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Engineered allosteric activation of kinases in living cells

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Studies of cellular and tissue dynamics benefit greatly from tools that can control protein activity with specificity and precise timing in living systems. Here we describe an approach to confer allosteric regulation specifically on the catalytic activity of protein kinases. A highly conserved portion of the kinase catalytic domain is modified with a small protein insert that inactivates catalytic activity but does not affect other protein functions (Fig. 1a). Catalytic activity is restored by addition of rapamycin or non-immunosuppresive rapamycin analogs. Molecular modeling and mutagenesis indicate that the protein insert reduces activity by increasing the flexibility of the catalytic domain. Drug binding restores activity by increasing rigidity. We demonstrate the approach by specifically activating focal adhesion kinase (FAK) within minutes in living cells and show that FAK is involved in the regulation of membrane dynamics. Successful regulation of Src and p38 by insertion of the rapamycin-responsive element at the same conserved site used in FAK suggests that our strategy will be applicable to other kinases.

Recently described methods for regulation of kinases with precise timing in living cells include induced dimerization, subcellular localization, proteolytic degradation and chemical rescue from an inactivating mutation[1–4]. Engineered allosteric regulation has also been used for precise control of protein activity[5–7]. Nonetheless, existing methods are limited to specific targets, inactivate rather than activate kinases and/or do not enable regulation of a particular domain within the target. Here we describe a method to activate specifically the catalytic domain within a multidomain protein kinase, using FAK as a model. FAK has been implicated in a wide variety of cell behaviors, including proliferation, apoptosis, migration and tumorigenesis[8–11]. It is a multidomain protein that functions as both a scaffold and a kinase[11], and relatively little is known about the role of its catalytic activity. It therefore served as a good test case for our method, which enabled us to specifically dissect the function of FAK kinase activity, controlling it with a temporal resolution of 1–2 min, without affecting the scaffolding function.

To allosterically regulate FAK's catalytic activity, we used a portion of the small protein FKBP12 (**Fig. 1a**). A previous study has shown that ligand binding to FKBP12 greatly increases its conformational rigidity[12], suggesting that insertion of FKBP12 near the catalytic site of kinases could be used to control the conformational mobility of the kinase active site. It was, however, unclear that FKBP12 could be inserted into the kinase sequence without disrupting kinase structure or FKBP12 binding interactions. We therefore tested truncated forms of FKBP12, leading to an FKBP12 derivative named insertable FKBP (iFKBP, **Fig. 1b**). In iFKBP, the N and C termini are positioned near one another for minimal perturbation of kinase secondary structure (**Fig. 1b**). Co-immunoprecipitation experiments showed that iFKBP binds rapamycin and FKBP12-rapamycin binding domain (FRB) as efficiently as wild-type FKBP12, even when inserted in the middle of the FAK molecule (**Fig. 1c** and **Supplementary Fig. 1**). Molecular dynamics studies of iFKBP indicated that its conformational fluctuation is reduced by interaction with rapamycin or by rapamycin-induced heterodimerization with FRB (**Fig. 1d** and **Supplementary Fig. 2**). Changes in conformational fluctuations were especially pronounced at the N and C termini where iFKBP would be linked to FAK, suggesting that the effects of rapamycin and FRB binding could be communicated to FAK.

Optimization of the insertion site and the linkers connecting iFKBP to FAK led to a version of FAK that was susceptible to regulation by rapamycin-induced FRB binding. The insertion of iFKBP at Glu445 (FAK-iFKBP445 construct) substantially reduced the catalytic activity of FAK. Rapamycin-induced binding to FRB restored activity (**Fig. 2a**). Treatment with rapamycin did not affect the activity of wild-type FAK or a construct with iFKBP attached to the FAK N terminus, demonstrating that regulation of catalytic activity is dependent on specific placement of the insert in the catalytic subunit. To optimize regulation of FAK by rapamycin, we introduced several modifications into the regions that connect iFKBP to FAK. iFKBP was positioned within the FAK loop Met442–Ala448, between two β-strands in the N-terminal lobe of the FAK catalytic domain (**Fig. 2b**). Replacing FAK residues Met442–Ala448 with iFKBP, using no linkers, negated the effect of iFKBP on FAK activity and dramatically reduced interaction with rapamycin and FRB (**Fig. 2a**,**b**, construct FAK-iFKBP442–448). Computational analysis revealed that the construct without linkers is locked in a distorted conformation that prevents ligand binding (**Supplementary Fig. 3**). In contrast, introduction of short linkers to connect iFKBP with the β-strands of the FAK catalytic domain led to the optimized structure used henceforth, rapamycin-regulated FAK (RapR-FAK). In RapR-FAK, activity in the absence of rapamycin was considerably lower than that of FAK-iFKBP445 (**Fig. 2a**). Rapamycin-induced FRB binding restored activity to near wild-type level. Activation of RapR-FAK catalytic activity was achieved in living cells within 2 min and with 50 nM rapamycin (**Fig. 2c**,**d**). Activation was also achieved by treatment

