JACS
JOURNAL OF THE AMERICAN CHEMICAL SOCIETY

Article

pubs.acs.org/JACS

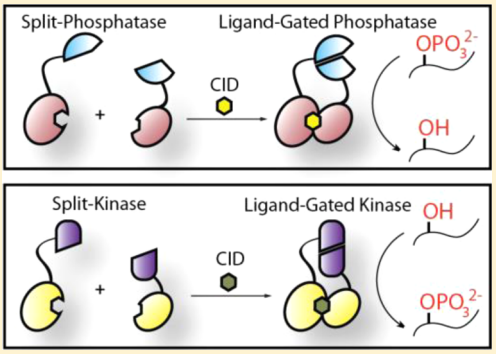
Small Molecule Gated Split-Tyrosine Phosphatases and Orthogonal Split-Tyrosine Kinases

Karla Camacho-Soto, Javier Castillo-Montoya, Blake Tye, Luca O. Ogunleye, and Indraneel Ghosh*

Department of Chemistry and Biochemistry, University of Arizona, 1306 East University Boulevard, Tucson, Arizona 85721, United States

Supporting Information

ABSTRACT: Protein kinases phosphorylate client proteins, while protein phosphatases catalyze their dephosphorylation and thereby in concert exert reversible control over numerous signal transduction pathways. We have recently reported the design and validation of split-protein kinases that can be conditionally activated by an added small molecule chemical inducer of dimerization (CID), rapamycin. Herein, we provide the rational design and validation of three split-tyrosine phosphatases (PTPs) attached to FKBP and FRB, where catalytic activity can be modulated with rapamycin. We further demonstrate that the orthogonal CIDs, abscisic acid and gibberellic acid, can be used to impart control over the activity of split-tyrosine kinases (PTKs). Finally, we demonstrate that designed split-phosphatases and split-kinases can be activated by orthogonal CIDs in mammalian cells. In sum, we provide a methodology that allows for post-translational orthogonal small molecule control over the activity of user defined split-PTKs and split-PTPs. This methodology has the long-term potential for both interrogating and redesigning phosphorylation dependent signaling pathways.



INTRODUCTION

The temporal and location specific activity of almost all proteins is controlled by myriad post-translational modifications. Of these exquisite chemical modifications, phosphorylation and dephosphorylation, catalyzed by protein kinases and phosphatases, respectively, regulate a diversity of cellular events from cell division to cell death.[1] The >500 human protein kinases and 147 protein phosphatases in tandem respond to a variety of both intra- and extracellular environmental cues.[2,3] The protein tyrosine kinases (PTKs), first discovered in 1979,[4] comprising >80 members, have been implicated in various diseases such as cancer,[5] metabolic disorders,[6] and inflammation.[7] Hence tyrosine kinases have emerged as important therapeutic targets.[8,9] Protein tyrosine phosphatases (PTPs), discovered in 1988,[10,11] comprising >100 members, have also been implicated in a plethora of human diseases.[12,13] Despite the importance of tyrosine phosphorylation driven signaling, decrypting the role of a specific PTK or PTP remains enormously challenging. Currently there are almost no uniquely selective small molecules for pharmacological perturbation of native PTKs[8,14−19] or PTPs.[20,21] Prevailing siRNA based genetic knockdown methods, which provide insight regarding the function of a specific enzyme, presently lack both spatial and temporal control, and details can be obscured by compensatory cellular mechanisms.[22,23] In order to impart post-translational control over PTKs, four different approaches that seek immediate temporal control of a specific PTK have been reported in the literature, that either turn a specific kinase off or on using small molecule ligands.

Turn-off control over unique PTKs was first realized with a now classic allele specific approach by Shokat and co-workers, where mutations or a "hole" at the gatekeeper residue proximal to the kinase ATP-binding cleft was complemented with the design of a "bumped" small molecule with unique selectivity.[24−26] Hahn and co-workers have developed a protein engineering approach that provides allosteric control over kinase activity by insertion of FKBP, which when bound by the small molecule rapamycin, and FKBP12-Rapamycin Binding protein (FRB), restores catalytic activity.[27,28] Chin and co-workers have gated kinase activity by incorporating a genetically encoded non-natural photocaged lysine that renders the kinase inactive, which upon uncaging leads to catalytic activity.[29] We have recently demonstrated a split-protein strategy for imparting small molecule control over protein kinase activity.[30] In this approach a protein kinase is split into two inactive fragments appended to FKBP and FRB, respectively, that can be induced to assemble into a ternary complex in the presence of rapamycin and restore catalytic activity (Figure 1A). This split-kinase method was shown to be applicable to several protein kinases. However, none of the current methods for modulating kinase activity have been shown to allow for orthogonal control over two or more user defined kinases.

In comparison to temporal control over PTKs, similar control over PTPs is as challenging due to the comparable promiscuity of existing small molecule inhibitors. To address

Received: August 6, 2014
Published: November 19, 2014

   dx.doi.org/10.1021/ja5080745 | J. Am. Chem. Soc. 2014, 136, 17078−17086

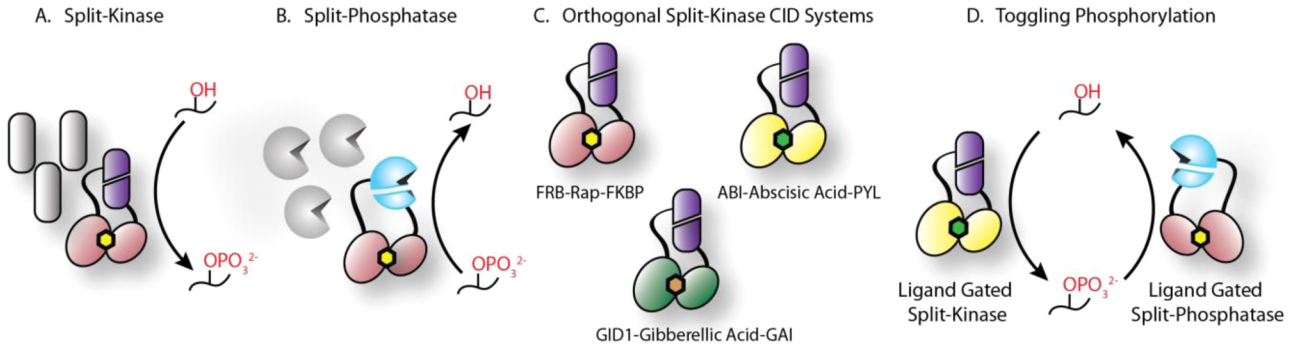


Figure 1. Ligand gated split-kinases and split-phosphatases. A) A specific protein kinase (purple) in the presence of other protein kinases (gray) can be gated, by creating a split-kinase, where the two fragments of the kinase are attached to conditionally interacting domains (pink) such as FKBP and FRB. Upon the addition of a CID (yellow), such as rapamycin, the protein kinase fragments will reassemble to catalyze the phosphorylation of its substrate. B) A specific protein phosphatase (blue) can be controlled by designing fragments (blue) attached to conditionally interacting domains (pink) that conditionally reassemble in the presence of a CID (yellow). C) Orthogonal control over user defined split-kinases can be accomplished through the use of the FRB-Rap-FKBP based CID system (pink) and the plant based CID systems, ABI/PYL (light yellow) activated by abscisic acid (dark green) and GID/GAI (green) activated by gibberellic acid (brown). D) Orthogonal CID gated split-kinases and split-phosphatases can potentially control the phosphorylation state of desired substrates.

this problem, Bishop and co-workers developed an allele specific approach, where specific residues within the catalytic domain of PTPs were mutated and selectively complemented with appropriate "knobbed" inhibitors.[31] They also developed an elegant allosteric strategy, where the insertion of a specific tetra-cysteine sequence within a PTP that binds a biarsenical probe, can enhance inhibition of a desired PTP.[32,33] We sought to complement these two existing avenues for the immediate turn-off of a PTP, by designing a potentially general turn-on approach for controlling the temporal activity of one or more PTPs.

The availability of small-molecule control over PTP and PTK activity has the potential for probing biology and enabling synthetic control over biological pathways (Figure 1). Herein, we report the identification of appropriate sites within the catalytic domain of PTPs that are amenable for fragmentation and reassembly by appended protein pairs that respond to specific CIDs, affording the first generation of ligand gated split-PTPs (Figure 1B). We further demonstrate that the split-protein approach, where appended proteins can be substituted as necessary, allows for the design of orthogonal small molecule control over split-PTKs (Figure 1C). This dual small molecule gating approach has the potential for exerting post-translational control over user defined PTK and PTP pairs (Figure 1D) and thereby the phosphorylation state of their substrates and cellular pathways.

