Supplementary tables

Supplementary table 1: Fitness scores and errors
Supplementary table 2: Fitted values of ΔΔGf and ΔΔGa
Supplementary table 3: Src surface pocket summary

Supplementary table 4: Plasmids

pGJJ133	aPCA empty	Available upon request (Material Transfer Agreement required)
pTB022	toxicity empty	https://benchling.com/s/seq-rGJbDMKRao9IdlAqhMoJ?m=slm-cUTBKgetTLCpJ3TveV5w
pTB198	sandwichPCA empty	https://benchling.com/s/seq-mve4HVJwo23HnO6BPNym?m=slm-lNfOeXfnOvLHoarUjhPK
pTB109	aPCA Src KD	https://benchling.com/s/seq-obS1wabcFstnPlwNCdPl?m=slm-an1b6yZtzRU1xIytarI5
pTB112	toxicity Src KD	https://benchling.com/s/seq-PUIubmPUQrNsuv9YkjJM?m=slm-KBT9Fq1il6zXODI1mvnj
pTB043	sandwichPCA full length	https://benchling.com/s/seq-0sA5ySViNYIlnltmMAFl?m=slm-27zYqdcFKogMjblGqR1y
pTB023	toxicity full length	https://benchling.com/s/seq-PdGjC1KRegS3IUGnMNPh?m=slm-CCYco9fv12T7nVaTxXhf

Supplementary table 5: Oligonucleotides

Amplification of SRC KD from full length gBlock	
oTB063	CAATATGCTAGCGATGCTTGGGAGATCCCTC
oTB064	TAATTTAAAGCTTCAAGTTCTCTC
Introduction of start codon in pTB112	
oTB214	atgGATGCTTGGGAGATCCCTC
oTB215	GCTAGCCTCCTTGACGTT
oPool and backbone amplification for Gibson assembly	
oTB447_b1_ins_F	GATGCTTGGGAGATCCCTC
oTB448_b1_ins_R	CACTCACAACTGCATACAATTG
oTB449_b2_ins_F	AGCACAAGTCATGAAGAAGC
oTB450_b2_ins_R	CACGGTGTACGTAATTCATTC
oTB451_b3_ins_F	CATGGCCGCCCAGATTG
oTB452_b3_ins_R	CGTGAACCTTCCATATAAGGC
oTB453_b4_ins_F	GTGCAAAGTTCCCCATCAAG
oTB454_b4_ins_R	CATAAGGTCGTGCAAGCTC
oTB455_b5_ins_F	GAACGTGGTTATAGAATGCC

oTB220_b5_ins_R	GCGTGACATAACTAATTTAAAGC
oTB457_b1_bb_F	CAATTGTATGCAGTTGTGAGTG
oTB458_b1_bb_R	GAGGGATCTCCCAAGCATC
oTB459_b2_bb_F	GAATGAATTACGTACACCGTG
oTB460_b2_bb_R	AGCTTCTTCATGACTTGTGC
oTB461_b3_bb_F	GCCTTATATGGAAGGTTCACG
oTB462_b3_bb_R	CAATCTGGGCGGCCATG
oTB463_b4_bb_F	GAGCTTGCACGACCTTATG
oTB464_b4_bb_R	CTTGATGGGGAACTTTGCAC
oTB241_b5_bb_F	AAGCTTTAAATTAGTTATGTCACG
oTB466_b5_bb_R	GGCATTCTATAACCACGTTC
qPCR quantification oligos	
oGJJ152	GCCTACATACCTCGCTCTGC
oGJJ153	CAACCCGGTAAGACACGACT
Frameshifting PCR1 oligos	
oTB302_b1_fs_F	ACACTCTTTCCCTACACGACGCTCTTCCGATCTATGCTTGGGAGATCCCTC
oTB303_302_+1	ACACTCTTTCCCTACACGACGCTCTTCCGATCTNATGCTTGGGAGATCCCTC
oTB304_302_+2	ACACTCTTTCCCTACACGACGCTCTTCCGATCTNCATGCTTGGGAGATCCCTC
oTB305_302_+3	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGGCATGCTTGGGAGATCCCTC
oTB306_302_+4	ACACTCTTTCCCTACACGACGCTCTTCCGATCTCTGNATGCTTGGGAGATCCCTC
oTB307_302_+5	ACACTCTTTCCCTACACGACGCTCTTCCGATCTNWWANATGCTTGGGAGATCCCTC
oTB308_b1_fs_R	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTCACTCACAACTGCATACAATTG
oTB309_308_+1	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGCACTCACAACTGCATACAATTG
oTB310_308_+2	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTAGCACTCACAACTGCATACAATTG
oTB311_308_+3	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTTAGCACTCACAACTGCATACAATTG
oTB312_308_+4	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTWTTGCACTCACAACTGCATACAATTG
oTB313_308_+5	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTSCATGCACTCACAACTGCATACAATTG
oTB471_449_b2_FS_F	ACACTCTTTCCCTACACGACGCTCTTCCGATCTAGCACAAGTCATGAAGAAGC

