

A Biosensor of Src Family Kinase Conformation by Exposable Tetracysteine Useful for Cell-Based Screening

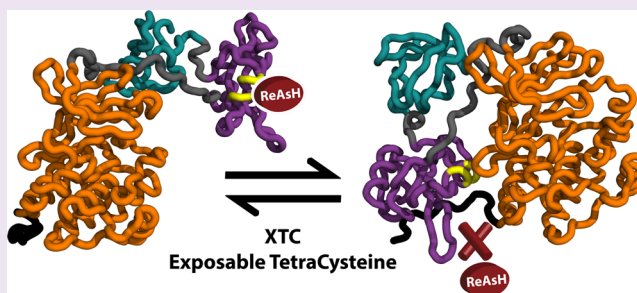
Sevgi Irtegun,^{†,||} Rebecca Wood,^{†,||} Kurt Lackovic,^{‡,§} Jörg Schweiggert,[†] Yasmin M. Ramdzan,[†] David C. S. Huang,^{‡,§} Terrence D. Mulhern,^{*,†} and Danny M. Hatters^{*,†}

[†]Department of Biochemistry and Molecular Biology and Bio21 Molecular Science and Biotechnology Institute and [§]Department of Medical Biology, University of Melbourne, Melbourne, Victoria 3010, Australia

[‡]Walter and Eliza Hall Institute of Medical Research, Parkville, Victoria 3052, Australia

S Supporting Information

ABSTRACT: We developed a new approach to distinguish distinct protein conformations in live cells. The method, exposable tetracysteine (XTC), involved placing an engineered tetracysteine motif into a target protein that has conditional access to biarsenical dye binding by conformational state. XTC was used to distinguish open and closed regulatory conformations of Src family kinases. Substituting just four residues with cysteines in the conserved SH2 domain of three Src-family kinases (c-Src, Lck, Lyn) enabled open and closed conformations to be monitored on the basis of binding differences to biarsenical dyes FAsH or ReAsH. Fusion of the kinases with a fluorescent protein tracked the kinase presence, and the XTC approach enabled simultaneous assessment of regulatory state. The c-Src XTC biosensor was applied in a boutique screen of kinase inhibitors, which revealed six compounds to induce conformational closure. The XTC approach demonstrates new potential for assays targeting conformational changes in key proteins in disease and biology.



Our knowledge of the mechanical operation of proteins in live cells has remained difficult to determine due to a dearth of methods. Fluorescence resonance energy transfer (FRET) has been a major method, particularly in the development of metabolite biosensors,¹ but is not suitable for all systems and contexts. For assessing conformational changes in proteins, FRET typically requires the expression of two fluorescent protein fusions to a target protein, which may be difficult to accommodate without major impediment to protein function. The emergence of chemical biology avenues offers much potential to greatly expand the repertoire of methods to address these challenges. One example is the capacity of a tetracysteine (TC) motif, CCXXCC, to bind biarsenical fluorophores FAsH and ReAsH with high affinity and specificity in live cells.² The binary nature of the system and small size of the dyes have enabled innovative applications for investigating protein self-association, folding, and oligomerization.^{3–5}

Here, we describe a new approach for tracking alternate conformations of Src family kinases (SFKs) using an exposable tetracysteine (XTC) sequence with conformation-specific biarsenical dye reactivity. SFKs modulate signaling pathways that drive cell growth, differentiation, proliferation, apoptosis, adhesion, and migration. They are overactive in a wide variety of human cancers, making them attractive therapeutic targets.⁶ SFKs have “open” active and “closed” inactive conformations that are well understood at an atomic level^{7,8} (Figure 1a). The

activity of c-Src in terms of the local balance of the upstream kinase and upstream phosphatase activities can now be measured spatially in live cells using FRET reporters of the phosphorylation status of a c-Src substrate;⁹ however, its regulatory state remains more challenging to monitor. Prior work established an approach involving microinjection of an extrinsic probe that selectively binds the SFK SH3 domain in the open conformation.¹⁰ This method is highly sensitive but not amenable for high-throughput assays and does not discriminate between different SFK members.¹¹

By introducing mutations R159C, E160C, R163C, and L164C in the SH2 domain of human c-Src, we created an exposable TC construct (c-Src^{XTC}) predicted to be solvent-exposed only in the open state and have a geometry permissive to FAsH and ReAsH binding^{7,8,12} (Figure 1a). To test whether c-Src^{XTC} bound biarsenical dyes in a conformation-specific manner, the construct was superimposed onto “open” active or “closed” inactive mutant backgrounds and transiently expressed in AD293 cells under a high-yielding CMV promoter. c-Src^{XTC}_{open} and c-Src^{XTC}_{closed} have additional mutations (open, Y530F; closed, Q531E, P532E, G533I) that alter the affinity of the C-tail to the SH2 domain, an interaction that stabilizes the closed form.¹³ To provide an independent measure of c-Src

