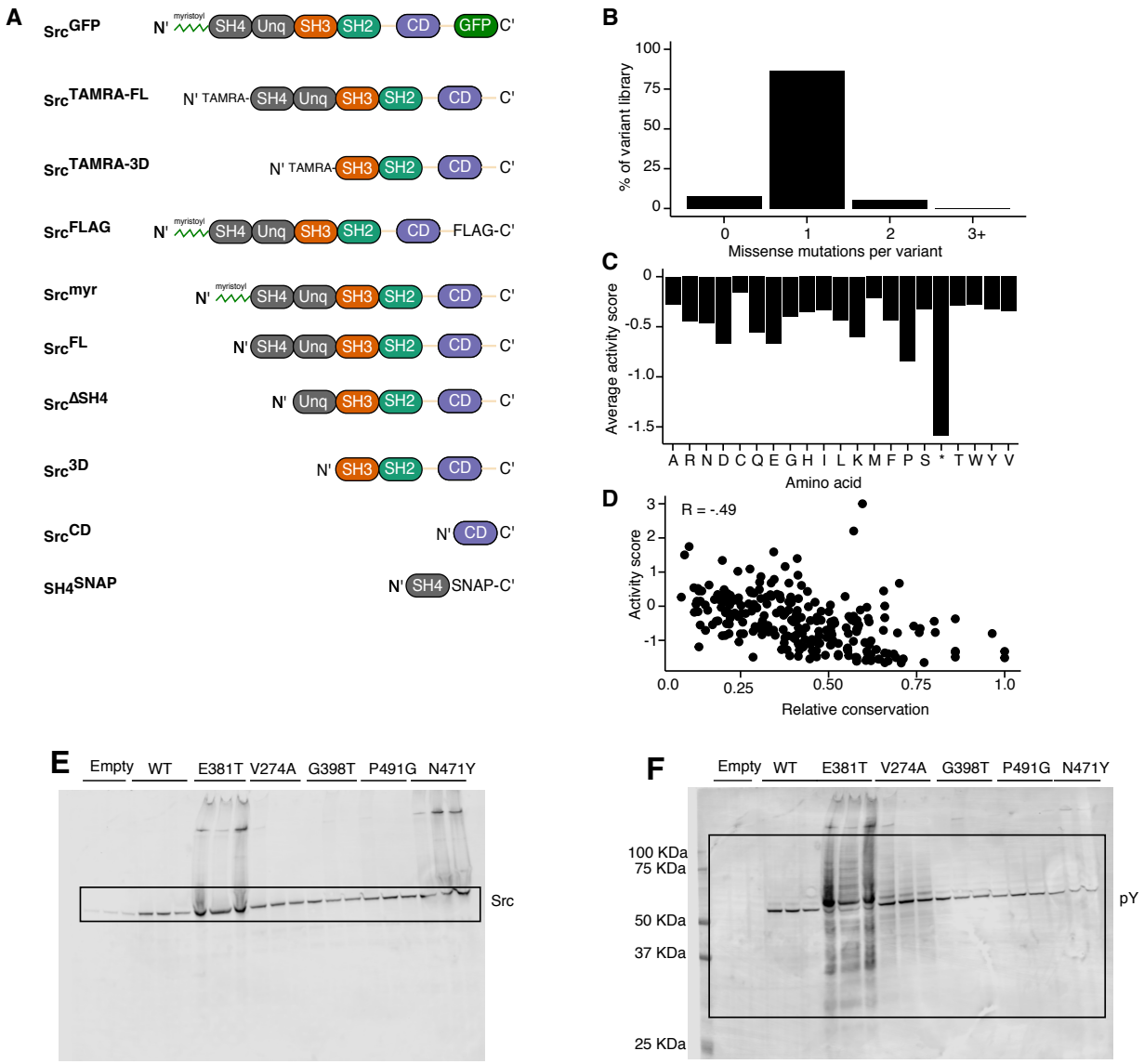


Figure S1. Deep mutational scan of Src's CD. Related to **Figure 2**.

A. Schematic of all Src constructs used in this manuscript. Src^{GFP} (Expressed in mammalian cells and used in **Figures 3G**, **S2F**, **5A-E**, and **S4A-D**): Src residues 1-536 with an 8 amino acid C-terminal linker fused to GFP. Src^{FLAG} (Expressed in mammalian cells and used in **Figures 4E**, **4H** (Hck), **S3B** (Hck), **S3G** (Hck), **5G**, **S4H-I**, **6B**, **S5S6B**, and **S5F**): Src residues 1-536 fused directly to a C-terminal FLAG tag. Src^{myr} (Expressed in mammalian cells and *S. cerevisiae*, and used in **Figures 2** (all), **S2D-E**, **S4E-G**, **7D** (Fyn), **S6C** (Fyn) and **S7D**): Src residues 1-536. Src^{FL} (Expressed in *E. coli* and used in **Figures 3C**, **3E**, **S2B-C**, **5F**, **6C-H**, **6K**, **S5C-D**, **S5G-I**, **S5N-O**,

7B (Fyn), **7C** (Fyn), and **S6B** (Fyn)): Src residues 1-536. Src^{3D} (Expressed in *E. coli* and *S. cerevisiae*, and used in **Figures 4C** (Src and Hck), **4G**, **S3A** (Src and Hck), **S3C**, **S3D** (Hck), **6C**, **6G-H**, **S5C-E**, **S5J**, **7B** (Fyn), **7C** (Fyn), **S6B** and **S6D**): Src residues 87-536. $Src^{\Delta SH4}$ (Expressed in *E. coli* and *S. cerevisiae*, and used in **Figures 6C-E**, **S5C-D**, **S5G-H**, and **S6D**): Src residues 19-536. Src^{CD} (Expressed in *E. coli* and used in **Figures 4D**, **S3E** (Hck) and **6I-K, S5E, S5K** and **S5M**): Src residues 261-536. $Src^{TAMRA-FL}$ (Expressed in *E. coli* and used in **Figure S5L**): Src residues 2-536 with an N-terminal TAMRA label. $Src^{TAMRA-3D}$ (Expressed in *E. coli* and used in **Figures S3C**, **7F** and **S6E**): Src residues 87-536 with an N-terminal TAMRA label. $SH4^{SNAP}$ (Expressed in *E. coli* and used in **Figures 6I-K**, **S5K-O**, **7F** and **S6E**): Src residues 1-18 fused to the N-terminus of SNAP-tag with a short LPETGG linker in between SH4 and SNAP. Relates to **Figures 2-7**. More details for each construct are provided in **Table S1**. **B.** Bar graph of percentage of library members that are WT or have one, two, or three or more amino acid mutations. **C.** Bar graph of average activity score of Src library grouped by mutant amino acid identity. **D.** Scatter plot comparing evolutionary conservation with average activity score at each residue (Pearson's R = -.49). **E.** Representative total Src immunoblot for Src^{myr} WT, Src^{myr} E381T, Src^{myr} V274A, Src^{myr} G398T, Src^{myr} P491G, and Src^{myr} N471Y transiently expressed in HEK293T cells for 24 hr. Relates to **Figure 2I**. **F.** Representative total phospho-tyrosine immunoblot for Src^{myr} WT, Src^{myr} E381T, Src^{myr} V274A, Src^{myr} G398T, Src^{myr} P491G, and Src^{myr} N471Y transiently expressed in HEK293T cells for 24 hr. Relates to **Figure 2I**.