BACKGROUND, RESULTS, AND DISCUSSION

The conditional reassembly of fragmented proteins,[34] such as split-GFP,[35] split-lactamase,[36] and split-luciferase,[37,38] has been primarily utilized for imaging protein partnerships and their inhibitors,[39] detecting a variety of macromolecules and their modifications,[40−42] and in the selections of protein pairs.[43] Rapamycin activated split-proteases for the conditional activation of caspases have also been utilized for understanding the details of apoptosis.[44] The identification of appropriate dissection sites and methods for identification of functionally reassembled proteins remains challenging, especially when life and death selection or readily measurable fluorescent or luminescent outputs are unavailable. Even with easily measured activity such as luminescence, incremental truncation was required for determining appropriate sites for firefly luciferase fragmentation.[37]

To design split-enzymes that can be gated by small molecules, we have recently developed a sequence dissimilarity based approach. This led to the facile identification of potential sites for fragmentation and CID dependent reassembly of split-kinases.[30] In our approach, the identification of sequences in the catalytic domain of protein kinases, particularly loops that harbor significant dissimilarities, guided the design of loop insertion mutants. We hypothesized and subsequently demonstrated that the catalytically active loop insertion mutants predicted sites for fragmentation of the parent enzyme to ultimately afford CID gated split-kinases. In order to design the first generation of ligand-gated split-PTPs, we sought to first identify sites in PTPs that would potentially be tolerant to 25-residue loop insertions.

Choice of Loop Insertions and Fragmentation Sites in PTPs. Human protein phosphatases are divided into three major functional classes (Tyr, dual specificity, or Ser/Thr) based on sequence similarity and catalytic mechanism.[45] Unlike protein kinases, phosphatases from different groups are not as structurally similar.[46] Among the three groups, PTPs comprise the largest group with 107 members classified into several subfamilies.[3] The classical PTPs are characterized by the signature motif VHCSXGXGR[T/S]G that recognizes phosphate within the active site cleft (Figure 2A and B).[47] The alignment of the human PTPs[47] (Supporting Information Figure S1) not only highlights well-conserved motifs but also more importantly provides us with sites that are dissimilar and particularly those with amino acid insertions. For instance, the loop separating the beta-strands β2-β3 contains insertions for SHP2, which correspond to gaps for the phosphatases SHP1, PTP1B, and PTPH1 (Figure 2C). We hypothesized that loops within the catalytic domain of PTPs that showed dissimilarities could potentially tolerate 25-residue loop insertions, which in turn would guide successful fragmentation into ligand gated split-phosphatases. An analysis of the sequence alignment suggested potential sites for loop insertions between β2-β3, β10-β11, β11-α3, and α3-β12 (Supporting Information Figure S1 and Figure 2A-B).

Loop Insertion Mutants of PTP1B. We began testing our hypothesis toward the generation of split-phosphatases with the

dx.doi.org/10.1021/ja5080745 | J. Am. Chem. Soc. 2014, 136, 17078−17086

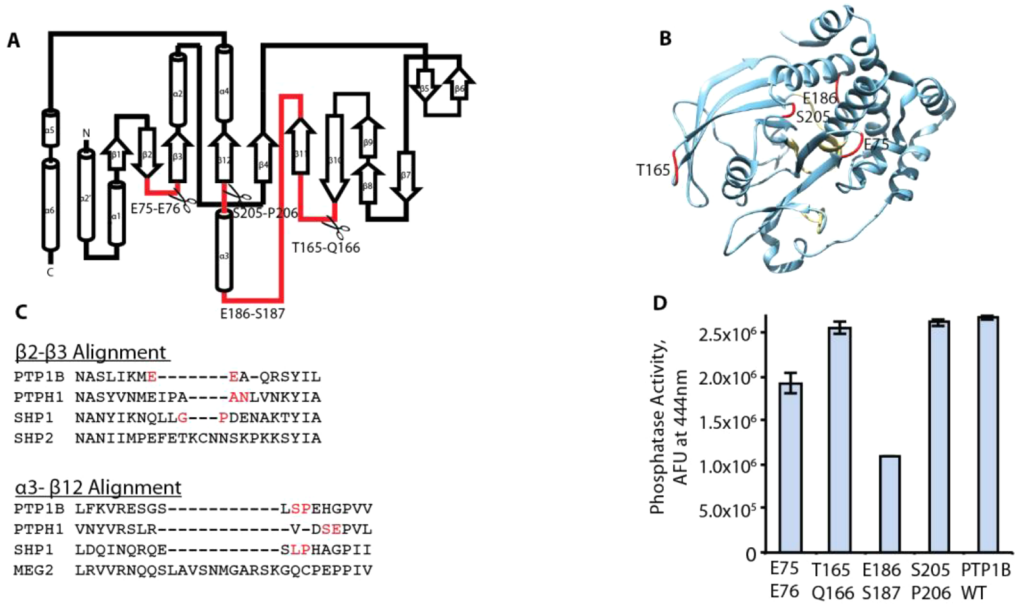


Figure 2. Identification of loop insertion sites for PTP1B. A) General topological representation of tyrosine phosphatases. Highlighted in red are sites identified as potential sites for loop insertions. Scissors represent sites where PTP1B was ultimately fragmented and attached to conditionally interacting domains. B) PTP1B crystal structure (PDB: 2NT7) with loop insertions highlighted in red and residues involved in catalysis in pale yellow. C) Partial alignment of PTPs chosen for testing. Loop insertion sites are depicted in red. D) Activity of PTP1B loop insertion mutants with the fluorescent substrate DiFMUP (6,8-Difluoro-4-Methylumbelliferyl Phosphate). Data presented after background correction of equivalent treatment of RRL, with excitation at 340 nm and emission at 444 nm.

first isolated PTP, PTP1B.[10,11] PTP1B is widely studied not only as the prototypical PTP but also for its role in leptin and insulin receptor mediated signaling.[48−50] We expressed the catalytic domain of PTP1B (residues 2−321) with a C-terminal hexa-histidine tag in cell free rabbit reticulocyte lysate (RRL),[30,51] and purified through Ni-NTA affinity chromatography. Success in isolation of a functional PTP1B was measured by its ability to dephosphorylate the fluorescent substrate DiFMUP (6,8-Difluoro-4-Methylumbelliferyl Phosphate) when compared to lysate alone. Having established a method for measuring activity of resin bound PTP1B (PTP1B-WT), we cloned and expressed the PTP1B loop insertion mutants based on our design (Figure 2A). The 25-residue loop insertion mutants corresponding to E75-E76, T165-Q166, E186-S187, and S205-P206 were expressed and purified, and their phosphatase activity was measured and compared to that of PTP1B-WT. We found that all loop insertion mutants were active. The T165-Q166 and S205-P206 mutants had similar activity to PTP1B-WT. The E75-E76 mutant showed lower activity, and the E186-S187 mutant was the least active (Figure 2D).

Ligand Gated Split-PTP1B. Having successfully identified loop insertion sites that retained activity, we designed split-PTP1B constructs corresponding to the three most active loop insertion mutants. We chose the classical CID, rapamycin, that induces heterodimerization of FKBP and FRB, which we have previously demonstrated as a viable CID for designing ligand gated split-kinases using cell free expression systems.[52−55] FRB was attached to the C-termini of E75, T165, and S205, while FKBP was attached to the N-termini of the E76, Q166, and P206 fragments, respectively, to yield the potential CID controllable split-phosphatase pairs (E75-FRB/FKBP-E76, T165-FRB/FKBP-Q166, and S205-FRB/FKBP-P206 (Figure 3A). We retained the 25-residue loop between the fragments and FKBP or FRB, to provide sufficient flexibility and proceeded without further optimization. The protein fragments were cotranslated in RRL in the presence or absence of 250 nM added rapamycin and subsequently purified and tested for their ability to dephosphorylate the DiFMUP substrate (Figure 3B).

The results from these experiments clearly demonstrate that the design strategy was successful and that the catalytic activity of the three designed split-PTP1Bs was conditional and restored only in the presence of 250 nM rapamycin. We note that addition of rapamycin has no effect upon any of the native PTPs or PTKs tested as expected (data not shown). The relative activities of the three split-PTP1Bs varied from 70- to 330-fold over their respective no-rapamycin controls (Figure 3B). In order to further demonstrate that activity is potentially small molecule tunable, the phosphatase activity for split-PTP1B (S205-FRB/FKBP-P206) was measured as a function of rapamycin concentration, and the data clearly demonstrates dose dependence (Figure 3C). This to our knowledge is the first designed split-PTP that can be gated by the addition of a small molecule.