Received: April 2, 2014

Accepted: May 14, 2014

Published: May 14, 2014

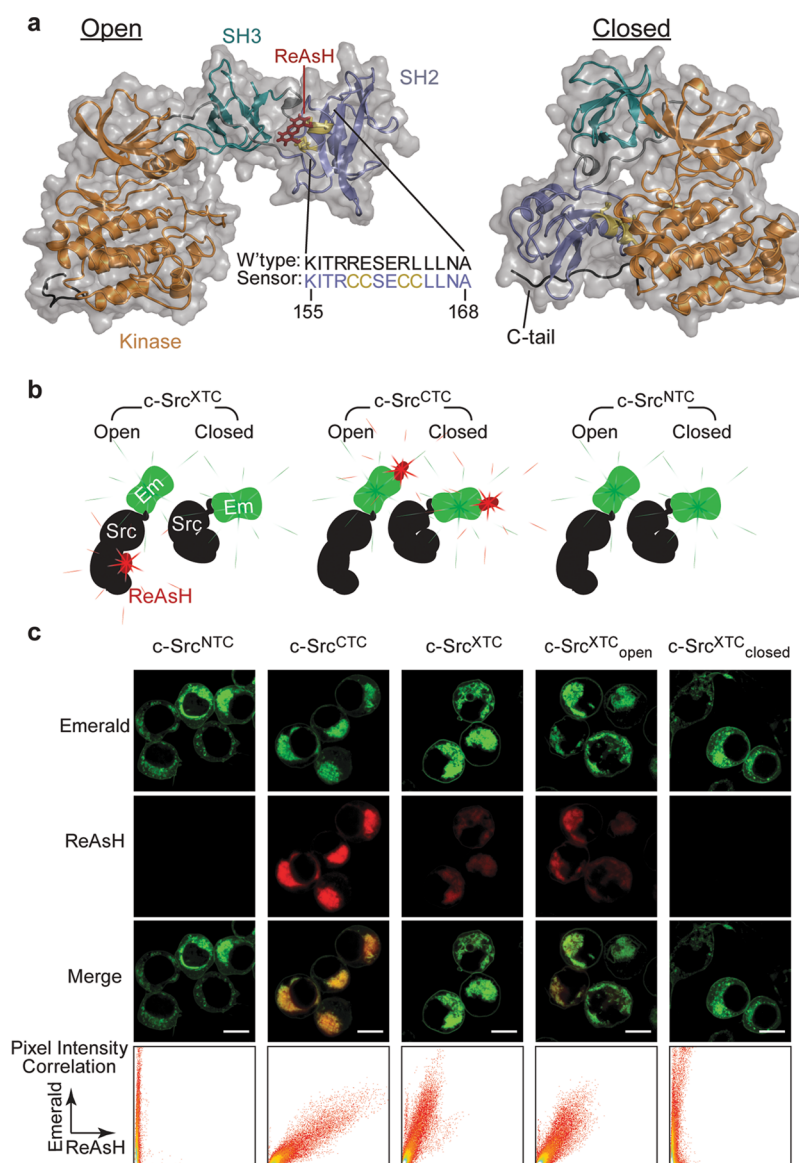


Figure 1. Designing tetracycline exposure to indicate c-Src conformation. (a) The location of cysteine mutations in the SH2 domain of the c-Src^{XTC} sensor are modeled on the crystal structures of open (1Y57) and closed (2SRC) c-Src with ReAsH predicted to selectively bind the open conformation. (b) Three basic variants were used in this study. c-Src^{XTC} (exposable TC) binds biarsenical dyes (FlAsH or ReAsH) only in the open conformation. c-Src^{CTC} (constitutive TC) binds biarsenical dyes regardless of conformation due to a tetracycline tag at the C-terminus of the fluorescent protein fusion. c-Src^{NTC} (no TC) lacks a tetracycline tag. (c) Confocal micrographs of AD293 cells expressing c-Src^{XTC} superimposed onto open or closed mutant backgrounds. All variants are fused to Emerald fluorescent protein.