oTB472_449_+1	ACACTCTTTCCCTACACGACGCTCTTCCGATCTTAGCACAAGTCATGAAGAAGC
oTB473_449_+2	ACACTCTTTCCCTACACGACGCTCTTCCGATCTCTAGCACAAGTCATGAAGAAGC
oTB474_449_+3	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGCTAGCACAAGTCATGAAGAAGC
oTB475_449_+4	ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNTAGCACAAGTCATGAAGAAGC
oTB476_449+5	ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNTTAGCACAAGTCATGAAGAAGC
oTB477_450_b2_FS_R	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTCACGGTGTACGTAATTCATTC
oTB478_450_+1	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGCACGGTGTACGTAATTCATTC
oTB479_450_+2	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTTGCACGGTGTACGTAATTCATTC
oTB480_450_+3	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTATGCACGGTGTACGTAATTCATTC
oTB481_450_+4	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTWSTTCACGGTGTACGTAATTCATTC
oTB482_450_+5	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTSWNWWCACGGTGTACGTAATTCATTC
oTB483_451_b3_FS_F	ACACTCTTTCCCTACACGACGCTCTTCCGATCTCATGGCCGCCCAGATTG
oTB484_451_+1	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGCATGGCCGCCCAGATTG
oTB485_451_+2	ACACTCTTTCCCTACACGACGCTCTTCCGATCTAGCATGGCCGCCCAGATTG
oTB486_451_+3	ACACTCTTTCCCTACACGACGCTCTTCCGATCTTTGCATGGCCGCCCAGATTG
oTB487_451_+4	ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNCATGGCCGCCCAGATTG
oTB488_451_+5	ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNWCATGGCCGCCCAGATTG
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oTB490_452_+1	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTACGTGAACCTTCCATATAAGGC
oTB491_452_+2	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGACGTGAACCTTCCATATAAGGC
oTB492_452_+3	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTTTACGTGAACCTTCCATATAAGGC
oTB493_452_+4	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTWSSACG

	TGAACCTTCCATATAAGGC
oTB494_452_+5	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTSWWWN CGTGAACCTTCCATATAAGGC
oTB495_453_b4_F S_F	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGTGCAAAG TTCCCCATCAAG
oTB496_453_+1	ACACTCTTTCCCTACACGACGCTCTTCCGATCTAGTGCAAA GTTCCCCATCAAG
oTB497_453_+2	ACACTCTTTCCCTACACGACGCTCTTCCGATCTTAGTGCAA AGTTCCCCATCAAG
oTB498_453_+3	ACACTCTTTCCCTACACGACGCTCTTCCGATCTCCCGTGCA AAGTTCCCCATCAAG
oTB499_453_+4	ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNAAGTGC AAAGTTCCCCATCAAG
oTB500_453_+5	ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNNGT GCAAAGTTCCCCATCAAG
oTB501_454_b4_F S_R	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTCATAAG GTCGTGCAAGCTC
oTB502_454_+1	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGCATAA GGTCGTGCAAGCTC
oTB503_454_+2	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTTGCATAA GGTCGTGCAAGCTC
oTB504_454_+3	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTATGCATA AGGTCGTGCAAGCTC
oTB505_454_+4	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNGCA TAAGGTCGTGCAAGCTC
oTB506_454_+5	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNSGC ATAAGGTCGTGCAAGCTC
oTB507_455_b5_F S_F	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGAACGTG GTTATAGAATGCC
oTB508_455_+1	ACACTCTTTCCCTACACGACGCTCTTCCGATCTTGAACGTG GTTATAGAATGCC
oTB509_455_+2	ACACTCTTTCCCTACACGACGCTCTTCCGATCTCTGAACGT GGTTATAGAATGCC
oTB510_455_+3	ACACTCTTTCCCTACACGACGCTCTTCCGATCTACTGAACG TGGTTATAGAATGCC
oTB511_455_+4	ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNCTGAAC GTGGTTATAGAATGCC
oTB512_455_+5	ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNTGAA CGTGGTTATAGAATGCC
oGJJ589_b5_R	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGCGTGA CATAACTAATTTAAAGC
oGJJ590_589_+1	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNGCGTG ACATAACTAATTTAAAGC