Split-SHP-1 and Split-PTPH1. Having successfully identified an approach for designing small molecule gated split-PTP1B, we next hypothesized that the rationale for choice of dissection sites and subsequent design of split-PTPs should be portable. We tested two other PTPs, SHP-1 and PTPH1, to explore the generality of our designed split-phosphatases. SHP-1, a protein tyrosine phosphatase from a different subfamily, shares 53% similarity with PTP1B within the catalytic domain. SHP-1 is much studied as a potential negative regulator of many signaling pathways, particularly in hematopoietic cells.[56,57] Loop insertion mutants of SHP-1 corresponding to the positions we had previously identified for PTP1B, namely G313-P314, N402-G403, E425-P426, and L443-P444 as well as SHP-1-WT, were translated and purified, and their phosphatase activity was measured by fluorescence using the DiFMUP substrate. The results show that the G313-P314 loop insertion

dx.doi.org/10.1021/ja5080745 | J. Am. Chem. Soc. 2014, 136, 17078−17086

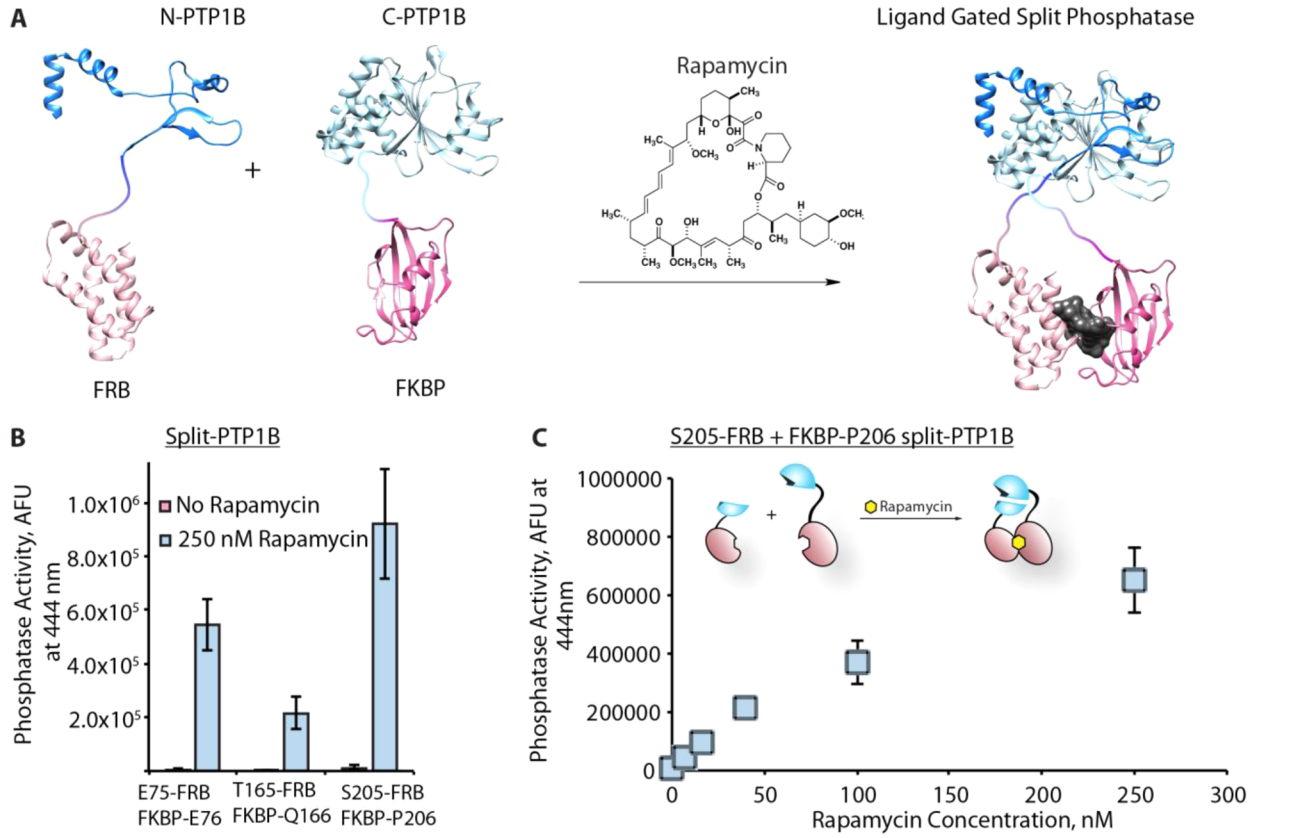


Figure 3. Small molecule gated Split-PTP1B. A) FRB (light pink) was attached to the N-terminal fragment of P-TP1B (dark blue) through a 25-residue linker. The C-terminal fragment of PTP1B (light blue) was attached to FKBP (dark pink) using a 25-residue linker. The addition of the chemical inducer of dimerization, rapamycin, results in a ternary complex and activates the split-PTP1B. B) Activity of different pairs of split-PTP1B fragments in the absence and presence of 250 nM rapamycin. Data presented after background correction of equivalent treatment of RRL at an emission of 444 nm (see Supporting Information, Figure S2). C) Activity of Split-PTP1B (S205-P206) as a function of increasing concentrations of rapamycin with excitation at 340 nm.

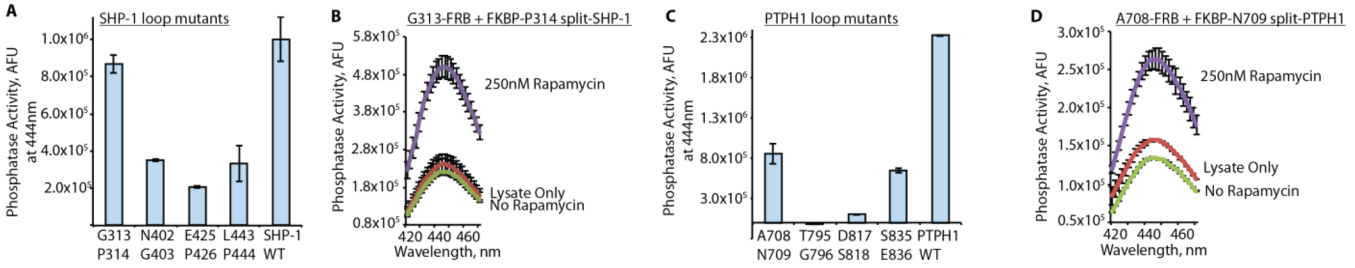


Figure 4. Small molecule gated Split-SHP-1 and split-PTPH1. A) Activity of SHP-1 loop insertion mutants after background correction of equivalently treated lysate. B) Activity of split-SHP-1 (G313-P314) in the absence or presence of 250 nM rapamycin. C) Activity of PTPH1 loop insertion mutants after background correction of equivalent treatment of RRL. D) Activity of split-PTPH1 (A708-N709) in the absence or presence of 250 nM rapamycin.

mutant retained activity similar to that of SHP1-WT, whereas the activities of the loop insertion mutants corresponding to N402-G403, E425-P426, and L443-P444 were compromised (Figure 4A). We designed split SHP1 constructs conjugated to FRB and FKBP corresponding to the G313-P314 site and tested the activity of the pairs in the presence or absence of rapamycin. The results from these experiments clearly demonstrate rapamycin dependent activity of SHP-1 (Figure 4B).

PTPH1 shares 52% similarity with SHP-1 and 53% similarity with PTP1B in its catalytic domain. PTPH1 is less well studied and has been implicated in both cognition and cancer.