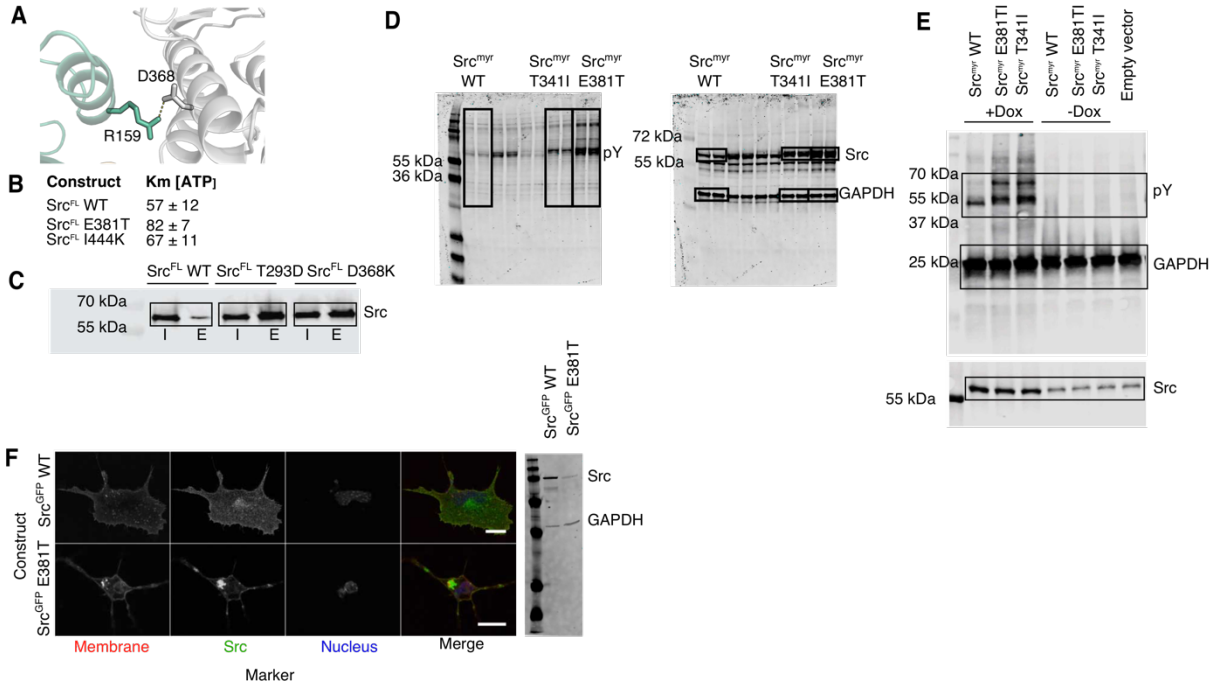


Figure S2. Interrogation of Src's intramolecular regulatory surfaces. Related to **Figure 3**.

A. Salt bridge formed between R159 (*green*) and D368 (*white*) in Src's closed conformation, PDB:2SRC. Relates to **Figure 3B**. **B.** Measured ATP Michaelis-Menten constants (K*m* [ATP]) for Src^{FL} WT, Src^{FL} E381T, and Src^{FL} I444K (n=3, mean ± s.e.m). Relates to **Figure 3C**. **C.** Total Src immunoblots for SH3 domain pulldown assays performed with Src^{FL} WT, Src^{FL} T293D, and Src^{FL} D368K (I = input Src construct; E = Src construct retained after SH3 pulldown). Retained Src was quantified by fitting the immunoblot signal intensity to a Src titration standard curve. Relates to **Figure 3E**. **D.** Immunoblots showing phospho-tyrosine (pY, (*left panel*)), total Src (*right panel*) and GAPDH of Src^{myr} WT, Src^{myr} T341I and Src^{myr} E381T transiently expressed in SYFs. **E.** Immunoblots showing phospho-tyrosine (pTyr), GAPDH and total Src of transiently expressed Src^{myr} WT, Src^{myr} E381T, or Src^{myr} T341I in HeLa cells under a doxycycline (Dox) inducible promoter. **F.** Representative micrographs comparing the localization of Src^{GFP} WT with Src^{GFP} E381T in SYFs (*left*). Total Src and GAPDH immunoblot of SYFs transiently expressing Src^{GFP} WT or Src^{GFP} E381T (same day transfection for both) (*right*).