oGJJ591_589_+2	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNGCGTGACATAACTAATTTAAAGC
oGJJ592_589_+3	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTHNHGCGTGACATAACTAATTTAAAGC
oGJJ593_589_+4	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTHWWHGCGTGACATAACTAATTTAAAGC
oGJJ594_589_+5	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTHWWAAGCGTGACATAACTAATTTAAAGC
oTB513_470+	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTCCCACCACCTCCactAAG
oTB514_470_+1	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTATCCCACCACCTCCactAAG
oTB515_470_+2	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTTGACCCACCACCTCCactAAG
oTB516_470_+3	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGAGTCCCACCACCTCCactAAG
oTB517_470_+4	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTSWTGDCCCACCACCTCCactAAG
oTB518_470_+5	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTWSNNDCCCACCACCTCCactAAG

Supplementary Table 6: Src sequences

Src kinase domain:
CTAGCGATGCTTGGGAGATCCCTCGTGAATCACTGCGTCTTGAGGTAAAGTTAGGCCAGGGATGC
TTTGGGGAGGTGTGGATGGGCACGTGGAACGGTACTACCAGGGTTGCAATTAAGACTCTGAAAC
CCGGAACCATGTCTCCTGAGGCGTTCCTGCAAGAAGCACAAGTCATGAAGAAGCTACGTCATGA
GAAGCTAGTGCAATTGTATGCAGTTGTGAGTGAAGAGCCGATCTACATTGTCACTGAGTACATGAG
CAAGGGTTCTTTGCTGGACTTCTTGAAGGGTGAAACCGGCAAATACCTGAGACTTCCCCAGTTGG
TAGACATGGCCGCCCAGATTGCATCCGGTATGGCTTACGTGGAGAGAATGAATTACGTACACCGT
GATCTAAGAGCTGCGAACATACTGGTTGGAGAAAACTTGGTATGTAAGGTCGCTGATTTCGGTCTG
GCGAGGCTTATTGAAGACAATGAATACACTGCACGTCAAGGTGCAAAGTTCCCCATCAAGTGGAC
GGCTCCAGAGGCTGCCTTATATGGAAGGTTCACGATAAAGTCCGATGTGTGGAGTTTCGGGATAT
TGTTAACAGAATTGACAACGAAGGACGTGTACCATATCCTGGCATGGTTAATAGAGAAGTACTTG
ACCAGGTAGAACGTGGTTATAGAATGCCATGCCCTCCGGAGTGTCCCGAGAGCTTGCACGACCTT
ATGTGTCAGTGTTGGAGGAAAGAGCCTGAGGAGAGGCCTACATTCGAGTATCTACAAGCATTCTT
AGAAGACTACTTCACGTCCACAGAACCACAGTACCAACCCGGAGAGAACTTGA

