**SUPPORTING INFORMATION**

# Rational Redesign of a Functional Protein Kinase-Substrate Interaction

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## SUPPLEMENTARY METHODS

### Site-directed mutagenesis

The following primers were used for Pim1 and BAD site-directed mutagenesis:

| Gene | Mutation | Primer pair |
|---|---|---|
| Pim1 | T134L | 5' CTCTTCGACTTCATCTTGGAAAGGGGAGCCCTGC 3'<br>5' GCAGGGCTCCCCTTTCCAAGATGAAGTCGAAGAG 3' |
| Pim1 | D170P | 5' CACCGCGACATCAAGCCCGAAAACATCCTTATCGAC 3'<br>5' GTCGATAAGGATGTTTTCGGGCTTGATGTCGCGGTG 3' |
| Pim1 | D234V,D239T | 5'GGGATCCTGCTGTATGTTATGGTGTGTGGAACTATTCCTTTCGAG 3'<br>5' CTCGAAAGGAATAGTTCCACACACCATAACATACAGCAGGATCCC 3' |
| Pim1 | D234I,D239Y | 5' GGGATCCTGCTGTATATTATGGTGTGTGGATATATTCCTTTCGAG 3'<br>5' CTCGAAAGGAATATATCCACACACCATAATATACAGCAGGATCCC 3' |
| Pim1 | T134V | 5'CTCTTCGACTTCATCGTGGAAAGGGGAGCCCTGC 3'<br>5' GCAGGGCTCCCCTTTCCACGATGAAGTCGAAGAG 3' |
| Pim1 | D234T,D239Y | 5' GGGATCCTGCTGTATACTATGGTGTGTGGATATATTCC 3'<br>5' GGAATATATCCACACACCATAGTATACAGCAGGATCCC 3' |
| BAD | S112A | 5' CGGAGTCGCCACAGTGCGTACCCAGCCGGGACC 3'<br>5' GGTCCCGGCTGGGTACGCACTGTGGCGACTCCG 3' |
| BAD | R107L | 5' GCTATGGAGACTCTGAGTCGCCACAGTTCG 3'<br>5' CGAACTGTGGCGACTCAGAGTCTCCATAGC 3' |
| BAD | Y113G | 5' GAGTCGCCACAGTTCGGGACCAGCGGGGACCG 3'<br>5' CGGTCCCCGCTGGTCCCGAACTGTGGCGACTC 3' |
| BAD | R107L,S112A, Y113G | 5' CTGAGTCGCCACAGTGCGGGACCAGCGG 3'<br>5' CCGCTGGTCCCGCACTGTGGCGACTCAG 3' |

### Protein expression and purification

Wild-type (WT) Pim1 and its mutants were expressed and purified from BL21 *E. coli* (Stratagene) by immobilized metal affinity chromatography as follows. An overnight starter culture of cells transformed with the Pim1 expression plasmid was expanded into 200 ml terrific broth [TB, 1.2% Bacto tryptone (BD Biosciences), 2.4% Bacto yeast extract (BD Biosciences), 0.4% glycerol, 17 mM $KH_2PO_4$, 72 mM $K_2HPO_4$] containing 33 µg/ml kanamycin. Protein expression was induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG) to 0.4 mM when cultures reached an OD value of 0.8–1.0 and incubated with shaking for an additional 2–3 hours at 37°C. Cells were collected by centrifugation, and pellets were resuspended in buffer containing 20 mM Tris, pH 7.5, 140 mM NaCl, 1 mM EDTA, 1 mM DTT, 10 µl/ml leupeptin. Lysozyme was added to 0.2 mg/ml, and cells were incubated on ice for 15 min before adding sodium deoxycholate (0.14%), PMSF (1 mM), DNAse I (0.03 U/ml) and $MgCl_2$ (13 mM) followed by rotation at room temperature for 15 minutes. Lysate was centrifuged at 20,000 X *g* at 4°C and the supernatant was rotated with 0.5 ml Talon immobilized metal affinity resin (Clontech) for 1 h at 4°C. Resin was then collected into a column equilibrated with phosphate buffered saline (PBS) containing 0.5% Igepal CA-630. The column was washed with PBS/0.5% Igepal CA-630 (3 X 0.5 ml followed by 1 X 2.5 ml) and then in the same way with buffer

