Focal Adhesion Kinase–Related Nonkinase Inhibits Vascular Smooth Muscle Cell Invasion by Focal Adhesion Targeting, Tyrosine 168 Phosphorylation, and Competition for p130Cas Binding

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Objective—Focal adhesion kinase–related nonkinase (FRNK), the C-terminal domain of focal adhesion kinase (FAK), is a tyrosine-phosphorylated, vascular smooth muscle cell (VSMC)–specific inhibitor of cell migration. FRNK inhibits both FAK and PYK2 in cultured VSMCs, and both kinases may be involved in VSMC invasion during vascular remodeling.

Methods and Results—Adenovirally mediated gene transfer of green fluorescent protein–tagged, wild-type (wt) FRNK into balloon-injured rat carotid arteries confirmed that FRNK overexpression inhibited both FAK and PYK2 phosphorylation and downstream signaling in vivo. To identify which kinase was involved in regulating VSMC invasion, adenovirally mediated expression of specific short hairpin RNAs was used to knock down FAK versus PYK2 in cultured VSMCs, but only FAK short hairpin RNA was effective in reducing VSMC invasion. The role of FRNK tyrosine phosphorylation was then examined using adenoviruses expressing nonphosphorylatable (Tyr168Phe−, Tyr232Phe−, and Tyr168,232Phe−) green fluorescent protein–FRNK mutants. wtFRNK and all FRNK mutants localized to FAs, but only Tyr168 phosphorylation was required for FRNK to inhibit invasion. Preventing Tyr168 phosphorylation also increased FRNK-paxillin interaction, as determined by coimmunoprecipitation, total internal reflection fluorescence microscopy, and fluorescence recovery after photobleaching. Furthermore, wtFRNK competed with FAK for binding to p130Cas (a critically important regulator of cell migration) and prevented its phosphorylation. However, Tyr168Phe-FRNK was unable to bind p130Cas.

Conclusion—We propose a 3-stage mechanism for FRNK inhibition: focal adhesion targeting, Tyr168 phosphorylation, and competition with FAK for p130Cas binding and phosphorylation, which are all required for FRNK to inhibit VSMC invasion.

Key Words: matrix signal transduction vascular biology vascular remodeling

Vascular remodeling requires a complex interaction between growth factor receptors, extracellular matrix components, and integrins. Key proteins are involved in integrating extracellular signals and promoting the intracellular signal transduction required for vascular remodeling. One of these proteins is focal adhesion kinase (FAK), which is activated by growth factor receptors and integrin clustering and which is critical for the assembly of a signaling complex within focal adhesions (FAs) that is required for cell migration and other aspects of the remodeling process.

In addition to FAK, FAK-related nonkinase (FRNK) is also a product of the PTK2 gene but is autonomously expressed under control of an alternative, intron-specific promoter. FRNK is composed of the noncatalytic, C-terminal region of FAK containing the focal adhesion targeting (FAT) sequence and proline-rich domains important for adaptor protein binding. FRNK is selectively expressed in vascular smooth muscle cells (VSMCs), with very high levels found in large arterioles and after arterial injury. Our laboratory has demonstrated that FRNK inhibits both FAK- and PYK2-dependent signaling in cultured VSMCs. We also showed that FRNK undergoes tyrosine phosphorylation at Tyr168 and Tyr232 after carotid artery injury in vivo and in response to angiotensin II (Ang II) in vitro. These phosphorylation sites are equivalent to the Tyr861 and Tyr925 phosphorylation sites within the C-terminal region of FAK.

The mechanisms responsible for FRNK inhibition of FAK and PYK2 signaling in VSMC invasion are uncertain. FAK localization to FAs is required for its activation, and its displacement from these sites results in decreased FAK activation. Because FRNK contains the identical FAT do-
main as FAK, we and others have proposed that FRNK inhibits FAK-dependent signaling by competitively displacing FAK from FA3s.8,9 However, another possibility is that FRNK inhibits FAK signaling by acting as a sink for FAK binding proteins.10 One candidate binding partner is p130Cas, which is a critical regulator of cell migration.11 p130Cas binds to the first of 2 proline-rich domains (residues 711–717; APPKPSR) in the C-terminal region of FAK,12,13 but its binding appears to be regulated by FAK phosphorylation at Tyr861.14 Our evidence that FRNK can undergo tyrosine phosphorylation independently of FAK suggests that other factors in addition to FRNK targeting are important for its inhibitory function. These factors may also be responsible for the phenotypic differences observed between FAK-null and FRNK-overexpressing cells.9,15–17

Our recent observations indicate that FRNK inhibition of FAK autophosphorylation at Tyr397 is not related to FRNK’s ability to inhibit cell migration, so long as FA targeting is preserved. For instance, FRNK mutated at Tyr168 (Tyr168Phe-FRNK) retains its ability to target to FAs and to inhibit FAK autophosphorylation, but it fails to inhibit VSMC spreading and migration.9 One possibility is that FRNK’s inhibitory effect depends on its ability to inhibit the autophosphorylation of PYK2, rather than FAK. PYK2 is the other member of the FAK-family of nonreceptor protein tyrosine kinases. PYK2 is highly expressed in VSMCs and shares both overlapping and distinct functions in integrin-dependent signaling. We now demonstrate that in addition to FAK, PYK2 is also upregulated in balloon-injured rat carotid arteries, and overexpression of FRNK by adenovirally mediated gene transfer immediately after injury reduces FAK, PYK2, and paxillin phosphorylation in vivo. To determine which kinase is involved in regulating cell injury reduces FAK, PYK2, and paxillin phosphorylation in balloon-injured rat carotid arteries, and overexpression of FRNK by adenovirally mediated gene transfer immediately after injury reduces FAK, PYK2, and paxillin phosphorylation in vivo.

**Methods**

**Experimental Animals**

Loyola University Medical Center’s Institutional Animal Care and Use Committee approved all procedures involving animals, which were handled in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health. Please see the supplemental data (available online at http://atvb.ahajournals.org) for a detailed description of the materials and methods used for these studies.