Full length Src:
ATGGGCAGCAATAAGTCAAAGCCGAAGGATGCAAGCCAAAGGCGTAGGTCTTTGGAGCCTGCCG
AGAATGTACATGGAGCTGGTGGTGGAGCTTTTCCGGCCAGCCAGACGCCCTCCAAACCCGCGTC
TGCTGATGGTCACCGTGGGCCAAGTGCTGCTTTTGCGCCCGCTGCAGCGGAGCCTAAGCTATTC
GGGGGTTTTAACAGTAGCGACACCGTAACGAGCCCGCAGAGAGCAGGTCCGTTGGCAGGGGGC
GTGACTACGTTCGTCGCCCTATACGACTACGAGTCTAGGACAGAGACTGACTTGAGCTTCAAAAA
GGGAGAACGTCTGCAGATCGTAAACAATACAGAGGGTGACTGGTGGCTTGCACATTCTCTTAGTA
CTGGGCAGACAGGTTATATTCCGAGCAACTATGTCGCACCGAGTGATTCAATACAGGCAGAAGAG
TGGTATTTTGGAAAAAATTACTCGTAGGGAGTCCGAGAGATTATTGCTTAACGCAGAGAACCCTCGT

GGGACGTTTCTGGTCAGGGAAAGCGAAACAACAAAAGGAGCGTACTGCTTAAGCGTAAGCGATT
TCGACAATGCCAAAGGTCTTAACGTTAAGCATTATAAGATTAGGAAGTTGGACTCCGGGGGCTTTT
ATATAACGAGCAGAACCCAATTTAACTCTCTACAGCAATTGGTTGCATATTACTCCAAACATGCAGA
CGGTCTATGTCATCGTTTGACAACTGTTTGCCCCACAAGTAAGCCTCAGACGCAAGGTTTAGCAA
AGGATGCTTGGGAGATCCCTCGTGAATCACTGCGTCTTGAGGTAAAGTTAGGCCAGGGATGCTTT
GGGGAGGTGTGGATGGGCACGTGGAACGGTACTACCAGGGTTGCAATTAAGACTCTGAAACCCG
GAACCATGTCTCCTGAGGCGTTCCTGCAAGAAGCACAAGTCATGAAGAAGCTACGTCATGAGAAG
CTAGTGCAATTGTATGCAGTTGTGAGTGAAGAGCCGATCTACATTGTCACTGAGTACATGAGCAAG
GGTTCTTTGCTGGACTTCTTGAAGGGTGAAACCGGCAAATACCTGAGACTTCCCCAGTTGGTAGA
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GTCAGTGTTGGAGGAAAGAGCCTGAGGAGAGGCCTACATTCGAGTATCTACAAGCATTCTTAGAA
GACTACTTCACGTCCACAGAACCACAGTACCAACCCGGAGAGAACTTGA

Supplementary Table 7: Library design

Block	start aa	end aa	Backgrounds (neutral, gain of function, loss of function)
1	268	326	WT, E273G, G282A, E283L, G287P, K298M, T304A, E313R, V316I, E323R
2	321	381	WT, T341I, L328I, K354R, D368V, A370L, K354P, S345P, G355V, M377F
3	376	435	WT, E381K, R382P, R391A, I395C, T420V, R412F, Y419A, P434H, I429A
4	431	491	WT, W431V, L454A, E473T, E489S, T443W, L458V, T443P, I444F, I444P
5	486	536	WT, D496Y, E508G, Y514R, P532W, Y530D, M498Q, F523K, Y514N, L494P

Library block 1

5' constant region:
GATGCTTGGGAGATCCCTCGT
Variable region:
GAATCACTGCGTCTTGAGGTAAAGTTAGGCCAGGGATGCTTTGGGGAGGTGTGGATGGGCACGT
GGAACGGTACTACCAGGGTTGCAATTAAGACTCTGAAACCCGGAACCATGTCTCCTGAGGCGTTC
CTGCAAGAAGCACAAGTCATGAAGAAGCTACGTCATGAGAAGCTAGTG

3' constant region:
CAATTGTATGCAGTTGTGAGTG

Library block 2
5' constant region:
AGCACAAGTCATGAAGAAGCTA
Variable region:
CGTCATGAGAAGCTAGTGCAATTGTATGCAGTTGTGAGTGAAGAGCCGATCTACATTGTCACTGAG
TACATGAGCAAGGGTTCTTTGCTGGACTTCTTGAAGGGTGAAACCGGCAAATACCTGAGACTTCC
CCAGTTGGTAGACATGGCCGCCCAGATTGCATCCGGTATGGCTTACGTGGAG
3' constant region:
AGAATGAATTACGTACACCGTG