containing 20 mM Tris, pH 7.5, 500 mM NaCl, and 10 mM imidazole. Protein was then eluted in 0.5 ml fractions with buffer containing 20 mM Tris, pH 7.5, 100 mM NaCl, 250 mM imidazole, 1 mM DTT and 10 μg/ml leupeptin. Protein-containing fractions were combined and dialyzed overnight at 4°C into buffer containing 20mM Tris, pH 8.0, 50 mM NaCl, 1 mM DTT, and 10% glycerol. Following dialysis, protein was recovered and quantified by Bradford assay (Bio-Rad). Protein purity was confirmed to be >90% by SDS-PAGE and Coomassie blue staining. Purified proteins were then snap frozen and stored at -80°C.

WT and mutant BAD was expressed and lysates were prepared in a similar way except that media contained 0.1 mg/ml ampicillin instead of kanamycin. BAD was then purified by sequential affinity purification using the $His_6$ and GST tags. Immobilized metal affinity chromatography using Talon resin was carried out as described above for kinase purification, and eluted protein was applied directly to Glutathione Sepharose 4B beads (125 ml, GE Healthcare) and incubated for 1 h at 4°C with rotation. Beads were washed twice with 5 ml cold PBS/0.5% Igepal CA-630, and once with 5 ml wash buffer (50 mM Tris, pH 8.0, 50 mM NaCl, 1 mM DTT, 0.01% Igepal CA-630, and 10% glycerol). Beads were then suspended in 1 ml wash buffer, transferred to a microcentrifuge tube, and pelleted. Purified protein was eluted from the resin by two rounds of incubation with 0.5 ml elution buffer (wash buffer containing 20 mM glutathione) by rotating incubation for 10 min at 4°C. Purified protein was frozen and stored as described above for kinase purification.

**Peptide synthesis and purification**

Synthetic peptides used in this study (ARARAASAALAKKK and ALARAASAALAKKK) were prepared using standard Fmoc solid phase peptide synthesis procedures[1] on a Tetras peptide synthesizer (Creosalus). Peptides were synthesized on 150 mg Wang resin preloaded with Fmoc-Lys (Advanced ChemTech) using DMF (American Bioanalytical) as the solvent and HBTU/HOBt (Anaspec) and DIPEA (Sigma-Aldrich) as coupling reagents. Fmoc-protected amino acids were purchased from Advanced ChemTech or Anaspec. Peptides were cleaved from the resin in 3 ml reagent K (83% trifluoroacetic acid, 2.5% tri-isopropylsilane, 2.5% 1,2-ethanedithiol, 4% thioanisole, 4% phenol and 4% water). Peptides were precipitated in diethyl ether, recovered by centrifugation and dried under a stream of argon. Peptides were purified by preparative reversed phase HPLC on a C18 column using a gradient of acetonitrile in water with 0.1% TFA as the solvent, and lyophilized to dryness. Peptide identity was verified by MALDI-TOF mass spectrometry.