**Results**

**FRNK Overexpression in Balloon-Injured Rat Carotid Artery Reduces FAK and PYK2 Autophosphorylation and Downstream Signaling**

In initial experiments, we made use of a carotid artery balloon injury model5 to assess endogenous FAK, FRNK, and PYK2 expression and to determine whether adenovirally mediated gene transfer of green fluorescent protein (GFP)–wild-type (wt) FRNK inhibited FAK and PYK2 activation and downstream signaling in vivo. Western blot analysis demonstrated that in addition to FAK and endogenous FRNK, PYK2 was markedly increased in rat carotid arteries 2 weeks after balloon injury (FAK, 3.5±0.9-fold; FRNK, 8.9±2.8-fold; PYK2, 6.0±2.2-fold; n=3 animals; Figure 1). Increased FAK and PYK2 expression was also noted 1 week after injury, which was accompanied by increased FAK-Tyr397 and PYK2-Tyr402 phosphorylation, along with increased paxillin phosphorylation and expression in contralateral (control) and 1-week-injured, uninfected (UI) arteries, and in injured arteries subjected to adenoviral (Adv) gene transfer with 1010 pfu of Adv-green fluorescent protein (GFP) or Adv-GFP–wild-type (wt) FRNK. D, Quantitative analysis of FAK-Tyr397, PYK2-Tyr402, and paxillin-Tyr118 phosphorylation in injured uninfected, injured-Adv-GFP-infected, and injured-Adv-GFP–wtFRNK-infected arteries (relative to uninjured uninfected control arteries) from n=4 rats. *P<0.05 vs uninjured infected.
Confirming our previous studies,4,5 FRNK overexpression in cultured VSMCs also inhibited basal and Ang II-induced FAK-Tyr397 autophosphorylation and FAK phosphorylation at Tyr861 and Tyr925 (Supplemental Figure I). FRNK was also basally phosphorylated at Tyr168 and Tyr232 (the equivalent C-terminal phosphorylation sites on FAK), and FRNK phosphorylation at both sites increased further in response to Ang II. However, FRNK’s inhibitory activity was not specific for FAK, as FRNK reduced basal and Ang II-induced PYK2-Tyr402 phosphorylation. Finally, FRNK overexpression was associated with a marked inhibition of VSMC invasion, as assessed in a three-dimensional (3D) Boyden chamber assay.

FRNK-Dependent Inhibition of VSMC Invasion Is Not Dependent on PYK2

To ascertain whether FRNK’s inhibitory effect on cell invasion was dependent on FAK or PYK2, we generated adenoviral (Adv) vectors that express short hairpin RNAs specific for each kinase. As seen in Figure 2, each pair of short hairpin RNA vectors successfully knocked down FAK or PYK2 without significantly affecting the expression of the other kinase. However, only FAK short hairpin RNAs reduced VSMC invasion in the 3D Boyden chamber assay, indicating that FRNK-mediated inhibition of cell invasion was predominantly dependent on its inhibitory activity against FAK rather than PYK2.

To further explore the role of FAK versus PYK2 in VSMC invasion, we compared the invasive potential of VSMCs expressing GFP-wtFRNK with cells expressing GFP-wtCRNK, the C-terminal domain of PYK2. CRNK is a naturally occurring inhibitor of PYK2, which, when overexpressed in HEK293 cells, blocks PYK2 but not FAK autophosphorylation.18 wt-CRNK also inhibits basal, endothelin-1, and H2O2-induced PYK2 activation in neonatal rat cardiomyocytes.19 As seen in Supplemental Figure II, both wtFRNK and wtCRNK inhibited VSMC invasion in the 3D Boyden chamber assay, but FRNK was significantly more effective, further indicating that FRNK’s inhibition of cell invasion was predominantly dependent on its inhibitory activity against FAK.

To explore the requirement for FAK phosphorylation on VSMC invasion, we made use of 2 different pharmacological agents that differentially inhibit FAK tyrosine phosphorylation.6,20 As seen in Supplemental Figure III, PF-573228, a FAK-specific kinase inhibitor20 reduced FAK autophosphorylation at Tyr397 and also indirectly prevented Src-dependent FAK phosphorylation at Tyr397 and also indirectly prevented Src-dependent FAK phosphorylation at Tyr397 and also indirectly prevented Src-dependent FAK phosphorylation at Tyr861 and Tyr925.6 In contrast, PP2, a Src-specific kinase inhibitor, reduced FAK phosphorylation at Tyr861 and Tyr925 but had no significant effect on basal or Ang II-induced FAK autophosphorylation at Tyr397. However, both agents significantly inhibited VSMC invasion, indicating that FRNK’s ability to inhibit FAK autophosphorylation at Tyr397 was not the mechanism responsible.
FRNK Tyrosine Phosphorylation at Tyr168 Is Necessary for FRNK Inhibition of VSMC Invasion

Because FRNK overexpression, PP2, and PF-573228 all blocked FAK tyrosine phosphorylation at Tyr861 and Tyr925, we next examined whether the same tyrosine phosphorylation sites on FRNK (i.e., Tyr168 and Tyr232) are required for FRNK inhibition of VSMC invasion. We generated replication-deficient Adv expressing GFP-tagged wtFRNK, Tyr168Phe-FRNK, Tyr232Phe-FRNK, and Tyr168,232Phe-FRNK. Their effects on FAK phosphorylation, as well as FRNK targeting, binding kinetics, and inhibition of cell invasion, were examined. As seen in Supplemental Figure IV, wtFRNK and the 3 FRNK phosphorylation mutants all displayed identical FA distribution patterns, as observed by total internal reflection fluorescence microscopy of living cells. FAT of wtFRNK and the 3 FRNK mutants also reduced basal and Ang II-induced FAK phosphorylation at Tyr397, Tyr861, and Tyr925 and markedly reduced FRNK (data not shown).

A critical requirement for FRNK-mediated inhibition of VSMC migration is its binding affinity to paxillin and other FA proteins. Therefore, we used total internal reflection fluorescence microscopy and fluorescence recovery after photobleaching (FRAP) analysis to examine the kinetics of wt and mutant FRNK binding to FAs. Surprisingly, we observed a small but statistically significant reduction in $k_{FRAP}$ for the Tyr168Phe and Tyr168,232Phe mutants (Figure 3 and Supplemental Figure V), indicating that their binding affinity was increased by rendering these sites nonphosphorylatable. These data were further analyzed by 2-way ANOVA, which indicated that mutation of the Tyr168 site was highly significant ($P=0.007$), whereas mutation of the Tyr232 site had no significant effect ($P=0.224$) on $k_{FRAP}$. Also, there was no statistically significant interaction between mutation of the Tyr168 and Tyr232 sites ($P=0.631$). That is, the effect of the Tyr168 mutation was unaffected by the mutational status of Tyr232. Finally, the significant reduction in $k_{FRAP}$ was associated with a substantial loss of the inhibitory effect of the Tyr168Phe and Tyr168,232Phe mutants on VSMC invasion.