Library block 3
5' constant region:
CATGGCCGCCCAGATTGCATCC
Variable region:
GGTATGGCTTACGTGGAGAGAATGAATTACGTACACCGTGATCTAAGAGCTGCGAACATACTGGTT
GGAGAAAACTTGGTATGTAAGGTCGCTGATTTCGGTCTGGCGAGGCTTATTGAAGACAATGAATAC
ACTGCACGTCAAGGTGCAAAGTTCCCCATCAAGTGGACGGCTCCAGAG
3' constant region:
GCTGCCTTATATGGAAGGTTCACG

Library block 4
5' constant region:
GTGCAAAGTTCCCCATCAAG
Variable region:
TGGACGGCTCCAGAGGCTGCCTTATATGGAAGGTTCACGATAAAGTCCGATGTGTGGAGTTTCGG
GATATTGTTAACAGAATTGACAACGAAAGGACGTGTACCATATCCTGGCATGGTTAATAGAGAAGTA
CTTGACCAGGTAGAACGTGGTTATAGAATGCCATGCCCTCCGGAGTGTCCC

3' constant region:
GAGAGCTTGCACGACCTTATG

Library block 5
5' constant region:
GAACGTGGTTATAGAATGCCA
Variable region:
TGCCCTCCGGAGTGTCCCGAGAGCTTGCACGACCTTATGTGTCAGTGTTGGAGGAAAGAGCCTG
AGGAGAGGCCTACATTCGAGTATCTACAAGCATTCTTAGAAGACTACTTCACGTCCACAGAACCAC
AGTACCAACCCGGAGAGAACTTG
3' constant region:
AAGCTTTAAATTAGTTATGTCACGC

Genetic background sequences:

Block 1:
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Block 3:
>WT
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Supplementary Table 8: Kinase Atlas known allosteric pockets from the literature

Site	Site Name Origin	Inhibitor Type	Source Kinase	PDB	Pocket Description	Present in Src
DFG	DFG motif	II	many	1IEP	Hydrophobic pocket that opens up when DFG motif switches to inactive "DFG-out" conformation; binding here may stabilize inactive kinase conformation	yes
MT3	MEK1/2 type III inhibitor	III	MEK1/2	4AN2	Adjacent to ATP and DFG-out pockets; binding disrupts salt bridge required for kinase activity	yes
PIF	PDK1 interacting fragment	IV	PDK1	4RQK	PDK1 regulates other AGC kinases by recruiting them through this site	no
MPP	MKK4 p38a peptide	IV	MKK4	3ALO	p38a peptide binding inhibits MKK4 by inducing conformational changes that lead to auto-inhibition	yes
CMP	c-Abl myristoyl pocket	IV	c-Abl	3K5V	Binding here leads to active or inactive state in c-Abl (depending on ligand size) by affecting SH domain binding	yes
PMP	PKA myristoyl pocket	IV	PKA	1CMK	Myristoyl binding here activates membrane binding in PKA	no
DRS	D-recruitment site	IV	all MAPKs	1UKI	Substrate docking site present in all MAP kinases	no
DEF	docking site for ERK FXF	IV	some MAPKs	3O2M	Substrate docking site present in some MAP kinases; located near MAPK insert	no

LBP	lipid binding pocket	IV	p38a MAPK	3NEW	Binding of different lipids here affects p38a MAPK's preference and activity for different substrates	no
PDIG	PDIG motif	IV	Chk1	3JVS	Substrate recognition site located near PDIG motif in Chk1	yes
AAS	Aurora A activation segment	IV	Aurora A	4C3P	An Aurora A monomer activates another through binding of its activation segment to this site	yes
EDI	EGFR dimerization interface	IV	EGFR	2RFE	An EGFR monomer activates another by binding at this interface on the C-terminal domain	yes