**Peptide library analysis**

WT Pim1 and all Pim1 mutants were subjected to arrayed positional scanning peptide library screening as previously described[2, 3]. The library consisted of 198 peptide

mixtures having the general sequence Y-A-X-X-X-X-X-S/T-X-X-X-X-A-G-K-K(biotin), where X represents an equimolar mixture of the 17 amino acids (excluding Ser, Thr and Cys) and S/T indicates an equimolar mixture of Ser and Thr. In each component of the library, one of the X residues was replaced with one of the 20 unmodified amino acids, phosphothreonine, or phosphotyrosine. Peptides were distributed to assay plates at a final concentration of 50 mM in 50 mM HEPES, pH 7.4, 10 mM $MgCl_2$, 1 mM DTT and 0.1% v/v Tween-20. After addition of kinase (final concentration ranged from 35 - 380 nM) and ATP (to 50 mM with 0.03 mCi/ml [g-$^{33}$P] ATP), plates were sealed and incubated 2 h at 30 °C. Following incubation, aliquots (200 nl) of each reaction were spotted onto streptavidin membrane (SAM2 biotin capture membrane, Promega), which was immersed in quenching buffer (10 mM Tris, pH 7.5, 140 mM NaCl, 0.1% SDS). The membrane was then washed successively in quenching buffer, 2 M NaCl, and 2 M NaCl with 1% $H_3PO_4$, rinsed with $H_2O$, dried, and exposed to a phosphor screen as described. Screens were scanned on a phosphor imager (BioRad) using QuantityOne software.