These initial studies were conducted using unstimulated cells which demonstrate somewhat lower levels of basal wtFRNK-Tyr168 and wtFRNK-Tyr232 phosphorylation (Supplemental Figure I). Because the invasion assays were conducted in the presence of Ang II, we repeated the total internal reflection fluorescence FRAP analysis in unstimulated cells expressing wtFRNK or Tyr168Phe-FRNK and in paired cells that were stimulated with Ang II. As seen in Figure 4, Ang II stimulation had no significant effect on the observed $k_{FRAP}$ for either wtFRNK or Tyr168Phe-FRNK ($P=0.177$; 2-way ANOVA). Only the presence of the Tyr168Phe mutation was significant ($P<0.001$), and there was no statistically significant interaction between Tyr168Phe and Ang II ($P=0.848$).

The 2-compartment analysis of wtFRNK and Tyr168Phe-FRNK binding kinetics also revealed that there was a significant increase in the percentage of Tyr168Phe-FRNK in the M2 or “slow” compartment, which was unaffected by Ang II stimulation (Figure 4). These kinetic data were confirmed by coimmunoprecipitation analysis of paxillin, which revealed a substantial increase in the amount of Tyr168Phe-FRNK as compared with wtFRNK that was bound to paxillin under basal conditions. Ang II stimulation had no further effect on steady-state paxillin binding to either wtFRNK or Tyr168Phe-FRNK (data not shown).

Tyrosine Phosphorylation At Tyr168 Is Critical for the Binding of FRNK to p130Cas

Like paxillin, p130Cas is a FA adaptor protein that binds to the first of 2 proline-rich regions in the C-terminal domain of
Because FRNK contains the same proline-rich regions, we examined whether FRNK and p130 Cas colocalize to VSMC FAs. As seen in Figure 5A, GFP-tagged wtFRNK (green) was readily detected by confocal microscopy within linear structures at the cell-substratum interface of fully spread VSMCs. The identical structures also contained p130 Cas (red). Colocalization was confirmed in the merged images (yellow).

Interestingly, a similar colocalization pattern was observed for GFP-tagged Tyr168Phe-FRNK. A substantial fraction of the p130 Cas also colocalized with both wtFRNK and Tyr168Phe-FRNK at the leading edge of VSMCs during FA formation. As seen in Supplemental Figure VIA, GFP-tagged wtFRNK and Tyr168Phe-FRNK were readily identified in linear structures at the cell-substratum interface of spreading VSMCs. Although most of the p130 Cas was centrally located around the nucleus of spreading cells, it was also readily identified in peripheral FAs, where it was found to colocalize with both wtFRNK and Tyr168Phe-FRNK. A similar distribution pattern was observed for paxillin and for wt and mutant FRNK (Supplemental Figure VIB).

**Interaction of p130 Cas With FAK and FRNK**

Coimmunoprecipitation was then used to examine the steady-state interaction of p130 Cas with wtFRNK and Tyr168Phe-FRNK. As seen in a representative experiment (Figure 5B), equal amounts of endogenous p130 Cas were immunoprecipitated with anti-paxillin monoclonal antibody (mAb), and immunoblots were probed with anti-GFP mAb (to detect GFP-wtFRNK and GFP-Tyr168Phe-FRNK). The position of molecular weight markers is indicated to the left of each blot. IP indicates immunoprecipitation; WB, Western blot. D, Quantitative analysis of GFP-wtFRNK and GFP-Tyr168Phe-FRNK coimmunoprecipitated with paxillin. Data are mean ± SEM; n = 4 experiments. *P < 0.05 vs wtFRNK.  

Figure 4. Mutation of Tyr168 enhanced FAT and paxillin interaction. Rat aortic smooth muscle cells (RASMCs) were infected (100 MOI, 24 hours) with adenovirus (Adv) expressing green fluorescent protein (GFP)-tagged wild-type (wt) or Tyr168Phe-focal adhesion kinase-related nonkinase (FRNK). Paired cultures were stimulated with angiotensin II (Ang II) (100 nmol/L, 15–45 minutes). A, Average kFRAP values (second⁻¹) for the 80 individual total internal reflection fluorescence (TIRF)-FRAP recordings were compared. B, Two-compartment analysis of wtFRNK and Tyr168Phe-FRNK binding kinetics. M1 and M2 represent the fraction of total fluorescence in the “fast” and “slow” compartments, respectively. C and D, RASMCs were infected (100 MOI, 24 hours) with Adv-GFP, Adv-GFP-wtFRNK, or Adv-GFP-Tyr168Phe-FRNK. C, Cell extracts (500 μg total protein) were coimmunoprecipitated with anti-paxillin monoclonal antibody (mAb), and immunoblots were probed with anti-GFP mAb (to detect GFP-wtFRNK and GFP-Tyr168Phe-FRNK). The position of molecular weight markers is indicated to the left of each blot. IP indicates immunoprecipitation; WB, Western blot. D, Quantitative analysis of GFP-wtFRNK and GFP-Tyr168Phe-FRNK coimmunoprecipitated with paxillin. Data are mean ± SEM; n = 4 experiments. *P < 0.05 vs wtFRNK.
Tyrosine phosphorylation at Tyr168 is critical for the binding of focal adhesion kinase–related nonkinase (FRNK) to p130Cas.

A. Rat aortic smooth muscle cells (RASMCs) were infected (100 MOI, 24 hours) with adenoviral (Adv)-green fluorescent protein (GFP)–wild-type (wt) FRNK, or Adv-GFP-Tyr168Phe-FRNK (green). Cells were then fixed, permeabilized, counterstained with anti-p130Cas monoclonal antibody (mAb) (red), mounted with 4',6-diamidino-2-phenylindole–containing mounting medium to detect cell nuclei (blue), and viewed by confocal microscopy. Colocalization of GFP fluorescence and p130Cas in these 1 μm optical sections at the cell-substratum interface is represented in the merged image (yellow). Scale bars=15 μm.

B. RASMCs were infected (100 MOI, 24 hours) with Adv-GFP, Adv-GFP-wtFRNK, or Adv-GFP-Tyr168Phe-FRNK. Cell extracts (1000 μg of total protein) were coimmunoprecipitated with anti-p130Cas mAb, and immunoblots were probed with anti-p130Cas, anti-GFP mAb (to detect GFP-wtFRNK and GFP-Tyr168Phe-FRNK), anti-N-terminal focal adhesion kinase (FAK) mAb, and pTyr165p130Cas. The positions of molecular weight markers are indicated to the left of each blot. IP indicates immunoprecipitation; WB, Western blot.