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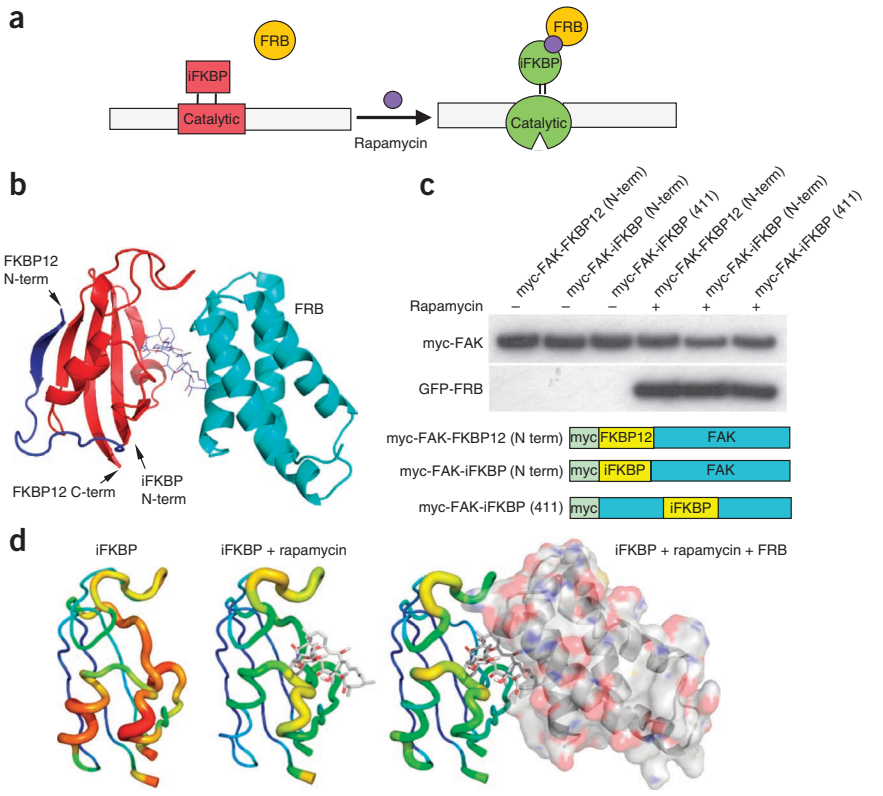

Figure 1 Design and generation of RapR-FAK. (**a**) Schematic representation of the approach used to regulate the catalytic activity of FAK. A fragment of FKBP is inserted at a position in the catalytic domain where it abrogates catalytic activity. Binding to rapamycin and FRB restores activity. (**b**) The truncated fragment of human FKBP12 (amino acids Thr22 through Glu108) inserted into the kinase domain. Blue and red, full-length FKBP12; red, proposed structure of the inserted fragment. The FKBP12 is shown in complex with rapamycin and FRB (cyan). (**c**) Immunoblot analysis of iFKBP interaction with rapamycin and FRB. Myc-tagged FKBP12 and iFKBP constructs were immunoprecipitated from cells treated for 1 h with either 200 nM rapamycin or ethanol (solvent control). Co-immunoprecipitation of co-expressed GFP-FRB was detected using anti-GFP antibody. (**d**) Changes in the molecular dynamics of iFKBP upon binding to rapamycin and FRB. Warmer colors and thicker backbone indicate increasing root mean square fluctuation.

with rapamycin alone, without co-expression of FRB (**Supplementary Fig. 4**). However, this required significantly higher concentrations of rapamycin (up to 4 μM), so the remaining studies described here were carried out using rapamycin-induced FRB binding. Computational analysis indicated that rapamycin alone does not stabilize iFKBP to the same extent as rapamycin together with FRB (**Fig. 1d** and **Supplementary Fig. 2**).