**Peptide kinase assays**

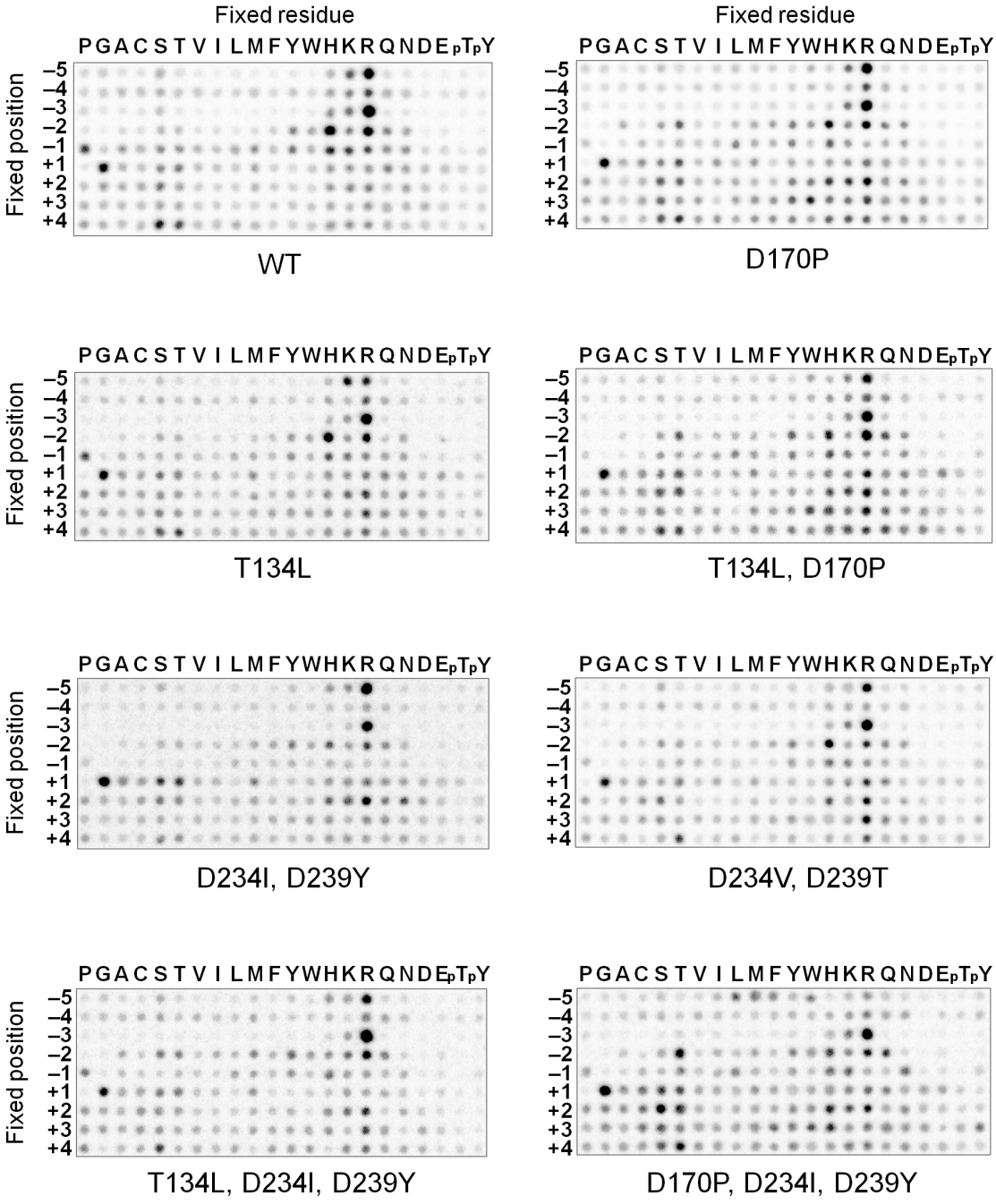
Assays were performed in duplicate at 500 nM WT or mutant Pim1 and 10 μM or 20 μM peptide in kinase assay buffer (20 mM HEPES, pH 7.5, 10 mM $MgCl_2$, 1 mM DTT). All reactions were started by the addition of ATP (final concentration 100 μM cold ATP and 0.25 μCi/μl [γ-$^{33}$P] ATP) and then incubated at 30 °C. At various times aliquots were spotted onto P81 phosphocellulose filters, which were quenched in 75 mM phosphoric acid. Filters were then washed for 3 x 4 min in 75 mM phosphoric acid, once briefly in acetone, and then air-dried, and counted by liquid scintillation. The amount of product formed was calculated from a standard curve made using [γ-$^{33}$P]ATP at the same specific activity. Specificity constants for peptide phosphorylation ($k_{cat}/K_M$) were calculated from initial rates of reaction using the equation: $k_{cat}/K_M = V/[E][S]$, obtained from the Michaelis-Menten equation under conditions where $K_M >> [S]$. Reaction rates were confirmed to be linear over the range of substrate concentration used.