C. Quantitative analysis of GFP-wtFRNK and GFP-Tyr168Phe-FRNK coimmunoprecipitated with p130Cas.

D. Quantitative analysis of FAK coimmunoprecipitated with p130Cas in the same pull-downs.

E. Quantitative analysis of pTyr165–p130Cas in the same pull-downs. Data are mean±SEM; n=4 experiments. *P<0.05 vs GFP control; +P<0.05 for wtFRNK vs Tyr168-FRNK.

Figure 5. Tyrosine phosphorylation at Tyr168 is critical for the binding of focal adhesion kinase–related nonkinase (FRNK) to p130Cas.

As predicted from its known structure, there was considerable steady-state interaction between p130Cas and wtFRNK. Surprisingly, this interaction was significantly reduced in cells expressing Tyr168Phe-FRNK. A quantitative analysis of wtFRNK versus Tyr168Phe-FRNK binding in n=4 experiments is depicted in Figure 5C. Of note, direct analysis by...
Western blotting of cell extracts before coimmunoprecipitation revealed equal expression levels of GFP-tagged wtFRNK and Tyr168Phe-FRNK, indicating that the dramatic difference in p130Cas binding was not due to differences in the expression or stability of wt versus mutant FRNK.

The same coimmunoprecipitates were then examined for the presence of endogenous FAK, which revealed evidence of competition for p130Cas binding between wtFRNK and FAK. A quantitative analysis of FAK/p130Cas binding from n = 4 experiments is depicted in Figure 5D. As is evident from the figure, there was a significant reduction in p130Cas-FAK steady-state interaction in cells expressing wtFRNK. In contrast, there was no significant difference in FAK binding to p130Cas in cells expressing Tyr168Phe-FRNK.

Once localized, FAK phosphorylates itself at a single tyrosine residue (Tyr397), which serves as a high-affinity binding site (pYAEI motif) for the SH2 domain of Src-family protein tyrosine kinases.21 Src also binds via its SH3 domain to the proline-rich region (RPLPSPP) of p130Cas,22 and Src then phosphorylates p130Cas at multiple sites within its substrate domain. Because p130Cas tyrosine phosphorylation is critical for downstream signaling required for cell migration,11 we examined whether the interaction of wtFRNK with p130Cas affected p130Cas phosphorylation at Tyr165. As seen in Figure 5B and 5E, the immunoprecipitated p130Cas was highly phosphorylated in control cells expressing GFP. wtFRNK expression markedly reduced p130Cas phosphorylation. However, p130Cas phosphorylation in cells expressing Tyr168Phe-FRNK was similar to control cells. Of note, the phosphorylation of p130Cas at Tyr165 was Src-dependent, as PP2 markedly suppressed basal- and Ang II-stimulated phosphorylation at this site (Supplemental Figure VIIA). We interpret these results to indicate that the residual p130Cas binding to FAK within FAs of cells expressing Tyr168Phe-FRNK was sufficient to maintain near normal levels of p130Cas phosphorylation by Src that is required for the initiation of cell migration.

To further examine the relationship between FAK, FRNK and p130Cas, we attempted to "rescue" wtFRNK-induced inhibition of VSMC invasion by overexpressing a "constitutively active" mutant of FAK, known as CD2-FAK.23 Fusion of CD2 to the FAK N-terminus caused hyperphosphorylation of its Tyr397 site (Figure 6A), perhaps by membrane anchor-
ing and unfolding its N-terminal autoinhibitory domain. As depicted in Figure 6B, this construct increased VSMC invasion in cells expressing GFP and also partially rescued the FRNK-mediated inhibition of cell invasion. Similarly, wt-p130Cas overexpression also partially reversed the inhibitory effect of GFP-wtFRNK but had no effect on VSMCs expressing GFP (Figure 6C). Finally, overexpression of a nonphosphorylatable mutant of p130Cas (ie, Cas-ΔSD) reduced endogenous p130Cas phosphorylation (Figure 6A) and mimicked the inhibitory phenotype of wtFRNK overexpression (Figure 6D).

Discussion
FRNK is a naturally occurring, smooth-muscle specific protein that is markedly upregulated in the vessel wall following vascular injury. In cultured VSMCs, FRNK overexpression has a number of effects, including inhibition of new protein synthesis, inhibition of cell proliferation and migration, and induction of serum- and transforming growth factor-β-stimulated smooth muscle marker gene expression. As FRNK can inhibit both FAK and PYK2, it is conceivable that some of FRNK’s inhibitory activity is mediated by inhibition of PYK2 rather than FAK. Indeed, both proteins have distinct as well as overlapping functions in cell signaling, including similar FAT sequences and proline-rich domains that are involved in protein-protein interactions. As we demonstrate in this report, the anti-invasive effect of FRNK is primarily due to its inhibitory effect on FAK, as FRNK overexpression, FAK knockdown, and PP-573228, a highly specific FAK kinase inhibitor, all showed similar results in our 3D Boyden chamber invasion assay. Nevertheless, it is conceivable that other FRNK effects, including its regulation of both the ERK1/2 and the phosphatidylinositol 3-kinase/Akt pathways that are involved in Ang II-induced VSMC protein synthesis, could be mediated by its inhibition of PYK2.

Although FRNK is a potent inhibitor of VSMC migration in two-dimensional and 3D culture, the mechanisms responsible for FRNK inhibition appear much more complicated than previously proposed. FRNK targeting to FAs is consistently required for FRNK inhibition of FAK-dependent signaling, including its effect on VSMC invasion. However, our data indicate that other factors, including FRNK tyrosine phosphorylation, are also necessary. In fact, rendering FRNK nonphosphorylatable at Tyr168 resulted in enhanced rather than reduced FAT, as made evident by a reduction in the dynamic exchange of FRNK between the cytoplasm and binding partners within VSMC FAs. Reduced exchange resulted in an increase in the steady-state interaction of FRNK with paxillin. Surprisingly, however, enhanced FAT did not correlate with enhanced inhibitory activity with respect to cell invasion. Rather, the Tyr168Phe mutation abolished FRNK’s ability to inhibit cell invasion, yet the mutated FRNK still retained its ability to inhibit FAK tyrosine phosphorylation at multiple sites, paxillin phosphorylation, and downstream signaling to the ERK1/2 cascade.

