (Supplemental Fig. 1). FAK S910 phosphorylation also decreased upon ERK inhibition (Supplemental Fig. 2), consistent with previous reports¹⁶.

Effects of S217A-FRNK expression on cell survival.

To determine the physiological significance of S217 phosphorylation we generated a non-phosphorylatable mutant by changing serine 217 to alanine. We compared the survival of cultured RASMs expressing equal levels of WT- or S217A-FRNK, evaluating survival by counting DAPI stained nuclei (Fig. 2A). Fig. 2B shows that uninfected cells were similar to GFP infected cells, while WT- and S217A-FRNK reduced cell number per microscopic field by 45% and 95% respectively. The data suggest that FRNK expression decreased cell survival, and this effect was increased by blocking S217A phosphorylation. In a complementary experiment we quantified cell apoptosis using flow cytometry. Uninfected and RASMs infected with β gal were used as negative controls and had an apoptotic population of 6% and 9% respectively. Significantly, WT- and S217A-FRNK infected RASMs had an apoptotic population of 17% and 96% respectively (Fig. 2C), suggesting that the decrease in cell survival with FRNK expression (Fig. 2A) was due to increased apoptosis. The data are consistent with a role for FRNK in negative regulation of FAK dependent survival pathways², and suggest that the S217 ERK phosphorylation site regulates this inhibitory function.

FRNK rendered nonphosphorylatable at S217 is a superinhibitor of FAK signaling.

To determine the functional role of FRNK S217 phosphorylation on FAK-dependent signaling, we measured FAK Y397 phosphorylation in RASMs expressing either WT- or S217A-FRNK. We and others have shown that FAK Y397 phosphorylation, the key activating step in FAK signaling, is reduced by increased FRNK expression^{4, 5, 34}. To determine the relative effect of S217A mutation we overexpressed both WT- and S217A-FRNK at increasing concentrations until FAK Y397 phosphorylation was reduced to near background levels (Fig. 2D). The S217A-FRNK dose required to inhibit FAK Y397 phosphorylation was remarkably lower than the required WT-FRNK dose, such that two different levels of exposures are required to show both the WT- and S217A-FRNK expression levels. Red boxes highlight samples with similar expression levels of WT- and S217A-FRNK, and these lanes are compared side-by-side in Fig. 2E. We concluded that the mutant is superinhibitory with respect to FAK Y397 phosphorylation; the data indicate a >10-fold increase in potency for S217A-FRNK.

FRNK expression does not displace FAK from focal adhesions as measured by TIRF microscopy.

FRNK has been proposed to inhibit FAK activity by binding to FAT domain binding sites within the focal adhesion, thereby displacing FAK from the specific environment of the focal adhesion where signaling occurs. Because S217A-FRNK expression resulted in more potent inhibition of FAK signaling and induction of apoptosis we hypothesized that S217A-FRNK was more effective at displacing FAK from focal adhesions. To test this hypothesis we measured YFP-FAK localization in focal adhesions with TIRF microscopy in cells expressing a wide range of concentrations of FRNK (Fig. 3A) or S217A-FRNK (Fig. 3B). Surprisingly, we did not observe displacement of FAK from focal adhesions over several orders of magnitude of FRNK expression, nor did cells expressing superinhibitory S217A-FRNK show different localization of YFP-FAK. Fig. 3 highlights similar YFP-FAK fluorescence in focal adhesions containing only FAK (yellow arrows) and focal adhesions with colocalization of FAK and FRNK (cyan arrows). Fig. 3C shows the quantification of FAK localization in focal adhesions quantified as a function of focal adhesion FRNK fluorescence intensity. We observed no correlation between FAK and FRNK localization nor a significant difference between WT- and S217A-FRNK. This result suggests that FRNK does not displace FAK from focal adhesions at concentrations that yield profound physiological effects. We note that previous reports of FAK displacement from focal adhesions by FRNK relied on coimmunoprecipitation experiments measuring FAK co-immunoprecipitation with paxillin as an index of focal adhesion anchoring^{19, 35, 36}, while the present study quantified FAK localization directly.

FRNK co-immunoprecipitates with FAK.