iFKBP-mediated FAK regulation was designed to specifically control catalytic activity without perturbing other FAK functions. Thus, it was important to test the effects of iFKBP insertion on normal FAK binding interactions and FAK regulation. FAK catalytic activity is regulated by an autoinhibitory interaction between the N-terminal FERM domain and the catalytic domain[13]. Two amino acids known to be involved in this interaction were mutated to alanines (Y180A and M183A, previously described[13]) to test if RapR-FAK remains regulated by autoinhibition. When activated by rapamycin, the Y180A/M183A construct (RapR-FAK-YM) demonstrates considerably higher activity than RapR-FAK (**Fig. 2e**), consistent with published results for constitutively active FAK[13] and demonstrating that RapR-FAK is still regulated by autoinhibition. RapR-FAK-YM is therefore solely regulated by rapamycin and not by endogenous mechanisms. To confirm that RapR-FAK phosphorylates substrates in a rapamycin-dependent manner in cells, phosphorylation of two known FAK substrates was tested before and after addition of rapamycin. Upon activation of RapR-FAK-YM, phosphorylation of paxillin on residue Tyr31 and autophosphorylation of FAK on residue Tyr397 are substantially increased (**Fig. 2f**). A control construct lacking catalytic activity (RapR-FAK-YM-KD, with additional mutation D546R) failed to demonstrate any change in phosphorylation. RapR-FAK and wild-type FAK showed similar binding to paxillin and Src in co-immunoprecipitation assays (**Supplementary Fig. 5**), indicating that introduction of iFKBP into the catalytic domain of FAK does not affect interaction with binding partners. Also, iFKBP insertion did not perturb the intracellular distribution of RapR-FAK; its localization was identical to that of wild-type FAK (**Supplementary Fig. 6**). Activation of RapR-FAK was accompanied by translocation of fluorescently labeled FRB into focal adhesions and co-localization with fluorescent RapR-FAK (**Supplementary Fig. 7**). The translocation of fluorescent FRB into adhesions served as a useful marker of FAK activation in live cells. Overall we conclude that RapR-FAK enables robust and specific activation of FAK catalytic activity in living cells without perturbation of other FAK properties.

FAK is known to be overexpressed and activated in human tumors[14–16], but the specific role of its catalytic activity remains unclear. To identify processes affected specifically by FAK catalytic activity, we examined the activation of RapR-FAK-YM in HeLa cells. The Y180A/M183A mutant was used to ensure the regulation of RapR-FAK by rapamycin only and to exclude modulation by endogenous upstream factors. Consistent with previous reports showing that catalytic activity is not required for FAK's role in growth factor–stimulated motility[17], activation of RapR-FAK-YM did not significantly affect cell movement (**Supplementary Fig. 8**). However, we did observe a distinct effect on membrane dynamics. HeLa cells normally show small peripheral ruffles that remain near the cell border. Upon addition of rapamycin, the extent of ruffling greatly increased, and very large and dynamic ruffles appeared across the dorsal surface (**Fig. 3a**,**b** and **Supplementary Movie 1**, 36/64 analyzed cells). In control studies, cells expressing similar levels of catalytically inactive RapR-FAK-YM-KD showed no change in normal ruffling activity (**Fig. 3b**, 34/35 analyzed cells). RapR-FAK was localized to these ruffles (**Supplementary Fig. 9**). Notably, wild-type FAK was also detected in ruffles stimulated by RapR-FAK and in those produced by platelet-derived growth factor (PDGF) treatment (**Supplementary Figs. 10–12**), indicating that dorsal ruffles are not an artifact of RapR-FAK mislocalization. Furthermore, FAK-null fibroblasts failed to produce PDGF-stimulated dorsal ruffles (158 cells analyzed), whereas 50% of control fibroblasts expressing FAK (59/118 analyzed) exhibited distinct dorsal ruffling under the same stimulation conditions. These data implicate FAK catalytic activity in the regulation of dorsal membrane protrusions.