As with SHP-1, we first tested four 27-residue loop insertion mutants (Figure 4C) and found 2 to be active when compared to lysate alone. Split-PTPH1 constructs were subsequently generated and tested for phosphatase activity in the absence or presence of rapamycin, and the A708-FRB/FKBP- N709 pair was found to be the most active (Figure 4D). These three examples of split-PTPs suggest that our methodology may potentially be general. Having successfully shown that CID controlled split-PTPs can be designed we next turned to address the issue of orthogonal control over the phosphoproteome. In principle, orthogonal control over protein phosphorylation and dephosphorylation would allow temporal control over a variety of regulatory pathways. Thus, we asked whether it

dx.doi.org/10.1021/ja5080745 | J. Am. Chem. Soc. 2014, 136, 17078−17086

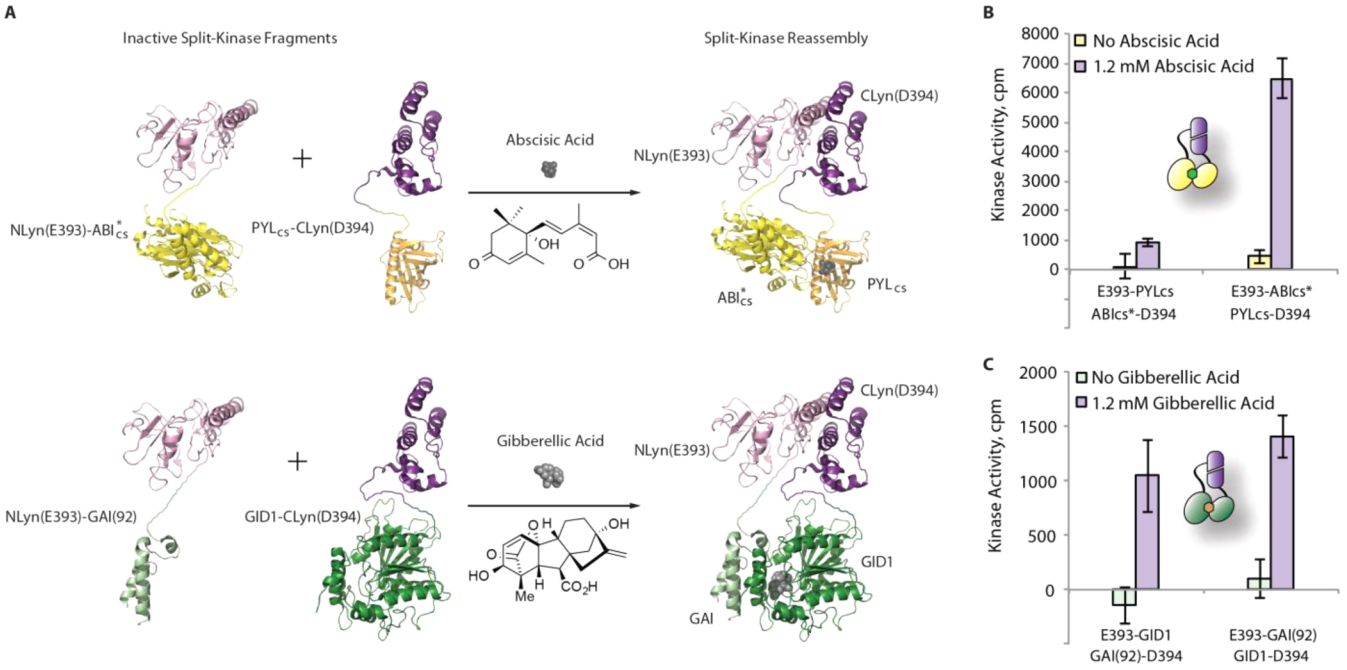


Figure 5. Orthogonal ligand gated split-kinases. (A) The N-terminal fragment of Lyn (purple) was attached through a 27-residue linker to either the PYL_{cs} (light orange) or ABI_{cs}* (yellow) domain for the abscisic acid triggered CID system or to the GID1 (green) or GAI(92) (light green) domain for the gibberellic acid triggered CID system. The C-terminal domain (dark purple) of Lyn was attached through a 25-residue linker to the complementary CID protein domain in each case. Only one of the two combinations is shown for each CID system. The addition of either abscisic or gibberellic acid results in the formation of the respective ternary complex PYL_{cs}-Abscisic Acid-ABI_{cs}* or GID1-Gibberellic Acid-GAI(92), leading to the reassembly of fragmented Lyn (Lyn PDB: 3A4O; PYL1/ABI1//ABA PDB: 3JRQ; GID1/GAI(92)/GA3 PDB: 2ZSH). (B) Activity of two PYL_{cs}/ABI_{cs}* orientations with split-Lyn demonstrates that kinase activity is recovered only in the presence of added abscisic acid. (C) Activity of two GID1/GAI(92) orientations with split-Lyn shows that kinase activity is recovered only in the presence of added gibberellic acid. Data presented after background correction of equivalent treatment of RRL.

would be possible to create split-PTKs that would respond to orthogonal small molecule CIDs.

Orthogonal Split-Phosphatase and Split-Kinase Pairs. We have very recently shown that designed split-kinases could be turned-on by utilizing the rapamycin gated FKBP/FRB heterodimer.[30] In order to provide orthogonal temporal control we would require that the split-kinase and split-phosphatase respond to different CIDs.[58,59] There are very few reported CIDs that can be successfully used in mammalian cells without perturbing native pathways. Even rapamycin, which is widely utilized, will eventually need to be replaced in our split-PTP and split-PTK systems with designed analogs, rapalogs, as it interferes with the mTOR pathway.[60,61] Two recent reports have demonstrated the potential utility of two plant-hormone based CID systems. One is based on modified proteins from the plant abscisic acid stress response pathway and consists of the two proteins pyrabactin resistance 1-like protein, PYL1 (PYL_{cs}, amino acids 33 to 209), and type 2C protein phosphatase, abscisic acid-insensitive 1 protein (ABI_{cs}*, amino acids 126 to 423, inactive D143A mutant), which bind only in the presence of the plant hormone *S*-(+)-abscisic acid (ABA).[62,63] The second orthogonal CID system is comprised of the two proteins, gibberellin insensitive dwarf1 receptor (GID1) and a truncated version of the protein gibberellin insensitive (GAI(92), amino acids 1 to 92), which bind in the presence of the plant hormone gibberellic acid (GA_3).[64,65] We first tested the viability of both of these CID systems in our reticulocyte lysate system using a variety of combinations of our split-luciferase constructs. Our initial studies demonstrated that the split-luciferase could be reassembled upon addition of abscisic acid or gibberellic acid in several orientations when expressed in RRL (Supporting Information, Figure S3).

With functional ligand dependent PYL_{cs}/ABI_{cs}*//ABA and GID1/GAI(92)//GA_3 systems in hand, we set out to test if these CID systems could replace the rapamycin CID system for turning on split-kinases. Our previous studies with split-kinases identified several functional fragmentation sites within the context of the protein tyrosine kinase, Lyn. We have also found that the N268-S269 site in Lyn is a viable site for dissection (Supporting Information, Figure S4).[27] We chose the E393/D394 fragmentation site for testing the viability of new CID systems. Constructs were designed, where the protein components of each CID system substituted for the corresponding FKBP or FRB in split-Lyn(E393/D394) (Figure 5A). The four resultant split-kinase pairs were tested in the presence or absence of the appropriate small molecule CIDs, for their ability to phosphorylate the Lyn substrate EDPIYEFLPAKKK. The PYL_{cs}/ABI_{cs}* pairs were tested in the presence or absence of 1.2 mM abscisic acid (Figure 5B). The GID1/GAI(92) pairs were tested with or without 1.2 mM gibberellic acid (Figure 5C). The results clearly showed that in the absence of ligand, either abscisic acid or gibberellic acid, the various split-Lyn pairs were catalytically inactive, with activity being conditionally restored upon addition of the appropriate CID (Figure 5B–C). The NLyn-ABI_{cs}*/PYL_{cs}-CLyn pair showed higher activity (15-fold) compared to the NLyn-PYL_{cs}/ABI_{cs}*-CLyn pair (8-fold). For the gibberellic acid dependent CID system, the NLyn-GAI(92)/GID1-CLyn showed higher activity (14-fold) compared to the NLyn-GID1/GAI(92)-CLyn pair (7-fold). These two new orthogonal