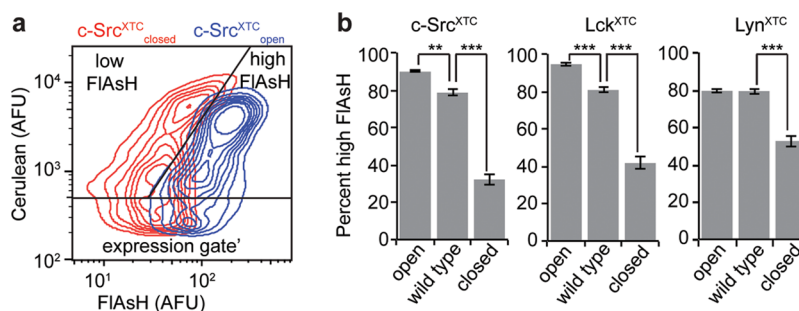


Figure 2. Exposable TC sensor is adaptable to multiple SFKs. (a) Gating strategy for flow cytometry analysis showing FIAsH-labeled AD293 cells expressing Cerulean-fused c-Src^{XTC}_{open} (blue) or c-Src^{XTC}_{closed} (red). Cells were gated first for high expression and then for high or low FIAsH. The percent of high expressers that are high FIAsH reactive was used for sample analysis. (b) c-Src^{XTC}, Lck^{XTC}, and Lyn^{XTC} biosensors successfully distinguish open and closed mutant backgrounds using flow cytometry. Shown are means $\pm$ SE, $n = 3$. ** $p < 0.01$; *** $p < 0.001$; one-tailed t test.

expression and localization, the monomeric GFP variant Emerald was fused to the C-terminus, via a linker sequence previously validated to preserve the major functionality of c-Src¹⁴ (Figure 1b).

c-Src^{XTC}, c-Src^{XTC_{open}}, and c-Src^{XTC_{closed}} were assessed for ReAsH red-fluorescent biarsenical dye reactivity in live cells, relative to a negative control (c-Src^{NTC}) with no TC tag and a positive control (c-Src^{CTC}) that had the TC tag appended in a highly accessible position on the C-terminus of Emerald (Figure 1b, Supplementary Table 1). c-Src^{XTC_{open}} had approximately 80% of the fluorescent yield of the positive control, c-Src^{XTC_{closed}} was similar to the negative control, and c-Src^{XTC} was intermediate between open and closed variants (Figure 1c). These data indicate that c-Src^{XTC} reports on c-Src conformation with good differential in the signal between the open and closed states.

For a more quantitative and high-throughput assessment using flow cytometry, we adapted the reporter color scheme for standard flow cytometry filter sets and laser combinations (to which ReAsH is not well-suited). The Emerald moiety was replaced with the monomeric Cerulean fluorescent protein, and the binding of the TC tag was examined with the green-fluorescent biarsenical dye FAsH. Cytograms plotting Pacific-Blue (for Cerulean) versus FITC (for FAsH) confirmed conformation-dependent differences in FAsH reactivity, similar to the patterns observed by microscopy with the Emerald/ReAsH combination (Figure 2a). A simple quantitative measure of “closure” was found by gating for high FAsH fluorescence and calculating the percent of cells within this gate (Figure 2a,b). The majority of cells expressing c-Src^{XTC_{open}} were high-FAsH-reactive, whereas the majority of those expressing c-Src^{XTC_{closed}} were low-FAsH-reactive (Figure 2a,b).

To make the reporter more generally useful as a tool for the study of SFKs, we examined whether other members of the family could be converted into biosensors of open and closed conformations using the same strategy. The exposable TC motif was introduced at the equivalent SH2 locations of Lyn and Lck, and the biosensors were superimposed onto open and closed mutant backgrounds. The Lck and Lyn sensors also exhibited conformation-sensitive FAsH reactivities (Figure 2b). All c-Src^{XTC}, Lck^{XTC}, and Lyn^{XTC} had FAsH reactivities significantly higher than those of their respective closed variants (Figure 2b). Because SFKs are largely inactive under basal conditions,¹⁵ our results raise the possibility that XTC mutations shift the conformational equilibrium toward the open state. Examination of the ability of the SFKs to phosphorylate substrate STAT3 found that the XTC mutations led to an increased basal kinase activity (Figure 3a). The SH2 domain of c-Src^{XTC} remained folded (Figure 3b) but had a 3-fold decreased binding affinity to phosphotyrosine (Figure 3c), which accounts for the destabilization of the closed state. In addition, we cannot exclude the possibility that the mutated residues abrogate direct or water-mediated electrostatic interactions with the C-lobe of the kinase domain of Src. This may limit the application of the biosensors to physiological problems. However, the shift toward the open state provides the dynamic range required to enable screening for compounds that induce closure.