Our current observations now explain the significance of FRNK Tyr168 phosphorylation and reveal its importance in regulating the competition of FRNK and FAK for p130Cas binding. Unlike paxillin, which binds to the FAT domain of FAK and FRNK and regulates targeting, p130Cas binds via its SH3 domain to the first of 2 proline-rich regions (APPKPSR) in both FAK and FRNK. SH3-domain-mediated binding of p130Cas to FAK is critically important in promoting cell migration through the coordinated activation of Rac at membrane extensions. Importantly, Lim et al showed that p130Cas binding to FAK was influenced by the phosphorylation state of FAK at Tyr861. We now clearly demonstrate that p130Cas binding to FRNK is highly dependent on the phosphorylation state of FRNK at Tyr168. When overexpressed, FRNK successfully competed for p130Cas binding as an essential mechanism for its inhibitory activity. However, FRNK overexpression also prevented the downstream phosphorylation of p130Cas, which is necessary for the initiation of cell migration. Therefore, we propose a 3-stage mechanism for FRNK inhibition: FAT, Tyr168 phosphorylation, and competition with FAK for p130Cas binding and phosphorylation, which are all required for FRNK to inhibit VSMC invasion. In this scenario, FRNK undergoes phosphorylation at Tyr168, targets to FAs, and competes with the FAK/Src complex for p130Cas. Because FRNK has no kinase activity or SH2 binding site for Src, it cannot phosphorylate p130Cas and initiate migration. This scenario is schematically depicted in Supplemental Figure VII.

Nevertheless, the relationship between FRNK-Tyr168/FAK-Tyr861 phosphorylation and p130Cas binding remains unknown. X-ray crystallographic analysis of the FAT domain has revealed a highly compact, 4-helix bundle that interacts via 2 hydrophobic patches with the LD2 and LD4 domains of paxillin. However, Campbell and colleagues have suggested that a similar mechanism may be operative in regulating the phosphorylation of paxillin by Src-dependent phosphorylation of FAK at Tyr925, thereby creating a recognition site for binding of the adaptor protein Grb2. Our recent data support this model, as reducing the affinity of FRNK for paxillin (by introduction of a hydrophilic Ser in place of a hydrophobic Leu at position 341) increased the phosphorylation of FRNK at Tyr232, which is equivalent to the Tyr925 site on FAK. These results also suggest that a similar mechanism may be operative in regulating p130Cas binding to FRNK and FAK. Src-dependent phosphorylation at Tyr168/Tyr861 may induce a more open conformation in the intervening sequences between the kinase and FAT domains, exposing the proline-rich domain, and thereby allowing p130Cas interaction with both FRNK and FAK. Interestingly, we observed that rendering FRNK nonphosphorylatable at Tyr168 not only reduced p130Cas-FRNK interaction but also increased FRNK’s interaction with paxillin. Thus, the Tyr168Phe mutation must also have favored the closed conformation of the FAT domain and reduced its dynamic exchange with paxillin in FAs.

There may be potential therapeutic value in pharmacologically manipulating FAK phosphorylation during vasculogenesis and neointima formation. However, the structural similarities between FAK and FRNK require caution in manipulating FAK-dependent signaling in VSMCs, as FAK and FRNK...
phosphorylation may occur by similar mechanisms but result in opposing effects on cell spreading, migration, and invasion.

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Disclosures
None.

References
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**SUPPLEMENTAL MATERIAL**

**SUPPLEMENTAL METHODS**

*Materials and reagents.* Tissue culture dishes and Permanox® chamberslides 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 (pY397), anti-FAK (pY861), anti-FAK (pY925), anti-paxillin (pY118), and anti-PYK2 (pY402) phosphospecific rabbit polyclonal antibodies (pAb) were from BioSource International (Camarillo, CA). Phospho-ERK1/2 pAb (pT183/pY185) was from Promega (Madison, WI). p130Cas mouse monoclonal antibody (mAb) for immunoprecipitation was from Millipore (Billerica, MA); phospho-p130Cas (pY165) pAb for Western blotting was from Cell Signaling (Danvers, MA), and rabbit p130Cas pAb for immunocytochemistry was from Santa Cruz (Santa Cruz, CA). Anti-GFP mAb was from Stressgen (Ann Arbor, MI). Anti-paxillin, anti-PYK2, and anti-N-terminal FAK mAb were from BD Transduction Laboratories (Lexington, KY). Anti-GAPDH mAb was purchased from Novus Biologicals (Littleton, CO). Horseradish peroxidase-conjugated goat anti-rabbit and anti-mouse IgG were from BioRad (Hercules, CA). Rhodamine-conjugated goat anti-rabbit and anti-mouse IgG were obtained from Invitrogen (Carlsbad, CA). Rainbow molecular weight standards were from Amersham (Arlington Heights, IL). Pico-West and LumiGLO Enhanced Chemiluminescence (ECL) kits were from Thermo-Scientific (Hudson, NH) and KPL (Gaithersburg, MD), respectively. Costar transwell polystyrene plates were from Corning Costar (Cambridge, MA). PF573,228 was obtained from Tocris Bioscience (Ellisville, MO). PP2 and PP3 were from EMD Chemicals (Gibbstown, NJ). All other reagents were of the highest grade commercially available and were obtained from Sigma Chemical (St. Louis, MO).
Cell culture. Rat aortic smooth muscle cells (RASMCs) were isolated as previously described\(^1\) and maintained in DMEM containing 10% FBS. Cells up to the 9th passage were used.