dx.doi.org/10.1021/ja5080745 | J. Am. Chem. Soc. 2014, 136, 17078–17086

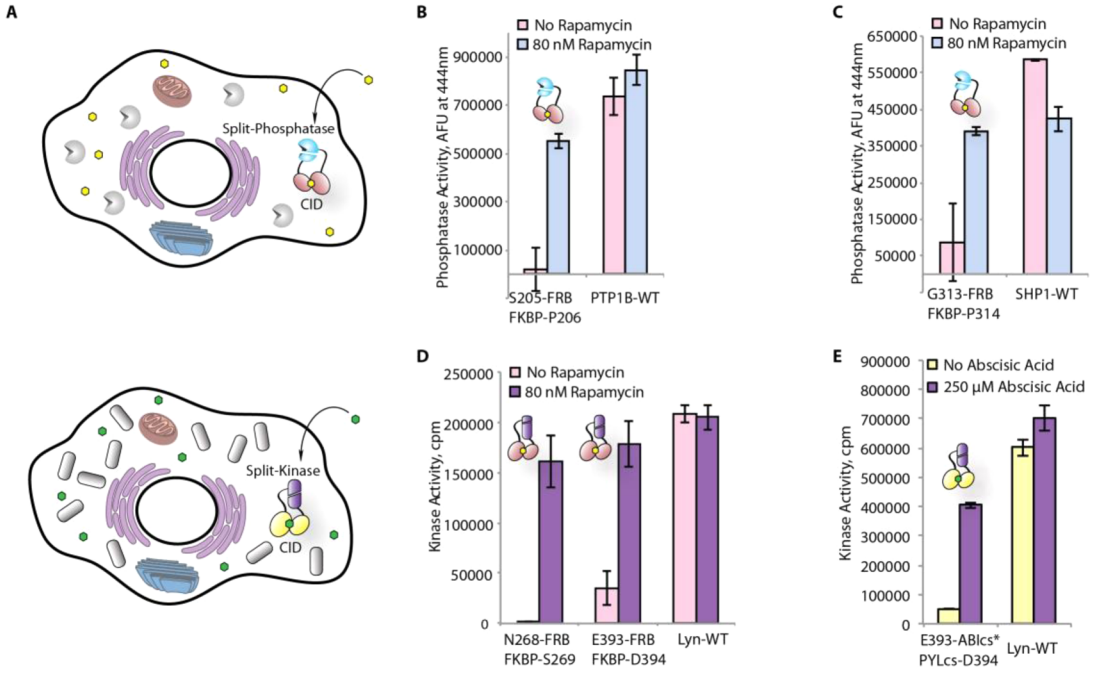


Figure 6. In-cellulo small molecule activation of a specific split-phosphatase or split-kinase. (A) Split-phosphatases or split-kinases were expressed in mammalian cells and reassembled by addition of a cell permeable small molecule. (B) Activity of split-PTP1B (205/206) and PTP1B-WT expressed in HEK293T cells in the absence or presence of 80 nM rapamycin. (C) Activity of split-SHP1(313/314) and SHP1-WT expressed in HEK293T cells in the absence or presence of 80 nM rapamycin. Activity was measured post pull-down using the fluorescent DiFMUP substrate. (D) Activity of split-Lyn kinase at two different fragmentation sites (268/269 and 393/394) and Lyn-WT expressed in HEK293T cells in the absence or presence of 80 nM rapamycin. (E) Activity of an orthogonal small molecule, abscisic acid, activated split-Lyn and WT Lyn expressed in HEK293T cells in the absence or presence of 250 μM abscisic acid. Activity was measured by ^{32}P incorporation in Lyntide post pull-down. All data are presented after subtraction of equivalent treatment of cells with empty vector.

CIDs for split-PTKs in concert with the earlier rapamycin based CIDs now provide a palette of CIDs that can be potentially used for temporal control over the activity of appropriately designed split-kinases.

In-Cellulo Activation of Split-Phosphatases and Split-Kinases. Having successfully showed in vitro expression of split kinases and phosphatases we next sought to investigate if our split-protein constructs were amenable for expression and small molecule activation in mammalian cells. We cotransfected HEK293T cells with equivalent amounts of the relevant constructs corresponding to split-PTP1B (S205-FRB/FKBP-P206), PTP1B-WT, split-SHP1 (G313-FRB/FKBP-P314), SHP1-WT, or empty pcDNA 3.1(+) vector as a control. Ten h post transfection, cells were treated with either 80 nM rapamycin or DMSO for 14 h. Subsequently, proteins were isolated from each population of treated and untreated cells, and 150 μg of total protein, based on BCA assay, was captured by Ni-NTA and subsequently interrogated for phosphatase activity using the DiFMUP substrate. Split-PTP1B and split-SHP1 showed significant phosphatase activity only in the presence of rapamycin (Figure 6B and 6C). The relative activity of the rapamycin induced split-phosphatases was 65% and 92% to that of an equivalent amount of Ni-NTA captured proteins from cells transfected with PTP1B-WT and SHP1-WT, respectively. Having successfully demonstrated the feasibility of small molecule induced activation of split-phosphatases, we next investigated whether the designed split-kinase, split-Lyn, could also be activated in mammalian cells. Two different versions of split-Lyn corresponding to fragmentation at 268/269 or 393/394 were first tested using the rapamycin CID system in HEK293T cells. Cells were transfected with constructs corresponding to split-Lyn (N268-FRB/FKBP-S269), split-Lyn (E393-FRB/FKBP-D394), Lyn-WT, or empty plasmid as a control. Both split-Lyn kinases showed significant activity only in the presence of rapamycin, and the relative activity was 79% and 87% for Split-Lyn (N268-FRB/FKBP-S269) and Split-Lyn (E393-FRB/FKBP-D394), respectively, when compared to equivalent amounts of protein isolated from cells transfected with Lyn-WT (Figure 6D). We next investigated the orthogonal abscisic acid inducible split-Lyn constructs, corresponding to fragmentation at 393/394, in HEK293T cells. Experiments with and without 250 μM abscisic acid clearly demonstrated that the NLyn-ABI$_{cs}$*/PYL$_{cs}$-CLyn pair was activated with added abscisic acid, and the relative activity was 58% when compared to equivalent amounts of protein isolated from cells transfected with Lyn-WT (Figure 6E). These experiments further demonstrate the feasibility of small molecule dependent control of split-phosphatases and split-kinases in mammalian cells. We are currently optimizing various combinations of the designed ligand gated split-PTPs and split-PTKs and their full-length counterparts for expression in mammalian cells (Figure 1), to answer questions in biology that are not accessible with current approaches.

■ CONCLUSIONS

Herein, we establish the design and experimental validation of the first generation of small molecule gated split-protein tyrosine phosphatases (split-PTPs) using a rapamycin dependent CID. We further demonstrate that previously designed split-protein tyrosine kinases (split-PTKs) can be rendered orthogonal and respond to a variety of CIDs including abscisic acid and gibberellic acid, which are potentially biologically silent

dx.doi.org/10.1021/ja5080745 | J. Am. Chem. Soc. 2014, 136, 17078−17086

in mammalian cells. Our convergent approach toward the design of split-PTPs and PTKs provides the necessary architectures to reversibly tune phosphorylation dependent signaling pathways using orthogonal sets of small molecule CIDs that can exert temporal control over user defined split-PTKs and split-PTPs (Figure 1). We anticipate that these split-enzymes may also be amenable to gating with light using phototriggered small molecules[28,29,66,67] as well as light dependent protein complexes.[68−72] In this vein, Hahn and co-workers have recently reported a LOV2 domain based design of light activated kinase inhibitors.[72]

The small molecule activated orthogonal control over specific enzyme catalyzed phosphorylation and dephosphorylation events described herein complements ongoing efforts toward understanding and controlling the phosphoproteome using a variety of traditional and nontraditional approaches.[18,19,73−82] More generally, the sequence dissimilarity based approach for identification of fragmentation sites to control split-protein function may also prove to be applicable to a variety of enzyme families involved in post-translational modifications such as acetylases and deacetylases;[83] methyltransferases and demethylases;[84,85] and ubiquitin ligases and deubiquitinases.[86−88] The ability to orthogonally tune subsets of proteins involved in post-translational events has the potential to allow new ways to study the details of signal transduction pathways while designing new synthetic circuits.[89−93]

MATERIALS AND METHODS

Restriction enzymes and T4 DNA Ligase were obtained from NEB. KAPA Hi-Fi was from KAPA Biosystems. RiboMAX Large Scale RNA production system was from Promega. Primers were from IDT. pDNR-PTPN1, pDNR-PTPN3, and pDNR-PTPN6 vectors were from DNAsu plasmid repository. pSV40p-AD-VP16-PYLcs-HA-IRES-GalDBD-ABIcs*-Flag, pEYFP-GID1, and pLyn-CFP-GAI vectors were from Addgene. Rabbit Reticulocyte Lysate (RRL) and reagents for cell free expression were from Luceome Biotechnologies. Ni-NTA agarose was from Macherey-Nagel. 6,8-Difluoro-4-Methylumbelliferyl Phosphate (DiFMUP) phosphatase substrate was from Life Technologies. Budget Solve Complete count was from RPI. Lyn substrate (EDPIYEFLPAKKK) was synthesized through solid phase peptide synthesis. ^{32}P-ATP was from PerkinElmer. Rapamycin, abscisic acid (ABA), and gibberellic acid (GA_3) were obtained from LC Laboratories, AG Scientific, and Acros Organics, respectively. DME/F12, FBS, and PBS were from Hyclone. PolyJet was obtained from Signagen. Protease inhibitors were from Sigma-Aldrich. Phosphatase inhibitors cocktail A and B were from Santa Cruz Biotechnologies. BCA quantification reagent and mPER were obtained from Thermo Scientific.