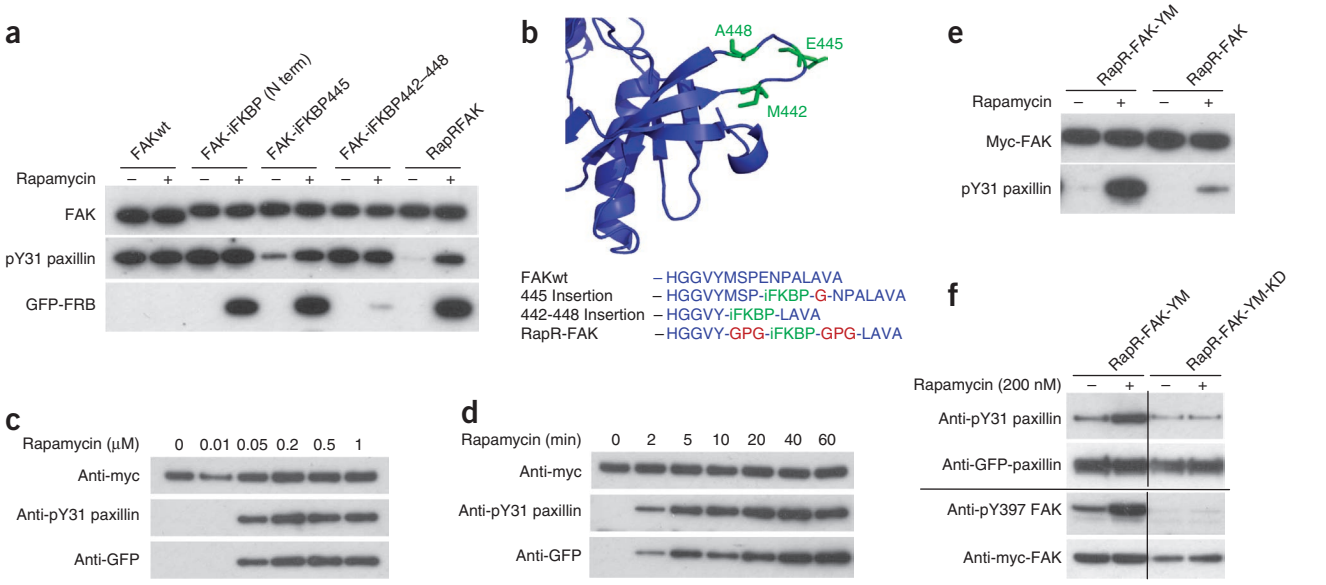

Figure 2 Development and biochemical characterization of RapR-FAK. (**a**) Rapamycin regulation of FAK variants with iFKBP inserted at different positions. HEK293T cells co-expressing myc-tagged FAK constructs and GFP-FRB were treated for 1 h with either 200 nM rapamycin or ethanol (solvent control). The activity of immunoprecipitated FAK variants was tested using the N-terminal fragment of paxillin as a substrate. (**b**) Sites of iFKBP insertion (green) and connecting linkers (red). (**c**,**d**) HEK293T cells co-expressing RapR-FAK and FRB were treated with the indicated amount of rapamycin for 1 h or with 200 nM rapamycin for the indicated period of time. The kinase was immunoprecipitated and its activity tested as described above. (**e**) FAK Y180A and M183A mutations were introduced to eliminate autoinhibitory interactions, thereby generating RapR-FAK-YM, which was tested as in **a**. (**f**) HEK293T cells co-expressing Cherry-FRB, GFP-paxillin and either myc-tagged RapR-FAK-YM or its kinase-inactive mutant (RapR-FAK-YM-KD) were treated with rapamycin or ethanol (solvent control) for 1 h. GFP-paxillin was immunoprecipitated and its phosphorylation was assessed using anti-phospho-Tyr31 antibody. Autophosphorylation of FAK on Tyr397 was analyzed using total cell lysate. (Full-length blots are provided in **Supplementary Fig. 19**.)

Published work has demonstrated that FAK autophosphorylation of Tyr397 plays an important role in FAK-mediated signaling, and that Tyr397 phosphorylation level correlates with FAK activation[18]. Because autophosphorylation of FAK on Tyr397 creates a binding site for Src family kinases[18,19], it has been proposed that interaction of FAK with Src leads to Src activation[18]. Furthermore, Src is involved in the formation of dorsal protrusions stimulated by PDGF[20]. Together these observations led us to hypothesize that the FAK-stimulated formation of dorsal protrusions occurs through activation of Src. In our studies, mutation of Tyr397 to phenylalanine in RapR-FAK completely abolished the formation of dorsal protrusions (**Fig. 3b**). To test the potential role of Src, cells were treated with PP2, an inhibitor of Src family kinases, after stimulation of RapR-FAK-YM. This abrogated the FAK-induced dorsal ruffling (**Fig. 3c** and **Supplementary Movie 2**). In contrast, control compound PP3, an inactive PP2 stereoisomer, or imatinib (Gleevec), an inhibitor of Abl kinase, had no effect (data not shown). Phosphorylation of Src Tyr418 (Tyr416 in avian Src) is known to occur upon Src activation[21,22]. Rapamycin addition to cells transfected with RapR-FAK-YM led to increased Src Tyr418 phosphorylation, whereas cells expressing RapR-FAK-YM with an