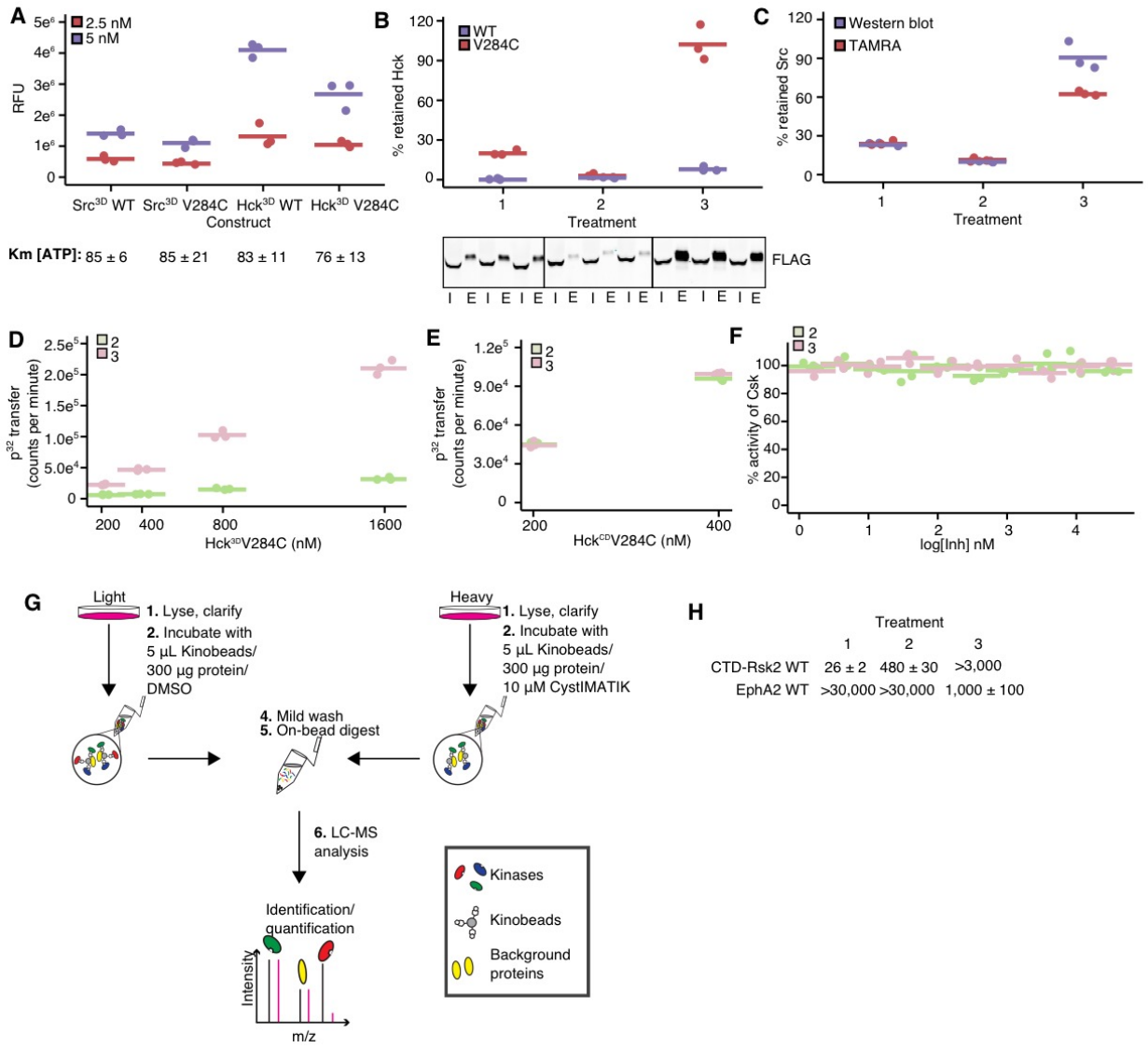


Figure S3. Development and characterization of CystIMATIK. Related to **Figure 4**.

A. Comparison of the catalytic activities (t*op*) and K*m* [ATP]s (*bottom*) for Src^{3D} WT and Src^{3D} V284C or Hck^{3D} WT and Hck^{3D} V284C (n=3). Catalytic activity was measured at either 2.5 nM (*red*) or 5 nM (*purple*) SFK^{3D} concentration (n=3). For catalytic activity comparisons, each SFK^{3D} was incubated with 20 µM of a self-reporting fluorescence SFK peptide (EEEIYGE-(DAP-Pyrene)-EA) and 1 mM ATP and relative fluorescence units (RFU) was measured after 60 min. Relates to **Figure 4C**. **B.** Quantification of percent retained Hck in the SH3 pulldown assay for HEK293Ts expressing Hck^{FLAG} V284C (*red*) (human Src numbering) or Hck^{FLAG} WT (*purple*) and treated with 10 µM **1**, **2**, or **3** (n=3). Hck was quantified with an anti-FLAG antibody. A representative FLAG

immunoblot for HEK293Ts expressing Hck^{FLAG} V284C and treated with **1**, **2**, or **3** is shown (I = input Hck^{FLAG} V284C; E = Hck^{FLAG} V284C retained after SH3 pulldown). **C.** Quantification of SH3 pulldown for purified Src^{3D} V284C (*purple*) or tetramethylrhodamine-labeled $Src^{TAMRA-3D}$ V284C (**Figure S1A** and **Table S1**, *red*) complexed to inhibitors **1**, **2**, or **3** (n=3). Retained Src was quantified by fitting the immunoblot signal intensity to a Src (using an anti-Src antibody) titration standard curve. **D.** SH2 domain accessibility assay of the Hck^{3D} V284C-**2** (*green*) and Hck^{3D} V284C-**3** (*pink*) complexes (n=3). Various concentrations of Hck^{3D} V284C-**2** and Hck^{3D} V284C-**3** were incubated with Csk (25 nM) and 0.4 µCi/mL γ^{32}P-ATP for 60 min. γ^{32}P transfer on Y530 (Src numbering) of Hck^{3D} V284C by Csk is represented as individual measurements and the horizontal lines indicate the mean of all measurements. Relates to **Figures 4F**, **4G**. **E.** SH2 domain accessibility assay of 200 or 400 nM Hck^{CD} V284C-**2** (*green*) and Hck^{CD} V284C-**3** (*pink*) complexes (n=3) in the presence of Csk (25 nM) and 0.4 µCi/mL γ^{32}P-ATP. γ^{32}P transfer on Y530 (Src numbering) of Hck^{CD} V284C by Csk is represented as individual measurements and the horizontal lines indicate the mean of all measurements. **F.** Activity assay of Csk and an exogenous peptide substrate (Csktide, KKKKEEIYFFFG) in the presence CystIMATIK probes **2** (*green*) or **3** (*pink*) at the concentrations shown (n=3). **G.** Schematic for Kinobead profiling. Hck^{FLAG} V284C-expressing HEK293 cells were grown for at least 5 cell doublings in SILAC medium and then lysed and clarified. Lysates were then incubated with a kinobead matrix and DMSO (*left*) or 10 µM CystIMATIK probes **1**, **2**, or **3** (*right*). After mild washing, kinobead matrices (*left* and *right*) were combined, subjected to on-bead digestion, and relative kinase enrichment quantified by LC-MS. **Table S7** lists SILAC ratios and intensity values for proteins and kinases identified and quantified in profiling experiments. Relates to **Figure 4H**. **H.** IC_{50} values of CystIMATIK probes **1**-**3** for WT off-target kinases. Probe **1** is a close structural analog of a selective inhibitor of the C-terminal catalytic domain of the p90 ribosomal protein S6 kinase Rsk2 (CTD-Rsk2; (Serafimova et al., 2012)). The CTD of Rsk2 contains a Cys residue at the position analogous to Val284 in Src and a Thr gatekeeper residue (**Figure 4A**) but Rsk2 was not one of