Cloning and mRNA Generation. N-terminal phosphatase PCR products were cloned with appropriate forward and reverse primers using EcoRI/NotI sites into a pRSFDuet-1 vector containing a 25 residue loop flanked multiple cloning sites in both sides. After identification of positive N-terminal-loop clones, C-terminal phosphatases PCR products were cloned using MfeI/Kpn1 for PTP1B, MfeI/XhoI for SHP-1 and EcoRV/Kpn1 for PTPH1. For the construction of split phosphatases, N-terminal and C-terminal PCR products were cloned into the pRSFD-FKBP-linker-MCS and pRSFD-MCS-linker-FRB plasmids generated previously.[30] PYL_{cs}/ABI_{cs}* and GID1/GAI(92) split kinases, pRSFD-PYL_{cs}-linker-MCS, ABI_{cs}*-linker-MCS, GID1-linker-MCS, and GAI(92)-linker-MCS plasmids were generated by cloning the respective PCR products with appropriate forward and reverse primers using BamHI/NotI sites into a pRSFDuet vector containing a 25 residue loop flanked by NotI and MfeI restriction sites. In a similar fashion, pRSFD-MCS-linker-PYL_{cs}, pRSFD-MCS-linker-ABI_{cs}*, pRSFD-MCS-linker-GID1, and pRSFD-MCS-linker-GAI plasmids were also generated by cloning the respective PCR products with appropriate forward and reverse primers using EcoRV/KpnI sites into a pRSFDuet vector containing a 27 residue loop flanked by NotI and MfeI restriction sites. With these plasmids in hand, respective N-terminal and C-terminal kinase PCR products were cloned using BamHI/NotI and MfeI/XhoI sites, respectively. For mammalian cell expression the relevant pRSFD constructs were PCR amplified using appropriate forward primers containing a Kozak sequence and subsequently cloned into pcDNA 3.1(+). Please see Supporting Information, Table S2, for a complete list of constructs used in this study. All positive clones were verified by dideoxyoligonucleotide sequencing. In vitro translation PCR products were generated with appropriate primers to contain a T7 RNA polymerase promoter, a mammalian Kozak sequence, and the fragment of interest. mRNA was produced according to manufacturer's protocol. Briefly, 3 μg of IVT-PCR products was transcribed using RiboMax Large Scale RNA production system (T7) for 4 h at 30 °C in a final volume of 25 μL. mRNA was further purified using G50-microcolumns (GE Healthcare).

Cell Free Protein Expression for WT and Loop Mutants. Proteins were expressed using RRL according to protocols. Briefly, 1 pmol of mRNA was translated in RRL for 90 min at 30 °C in a final volume of 25 μL.

Cell Free Protein Expression for Split Proteins. One pmol of each split fragment was coexpressed in RRL in the presence of 250 nM of rapamycin (FKBP/FRB systems), 1.2 mM ABA (PYL_{cs}/ABI_{cs}* systems), 1.2 mM GA_3 (GID1/GAI(92) systems), or 0.1% DMSO for controls.

Protein Purification. Proteins were purified as described previously. Briefly, 5 μL of Ni-NTA resin was equilibrated in Buffer A (20 mM Tris, 250 mM NaCl, 20 mM imidazole, pH 8) for 30 min at 4 °C. Protein translation products were diluted to a final volume of 100 μL in Buffer A and bound to equilibrated Ni-NTA resin for 1 h at 4 °C; PYL_{cs}/ABI_{cs}* and GID1/GAI(92) proteins were incubated at RT for 1 h after *in vitro* translation and before Ni-NTA resin binding. For phosphatase experiments, the resin was washed for 4 min with the following buffers: 1x Buffer A, 4x Wash Buffer B (20 mM Tris, 250 mM NaCl, 20 mM imidazole, pH = 7), 1x Wash Buffer C (60 mM HEPES pH 7.2, 150 mM NaCl, 0.83% glycerol, 0.002% Brij-35 for PTP1B and PTH1 and 32 mM HEPES pH 7.2, 50 mM NaCl, 0.83% glycerol, 0.002% Brij-35 for SHP-1). For kinase experiments, the resin was washed for 4 min with the following buffers: 1x Wash Buffer A, 2x Wash Buffer B, 1x Kinase Reaction Buffer (20 mM MOPS pH 7, 1 mM EDTA, 10 mM $MgCl_2$). All buffers were supplemented with 250 nM rapamycin (FKBP/FRB systems), 1.2 mM ABA (PYL_{cs}/ABI_{cs}* systems), 1.2 mM GA_3 (GID1/GAI(92) systems), or 1% DMSO (controls) for purification of split proteins.

Phosphatase Assay. All measurements were carried out in duplicates and repeated on different days. Proteins on resin were incubated with Reaction Buffer (60 mM HEPES pH 7.2, 150 mM NaCl, 0.83% glycerol, 0.002% Brij-35, 1 mM EDTA, 0.17 mM DTT, 0.017% BSA for PTP1B and PTPH1 and 32 mM HEPES pH 7.2, 50 mM NaCl, 0.83% glycerol, 0.002% Brij-35, 2.5 mM EDTA, 0.17 mM DTT, 0.017% BSA for SHP-1) and 200 μM DiFMUP for 4 h at RT in a final volume of 30 μL supplemented with 250 nM rapamycin or 0.1% DMSO. Reactions were further diluted to 60 μL in Reaction Buffer and supernatant transferred to a new microcentrifuge tube. Fluorescence readings were acquired using a Photon Technology International fluorimeter. All samples were excited at 340 nm, and emission scans were taken from 420 to 470 nm with maximum emission at 444 nm.

Kinase Assays. All radioactive ^{32}P incorporation assays were carried out in duplicate and repeated on different days. Proteins were expressed in RRL and purified on resin and subsequently incubated with a peptide substrate (Lyntide: EDPIYEFLPAKKK at 24 μM) and Kinase Reaction Buffer (supplemented with 0.1 % DMSO for negative controls, 250 nM rapamycin for FKBP/FRB splits, 1.2 mM ABA for PYL_{cs}/ABI_{cs}* splits and 1.2 mM GA_3 for GID1/GAI(92) splits) for 30 min at RT. 10 μL of radioactive ATP mix (100 μM, $^1/_{24}$ ^{32}P-ATP, $^{23}/_{24}$ ATP) was added to the protein substrate mix and incubated for 4 h at

dx.doi.org/10.1021/ja5080745 | J. Am. Chem. Soc. 2014, 136, 17078−17086

RT. The reaction was spotted on P81 paper, which was washed for 3 min 3× with 500 mL of 0.85% phosphoric acid and 1× with 500 mL of acetone. The P81 paper was immersed in 10 mL of scintillation cocktail, and the radioactive counts were measured. All data are presented without normalization as total radioactive counts subtracted from reactions containing only lysate.

Experiments in Mammalian Cells. HEK293T cells were maintained in DME/F12 (1:1) media supplemented with 10% FBS, 100 U/mL Penicillin, 100 μg Streptomycin, and 2.5 μg/mL Amphotericin B in a 5% CO_2 incubator. HEK293T cells were plated at a density of 1×10^6 cells in 6 well plates for 18−24 h prior to transfection. In general, cells were cotransfected with 1 μg of total plasmid DNA using a PolyJet transfection reagent according to manufacturer protocol. For split-constructs, 0.5 μg of each plasmid was contransfected and compared to transfections with 0.5 μg of wild-type and 0.5 μg of empty vector. Ten h post-transfection, transfection complexes were removed, and wells were replenished with media containing 80 nM rapamycin in DMSO or equivalent DMSO for negative controls. Cells were incubated with rapamycin for 14 h at 37 °C. Twenty-four h post-transfection each well was washed with 1 mL of PBS, and cells were lysed in 100 μL of Lysis Buffer (mPER supplemented with protease and phosphatase inhibitors for kinases or protease inhibitors only for phosphatases and 250 nM rapamycin for FKBP-FRB CID, 250 μM abscisic acid for ABI-PYL CID or DMSO for negative controls) for 20 min at 4 °C. Cell lysate was cleared at 14,000 rcf for 10 min at 4 °C. Total protein concentration was quantified using BCA reagent. 150 μg of total protein for each experiment was loaded on 5 μL of Ni-NTA resin and bound for 1 h at 4 °C. The resin was washed for 4 min with each of the following buffers: 2x 100 μL Lysis Buffer, 3x 100 μL Wash Buffer B, and 1x with 100 μL Reaction Buffer (buffers were supplemented with 250 nM rapamycin, 1 mM abscisic acid, or DMSO for controls). All kinase and phosphatase assays were carried out as previously described.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Complete alignments, list of constructs, controls, and experimental procedure. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

ghosh@email.arizona.edu

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank members of the Ghosh lab, Dr. Reena Zutshi, and Dr. John Jewett for helpful comments and reagents.