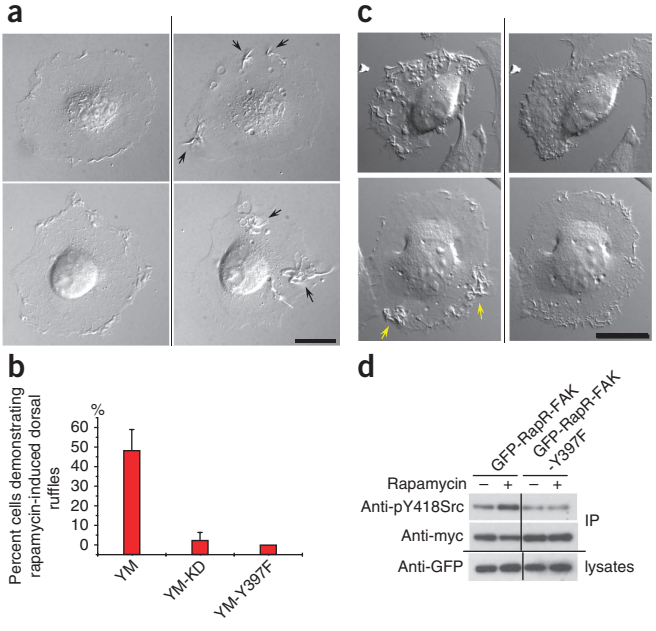

Figure 3 Activation of FAK catalytic activity initiates large dorsal ruffles through the activation of Src. (**a**) Rapamycin treatment of HeLa cells co-expressing RapR-FAK-YM and FRB caused formation of large dorsal ruffles. Scale bars in **a** and **c**, 20 μm. (**b**) HeLa cells expressing either GFP-RapR-FAK-YM (YM, 64 cells), GFP-RapR-FAK kinase-dead mutant (YM-KD, 35 cells) or GFP-tagged Y397F mutant (YM-Y397F, 47 cells) were scored for ruffle induction by rapamycin. No dorsal ruffles were seen before rapamycin addition. (**c**) Inhibition of Src family kinases eliminated the FAK-induced ruffles. Cells co-expressing GFP-RapR-FAK-YM and Cherry-FRB were treated with rapamycin for 1 h and imaged before and after addition of Src family kinase inhibitor PP2. PP2 addition stopped dorsal protrusion in all cells analyzed (16 cells). (**d**) Activation of FAK leads to activation of Src. HeLa cells co-expressing myc-tagged Src, Cherry-FRB and either GFP-RapR-FAK-YM or its Y397F mutant were treated with rapamycin for 1 h. Src was immunoprecipitated using anti-myc antibody, and its phosphorylation on Tyr418 was assessed by immunoblotting. (Full-length blot is provided in **Supplementary Fig. 19**.)