the ~220 enriched kinases in our kinobead profiling experiment. Therefore, the IC_{50} values of CystIMATIK probes **1**-**3** for CTD-Rsk2 (substrate used: CTDide, sequence RRQLFRGFSFVAK) were also determined. We also determined the IC_{50} values of **1**-**3** for WT EphA2. IC_{50} value determinations for both kinases were performed in the presence of 0.08 µCi/mL γ^{32}P-ATP. (n=3, mean ± s.e.m.) Points represent individual measurements and the horizontal lines indicate the mean of all measurements.

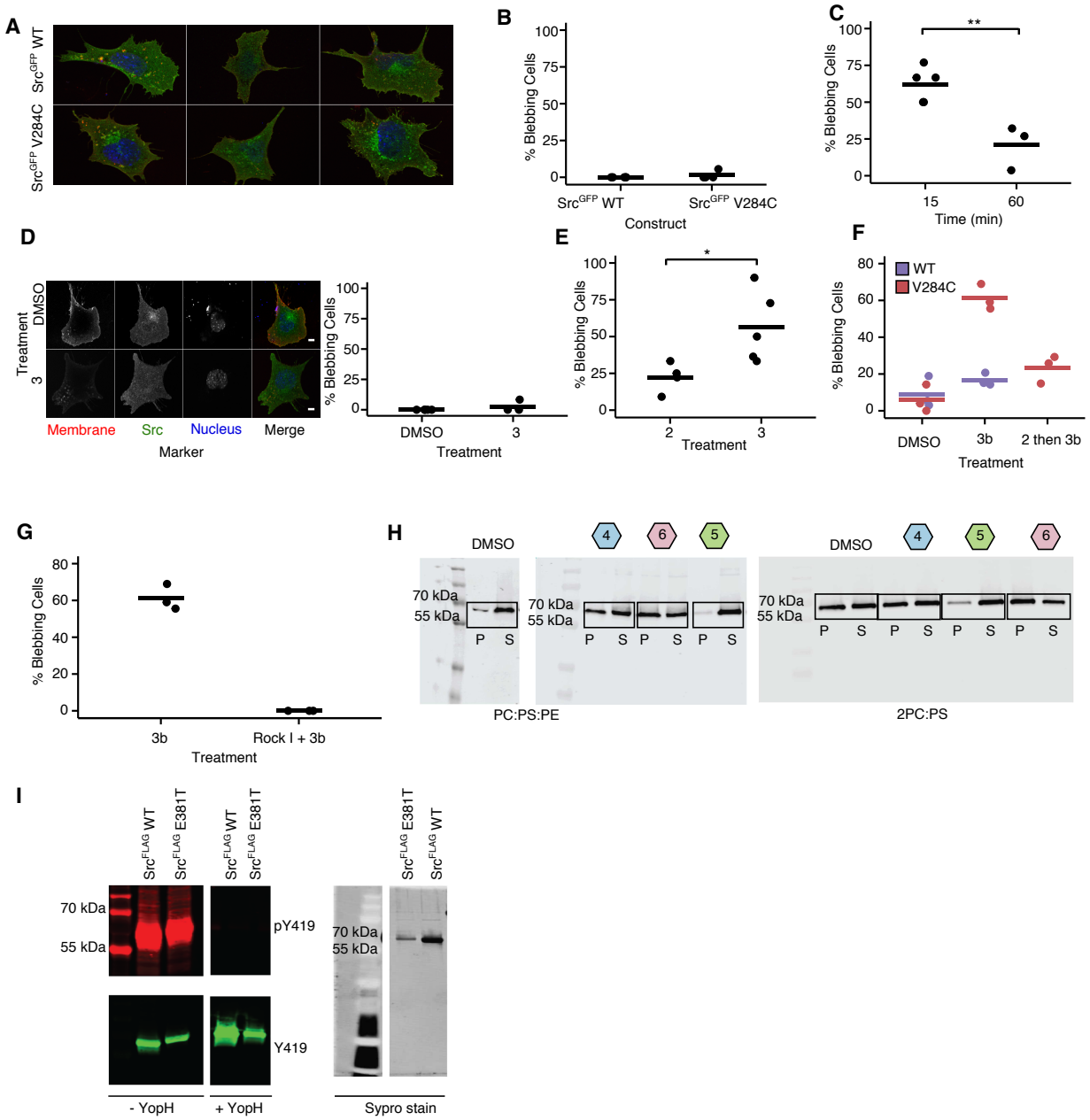
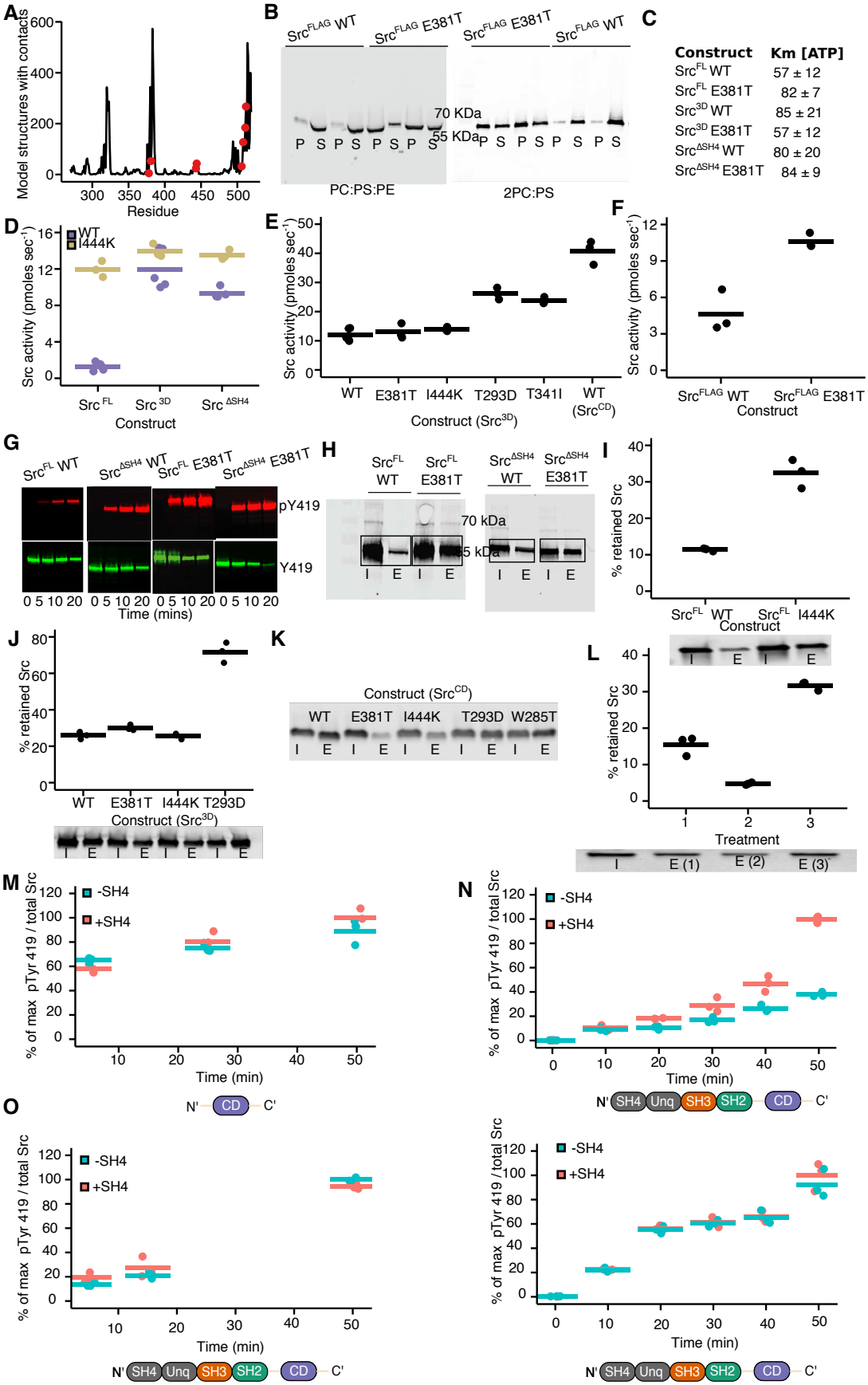