■ REFERENCES

(1) Hunter, T. *Cell* **1995**, *80*, 225.
(2) Manning, G.; Whyte, D. B.; Martinez, R.; Hunter, T.; Sudarsanam, S. *Science* **2002**, *298*, 1912.
(3) Alonso, A.; Sasin, J.; Bottini, N.; Friedberg, I.; Osterman, A.; Godzik, A.; Hunter, T.; Dixon, J.; Mustelin, T. *Cell* **2004**, *117*, 699.
(4) Eckhart, W.; Hutchinson, M. A.; Hunter, T. *Cell* **1979**, *18*, 925.
(5) Cohen, P. *Nat. Rev. Drug Discovery* **2002**, *1*, 309.
(6) Hotamisligil, G. S. *Nature* **2006**, *444*, 860.
(7) Kyriakis, J. M.; Avruch, J. *Physiol. Rev.* **2012**, *92*, 689.
(8) Knight, Z. A.; Lin, H.; Shokat, K. M. *Nat. Rev. Cancer* **2010**, *10*, 130.
(9) Zhang, J.; Yang, P. L.; Gray, N. S. *Nat. Rev. Cancer* **2009**, *9*, 28.
(10) Tonks, N. K.; Diltz, C. D.; Fischer, E. H. *J. Biol. Chem.* **1988**, *263*, 6731.
(11) Tonks, N. K.; Diltz, C. D.; Fischer, E. H. *J. Biol. Chem.* **1988**, *263*, 6722.
(12) Tonks, N. K. *Nat. Rev. Mol. Cell Biol.* **2006**, *7*, 833.
(13) Julien, S. G.; Dube, N.; Hardy, S.; Tremblay, M. L. *Nat. Rev. Cancer* **2011**, *11*, 35.
(14) Fabian, M. A.; Biggs, W. H.; Treiber, D. K.; Atteridge, C. E.; Azimioara, M. D.; Benedetti, M. G.; Carter, T. A.; Ciceri, P.; Edeen, P. T.; Floyd, M.; Ford, J. M.; Galvin, M.; Gerlach, J. L.; Grotzfeld, R. M.; Herrgard, S.; Insko, D. E.; Insko, M. A.; Lai, A. G.; Lelias, J. M.; Mehta, S. A.; Milanov, Z. V.; Velasco, A. M.; Wodicka, L. M.; Patel, H. K.; Zarrinkar, P. P.; Lockhart, D. J. *Nat. Biotechnol.* **2005**, *23*, 329.
(15) Lamba, V.; Ghosh, I. *Curr. Pharm. Des.* **2012**, *18*, 2936.
(16) Zhao, Z.; Wu, H.; Wang, L.; Liu, Y.; Knapp, S.; Liu, Q.; Gray, N. S. *ACS Chem. Biol.* **2014**, *9*, 1230.
(17) Jester, B. W.; Gaj, A.; Shomin, C. D.; Cox, K. J.; Ghosh, I. *J. Med. Chem.* **2012**, *55*, 1526.
(18) Ranjitkar, P.; Perera, B. G. K.; Swaney, D. L.; Hari, S. B.; Larson, E. T.; Krishnamurty, R.; Merritt, E. A.; Villen, J.; Maly, D. J. *J. Am. Chem. Soc.* **2012**, *134*, 19017.
(19) Shi, H.; Zhang, C.-J.; Chen, G. Y. J.; Yao, S. Q. *J. Am. Chem. Soc.* **2012**, *134*, 3001.
(20) He, R.; Zeng, L.-F.; He, Y.; Zhang, S.; Zhang, Z.-Y. *FEBS J.* **2013**, *280*, 731.
(21) Combs, A. P. *J. Med. Chem.* **2010**, *53*, 2333.
(22) Hannon, G. J. *Nature* **2002**, *418*, 244.
(23) Jackson, A. L.; Bartz, S. R.; Schelter, J.; Kobayashi, S. V.; Burchard, J.; Mao, M.; Li, B.; Cavet, G.; Linsley, P. S. *Nat. Biotechnol.* **2003**, *21*, 635.
(24) Bishop, A. C.; Kung, C. Y.; Shah, K.; Witucki, L.; Shokat, K. M.; Liu, Y. *J. Am. Chem. Soc.* **1999**, *121*, 627.
(25) Liu, Y.; Shah, K.; Yang, F.; Witucki, L.; Shokat, K. M. *Chem. Biol.* **1998**, *5*, 91.
(26) Bishop, A. C.; Ubersax, J. A.; Petsch, D. T.; Matheos, D. P.; Gray, N. S.; Blethrow, J.; Shimizu, E.; Tsien, J. Z.; Schultz, P. G.; Rose, M. D.; Wood, J. L.; Morgan, D. O.; Shokat, K. M. *Nature* **2000**, *407*, 395.
(27) Karginov, A. V.; Ding, F.; Kota, P.; Dokholyan, N. V.; Hahn, K. M. *Nat. Biotechnol.* **2010**, *28*, 743.
(28) Karginov, A. V.; Zou, Y.; Shirvanyants, D.; Kota, P.; Dokholyan, N. V.; Young, D. D.; Hahn, K. M.; Deiters, A. *J. Am. Chem. Soc.* **2011**, *133*, 420.
(29) Gautier, A.; Deiters, A.; Chin, J. W. *J. Am. Chem. Soc.* **2011**, *133*, 2124.
(30) Camacho-Soto, K.; Castillo-Montoya, J.; Tye, B.; Ghosh, I. *J. Am. Chem. Soc.* **2014**, *136*, 3995.
(31) Hoffman, H. E.; Blair, E. R.; Johndrow, J. E.; Bishop, A. C. *J. Am. Chem. Soc.* **2005**, *127*, 2824.
(32) Davis, O. B.; Bishop, A. C. *Bioconjugate Chem.* **2012**, *23*, 272.
(33) Zhang, X.-Y.; Bishop, A. C. *J. Am. Chem. Soc.* **2007**, *129*, 3812.
(34) Johnsson, N.; Varshavsky, A. *Proc. Natl. Acad. Sci. U. S. A.* **1994**, *91*, 10340.
(35) Ghosh, I.; Hamilton, A. D.; Regan, L. *J. Am. Chem. Soc.* **2000**, *122*, 5658.
(36) Galarneau, A.; Primeau, M.; Trudeau, L. E.; Michnick, S. W. *Nat. Biotechnol.* **2002**, *20*, 619.
(37) Luker, K. E.; Smith, M. C. P.; Luker, G. D.; Gammon, S. T.; Piwnica-Worms, H.; Piwnica-Worms, D. P. *Proc. Natl. Acad. Sci. U. S. A.* **2004**, *101*, 12288.
(38) Paulmurugan, R.; Gambhir, S. S. *Anal. Chem.* **2003**, *75*, 1584.
(39) Michnick, S. W.; Ear, P. H.; Manderson, E. N.; Remy, I.; Stefan, E. *Nat. Rev. Drug Discovery* **2007**, *6*, 569.
(40) Stains, C. I.; Furman, J. L.; Porter, J. R.; Rajagopal, S.; Li, Y.; Wyatt, R. T.; Ghosh, I. *ACS Chem. Biol.* **2010**, *5*, 943.
(41) Furman, J. L.; Mok, P.-W.; Badran, A. H.; Ghosh, I. *J. Am. Chem. Soc.* **2011**, *133*, 12518.
(42) Herbst, K. J.; Allen, M. D.; Zhang, J. *J. Am. Chem. Soc.* **2011**, *133*, 5676.
(43) Shekhawat, S. S.; Ghosh, I. *Curr. Opin. Chem. Biol.* **2011**, *15*, 789.
(44) Gray, D. C.; Mahrus, S.; Wells, J. A. *Cell* **2010**, *142*, 637.