Since focal adhesion displacement did not account for the observed physiological activity of FRNK and superinhibitory S217A-FRNK, we considered the possibility that FRNK may inhibit FAK activity by alternative mechanisms. In particular, we hypothesized that FRNK may be able to bind directly to FAK within focal adhesions and disrupt FAK signaling by forming an inhibitory complex. Immunoprecipitation was performed with a FAK-specific antibody (BD #610087) directed toward the FAK kinase domain. This domain is absent from FRNK. After immunoprecipitation, FAK and any co-immunoprecipitated binding partners were subjected to SDS-PAGE and Western blot. FAK and FRNK were detected on the blot using a pan-specific antibody (sc-558) that recognizes a C-terminal epitope that is shared by FAK and FRNK. As we and others have previously observed^{21, 30, 35–39}, FRNK migrated as multiple bands. Notably, S217A mutation increased FRNK co-immunoprecipitation by 5 fold. Overall, the data suggest a direct interaction of FRNK with FAK and that is negatively regulated by FRNK phosphorylation at S217.

S217A-FRNK binds to FAK within focal adhesions.

Since a large portion of FAK signaling is mediated by the fraction of FAK that is localized in focal adhesions^{3, 5} and FAK dimerization and activation occurs in focal adhesions²², we tested the hypothesis that FRNK binds directly to FAK using TIRF imaging with progressive acceptor photobleaching to quantify FRET from FRNK to FAK in the focal adhesions of living RASMs. Cells were first subjected to CFP/YFP imaging for five minutes to establish a

stable baseline. Initiation of acceptor-selective photobleaching resulted in a progressive decrease in YFP-FAK fluorescence. While this treatment did not significantly increase Cer-WT-FRNK donor fluorescence (Fig. 5A,C), we observed a marked increase in the fluorescence of the donor fluorescent protein fused to superinhibitory S217A-FRNK consistent with robust FRET from CFP-S217A-FRNK to FAK (Fig. 5B,D). From the magnitude of the donor fluorescence intensity changes after photobleaching, we calculated a remarkably high FRET efficiency of $53.01 \pm 3.3\%$ for S217A-FRNK suggesting a direct interaction of nonphosphorylatable FRNK with FAK. We did not detect FRET from WT-FRNK to FAK. We did detect a very low level (8%) of WT-FRNK-FAK FRET using fluorescence lifetime analysis (not shown), but overall the interaction of WT-FRNK appears to bind poorly to FAK, likely because of high basal phosphorylation of FRNK S217. Taken together, FRET and co-immunoprecipitation data suggest that phosphorylated FRNK interacts only weakly with FAK and this interaction is markedly increased upon dephosphorylation. Because of the correlation of increased binding of S217A-FRNK with FAK (Fig. 4, 5) with increased inhibitory potency (Fig. 2) we hypothesize that the FRNK-FAK interaction is the mechanistic basis for FAK inhibition.

FRNK-FAK complex stoichiometry.

The observed 53% FRET efficiency for CFP-S217A-FRNK and YFPFAK is unusually high for a fluorescent protein FRET pair. For comparison, a control Cerulean-Venus fusion construct with a very short (5 residue) linker has a FRET efficiency of approximately 40%^{33, 40}. One possible explanation is that S217A-FRNK interacts with an oligomeric FAK complex containing multiple acceptors. To test this possibility, we compared the progressive increase in donor fluorescence with the progressive decrease in acceptor fluorescence (Fig. 5F), an analysis that we have used previously to probe the stoichiometry of oligomeric complexes^{33, 41–44}. As diagrammed in Fig. 5E, a 1:1 FRNK-FAK complex (heterodimer) is expected to yield a linear increase in donor fluorescence as acceptor fluorescence decreases because each acceptor (“A”) that is bleached results in one donor (“D”) getting brighter. In contrast, a higher order oligomer with multiple acceptors yields a curved relationship. Even after photobleaching of the first acceptor (“A₁”) the donor is still quenched by efficient energy transfer (black arrows) to the remaining acceptors (“A₂, A₃”). Thus, donor fluorescence increases slowly at first, then more rapidly as the last acceptors in the complex are photobleached. We observed that the CFP/YFP relationship was highly curved for non-phosphorylatable FRNK (Fig. 5F, black points), consistent with high order oligomerization stoichiometry. For comparison, Fig. 5F shows the expected relationship for a dimer (grey line) and the profile for the highly phosphorylated WT-FRNK (red points) for which we detected no FRET. Overall, the analysis suggests that FRNK dephosphorylated at S217 interacts with clusters of FAK, which is consistent with reports that FAK can form complexes containing up to eight FAK molecules²³.