Adenoviral constructs. Wildtype (wt) chick FRNK was kindly provided by Dr. Tom Parsons, University of Virginia, and cloned in-frame into pEGFP expression plasmid (Clontech, Palo Alto, CA) as previously described.\(^2\) Mutagenesis of the GFP-wtFRNK expression plasmid was then performed using the Stratagene QuikChange Kit (Stratagene, La Jolla, CA). Two sets of 35mer oligo primers were used to generate the desired mutations (Y168F, Y232F, and Y168,232F mutations, respectively) which were confirmed by DNA sequencing. Replication-defective adenoviruses (Adv) expressing GFP, GFP-wtFRNK, GFP-Y168F-FRNK, GFP-Y232F-FRNK, and GFP-Y168,232F-FRNK were then generated using the AdEasy XL system (Agilent Technologies, Santa Clara, CA), amplified, and purified as previously described.\(^2\)

Two replication-deficient Adv expressing shRNAs specific for rat FAK\(^3,4\) and PYK2\(^5\) were also generated. The siRNA sequences were designed using GenScript siRNA Construct Builder. Each siRNA sequence had a MluI site at the 5' end, and a HindIII site at the 3' end. Sequences for each different target gene are shown in Supplemental Table I. The oligos were PAGE-purified and dissolved in H\(_2\)O to a concentration of 1\(\mu\)g/\(\mu\)l. Top and bottom oligos (1\(\mu\)g) were mixed in annealing buffer (1xSSC) and boiled at 95°C for 10 min. The mixture was then incubated at room temperature for 1h and diluted to a final concentration of 40ng/\(\mu\)l. The annealed oligos were then subcloned into pRNAT-H1.1/Adeno shuttle vector (GeneScript, Piscataway, NJ) for use with the AdEasy Adenoviral XL Vector System. The cloning results were confirmed by DNA sequencing. An Adv expressing shRNA specific for luciferase (Adv-Luci-shRNA) was similarly generated, and used to control for nonspecific effects of Adv infection in the gene silencing experiments.\(^6\)

A replication-deficient Adv expressing GFP-CRNK was generated by PCR-cloning rat CRNK\(^7\) from rat PYK2 cDNA, which was kindly provided by Dr. T. Sasaki, Sapporo Medical
University School of Medicine, Sapporo, Japan. The PCR product was ligated in frame into pEGFP expression plasmid, verified by DNA sequencing, and the GFP-CRNK cDNA was then subcloned into the AdEasy XL pShuttle vector for preparation of Adv-GFP-CRNK. A replication-defective Adv expressing CD2-FAK\textsuperscript{8} was constructed using human CD2-FAK cDNA, kindly provided by Dr. Steve Nadler, Bristol-Myers Squibb Co, Princeton, NJ. Replication-deficient Adv expressing wt-p130\textsuperscript{Cas} (wtCas) and a nonphosphorylatable mutant of p130\textsuperscript{Cas} (i.e., Cas-\textDelta SD)\textsuperscript{9} were kindly provided by Dr. Jody Martin, Loyola University Medical Center, Maywood, IL. In some experiments, an Adv expressing nuclear-encoded β-galactosidase (neβgal) was also used to control for nonspecific effects of Adv infection.

For all Adv, the multiplicity of viral infection (moi) was determined by dilution assay in HEK293 cells grown in 96 well clusters. RASMCs were growth-arrested in serum-free culture medium for at least 1h prior to infection. Cells were incubated (24h, 37°C) with Adv in serum-free medium, and the medium was replaced with serum-free DMEM for an additional 24-96h.

Immunoprecipitation, co-immunoprecipitation, SDS-PAGE and Western blotting. For Western blotting experiments, cells were homogenized in lysis buffer\textsuperscript{10} and protein concentration was determined by bicinchoninic acid protein assay (Pierce Chemical Co., Rockford IL) using bovine serum albumin as standard. Equal amounts of extracted proteins (50µg) were directly separated by SDS-PAGE on 10% gels. Separated proteins were transferred to nitrocellulose, and the membranes were probed with the appropriate phosphospecific and total antibodies. Primary antibody binding was detected with horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse secondary antibody, and visualized by ECL. Band intensity was quantified using an HP Scanjet 4890 flatbed scanner and UN-SCAN-IT Gel, Ver. 6.1 software. The ratios of phosphospecific to total FAK, PYK2, p130\textsuperscript{Cas}, and GFP-FRNK were analyzed. In some experiments, equal loading was confirmed by quantifying GAPDH in each sample.
For immunoprecipitation experiments, cells were homogenized in lysis buffer and equal amounts of extracted proteins (1000μg) were incubated (18h, 4°C) with anti-p130Cas mAb. Immune complexes were recovered by adding protein A/G beads (2h, 4°C) followed by centrifugation (10,000g; 10min). The complexes were then washed, resuspended in electrophoresis sample buffer, and separated by SDS-PAGE and Western blotting.

For co-immunoprecipitation experiments, cells were scraped in homogenization buffer (25mM HEPES, pH 7.4, containing 150mM NaCl, 1.5mM MgCl₂, 1mM EDTA, 10mM sodium pyrophosphate, 10mM NaF, 0.1mM sodium orthovanadate, 0.5% Nonidet P-40, and 1% sodium deoxycholate). Following centrifugation (10,000g, 20 min), equal amounts of extracted cellular proteins (500-1000μg) were co-immunoprecipitated with either nonimmune IgG, anti-paxillin mAb or anti-p130Cas mAb overnight at 4°C followed by the addition of protein A/G beads (4h, 4°C) and centrifugation (10,000g; 10min). The complexes were then washed, resuspended in electrophoresis sample buffer, and separated by SDS-PAGE and Western blotting.

Carotid artery balloon injury. Balloon injury of the right common carotid artery was accomplished using a 2.5F double-lumen balloon catheter (NuMED, Inc., Hopkinton, NY), by a modification of the method of Clowes et al. Briefly, adult male Sprague-Dawley rats (~400g) were anesthetized, and the right common and external carotid arteries were surgically exposed. The external carotid artery was proximally ligated, the catheter was introduced through an arteriotomy, and advanced into the common carotid artery. The balloon was inflated to 4 atm for 1min to rupture the internal elastic lamina. The catheter was then drawn backwards and forwards three times to denude the endothelial cell layer. In some experiments, replication-deficient adenoviruses (Adv) expressing GFP or GFP-wtFRNK (~10^10 pfu in 0.1ml) were introduced via the central lumen of the catheter and incubated for 30min. Then, the catheter was removed and the external carotid artery ligated distal to the arteriotomy site. This procedure ensured that blood flow was maintained through the common, to the internal carotid artery. One
or 2wks later, rats were re-anesthetized, and the injured and contralateral control arteries were removed. The arteries were homogenized in lysis buffer for analysis of protein expression and phosphorylation by Western blotting.

RASMC invasion assay. Matrigel transwell invasion assays were performed as previously described. Briefly, equal numbers of Adv-infected cells were suspended in serum-free medium, and placed in the upper chamber of Matrigel-coated Boyden chambers. AngII (100nM) was placed in the lower chamber. Cells were allowed to migrate for 2h, and cells expressing GFP, wildtype and mutant GFP-FRNK, GFP-CRNK, or FAK, PYK2 and Luciferase shRNAs were detected by fluorescence microscopy and counted using the multi-wavelength cell scoring application of Metamorph (Molecular Devices Corp., Downingtown, PA), which identifies objects on the basis of brightness and size. For all samples, the minimum brightness criterion was set to 50 counts above local background, and the maximum and minimum object diameter criteria were set to 20 and 5 pixels, respectively. For this magnification (10X objective), image scale is 1.60 μm/pixel.