Figure S4. **Modulation of phosphotransferase-independent function of Src with CystIMATIK probes.** Related to **Figure 5**.

A. Three representative micrographs of untreated SYFs expressing Src^{GFP} WT or Src^{GFP} V284C. **B.** Percentage of untreated SYFs expressing expressing Src^{GFP} WT or Src^{GFP} V284C with blebs. **C.** Percentage of SYFs expressing Src^{GFP} V284C with blebs 15 or 60 min after treatment with **3**. Representative images from live cell microscopy are shown in **Figure 5B** and movies are provided as **Movie S1-2**. **D.** Representative micrographs of Src^{GFP} WT-expressing SYFs treated for 15 min with DMSO or **3** (*left panels*). Percentage of Src^{GFP} WT-expressing SYFs with blebs after treatment with DMSO or **3** for 15 minutes. **E.** Percentage of Src^{myr} V284C-expressing SYFs with blebs after treatment with **2** or **3** for 15 min. **F.** Percentage of HeLa cells expressing Src^{myr} WT or Src^{myr} V284C with blebs after treatment with DMSO, 5 μM **3b**, or pre-treated with **2** for 15 min, followed by **3b** (**3b** is an analog of CystIMATIK probe **3** that lacks a methyl group on the cyanoacrylamide moiety). **G.** Percentage of HeLa cells expressing Src^{myr} V284C pre-treated with DMSO or Rock inhibitor GSK429286A, and then treated with **3b**. **H**. Representative Src (anti-Src) immunoblots for the co-sedimentation assays quantified in **Figure 5G**. (P) = pelleted and (S) = soluble Src fractions after sedimentation. Representative data for *apo* Src^{FLAG} WT (DMSO), Src^{FLAG} WT-**4**, Src^{FLAG} WT-**5** or Src^{FLAG} WT-**6** with liposomes composed of 1:1:1 phosphatidylcholine, phosphatidylserine and phosphatidylethanolamine (PC:PS:PE) or 2:1 PC and PS (2PC:PS) are shown. Pelleted and soluble Src were quantified by fitting the immunoblot signal intensity to a Src (using an anti-Src antibody) titration standard curve. **I.** Purification and characterization of non-phosphorylated Src^{FLAG} WT and Src^{FLAG} E381T (see **Figure S1A** and **Table S1**) from HEK293T cells. The left panels show phosphorylated (anti-pTyr419 SFK) and non-phosphorylated (anti-Tyr419 SFK) blots before (- YopH) and after (+ YopH) treatment with *Yersinia pestis* protein tyrosine phosphatase (YopH). The right panels show total protein (Sypro staining) after dephosphorylation and purification. Relates to **Figure 5G**. For **S4B**-S4**G**, each point represents a replicate treatment with multiple cells imaged and scored in a double-blind fashion.

Points represent individual measurements and the horizontal lines indicate the mean of all measurements. See **Table S5** for the total number of replicates and cells analyzed. *p <0.05; **p < 0.01.