DISCUSSION

Alternative mechanistic models-

The principle goal of the present study was to test the hypothesis that the FAK inhibitor, FRNK, binds directly to its target in an inhibitory complex. Co-immunoprecipitation (Fig. 4)

and FRET experiments (Fig. 5) suggest FRNK-FAK binding does indeed occur in vascular smooth muscle cells. We determined this interaction is regulated by ERK phosphorylation of FRNK at S217. The regulatory significance of the FRNK-FAK interaction is suggested by the observation that the nonphosphorylatable mutant S217A-FRNK is a superinhibitor of FAK activation (Fig. 2D) that strongly induces apoptosis of smooth muscle cells (Fig. 2C). The present data do not support the commonly cited model of FAK inhibition through competition of FRNK for anchoring sites in focal adhesions. Rather, we observed strong focal adhesion localization of FAK in smooth muscle cells expressing WT-FRNK or superinhibitory S217A-FRNK (Fig. 3, 5). We cannot discount the possibility that displacement of FAK from focal adhesions may occur at even higher levels of FRNK expression, but we note that we obtained FRNK concentrations that strongly inhibited FAK activity and cell survival (Fig. 2).

FAK in health and disease-

The specific mechanism of FRNK inhibition of FAK is of interest because of the central role of FAK in normal physiology and in several pathological conditions. We are particularly interested in the relevance of the putative inhibitory complex in vascular smooth muscle cells as we have previously demonstrated the significance of FRNK in the context of neointima formation³⁰. This process underlies vascular restenosis, a complication of angioplasty and stenting of coronary or peripheral arteries⁴⁵. In contrast to primary stenotic lesions restenosis is characterized by growth and migration of vascular smooth muscle cells^{31,46}, but current drug-eluting stents are poorly specific for smooth muscle and collateral damage to endothelium increases thrombosis. In this context, FRNK is a tantalizing target- it is expressed primarily in vascular smooth muscle cells²⁹, it is upregulated in injured vascular smooth muscle cells³⁰, and it potently inhibits cell migration¹⁹ and survival. The present study highlights several new points of intervention, specifically the FAK FRNK regulatory complex and the regulation of this complex by ERK mediated S217 phosphorylation (Fig. 5). The potent inhibitory character of FRNK also has important implications for the physiological consequences of FAK degradation by proteolysis. FAK contains two caspase cleavage sites⁴⁷ in the kinase-FAT linker region and we have observed cleavage of FAK into smaller species³⁰. If, as seems likely, these fragments have inhibitory capacity, activation of pro-apoptotic pathways may feed back to further inhibit FAK survival pathways. Moreover, tissues that do not express endogenous FRNK could regulate FAK signaling through proteolytic production of inhibitory FRNK-like species. Future studies may reveal whether FAK inhibition by FRNK and proteolytic surrogates is a general feature of FAK signaling.

Regulation of FAK signaling pathways –

FRNK is known as a dominant negative inhibitor FAK, a broad description that includes a wide range of possible molecular mechanisms. While the present data do not support inhibition by competition of FRNK for FAK focal adhesion anchoring sites per se, it is clear that the two species are rivals for key downstream targets such as paxillin^{36, 48, 49} and p130Cas¹⁹. These downstream regulatory interactions are regulated by specific residues of FRNK, with paxillin binding altered by mutating leucine 341 to serine³⁶ and p130Cas binding altered by phosphorylating tyrosine 168¹⁹. Y168F-FRNK altered FAK p130Cas

binding and cell invasion but had no effect on FAK Y397 phosphorylation status or apparent cell death¹⁹. Interestingly, FRNK S217 controls FAK binding, FAK Y397 phosphorylation, and apoptosis. Thus, the present results add to the diversity of structural determinants of FRNK inhibitory potency. The FRNK-FAK protein-protein interaction may exert a dominant negative activity by preventing FAK dimerization and auto-phosphorylation and thereby reducing FAK Y397 phosphorylation levels (Fig. 2). The FRNK-FAK complex may also nucleate additional signaling proteins in a phosphorylation dependent manner. For example, Zheng et. al. showed that phosphorylation of FAK at S910 brings the phosphatase PTP-PEST into the FAK scaffold, and this phosphatase can dephosphorylate FAK Y397¹⁶. The structurally equivalent site on FRNK, S217, may similarly recruit PTP-PEST and bring it into close proximity to FAK. In addition to these possibilities, FRNK binding may induce a conformational change that prevents FAK Y397 autophosphorylation.