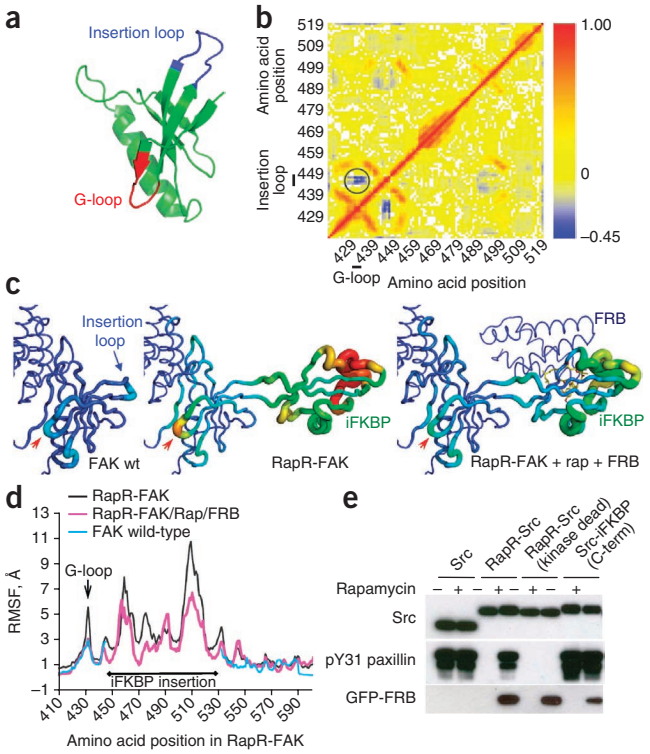

Figure 4 Mechanism of regulation by iFKBP; Src regulation. (**a**) The portion of the FAK catalytic domain targeted for insertion of iFKBP (blue) and the G-loop (red). (**b**) Dynamic correlation analysis of the wild-type FAK catalytic domain (red, positive correlation; blue, negative correlation). The circled region indicates strong negative correlation between the movement of the insertion loop and the G-loop. (**c**) Tube representation depicting changes in the dynamics of the FAK catalytic domain's N-terminal lobe, based on molecular dynamics simulations. Warmer colors and thicker backbone correspond to higher root mean squared fluctuation (RMSF) values, reflecting the degree of free movement within the structure. The red arrows points to the G-loop. (**d**) RMSF of amino acids in FAK and RapR-FAK (arrow indicates G-loop). The break in the wild-type FAK graph corresponds to the iFKBP insert in RapR-FAK. (**e**) Regulation of Src kinase by insertion of iFKBP. HEK293T cells co-expressing the indicated myc-tagged Src construct and GFP-FRB were treated with either 200 nM rapamycin or ethanol solvent control. The kinase activity of immunoprecipitated Src was tested as in **Figure 2a**. (Full-length blot is provided in **Supplementary Fig. 19**.)

additional mutation that abolishes Src binding (Y397F mutation) showed no effect (**Fig. 3d**). Together, these data directly demonstrate that FAK catalytic activity stimulates Src, and that this in turn leads to dorsal protrusions. Dorsal protrusions are important in the invasive migration of cells into extracellular matrix[23] and enhanced FAK expression in tumor cells is associated with cell invasiveness. Our data suggest a mechanism whereby FAK overexpression contributes to the invasive nature of tumors.

To understand the molecular mechanism of RapR-FAK allosteric regulation and explore the generalizability of the approach, we carried out molecular dynamics simulations[24,25]. Combined with the biochemical data, the computational analysis indicated a mechanism for iFKBP-mediated regulation. The iFKBP insertion site connects via a β-strand to FAK's glycine loop (G-loop), a structural feature critical for positioning the ATP phosphate groups in the catalytic site (**Fig. 4a**)[26]. Molecular dynamics analysis indicated that the conformational mobility of the G-loop is correlated with that of the FAK region where iFKBP is inserted (the 'insertion loop', **Fig. 4b**), suggesting that the dynamics of the insertion loop could affect the dynamics of the G-loop and thereby change the catalytic activity. Comparison of wild-type FAK and RapR-FAK dynamics indicated that the amplitude of G-loop conformational dynamics is dramatically increased when iFKBP is inserted in the catalytic domain. These dynamics decreased back to wild-type levels upon binding to rapamycin and FRB (**Fig. 4c**,**d** and **Supplementary Movie 3**). Based on this analysis, we postulate that the effectiveness of the G-loop in the phosphate transfer reaction is reduced owing to greater conformational flexibility produced by insertion of iFKBP. Interaction with rapamycin and FRB stabilizes the G-loop to rescue FAK catalytic activity. Molecular dynamics analysis was consistent with empirical measurements; dynamics analysis of the FAK-iFKBP445 variant suggested that its longer linkers decreased coupling between the iFKBP insert and G-loop dynamics (**Supplementary Fig. 13**), resulting in the less effective FAK inhibition observed in biochemical studies (**Fig. 2a**, FAK-iFKBP445 construct). In contrast, insertion of iFKBP without any linkers restricted the structural dynamics of iFKBP, consistent with the observed minimal effects on catalytic activity (**Supplementary Fig. 13**, FAK-iFKBP442–448 construct). In summary, computational analysis indicates that the allosteric modulation of RapR-FAK activity results from dynamic coupling of the optimized iFKBP insertion and the kinase G-loop, highly conserved structural features in all known kinases[26].

Because the mechanism of allosteric regulation is based on coupling of highly conserved structural elements, the rapamycin-mediated regulation approach may well be applicable to other kinases. We explored this by inserting iFKBP into a tyrosine kinase, Src, and into a serine/threonine kinase, p38, at a site analogous to that used in FAK (Gly288 in Src, Lys45 in p38α; **Supplementary Fig. 14**). In both Src and p38, insertion of iFKBP strongly inhibited activity, and activity was rescued by interaction with rapamycin and FRB (**Fig. 4e** and **Supplementary Fig. 15**). Treatment with rapamycin did not affect wild-type Src or control Src constructs in which iFKBP was added to the C terminus, nor did it have any significant effect on wild-type p38. In molecular dynamics simulations, Src showed the same coupling between iFKBP and the G-loop that was observed for FAK (**Supplementary Figs. 16** and **17**). These data suggest that the iFKBP cassette can be used for allosteric regulation of a wide variety of both tyrosine and serine/threonine kinases.