A
Model structures with contacts
Residue
B
Src^FLAG WT
Src^FLAG E381T
Src^FLAG E381T
Src^FLAG WT
70 KDa
55 KDa
P S P S P S P S P S P S P S P S
PC:PS:PE
2PC:PS
C
Construct | Km [ATP]
Src^FL WT | 57 ± 12
Src^FL E381T | 82 ± 7
Src^3D WT | 85 ± 21
Src^3D E381T | 57 ± 12
Src^ΔSH4 WT | 80 ± 20
Src^ΔSH4 E381T | 84 ± 9
D
WT
I444K
Src activity (pmoles sec-1)
Src FL
Src 3D
Src ΔSH4
Construct
E
Src activity (pmoles sec-1)
WT
E381T
I444K
T293D
T341I
WT (SrcCD)
Construct (Src3D)
F
Src activity (pmoles sec-1)
Src^FLAG WT
Src^FLAG E381T
Construct
G
Src^FL WT
Src^ΔSH4 WT
Src^FL E381T
Src^ΔSH4 E381T
pY419
Y419
0 5 10 20 0 5 10 20 0 5 10 20 0 5 10 20
Time (mins)
H
Src^FL WT
Src^FL E381T
Src^ΔSH4 WT
Src^ΔSH4 E381T
70 kDa
55 kDa
I E I E I E I E
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% retained Src
Src^FL WT
Src^FL I444K
Construct
J
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E381T
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T293D
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T293D
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L
% retained Src
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Treatment
I
E (1)
E (2)
E (3)
M
% of max pTyr 419 / total Src
-SH4
+SH4
Time (min)
N'
CD
C'
N
% of max pTyr 419 / total Src
-SH4
+SH4
Time (min)
N'
SH4
Unq
SH3
SH2
CD
C'
O
% of max pTyr 419 / total Src
-SH4
+SH4
Time (min)
N'
SH4
Unq
SH3
SH2
CD
C'
% of max pTyr 419 / total Src
-SH4
+SH4
Time (min)
N'
SH4
Unq
SH3
SH2
CD
C'

Figure S5. Characterization of the SH4 domain/αF pocket interaction. Related to **Figure 6**.

A. 4,528 N-tail model structures were created with Rosetta based on the 2SRC crystal structure. Plot of the number of N-tail model structures that contact each catalytic domain residue. The red dots indicate αF pocket residues. **B.** Representative Src (anti-Src) immunoblots for the co-sedimentation assays quantified in **Figure 6B**. P = pelleted and S = soluble Src fractions after sedimentation. Representative data for *apo* Src^{FLAG} WT (DMSO) and *apo* Src^{FLAG} E381T with liposomes composed of 1:1:1 phosphatidylcholine, phosphatidylserine and phosphatidylethanolamine (PC:PS:PE) or 2:1 PC and PS (2PC:PS) are shown. Pelleted and soluble Src were quantified by fitting the immunoblot signal intensity to a Src (using an anti-Src antibody) titration standard curve. **C.** K*m* [ATP] for Src^{FL} WT, Src^{FL} E381T, Src^{3D} WT, Src^{3D} E381T, $Src^{\Delta SH4}$ WT and $Src^{\Delta SH4}$ E381T (n=3, mean ± s.e.m). **D.** Phosphotransferase activity of purified Src^{FL}, Src^{3D} or $Src^{\Delta SH4}$ with either the WT or I444K sequence. (n=4-6). Values for WT (FL and truncations) were used previously in **Figure 6C**. **E.** Phosphotransferase activity of purified Src^{3D} comparing WT, E381T, I444K, T293D and T341I sequence (n=3-5). The phosphotransferase activity of Src^{CD} WT is provided as reference for Src's activity in the absence of regulatory SH2 and SH3 domains. Values for Src^{3D} WT, E381T, and I444K were shown previously in **Figures 6C**, **S5D**. **F.** Phosphotransferase activity of Src^{FLAG} with either the WT or E381T sequence (n=3) purified from HEK293T cells. **G.** Representative (anti-pTyr419 SFK) (*top*) and non-phosphorylated (anti-Tyr419 SFK) (*bottom*) Src immunoblots for autophosphorylation assays quantified in **Figure 6D**. Non-phosphorylated Src variants were incubated with ATP (30 μM) for varying times and then the ratio of pTyr419 and Tyr419 was quantified. **H.** Representative Src immunoblots for SH3 pulldown assays performed with $Src^{\Delta SH4}$ WT or E381T and Src^{FL} WT or E381T and quantified in **Figure 6E**. (I = input Src construct; E = Src construct retained after SH3 pulldown). Retained Src was quantified by fitting the immunoblot signal intensity to a Src titration standard curve. **I.** Quantification (*top panel*) and representative immunoblots (*bottom panel*) for

SH3 pulldown assays performed with Src^{FL} WT or I444K (n=3, I = input Src construct; E = Src construct retained after SH3 pulldown). Values for Src^{FL} WT were used previously in **Figure 6E**. **J.** Quantification (*top panel*) and representative immunoblots (*bottom panel*) for SH3 pulldown assays performed with Src^{3D} WT, E381T, I444K and T293D (n=3, I = input Src construct; E = Src construct retained after SH3 pulldown). **K.** Representative immunoblots for SH4 pulldown assays performed with Src^{3D} WT, E381T, I444K, T293D and W285T (I = input Src construct; E = Src construct retained after SH4 pulldown). Retained Src was quantified by fitting the immunoblot signal intensity to a Src titration standard curve. Relates to **Figure 6J**. **L.** Quantification (*top panel*) and representative fluorescent gel (*bottom panel*) for SH4 pulldown assays performed with tetramethylrhodamine-labeled $Src^{TAMRA\text{-}FL}$ V284C (**Figure S1A** and **Table S1**) complexed to 5 μM inhibitor **1**, **2**, or **3** (n=3, I = input $Src^{TAMRA\text{-}FL}$ V284C; E = $Src^{TAMRA\text{-}FL}$ V284C retained after SH4 pulldown). Input and eluted Src was quantified using fluorescence gel scanning. **M.** Autophosphorylation quantification of Src^{CD} WT in the presence of $SH4^{SNAP}$ (residues 1-18 of Src, +SH4, *red*) or control SNAP (-SH4, *blue*) at various time points after the addition of ATP (n=3). **N.** Autophosphorylation quantification of Src^{FL} WT in the presence of $SH4^{SNAP}$ (residues 1-18 of Src, +SH4, *red*) or control SNAP protein (-SH4, *blue*) at various time points after the addition of ATP (n=3). **O.** Autophosphorylation quantification of Src^{FL} E381T (*left*) and I444K (*right*) in the presence of $SH4^{SNAP}$ (residues 1-18 of Src, +SH4, *red*) or control SNAP protein (-SH4, *blue*) at various time points after the addition of ATP (n=3). For **S5M**-**S5O**, autophosphorylation was quantified by measuring the ratio of pTyr419 (anti-pTyr419 SFK) to total Src (anti-Src) by immunoblotting. Points represent individual measurements and the horizontal lines indicate the mean of all measurements.