Summary-

We conclude FRNK regulates FAK signaling by binding directly to FAK oligomers within focal adhesions and forming an inhibitory complex. This interaction is highly sensitive to FRNK phosphorylation at position S217, which also potentially regulates FAK Y397 phosphorylation (activation) and apoptosis. Fig. 6 integrates the present findings in a simplified diagram of functional regulation of FAK signaling by FRNK. In tissues where FAK pathways are activated FAK forms oligomers in focal adhesions and FAK autophosphorylation stimulates scaffolding of downstream targets that initiate and sustain growth, migration, and survival signaling. The pathways are unrestrained when FRNK expression is low and FRNK is highly phosphorylated by ERK at S217. FAK pathway inhibition is accomplished by increased expression of dephosphorylated FRNK, which forms high-order hetero-oligomers with FAK, inhibiting FAK autophosphorylation. This limits growth, migration, and survival. We regard this mechanism as a high-value target for pharmacological intervention due to FRNK's unique expression profile in injured vascular smooth muscle cells. More generally, the findings provide insight into the mechanism of regulation of FAK signaling in growth, migration, and survival pathways.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

Flow cytometry analysis and sorting services were performed by the Loyola University Chicago Flow Cytometry (FACS) Core Facility. The authors thank Dan Blackwell and Olga Raguimova for technical advice, Taehoon Kim and Jody Martin for production and titration of adenovirus. This work was supported by resources from the Cardiovascular Research Institute, Health Sciences Division, Loyola University Chicago.

Sources of Funding

NIH: R01-HL092321 and R01-HL106189 to S.L.R., P01-HL62426 to A.M.S., and F32-HL096143 to Y.E.K.

Abbreviations:

S217

Serine 217

RASM	Rat aortic smooth muscle cell
GFX	GF 109203X
PD98	PD98059
SP600	SP600125
Y27	Y-27632
SB203	SB 203580
AT II	Angiotensin II
TIRF	total internal reflection fluorescence
P130Cas	130 kDa Crk-associated substrate
Src	cellular Src kinase

REFERENCES

- [1]. Govindarajan G, Eble DM, Lucchesi PA, and Samarel AM (2000) Focal adhesion kinase is involved in angiotensin II-mediated protein synthesis in cultured vascular smooth muscle cells, *Circ. Res* 87, 710–716. [PubMed: 11029408]
- [2]. Heidkamp MC, Bayer AL, Kalina JA, Eble DM, and Samarel AM (2002) GFP-FRNK disrupts focal adhesions and induces anoikis in neonatal rat ventricular myocytes, *Circ. Res* 90, 1282–1289. [PubMed: 12089066]
- [3]. Walkiewicz KW, Girault JA, and Arold ST (2015) How to awaken your nanomachines: Site-specific activation of focal adhesion kinases through ligand interactions, *Prog. Biophys. Mol. Biol* 119, 60–71. [PubMed: 26093249]
- [4]. Parsons JT (2003) Focal adhesion kinase: the first ten years, *J. Cell Sci* 116, 1409–1416. [PubMed: 12640026]
- [5]. Mitra SK, Hanson DA, and Schlaepfer DD (2005) Focal adhesion kinase: in command and control of cell motility, *Nat. Rev. Mol. Cell Biol* 6, 56–68. [PubMed: 15688067]
- [6]. Ilic D, Furuta Y, Kanazawa S, Takeda N, Sobue K, Nakatsuji N, Nomura S, Fujimoto J, Okada M, and Yamamoto T (1995) Reduced cell motility and enhanced focal adhesion contact formation in cells from FAK-deficient mice, *Nature* 377, 539–544. [PubMed: 7566154]
- [7]. Burridge K, and Guilluy C (2016) Focal adhesions, stress fibers and mechanical tension, *Exp. Cell Res* 343, 14–20. [PubMed: 26519907]
- [8]. Samarel AM (2014) Focal adhesion signaling in heart failure, *Pflugers Arch.* 466, 1101–1111. [PubMed: 24515292]
- [9]. Geiger B, Spatz JP, and Bershadsky AD (2009) Environmental sensing through focal adhesions, *Nat. Rev. Mol. Cell Biol* 10, 21–33. [PubMed: 19197329]
- [10]. Nolan K, Lacoste J, and Parsons JT (1999) Regulated expression of focal adhesion kinase-related nonkinase, the autonomously expressed C-terminal domain of focal adhesion kinase, *Mol. Cell. Biol* 19, 6120–6129. [PubMed: 10454559]
- [11]. Juliano RL (1994) Integrin signals and tumor growth control, *Princess Takamatsu symp* 24, 118–124. [PubMed: 8983069]
- [12]. Hecker TP, and Gladson CL (2003) Focal adhesion kinase in cancer, *Front. Biosci* 8, s705–714. [PubMed: 12700131]
- [13]. Schwock J, Dhani N, Cao MP, Zheng J, Clarkson R, Radulovich N, Navab R, Horn LC, and Hedley DW (2009) Targeting focal adhesion kinase with dominant-negative FRNK or Hsp90 inhibitor 17-DMAG suppresses tumor growth and metastasis of SiHa cervical xenografts, *Cancer Res.* 69, 4750–4759. [PubMed: 19458065]