Although we saw no effects of rapamycin in the absence of rapamycin-regulated kinases, we were concerned that some potential studies could be complicated by the known immunosuppressive effects of rapamycin[27]. We therefore tested the ability to regulate rapamycin-regulated kinases using known non-immunosuppresive analogs of rapamycin, iRap and AP21967. Both compounds regulated RapR-FAK activity at concentrations comparable to those reported previously for dimerization of proteins in living cells[28] (**Supplementary Fig. 18**). AP21967 and a similar analog of rapamycin (C20-MaRap) have been successfully used for experiments in animals[29,30], indicating that the RapR method can be applied in live animal studies. The F36V mutant of FKBP, which interacts tightly with the Shield 1 compound[4], could potentially eliminate the requirement for FRB and minimize effects on endogenous FKBP12 function.

In summary, we describe a protein modification to confer rapamycin sensitivity specifically on the catalytic activity of kinases. The approach is based upon addition of a small protein insert into highly conserved regions of either serine/threonine or tyrosine kinases, promising broad applicability. It can be used with non-immunosuppresive rapamycin analogs suitable for *in vivo* studies. The approach

combines the temporal resolution of small molecule inhibitors with the absolute specificity of genetic approaches and enables allosteric regulation of a single domain in a multidomain protein. A mechanistic model based on molecular dynamics and application to analogous sites in FAK, Src and p38α indicate that rapamycin exerts its effect by modulating the conformational flexibility of the conserved catalytic subunit. By selectively activating FAK catalytic activity in living cells, we directly demonstrated that FAK catalysis activates Src to trigger large dorsal protrusions, a potential mechanism explaining how overexpression and activation of FAK contributes to tumor progression.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturebiotechnology/.

Note: Supplementary information is available on the Nature Biotechnology website.

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AUTHOR CONTRIBUTIONS

A.V.K. initiated the project, developed and validated regulation of RapR-kinases and performed the studies of FAK biological function. F.D. performed molecular modeling of FKBP12 variants, RapR-FAK and RapR-Src. P.K. performed biochemical characterization of RapR-p38. N.V.D. coordinated molecular dynamics studies. K.M.H. coordinated the study and wrote the final version of the manuscript, based on contributions from all authors.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Antibodies and reagents. Anti-phospho-paxillin (Tyr31), anti-phospho-FAK (Tyr397), anti-phospho-Src (Tyr418) and anti-GFP antibodies were purchased from Invitrogen. Anti-myc antibodies and IgG-coupled agarose beads were purchased from Millipore. Anti-paxillin antibodies were a gift from M. Schaller. Rapamycin was purchased from Sigma. All restriction enzymes were purchased from New England Biolabs. iRap was a gift from T. Wandless. AP21697 was provided by Ariad Pharmaceuticals.

Molecular biology. The construct for myc-tagged mouse FAK was a gift from S. K. Hanks. The construct expressing the GST-tagged N-terminal fragment of paxillin was a gift from M. Schaller. The mouse Src and was purchased from Upstate. The flag-tagged mouse p38α, human FKBP12 and FRB domain of human FRAP1 DNA constructs were a gift from G. Johnson. The iFKBP domain consisted of amino acids Thr22 through Glu108 of human FKBP12. Insertion of wild-type FKBP12 or iFKBP at the ends or in the middle of FAK, p38 and Src genes was performed using a modification of the QuikChange site-directed mutagenesis kit (Stratagene). The FKBP12 and iFKBP inserts were created by PCR such that their 5′- and 3′-end sequences annealed at the desired insertion site within the p38, Src and FAK genes. The PCR products were used as mega-primers for QuikChange mutagenesis reactions. In the RapR Src construct, the iFKBP insert contained GPG linkers on both sides. In RapR-p38, iFKBP was flanked by PE and NP linkers at the N and C temini, respectively. The FRB domain of human FRAP1 protein was cloned into pmCherry-CI vector using EcoRI/BamHI cloning sites. GFP-tagged FAK variants were created by subcloning the FAK gene into pEGFP-CI vector (Clontech) using BglII/BamHI cloning sites. The myc-tagged Src gene was constructed by insertion of a myc-tag sequence at the 3′-end of the Src gene using the QuikChange mutagenesis kit.