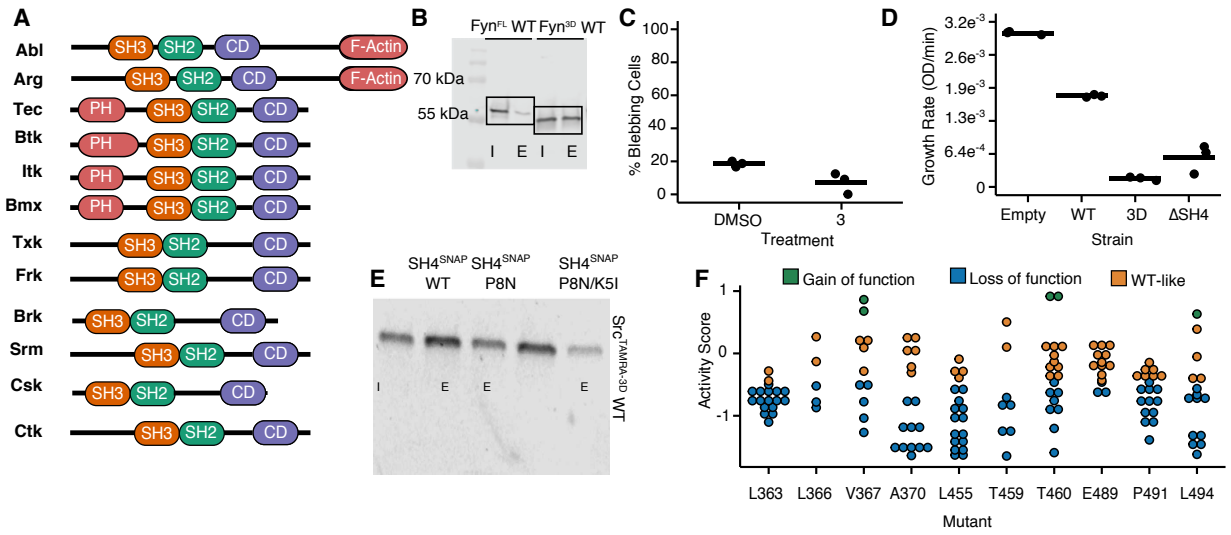

Figure S6. Conservation of the SH4 domain regulatory interaction amongst the SFKs. Related to **Figure 7**.

A. Domain organization of SH4 domain-lacking (SH4-) human non-receptor tyrosine kinases that contain an SH3/SH2/CD architecture. Relates to **Figure 7A**. **B.** Representative Fyn immunoblots (anti-Tyr419 SFK) for SH3 pulldown assays performed with Fyn^{FL} WT or Fyn^{3D} WT. Quantified in **Figure 7C**. (I = input Fyn construct; E = Fyn construct retained after SH3 pulldown). Retained Fyn was quantified by fitting the immunoblot signal intensity to a Fyn (anti-Tyr419 SFK) titration standard curve. **C.** Percentage of Fyn^{myr} WT-expressing SYF cells with blebs after treatment with DMSO or **3**. **D.** Quantification of growth rates in yeast strains expressing Src^{FL} WT, Src^{3D} WT, $Src^{\Delta SH4}$ WT, or empty vector control (n=3). **E.** Representative fluorescent gel of SH4 pulldown assays performed with tetramethylrhodamine-labeled $Src^{TAMRA\text{-}3D}$ WT and immobilized $SH4^{SNAP}$ (residues 1-18 of Src) variants (WT, P8N or P8N/K5I). Quantified in **Figure 7F** (n=3, I = input $Src^{TAMRA\text{-}3D}$ WT; E = $Src^{TAMRA\text{-}3D}$ WT retained after SH4 pulldown). **F.** Activity scores of Src residues that overlay with Abl's myristoyl-binding pocket (as defined in Cowan-Jacob, 2005). Most variants in this region are loss-of-function or WT-like. Points represent individual measurements and the horizontal lines indicate the mean of all measurements. See **Table S5** for the number of cells for all cellular blebbing quantifications.

Table S1. List of constructs and mutations used in manuscript. Relates to all Figures.

Construct	Expressed in	Residue numbers	Global Conformation relative to WT or FL	Activity relative to WT or FL	Vectors used in	Notes	Citation
Src WT	E. coli, S. cerevisiae, HeLa, SYF, HEK293T	1-536	Same	Same	P415 GAL1, pMCSG7, pcDNA3.3, pEF1A, pcDNA5		
Src V284C	E. coli, HEK293T, HeLa, SYF	/	Not measured	Same	pET-28a, pMCSG7, pEF1A	CystIMATIK mutation	This study
Src K298M	S. cerevisiae	/	Not measured	Less	P415 GAL1	Catalytically-inactive mutant	Florio *et al*. 1994
Src T341I	S. cerevisiae, HeLa	/	Not measured	More	P415 GAL1, pcDNA5	Gatekeeper mutant	Azam *et al*. 2008
Src E381T	E. coli, S. cerevisiae, HEK293T, HeLa, SYF	/	More open	More	pMCSG7, P415 GAL1, pcDNA3.3, pEF1A, pcDNA5	αF pocket mutant	This study
Src I444K	E. coli, HEK293T	/	More open	More	pMCSG7	αF pocket mutant	This study
Src T293D	E. coli	/	More open	More	pMCSG7	SH3-interface mutant	This study
Src D368K	E. coli	/	More open	More	pMCSG7	SH2-interface	This study
Src N471Y	S. cerevisiae, HEK293T	/	Not measured	Less	P415 GAL1, pEF1A	Activity score = -1.3	This study
Src P491G	S. cerevisiae, HEK293T	/	Not measured	Less	P415 GAL1, pEF1A	Activity score = -0.9	This study
Src G398T	S. cerevisiae, HEK293T	/	Not measured	Same	P415 GAL1, pEF1A	Activity score = 0	This study
Src V274A	S. cerevisiae, HEK293T	/	Not measured	More	P415 GAL1, pEF1A	Activity score = 1.9	This study
Src G2A	SYF	/	Not measured	Less	pEF1A	Unable to be	Kamps *et al*. 1985