- [14]. Kim I, Kim HG, Moon SO, Chae SW, So JN, Koh KN, Ahn BC, and Koh GY (2000) Angiopoietin-1 induces endothelial cell sprouting through the activation of focal adhesion kinase and plasmin secretion, *Circ. Res* 86, 952–959. [PubMed: 10807867]
- [15]. Nowakowski J, Cronin CN, McRee DE, Knuth MW, Nelson CG, Pavletich NP, Rogers J, Sang BC, Scheibe DN, Swanson RV, and Thompson DA (2002) Structures of the cancer-related Aurora-A, FAK, and EphA2 protein kinases from nanovolume crystallography, *Structure* 10, 1659–1667. [PubMed: 12467573]
- [16]. Zheng Y, Xia Y, Hawke D, Halle M, Tremblay ML, Gao X, Zhou XZ, Aldape K, Cobb MH, Xie K, He J, and Lu Z (2009) FAK phosphorylation by ERK primes ras-induced tyrosine dephosphorylation of FAK mediated by PIN1 and PTP-PEST, *Mol. Cell* 35, 11–25. [PubMed: 19595712]
- [17]. Chu M, Iyengar R, Koshman YE, Kim T, Russell B, Martin JL, Heroux AL, Robia SL, and Samarel AM (2011) Serine-910 phosphorylation of focal adhesion kinase is critical for sarcomere reorganization in cardiomyocyte hypertrophy, *Cardiovasc. Res* 92, 409–419. [PubMed: 21937583]
- [18]. Taylor JM, Mack CP, Nolan K, Regan CP, Owens GK, and Parsons JT (2001) Selective expression of an endogenous inhibitor of FAK regulates proliferation and migration of vascular smooth muscle cells, *Mol. Cell Biol* 21, 1565–1572. [PubMed: 11238893]
- [19]. Koshman YE, Chu M, Engman SJ, Kim T, Iyengar R, Robia SL, and Samarel AM Focal adhesion kinase-related nonkinase inhibits vascular smooth muscle cell invasion by focal adhesion targeting, tyrosine 168 phosphorylation, and competition for p130(Cas) binding, *Arterioscler., Thromb., Vasc. Biol* 31, 2432–2440.
- [20]. Hayashi I, Vuori K, and Liddington RC (2002) The focal adhesion targeting (FAT) region of focal adhesion kinase is a four-helix bundle that binds paxillin, *Nat. Struct. Biol* 9, 101–106. [PubMed: 11799401]
- [21]. Sayers RL, Sundberg-Smith LJ, Rojas M, Hayasaka H, Parsons JT, Mack CP, and Taylor JM (2008) FRNK expression promotes smooth muscle cell maturation during vascular development and after vascular injury, *Arterioscler., Thromb., Vasc. Biol* 28, 2115–2122. [PubMed: 18787183]
- [22]. Brami-Cherrier K, Gervasi N, Arsenieva D, Walkiewicz K, Boutterin MC, Ortega A, Leonard PG, Seantier B, Gasmi L, Bouceba T, Kadare G, Girault JA, and Arold ST FAK dimerization controls its kinase-dependent functions at focal adhesions, *EMBO J.* 33, 356–370. [PubMed: 24480479]
- [23]. Goni GM, Epifano C, Boskovic J, Camacho-Artacho M, Zhou J, Bronowska A, Martin MT, Eck MJ, Kremer L, Grater F, Gervasio FL, Perez-Moreno M, and Lietha D (2014) Phosphatidylinositol 4,5-bisphosphate triggers activation of focal adhesion kinase by inducing clustering and conformational changes, *Proc. Natl. Acad. Sci. U. S. A* 111, E3177–3186. [PubMed: 25049397]
- [24]. Arold ST, Hoellerer MK, and Noble ME (2002) The structural basis of localization and signaling by the focal adhesion targeting domain, *Structure* 10, 319–327. [PubMed: 12005431]
- [25]. Hanks SK, Calalb MB, Harper MC, and Patel SK (1992) Focal adhesion protein-tyrosine kinase phosphorylated in response to cell attachment to fibronectin, *Proc. Natl. Acad. Sci. U. S. A* 89, 8487–8491. [PubMed: 1528852]
- [26]. Biscardi JS, Maa MC, Tice DA, Cox ME, Leu TH, and Parsons SJ (1999) c-Src-mediated phosphorylation of the epidermal growth factor receptor on Tyr845 and Tyr1101 is associated with modulation of receptor function, *J. Biol. Chem* 274, 8335–8343. [PubMed: 10075741]
- [27]. Owens LV, Xu L, Craven RJ, Dent GA, Weiner TM, Kornberg L, Liu ET, and Cance WG (1995) Overexpression of the focal adhesion kinase (p125FAK) in invasive human tumors, *Cancer Res.* 55, 2752–2755. [PubMed: 7796399]
- [28]. Ding Q, Cai GQ, Hu M, Yang Y, Zheng A, Tang Q, Gladson CL, Hayasaka H, Wu H, You Z, Southern BD, Grove LM, Rahaman SO, Fang H, and Olman MA FAK-related nonkinase is a multifunctional negative regulator of pulmonary fibrosis, *Am. J. Pathol* 182, 1572–1584.
- [29]. Hayasaka H, Simon K, Hershey ED, Masumoto KH, and Parsons JT (2005) FRNK, the autonomously expressed C-terminal region of focal adhesion kinase, is uniquely regulated in vascular smooth muscle: analysis of expression in transgenic mice, *J. Cell. Biochem* 95, 1248–1263. [PubMed: 15962314]