## SUPPORTING FIGURE

a

| | |
|---|---|
| Y-A-**Z**-X-X-X-X-S/T-X-X-X-X-A-G-K-K(biotin) | –5 set |
| Y-A-X-**Z**-X-X-X-S/T-X-X-X-X-A-G-K-K(biotin) | –4 set |
| Y-A-X-X-**Z**-X-X-S/T-X-X-X-X-A-G-K-K(biotin) | –3 set |
| Y-A-X-X-X-**Z**-X-S/T-X-X-X-X-A-G-K-K(biotin) | –2 set |
| Y-A-X-X-X-X-**Z**-S/T-X-X-X-X-A-G-K-K(biotin) | –1 set |
| Y-A-X-X-X-X-X-S/T-**Z**-X-X-X-A-G-K-K(biotin) | +1 set |
| Y-A-X-X-X-X-X-S/T-X-**Z**-X-X-A-G-K-K(biotin) | +2 set |
| Y-A-X-X-X-X-X-S/T-X-X-**Z**-X-A-G-K-K(biotin) | +3 set |
| Y-A-X-X-X-X-X-S/T-X-X-X-**Z**-A-G-K-K(biotin) | +4 set |

b



b (cont.)

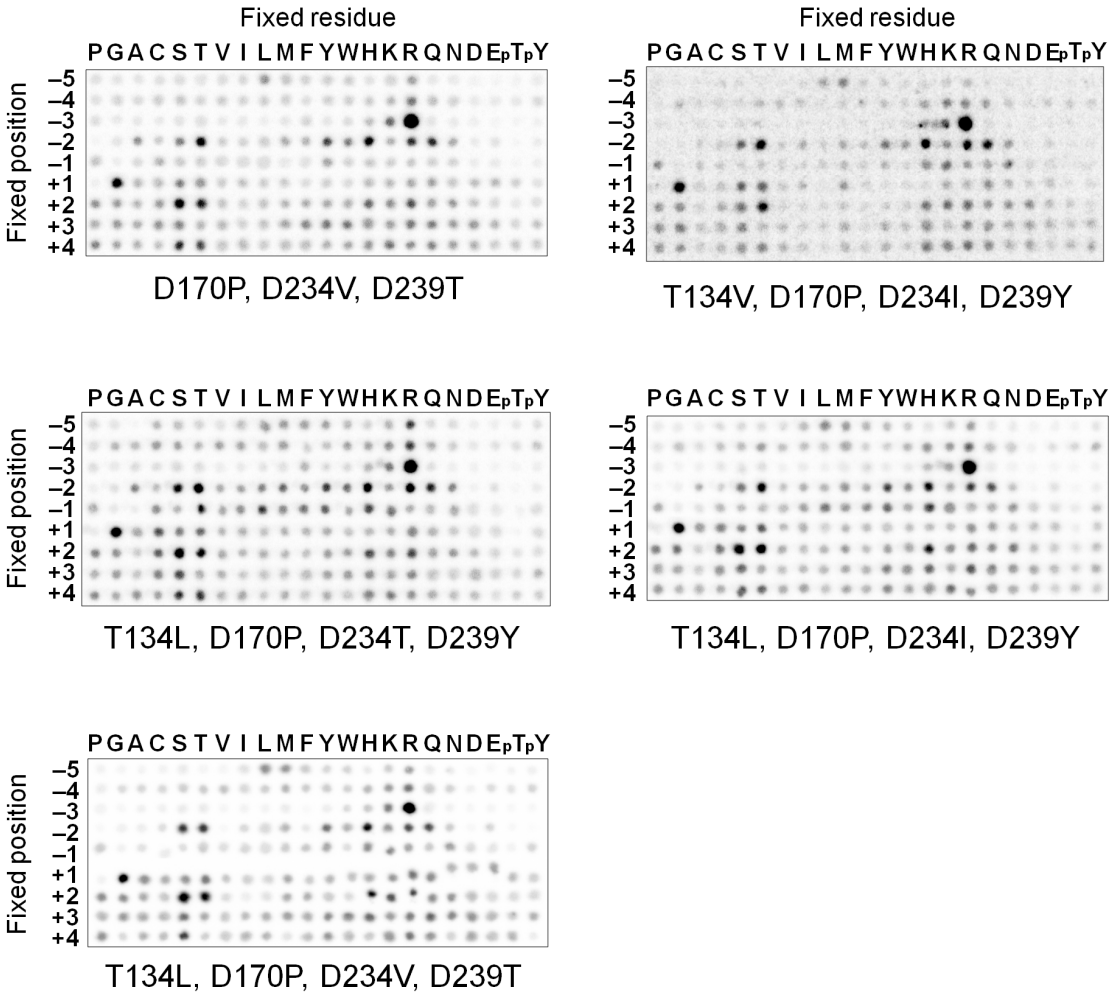



**Figure S1. Peptide library analysis of Pim1 mutants.** Purified, bacterially expressed Pim1 mutants were subjected to arrayed positional scanning peptide library screening as described[2]. (**a**) Peptide library sequences. X indicates an approximately equimolar mixture of the 17 proteogenic amino acids excluding Ser, Thr and Cys. In each well of the array, the peptide mixture included a single residue Z at one of the nine variable positions surrounding the phosphorylation site that was fixed as one of the 20 unmodified amino acids, phosphothreonine (pT) or phosphotyrosine (pY). (**b**) Peptide array results for WT Pim1 and the 12 Pim1 mutants analyzed in this study. Peptide library analysis was done by radiolabel assay of the peptides shown in (**a**) as described in the main text.

## SUPPLEMENTARY REFERENCES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