						myristoylated	
Src K445C	E. coli	/	Not measured	Same	pMCSG7	Used in maleimide labeling	This study
Src^{GFP}	SYF, HeLa	1-536 with full length GFP	Not measured	Not measured	pEF1A	C-terminal GFP tag with GSGTGSGTAT	Patwardhan and Resh, 2010
Src^{FLAG}	HEK293T	1-536 with FLAG tag	Not measured	More	pcDNA3.3	C-terminal FLAG with linker sequence GSGT in between Src and FLAG tag	
Src^{myr}	S. cerevisiae, HEK293T, HeLa	1-536	Not measured	Same	P415 GAL1, pcDNA5	Co-translationally myristoylated on G2	
Src^{FL}	E. coli	2-536	Same	Same	pMCSG7	N-terminal His6-SUMO tag gets cleaved	This study
$Src^{\Delta SH4}$	E. coli, S. cerevisiae	19-536	More open	More	pMCSG7, P415 GAL1	N-terminal His6-SUMO tag gets cleaved	This study
Src^{3D}	E. coli, S. cerevisiae	87-536	More open	More	pMCSG7, P415 GAL1	N-terminal His6-SUMO tag gets cleaved	
Src^{CD}	E. coli	261-536	NA	More	pET28a, pMCSG7	N-terminal His6-SUMO tag gets cleaved	This study
Fyn^{myr}	SYF	1-537	Not measured	Not measured	pEF1A	C-terminal IRES-EGFP	This study

Fyn^{FL}	E. coli	2-537	Not measured	Same	pMCSG7	N-terminal His6-SUMO tag gets cleaved	This study
Fyn^{3D}	E. coli	85-537	Not measured	More	pMCSG7	N-terminal His6-SUMO tag gets cleaved	This study
$Src^{TAMRA-FL}$	E. coli	2-536	Same	Not measured	pMCSG7	N-terminal TAMRA label with LPYTG sequence prior to G2	This study
$Src^{TAMRA-3D}$	E. coli	87-536			pMCSG7	N-terminal TAMRA label with LPYTG sequence prior to T87	This study
$SH4^{SNAP}$	E.coli	2-18	NA	NA	pMCSG7	SH4 domain with C-terminal SNAP tag with a linker LPETGG in between	This study

Table S3. Essential Src catalytic domain residues (defined as >90% of nonsynonymous mutations are classified as “loss of function”). At least 5 mutations must be measured. Relates to Figure 2F.

Residue	Notes
G277	Glycine-rich loop
G279	Glycine-rich loop
F281	Glycine-rich loop
G282	Glycine-rich loop
V284	C-spine
A296	C-spine
K298	Involved in catalytic B3-αC salt bridge
M305	
F310	
E313	Involved in catalytic B3-αC salt bridge
A314	
M317	R-spine
K318	
L320	
L328	R-spine
P336	
G376	
H387	R-Spine, HRD motif, catalytic loop
R388	HRD motif, catalytic loop
D389	HRD motif, catalytic loop
R391	Catalytic loop
N394	Catalytic loop
L396	C-spine
V397	C-spine
A406	xDFG residue
D407	DFG motif
F408	DFG motif

G409	DFG motif
L410	
A411	
R412	Electrostatic switch
Y419	Activation loop tyrosine
A421	Anchor point 2
G424	
F427	
P428	Interacts with substrate
K430	P + 1 loop
W431	P + 1 loop
T432	P + 1 loop
E435	P + 1 loop
S446	
D447	R-spine
E457	
P465	
N471	
R509	Conserved salt bridge with D407

Table S5. Values for cellular blebbing. Relates to Figures 3G, 5A, 5C, 5D, 5E, 7D, S4B, S4C, S4D, S4E, S4F, and S6C.

Figure	Condition (in SYF cells unless otherwise noted)	n	Total # cells scored
3G	Src^{FL} WT	6	176
3G	Src^{FL} E381T	11	140
5A	Src^{GFP} V284C +DMSO	4	51
5A	Src^{GFP} V284C + 1	3	51
5A	Src^{GFP} V284C + 2	9	138
5A	Src^{GFP} V284C +3	5	88
5A	Src^{GFP} V284C 2 then 3	5	56
5C	Src^{GFP} V284C +DMSO	4	51
5C	Src^{GFP} V284C + 1 μM	4	45
5C	Src^{GFP} V284C + 3 μM	3	35
5C	Src^{GFP} V284C + 10 μM	5	88
5D	Src^{GFP} V284C + Rock I	5	58
5D	Src^{GFP} V284C + Rock I + 3	3	33
5E	Src^{GFP} G2A/V284C + DMSO	3	64
5E	Src^{GFP} G2A/V284C + 3	3	39
7D	Fyn^{myr} V284C + DMSO	4	53
7D	Fyn^{myr} V284C + 3	7	96
S4B	Src^{GFP} WT	6	176
S4B	Src^{GFP} V284C	4	51
S4C	Src^{GFP} V284C + 15 min	5	88
S4C	Src^{GFP} V284C + 60 min	3	81
S4D	Src^{GFP} WT + DMSO	5	176
S4D	Src^{GFP} WT + 3	3	59
S4E	Src^{myr} V284C + 2	4	48
S4E	Src^{myr} V284C + 3	5	51
S4F	Src^{myr} V284C +DMSO (HeLa)	3	80
S4F	Src^{myr} V284C +3b (HeLa)	3	182
S4F	Src^{myr} V284C +2 then 3b (HeLa)	3	99
S4F	Src^{myr} WT+DMSO (HeLa)	3	107
S4F	Src^{myr} WT +3b (HeLa)	3	80
S6C	Fyn^{myr} WT + DMSO	3	38
S6C	Fyn^{myr} WT + 3	3	30