- [30]. Koshman YE, Engman SJ, Kim T, Iyengar R, Henderson KK, and Samarel AM Role of FRNK tyrosine phosphorylation in vascular smooth muscle spreading and migration, *Cardiovasc. Res* 85, 571–581. [PubMed: 19793767]
- [31]. Hibbert B, Chen YX, and O'Brien ER (2004) c-kit-immunopositive vascular progenitor cells populate human coronary in-stent restenosis but not primary atherosclerotic lesions, *Am. J. Physiol.: Heart Circ. Physiol* 287, H518–524. [PubMed: 15277195]
- [32]. Sabri A, Govindarajan G, Griffin TM, Byron KL, Samarel AM, and Lucchesi PA (1998) Calcium- and protein kinase C-dependent activation of the tyrosine kinase PYK2 by angiotensin II in vascular smooth muscle, *Circ. Res* 83, 841–851. [PubMed: 9776731]
- [33]. Kelly EM, Hou Z, Bossuyt J, Bers DM, and Robia SL (2008) Phospholamban oligomerization, quaternary structure, and sarco(endo)plasmic reticulum calcium ATPase binding measured by fluorescence resonance energy transfer in living cells, *J. Biol. Chem* 283, 12202–12211. [PubMed: 18287099]
- [34]. Sundberg LJ, Galante LM, Bill HM, Mack CP, and Taylor JM (2003) An endogenous inhibitor of focal adhesion kinase blocks Rac1/JNK but not Ras/ERK-dependent signaling in vascular smooth muscle cells, *J. Biol. Chem* 278, 29783–29791. [PubMed: 12782622]
- [35]. Heidkamp MC, Bayer AL, Kalina JA, Eble DM, and Samarel AM (2002) GFP-FRNK disrupts focal adhesions and induces anoikis in neonatal rat ventricular myocytes, *Circ. Res* 90, 1282–1289. [PubMed: 12089066]
- [36]. Koshman YE, Kim T, Chu M, Engman SJ, Iyengar R, Robia SL, and Samarel AM FRNK inhibition of focal adhesion kinase-dependent signaling and migration in vascular smooth muscle cells, *Arterioscler., Thromb., Vasc. Biol* 30, 2226–2233.
- [37]. Schaller MD, Borgman CA, and Parsons JT (1993) Autonomous expression of a noncatalytic domain of the focal adhesion-associated protein tyrosine kinase pp125FAK, *Mol. Cell. Biol* 13, 785–791. [PubMed: 8423801]
- [38]. DiMichele LA, Hakim ZS, Sayers RL, Rojas M, Schwartz RJ, Mack CP, and Taylor JM (2009) Transient expression of FRNK reveals stage-specific requirement for focal adhesion kinase activity in cardiac growth, *Circ. Res* 104, 1201–1208. [PubMed: 19372463]