Immunoprecipitation and kinase assay. Cells expressing FAK or Src were treated with either rapamycin or equivalent volumes of ethanol (solvent control). After treatment, cells were washed with ice-cold PBS and lysed with lysis buffer (20 mM HEPES-KOH, pH 7.8, 50 mM KCl, 100 mM NaCl, 1 mM EGTA, 1% NP40, 1 mM NaF, 0.1 mM Na3VO4, 0.033% ethanol). Cells treated with rapamycin were lysed with lysis buffer containing 200 nM rapamycin. Cleared lysates were incubated for 2 h with IgG-linked agarose beads pre-bound with antibody used for immunoprecipitation. The beads were washed two times with wash buffer (20 mM HEPES-KOH, pH 7.8, 50 mM KCl, 100 mM NaCl, 1 mM EGTA, 1% NP40) and two times with kinase reaction buffer (25 mM HEPES, pH7.5, 5 mM $MgCl_2$, 5 mM $MnCl_2$, 0.5 mM EGTA, 0.005% BRIJ-35). No $MnCl_2$ was used in the kinase reaction buffer for Src kinase immunoprecipitation and assay. Bead suspension (20 μl) was used in kinase assays using the N-terminal fragment of paxillin as previously described[31]. Kinase assay for p38α was performed as previously described[32].

Cell imaging. Cells were plated on fibronectin-coated coverslips (10 mg/l fibronectin) 2 h before imaging, then transferred into L-15 imaging medium (Invitrogen) supplemented with 5% FBS. Live cell imaging was performed in an open heated chamber (Warner Instruments) using an Olympus IX-81 microscope equipped with an objective-based total internal reflection fluorescence (TIRF) system and a PlanApo N 60× TIRFM objective (NA 1.45). All images were collected using a PhotometrixCoolSnap ES2 CCD camera controlled by Metamorph software. The 468 nm and 568 nm lines from an omnichrome series 43 Ar/Kr laser were used for TIRF imaging. Epifluorescence images were taken using a high-pressure mercury arc light source. Cells expressing GFP-RapRFAK constructs and mCherry-FRB were selected using epifluorescence imaging. Time-lapse movies were taken at 1 min time intervals. GFP-RapR-FAK expression level quantification and other image analysis were performed using Metamorph software.

Thermodynamics study of FKBP, FKBP deletion mutant with and without binding partners. We performed replica exchange discrete molecular dynamics (DMD) simulations of various molecular systems to estimate the thermostabilities and to study the conformational dynamics of FKBP and its deletion mutant, dFKBP. Details of the DMD method and simulation protocols can be found in previous studies[24,25]. Briefly, DMD is an efficient conformational sampling algorithm and an all-atom DMD model has been shown to fold several small proteins to their native states *ab initio*[25]. Using replica exchange DMD simulations, the folding thermodynamics of superoxide dismutase (SOD1) and its variants were computationally characterized in agreement with experiments[24]. We applied a similar method to study the folding thermodynamics and conformational dynamics of FKBP and dFKBP bound to either rapamycin or both rapamycin and FRB. The X-ray crystal structure of FKBP, FRB and rapamycin was used to set up the simulations (PDB code: 3FAP).

Model construction of chimeric kinase. To model FAK with dFKBP insertion, we first manually positioned the dFKBP with various linkers in the proximity of insertion loci of FAK (PDB code: 2J0J) using PyMol (http://www.pymol.org/). To model the relative orientation of iFKBP with respect to FAK, we performed all-atom DMD simulations at 27 °C (ref. 25) with the FAK molecule kept static, whereas dFKBP and linkers were allowed to move. As the simulation temperature is below the folding transition temperature of dFKBP, the inserted domain stays folded while the DMD simulation optimizes its relative orientation with respect to FAK. By clustering the snapshot conformations from equilibrium DMD simulations, the centroid structure was identified. We modeled the chimera in complex with rapamycin and FRB by aligning the corresponding FKBP domains in the chimera and in the complex structure of FKBP, rapamycin and FRB. Similarly, we also constructed the model of FKBP insertion into Src kinase (PDB code: 1Y57).

DMD simulations of chimeric kinases. We performed equilibrium DMD simulations of FKBP-dFKBP chimera with different linkers at 27 °C. We also studied wild-type FAK, FAK-dFKBP chimera and FAK-dFKBP chimera in complex with rapamycin and FRB. To reduce the computational overhead, we kept the distal FERM domain of FAK and alpha-helical subdomain of the catalytic domain fixed. We allowed the inserted FKBP and the directly modified catalytic subdomain to sample their conformational space. Similarly, we also studied the Src-dFKBP chimera.

The dynamic coupling of the wild-type FAK was obtained by computing the normalized correlation matrix[33,34] from DMD simulation trajectories. In the calculation of the dynamics coupling and RMSF, the translational and rotational freedom was reduced by translating the center of mass to the origin and then aligning each snapshot with respect to the average structure.

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