Total internal reflection fluorescence (TIRF)-microscopy and fluorescence recovery after photobleaching (FRAP). RASMCs grown on 1mm glass coverslips were infected (24h, 100moi) with Adv-GFP-wtFRNK, Adv-GFP-Y168F-FRNK, Adv-GFP-Y232F-FRNK or Adv-GFP-Y168,232F-FRNK. Cells were then viewed under an inverted TIRF-microscope. TIRF-illumination was performed by a 445nm diode laser and/or multiline 300mW Argon laser via a Nikon TIRF II illuminator coupled with a custom beam combiner launch. For FRAP, the Argon laser was directed to the sample with a 10/90 beam splitter mounted in a second filter cube carousel positioned above the excitation dichroic. Bleach spot sizing was accomplished with a Keplerian telescope mounted on an XYZ translation stage. Laser routing and photobleaching exposure time were controlled by Uniblitz shutters driven with a shutter/filter wheel controller.
interfaced to a computer. A single peripheral FA in an individual cell (20 cells per group) was randomly selected, and subjected to FRAP analysis. Fluorescence intensity data were acquired every 22.9 msec for up to 20 sec after photobleaching a small region of interest encompassing a single FA site. Fluorescence intensity data immediately after the laser flash (F) were then normalized to the initial fluorescence intensity (F₀) and plotted as a function of time (sec). Data were then fit to the following double-exponential function:

\[ \frac{F}{F_0} = Y_0 + M1*(1-e^{k1t}) + M2*(1-e^{k2t}) \]

where \( Y_0 \) was the best-fitting value for \( F/F_0 \) at \( t=0 \) (i.e., immediately after the flash), \( M1 \) and \( M2 \) were the fraction of total fluorescence in the “fast” and “slow” compartments, respectively, and \( k1 \) and \( k2 \) were the first-order rate constants (sec\(^{-1}\)) describing the rate of rise of \( F/F_0 \) in each of the two compartments. Unweighted data were fit using SigmaStat Ver. 3.1 software. Best-fitting values for \( M1, k1, M2 \) and \( k2 \) were then used to calculate a single “average” rate constant for each cell (\( k_{FRAP, \text{sec}^{-1}} \)) describing the rate of rise of \( F/F_0 \) to plateau:

\[ \text{Average } k_{FRAP} = M1*k1 + M2*k2 \]

Cell fixation and confocal microscopy. RASMCs grown on Permanox® chamberslides were infected with Adv expressing GFP, GFP-wtFRNK and GFP-Y168F-FRNK (100 moi, 24h) Cells were fixed in 2% paraformaldehyde in PBS, permeabilized with 1% Triton X-100 in PBS, and stained with anti-p130Cas or anti-paxillin antibodies. Fluorescently labeled cells were viewed with a Leica TCS SP5 laser scanning confocal microscope.
Statistical analysis. Results were expressed as means±SEM. Data for multiple groups were compared by 2-ANOVA followed by the Holm-Sidak test, and 1-way ANOVA or 1-way ANOVA on Ranks followed by the Student-Newman-Keuls test. Data for 2 groups were compared by unpaired t-test or Mann-Whitney Rank Sum test, where appropriate. Differences among means were considered significant at \(P<0.05\). Data were analyzed using the SigmaStat Statistical Software Package, Ver. 3.1 (Systat Software, San Jose, CA).

SUPPLEMENTAL REFERENCES


Supplemental Figure I. **FRNK inhibits VSMC FAK and PYK2 phosphorylation and cell invasion.** Rat aortic smooth muscle cells (RASMCs) were infected (100moi, 24h) with Adv-GFP or Adv-GFP-wtFRNK. Paired cultures were then stimulated with AngII (100nM; 30min). (A) FAK-Y397, FAK-Y861 and FAK-Y925 phosphorylations were analyzed by Western blotting with phosphospecific antibodies. An N-terminal FAK antibody was used to ensure equal loading. (B) FRNK-Y168 and FRNK-Y232 phosphorylations were analyzed by Western blotting with the same phosphospecific antibodies. GFP and GAPDH antibodies were used to ensure equal loading. (C) PYK2-Y402 phosphorylation was analyzed by Western blotting, and a C-terminal PYK2 antibody was used to ensure equal loading. The position of molecular weight markers is indicated to the left of each blot. (D) Equal numbers of Adv-infected cells (100moi, 24h) were suspended in serum-free medium, and placed in the upper chamber of Matrigel-coated Boyden chambers. AngII (100nM) was placed in the lower chamber. Cells were allowed to migrate for 2h, and cells expressing GFP or GFP-FRNK were detected by fluorescence microscopy and counted using an automatic cell scoring application. Representative object scoring maps from a single experiment are depicted.

Supplemental Figure II. **Effects of GFP-wtFRNK and GFP-wtCRNK overexpression on VSMC invasion.** RASMCs were infected (100moi, 24h) with Adv-GFP, Adv-GFP-wtFRNK, or Adv-GFP-wtCRNK. Equal numbers of Adv-infected cells were suspended in serum-free medium, and placed in the upper chamber of Matrigel-coated Boyden chambers. AngII (100nM) was placed in the lower chamber. Cells were allowed to migrate for 2h, and cells expressing GFP, GFP-wtFRNK or GFP-wtCRNK were detected by fluorescence microscopy and counted using an automatic cell scoring application. (A) Representative object scoring maps from a single
experiment are depicted. (B) Quantitative analysis of 20 images per group from 3 separate experiments were analyzed. Data are mean±SEM; *P<0.05 vs. GFP; #P<0.05 vs. GFP-wtFRNK.