- [39]. Ahn S, Kim HJ, Chi SG, and Park H (2012) XIAP reverses various functional activities of FRNK in endothelial cells, *Biochem. Biophys. Res. Commun* 419, 419–424. [PubMed: 22366087]
- [40]. Koushik SV, Chen H, Thaler C, Puhl HL 3rd, and Vogel SS (2006) Cerulean, Venus, and VenusY67C FRET reference standards, *Biophys. J* 91, L99–L101. [PubMed: 17040988]
- [41]. Lietha D, Cai X, Ceccarelli DF, Li Y, Schaller MD, and Eck MJ (2007) Structural basis for the autoinhibition of focal adhesion kinase, *Cell* 129, 1177–1187. [PubMed: 17574028]
- [42]. Slack-Davis JK, Martin KH, Tilghman RW, Iwanicki M, Ung EJ, Autry C, Luzzio MJ, Cooper B, Kath JC, Roberts WG, and Parsons JT (2007) Cellular characterization of a novel focal adhesion kinase inhibitor, *J. Biol. Chem* 282, 14845–14852. [PubMed: 17395594]
- [43]. Yoon H, Dehart JP, Murphy JM, and Lim ST (2015) Understanding the roles of FAK in cancer: inhibitors, genetic models, and new insights, *J. Histochem. Cytochem* 63, 114–128. [PubMed: 25380750]
- [44]. Infante JR, Camidge DR, Mileskin LR, Chen EX, Hicks RJ, Rischin D, Fingert H, Pierce KJ, Xu H, Roberts WG, Shreeve SM, Burris HA, and Siu LL (2012) Safety, pharmacokinetic, and pharmacodynamic phase I dose-escalation trial of PF-00562271, an inhibitor of focal adhesion kinase, in advanced solid tumors, *J. Clin. Oncol* 30, 1527–1533. [PubMed: 22454420]
- [45]. Byrne RA, Joner M, and Kastrati A (2015) Stent thrombosis and restenosis: what have we learned and where are we going? The Andreas Gruntzig Lecture ESC 2014, *Eur. Heart J* 36, 3320–3331. [PubMed: 26417060]
- [46]. McDonald AI, and Iruela-Arispe ML (2015) Healing arterial ulcers: Endothelial lining regeneration upon vascular denudation injury, *Vasc. Pharmacol* 72, 9–15.
- [47]. Lemmon MA, Schlessinger J, and Ferguson KM (2014) The EGFR family: not so prototypical receptor tyrosine kinases, *Cold Spring Harbor Perspect. Biol* 6, a020768.
- [48]. Sieg DJ, Hauck CR, and Schlaepfer DD (1999) Required role of focal adhesion kinase (FAK) for integrin-stimulated cell migration, *J. Cell Sci* 112 (Pt 16), 2677–2691. [PubMed: 10413676]