(45) Moorhead, G. B. G.; Trinkle-Mulcahy, L.; Ulke-Lemee, A. *Nat. Rev. Mol. Cell Biol.* **2007**, *8*, 234.
(46) Fischer, E. H.; Charbonneau, H.; Tonks, N. K. *Science* **1991**, *253*, 401.
(47) Andersen, J. N.; Mortensen, O. H.; Peters, G. H.; Drake, P. G.; Iversen, L. F.; Olsen, O. H.; Jansen, P. G.; Andersen, H. S.; Tonks, N. K.; Møller, N. P. H. *Mol. Cell. Biol.* **2001**, *21*, 7117.
(48) Elchebly, M.; Payette, P.; Michaliszyn, E.; Cromlish, W.; Collins, S.; Loy, A. L.; Normandin, D.; Cheng, A.; Himms-Hagen, J.; Chan, C. C.; Ramachandran, C.; Gresser, M. J.; Tremblay, M. L.; Kennedy, B. P. *Science* **1999**, *283*, 1544.
(49) Zabolotny, J. M.; Bence-Hanulec, K. K.; Stricker-Krongrad, A.; Haj, F.; Wang, Y. P.; Minokoshi, Y.; Kim, Y. B.; Elmquist, J. K.; Tartaglia, L. A.; Kahn, B. B.; Neel, B. G. *Dev. Cell* **2002**, *2*, 489.
(50) Yip, S.-C.; Saha, S.; Chernoff, J. *Trends Biochem. Sci.* **2010**, *35*, 442.
(51) Porter, J. R.; Stains, C. I.; Jester, B. W.; Ghosh, I. *J. Am. Chem. Soc.* **2008**, *130*, 6488.
(52) Chen, J.; Zheng, X. F.; Brown, E. J.; Schreiber, S. L. *Proc. Natl. Acad. Sci. U. S. A.* **1995**, *92*, 4947.
(53) Choi, J. W.; Chen, J.; Schreiber, S. L.; Clardy, J. *Science* **1996**, *273*, 239.
(54) Banaszynski, L. A.; Liu, C. W.; Wandless, T. J. *Am. Chem. Soc.* **2005**, *127*, 4715.
(55) Spencer, D. M.; Wandless, T. J.; Schreiber, S. L.; Crabtree, G. R. *Science* **1993**, *262*, 1019.
(56) Doody, G. M.; Justement, L. B.; Delibrias, C. C.; Matthews, R. J.; Lin, J. J.; Thomas, M. L.; Fearon, D. T. *Science* **1995**, *269*, 242.
(57) Cornall, R. J.; Cyster, J. G.; Hibbs, M. L.; Dunn, A. R.; Otipoby, K. L.; Clark, E. A.; Goodnow, C. C. *Immunity* **1998**, *8*, 497.
(58) Fegan, A.; White, B.; Carlson, J. C. T.; Wagner, C. R. *Chem. Rev.* **2010**, *110*, 3315.
(59) Corson, T. W.; Aberle, N.; Crews, C. M. *ACS Chem. Biol.* **2008**, *3*, 677.
(60) Bayle, J. H.; Grimley, J. S.; Stankunas, K.; Gestwicki, J. E.; Wandless, T. J.; Crabtree, G. R. *Chem. Biol.* **2006**, *13*, 99.
(61) Czlapinski, J. L.; Schelle, M. W.; Miller, L. W.; Laughlin, S. T.; Kohler, J. J.; Cornish, V. W.; Bertozzi, C. R. *J. Am. Chem. Soc.* **2008**, *130*, 13186.
(62) Liang, F.-S.; Ho, W. Q.; Crabtree, G. R. *Sci. Signaling* **2011**, *4*, rs2.
(63) Miyazono, K.-i.; Miyakawa, T.; Sawano, Y.; Kubota, K.; Kang, H.-J.; Asano, A.; Miyauchi, Y.; Takahashi, M.; Zhi, Y.; Fujita, Y.; Yoshida, T.; Kodaira, K.-S.; Yamaguchi-Shinozaki, K.; Tanokura, M. *Nature* **2009**, *462*, 609.
(64) Miyamoto, T.; DeRose, R.; Suarez, A.; Ueno, T.; Chen, M.; Sun, T.-p.; Wolfgang, M. J.; Mukherjee, C.; Meyers, D. J.; Inoue, T. *Nat. Chem. Biol.* **2012**, *8*, 465.
(65) Murase, K.; Hirano, Y.; Sun, T.-p.; Hakoshima, T. *Nature* **2008**, *456*, 459.
(66) Hahn, M. E.; Muir, T. W. *Angew. Chem., Int. Ed.* **2004**, *43*, 5800.
(67) Klan, P.; Solomek, T.; Bochet, C. G.; Blanc, A.; Givens, R.; Rubina, M.; Popik, V.; Kostikov, A.; Wirz, J. *Chem. Rev.* **2013**, *113*, 119.
(68) Strickland, D.; Lin, Y.; Wagner, E.; Hope, C. M.; Zayner, J.; Antoniou, C.; Sosnick, T. R.; Weiss, E. L.; Glotzer, M. *Nat. Methods* **2012**, *9*, 379.
(69) Kennedy, M. J.; Hughes, R. M.; Peteya, L. A.; Schwartz, J. W.; Ehlers, M. D.; Tucker, C. L. *Nat. Methods* **2010**, *7*, 973.
(70) Wu, Y. I.; Frey, D.; Lungu, O. I.; Jaehrig, A.; Schlichting, I.; Kuhlman, B.; Hahn, K. M. *Nature* **2009**, *461*, 104.
(71) Levskaya, A.; Weiner, O. D.; Lim, W. A.; Voigt, C. A. *Nature* **2009**, *461*, 997.
(72) Yi, J. J.; Wang, H.; Vilela, M.; Danuser, G.; Hahn, K. M. *ACS Synth. Biol.* **2014**, *3*, 788−795 DOI: 10.1021/sb5001356.
(73) Lu, C. H. S.; Liu, K.; Tan, L. P.; Yao, S. Q. *Chem.—Eur. J.* **2012**, *18*, 28.
(74) Maly, D. J.; Allen, J. A.; Shokat, K. M. *J. Am. Chem. Soc.* **2004**, *126*, 9160.
(75) Statsuk, A. V.; Maly, D. J.; Seeliger, M. A.; Fabian, M. A.; Biggs, W. H., III; Lockhart, D. J.; Zarrinkar, P. P.; Kuriyan, J.; Shokat, K. M. *J. Am. Chem. Soc.* **2008**, *130*, 17568.
(76) Green, K. D.; Pflum, M. K. H. *J. Am. Chem. Soc.* **2007**, *129*, 10.
(77) Flint, A. J.; Tiganis, T.; Barford, D.; Tonks, N. K. *Proc. Natl. Acad. Sci. U. S. A.* **1997**, *94*, 1680.
(78) Parang, K.; Till, J. H.; Ablooglu, A. J.; Kohanski, R. A.; Hubbard, S. R.; Cole, P. A. *Nat. Struct. Biol.* **2001**, *8*, 37.
(79) Meyer, S. C.; Shomin, C. D.; Gaj, T.; Ghosh, I. *J. Am. Chem. Soc.* **2007**, *129*, 13812.
(80) Hill, Z. B.; Perera, B. G. K.; Maly, D. J. *J. Am. Chem. Soc.* **2009**, *131*, 6686.
(81) Garaud, M.; Pei, D. *J. Am. Chem. Soc.* **2007**, *129*, 5366.
(82) Xiao, Q.; Luechapanichkul, R.; Zhai, Y.; Pei, D. *J. Am. Chem. Soc.* **2013**, *135*, 9760.
(83) Choudhary, C.; Kumar, C.; Gnad, F.; Nielsen, M. L.; Rehman, M.; Walther, T. C.; Olsen, J. V.; Mann, M. *Science* **2009**, *325*, 834.
(84) Arrowsmith, C. H.; Bountra, C.; Fish, P. V.; Lee, K.; Schapira, M. *Nat. Rev. Drug Discovery* **2012**, *11*, 384.
(85) Kooistra, S. M.; Helin, K. *Nat. Rev. Mol. Cell Biol.* **2012**, *13*, 297.
(86) Varshavsky, A. *Trends Biochem. Sci.* **2005**, *30*, 283.
(87) Deshaies, R. J.; Joazeiro, C. A. P. *Annu. Rev. Biochem.* **2009**, *78*, 399.
(88) Komander, D.; Clague, M. J.; Urbe, S. *Nat. Rev. Mol. Cell Biol.* **2009**, *10*, 550.
(89) Howard, P. L.; Chia, M. C.; Del Rizzo, S.; Liu, F. F.; Pawson, T. *Proc. Natl. Acad. Sci. U. S. A.* **2003**, *100*, 11267.
(90) Park, S. H.; Zarrinpar, A.; Lim, W. A. *Science* **2003**, *299*, 1061.
(91) Zellefrow, C. D.; Griffiths, J. S.; Saha, S.; Hodges, A. M.; Goodman, J. L.; Paulk, J.; Kritzer, J. A.; Schepartz, A. *J. Am. Chem. Soc.* **2006**, *128*, 16506.
(92) Hobert, E. M.; Schepartz, A. *J. Am. Chem. Soc.* **2012**, *134*, 3976.
(93) Cameron, D. E.; Bashor, C. J.; Collins, J. J. *Nat. Rev. Microbiol.* **2014**, *12*, 381.