**Supplemental Figure III.** *FAK tyrosine phosphorylation at Y861 and Y925 is critical for VSMC invasion.* (A) Paired cultures were stimulated with AngII (100nmol/L, 10min) in the absence or presence of PF (100mM, 1 hr pre-treatment), PP2 or PP3 (20µM, 1h pre-treatment). FAK Y397, Y861 and Y925 phosphorylation were analyzed by Western blotting with phosphospecific antibodies. (B) Paired cultures were stimulated with AngII (100nmol/L, 30min) in the absence or presence of PF, PP2 or PP3 (20µM, 1h pre-treatment). FAK Y397, Y861 and Y925 phosphorylation were analyzed by Western blotting with phosphospecific antibodies. (C) Equal numbers of Adv-infected cells were suspended in serum-free medium, and placed in the upper chamber of Matrigel-coated Boyden chambers. AngII (100nmol/L) was placed in the lower chamber, in the absence or presence of PF (100mM, 1 hr pre-treatment) or PP2 or PP3 (20µM, 1h pre-treatment) (D). Cells were allowed to migrate for 2h, and fluorescently labeled cells were visualized and counted. (C-D) Representative fluorescent images from a single experiment. (E) Quantitative analysis of 20 images per group from 3 separate experiments. Data are mean±SEM; *P<0.05 UT vs PF. (F) Quantitative analysis of 20 images per group from 3 separate experiments. Data are mean±SEM; *P<0.05 PP2 vs UT or PP3.

**Supplemental Figure IV.** *FRNK tyrosine phosphorylation is not required for efficient FA targeting and inhibition of certain aspects of FAK-dependent signaling.* (A) Live TIRF microscopic images of RASMCs infected (100moi, 24h) with Adv expressing GFP-tagged wt, Y168F-, Y232F- or Y168,232F-FRNK. (B) Tyrosine phosphorylation of endogenous FAK was analyzed by Western blotting in RASMCs infected (100moi, 24h) with Adv expressing GFP, or GFP-tagged wt, Y168F-, Y232F-, or Y168,232F-FRNK. Paired cultures were stimulated with AngII (100nM, 30min). FAK-Y397, FAK-Y861 and FAK-Y925 phosphorylations were analyzed
by Western blotting with phosphospecific antibodies, and with N-terminal FAK antibody. Pax-Y118 and ERK1/2 phosphorylations were also analyzed by Western blotting with phosphospecific antibodies. Samples were probed for GAPDH to ensure equal loading. The position of molecular weight markers is indicated to the left of each blot. (C-E) Quantitative analysis of FAK-Y397, FAK-Y861 and FAK-Y925 phosphorylations from n=4 experiments. Data are means±SEM; *P<0.05 vs. GFP.

**Supplemental Figure V.** *TIRF-microscopy and FRAP analysis of wtFRNK and FRNK phosphorylation mutants.* Representative images of the recovery of GFP fluorescence at 0, 0.5, 1.0 and 5.0 sec after photobleaching are depicted for wtFRNK, Y168F-FRNK, Y232F-FRNK and Y168,232F-FRNK. The dashed circle represents the region of interest from which the quantitative analysis of GFP fluorescence recovery was obtained.

**Supplemental Figure VI.** *p130Cas and paxillin co-localize with GFP-wtFRNK and GFP-Y168-FRNK in spreading VSMCs.* RASMCs were infected (100moi, 24h) with Adv-GFP-wtFRNK or Adv-Y168F-FRNK (green). Cells were then trypsinized, replated onto fibronectin-coated chamber slides, and allowed to attach and spread at 37°C for 60min. Cells were then fixed, permeabilized, counterstained with anti-p130Cas mAb (red; Panel A), or anti-paxillin mAb (red; Panel B), mounted with DAPI-containing mounting medium to detect cell nuclei (blue), and viewed by confocal microscopy. Co-localization of GFP fluorescence and p130Cas or paxillin in these 1μm optical sections at the cell-substratum interface was represented in the merged image (yellow). The bar indicates 10μm.

**Supplemental Figure VII.** *pY165p130Cas phosphorylation is Src-dependent.* (A) RASMCs were infected (100moi, 24h) with Adv-GFP, Adv-GFP-wtFRNK and Adv-Y168F-FRNK. Paired cultures were then stimulated with AngII (100 nM; 30min) in the absence or presence of PP2
p130$^{\text{Cas}}$-Y165 phosphorylation was analyzed by Western blotting with phosphospecific antibody. (B) Model mechanism of FRNK-mediated inhibition of VSMC invasion. (Left Panel) With little or no FRNK, VSMCs migrate as a result of FAK transphosphorylation at Y397, Src-dependent FAK phosphorylation at Y861, and binding and subsequent phosphorylation of p130$^{\text{Cas}}$ by the FAK/Src complex. (Right Panel) With wtFRNK overexpression, FRNK undergoes Src-dependent Y168 phosphorylation, targets to FAs, and subsequently binds to p130$^{\text{Cas}}$, but since FRNK lacks the kinase domain and Y397 autophosphorylation site present in full-length FAK, it fails to form an equivalent FRNK/Src complex which is required for p130$^{\text{Cas}}$ phosphorylation and initiation of cell invasion.
### Supplemental Table I. shRNA sequences for FAK and PYK2

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Supplemental Figure II

A.

Adv-GFP  Adv-wtFRNK  Adv-wtCRNK

B.

No. of Objects per HPF

GFP  wtFRNK  wtCRNK

*  #
**Figure III**

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**WB:**
- pY397FAK
- pY861FAK
- pY925FAK
- FAK

### B.

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**WB:**
- pY397FAK
- pY861FAK
- pY925FAK
- FAK

### C.

- UT
- PF-573228

### D.

- UT
- PP2
- PP3

### E.

![Graph showing the number of objects per HPF](graph_E.png)

- UT
- PF-573228

### F.

![Graph showing the number of objects per HPF](graph_F.png)

- UT
- PP2
- PP3
Supplemental Figure IV

A. 

wtFRNK  

Y168F-FRNK  

Y232F-FRNK  

Y168,232F-FRNK

B. 

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C. 

pY397 FAK/FAK ratio (normalized to GFP control)

D. 

pY861 FAK/FAK ratio (normalized to GFP control)

E. 

pY925 FAK/FAK ratio (normalized to GFP control)
Supplemental Figure V

Pre-Flash  0 sec  0.5 sec  1.0 sec  5.0 sec

wtFRNK

Y168F-FRNK

Y232F-FRNK

Y168, 232F-FRNK
Supplemental Figure VI-A

60' cell spreading on Fibronectin

GFP

p130Cas

Merged

Adv-GFP-wtFRNK

Adv-GFP-Y168F-FRNK
60' cell spreading on Fibronectin

Supplemental Figure VI-B

GFP

Paxillin

Merged

Adv-GFP-wtFRNK  Adv-GFP-Y168F-FRNK
Supplemental Figure VII

A.

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WB: pY165p130Cas

WB: p130Cas

B.

A. wtFAK

B. wtFRNK