- [49]. Schlaepfer DD, Mitra SK, and Ilic D (2004) Control of motile and invasive cell phenotypes by focal adhesion kinase, *Biochim. Biophys. Acta* 1692, 77–102. [PubMed: 15246681]

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Fig. 1. FRNK serine 217 is highly phosphorylated by ERK.

(A) Schematic cartoon comparing FAK to FRNK including the phosphorylation sites tyrosine 397 and serine 910 (217 on FRNK) (B) Western blot of RASMs treated with various kinase inhibitors probed with P-S217 or total FRNK antibodies. (C) Quantification of (B) for N = 4 biological replicates revealing that ERK inhibition reduced FRNK S217 phosphorylation. (D) Kinase inhibitors reduced FRNK S217 phosphorylation while angiotensin II stimulation resulted in no significant increase in phosphorylation. UI = uninfected cells. (E) Quantification of (D) for N = 3 biological replicates show FRNK is highly phosphorylated at position S217 by ERK. * indicates $p < 0.05$ and ** indicates $p < 0.005$.

Fig. 2. S217A-FRNK is a super inhibitory mutant with respect to FAK Y397 phosphorylation and survival.

(A) Fluorescence microscopy of cells infected with a control virus (GFP), WT-FRNK, or S217A-FRNK and stained with DAPI. (B) Quantification of A for N = 4 biological replicates demonstrated that apparent cell survival was strongly reduced by S217A-FRNK compared to WT-FRNK. Data shown are mean $\pm$ SE. (C) RASMs expressing β -gal, Cer-FRNK, or CFP-S217A-FRNK, were subjected to flow cytometry apoptosis analysis. Double negative cells indicate viable cells (V). Annexin V positive, PI negative cells indicate apoptotic cells (A), and PI positive, Annexin V negative cells indicate dead cells (D). The data show that the non-phosphorylatable FRNK has increased potency for inducing apoptosis compared to wild type FRNK. (D) 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 (E) for side-

by-side comparison. UI = uninfected cells. GAPDH was used as a loading control. * indicates $p < 0.05$ and ** indicates $p < 0.005$.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Fig. 3. FRNK does not displace FAK from focal adhesions, as measured by TIRF microscopy. Fluorescence microscopy of cells infected with WT-FRNK (A) or S217A-FRNK (B) and co-infected with YFP-FAK. YFP-FAK localized strongly to focal adhesions whether they contained FRNK (cyan arrow) or not (yellow arrow). (C) Quantification of FAK anchoring in focal adhesions (N=74) demonstrated that increasing FRNK expression did not decrease FAK focal adhesion localization, nor was there a significant difference between WT-FRNK and superinhibitory S217A-FRNK.

Fig. 4. FRNK co-immunoprecipitates with FAK.

RASMs infected with Cer-WT-FRNK or CFP-S217AFRNK were lysed in a non-denaturing buffer and endogenous FAK was immunoprecipitated with a FAK-specific antibody that does not recognize FRNK. FAK and co-immunoprecipitated FRNK were detected on the blot using a different antibody that is pan-specific, recognizing both FAK and FRNK. S217A-FRNK exhibited approximately five-fold increased co-immunoprecipitation with FAK compared to wild type. GAPDH was used as a loading control.

Fig. 5. FAK and FRNK form oligomeric complexes.

(A, B) TIRF microscopy revealed YFP-FAK localization in focal adhesions in cells coexpressing WT- or S217A-FRNK. Acceptor-selective photobleaching of YFP-FAK resulted in increased fluorescence of non-phosphorylatable FRNK, but not WT-FRNK. (C, D) Quantification of acceptor photobleaching experiments (N=24) for WT- or S217A-FRNK coexpressed with YFP-FAK. Red arrows indicate initiation of photobleaching. Data shown are mean $\pm$ SE. (E) 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. **(F)** The data in (C, D) plotted as donor vs. acceptor intensity revealed significant deviation from a linear relationship (grey line) for non-phosphorylatable FRNK, suggesting multiple acceptors in complex with each donor. The green arrow denotes the direction of change over time. Data are mean $\pm$ SE.

Fig. 6. Summary model of FAK activation.

FAK translocates from the cytoplasm to focal adhesions where it forms homo-oligomers. Activated by diverse stimuli, FAK autophosphorylates at Y397, activating growth, survival, and migration signaling pathways. Unchecked activity can lead to pathological conditions such as cancer or excessive neointimal formation as seen in restenosis. FRNK interacts directly with FAK, forming an inhibitory hetero-oligomeric complex in the focal adhesion. Inhibition is relieved by ERK phosphorylation of FRNK on S217.