Therefore, we next assessed whether the XTC sensors can be used to assay drugs for their effect on c-Src conformation. To our knowledge, there are no high-throughput drug screening platforms that assess changes in the regulatory state of SFKs in live cells, which have the potential to be used to identify

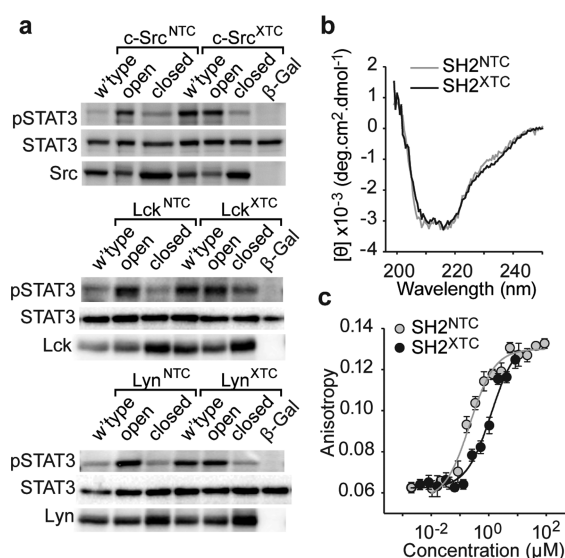


Figure 3. Effect of exposable TC mutations on structure and activity of SFKs. (a) Kinase activity of SFKs as indicated by levels of phosphorylated substrate STAT3 (pSTAT3), compared to total STAT3. (b) Circular dichroism spectra of the purified no-TC (NTC) and TC-containing (XTC) SH2 domains indicate the tetracycysteine-containing domain folds properly. (c) Binding curves for the interaction of the SH2 domains with the high affinity pYEEI peptide ligand. The fluorescence anisotropy of FITC-labeled peptide was measured versus protein concentration. Error bars on data points indicate SE from five repeated measures. The TC mutation caused a decrease in affinity of the SH2 for the peptide (K_d from 0.43 ± 0.07 μM to 1.50 ± 0.21 μM ; errors indicate SE of the estimate from the fit).

inhibitors with novel mechanisms of action. To test for this capability, the c-Src^{XTC} biosensor was screened against a library of 72 kinase inhibitors that had no inherent fluorescence based on a prescreen on untransfected cells. After a 2-h treatment with the drugs at 5 μM , COS-1 cells were labeled with FAsH and analyzed by flow cytometry. The screen identified drugs that appeared to induce c-Src closure as well as those that appeared to open c-Src; however, the majority had no statistically significant effect on c-Src^{XTC} conformation, including compounds known to inhibit c-Src (Supplementary Table 2). This is unsurprising since the majority of kinase inhibitors target the ATP binding site in the active DFG-in conformation (as opposed to the catalytically inactive DFG-out conformation)¹⁶ and would therefore not be expected to induce c-Src closure. For example, the clinical c-Src and BCR-Abl inhibitor bosutinib binds c-Src in the DFG-in conformation¹⁷ and produced no significant change in FAsH binding in the drug screen ($p = 0.64$; Supplementary Table 2). By assaying conformation rather than activity, the biosensor has the potential to identify inhibitors with novel mechanisms of action and potentially how changes in the cellular signaling landscape impact on Src conformation through indirect pathways. Six compounds that increased c-Src closure were identified: PP1, PP2, tozasertib, CYC116, vandetanib, and N2-[4-(aminomethyl)phenyl]-5-fluoro-N4-phenylpyrimidine-2,4-diamine. Vandetanib targets EGFR and VEGFR;¹⁸ however, it has been shown to bind and inhibit c-Src.¹⁹ Tozasertib and CYC116 target Aurora kinases^{20,21} and are reported to inhibit c-Src.²² N2-[4-(Aminomethyl)phenyl]-5-fluoro-N4-phenylpyrimidine-2,4-diamine is a pan kinase inhibitor; however, to our knowledge there is no available data specific to c-Src. PP1 and

PP2 are gold standard c-Src inhibitors, with IC_{50} values of 50 and 100 nM, respectively in *in vitro* kinase assays.²³

We further validated PP1 and PP2 by concentration–response curves to calculate the half maximal effective concentration (EC_{50}) for c-Src^{XTC} conformational shift (Figure 4). Both PP1 and PP2 EC_{50} values were estimated to be in the

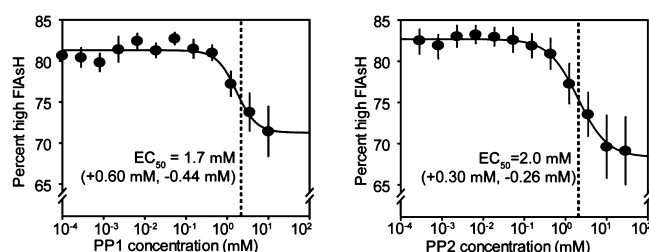


Figure 4. c-Src^{XTC} detects inhibitor-induced conformational closure. Concentration–response curves are shown for two hits from the drug screen, SFK inhibitors PP1 and PP2. Error bars show means $\pm$ SE ($n = 8$); reported error in EC_{50} values are SE of the estimate from the fit.

low micromolar range. These values agree with other cell-based dose–response data for PP2;²⁴ however, they are over 2 orders of magnitude greater than the *in vitro* IC_{50} values. These results demonstrate limitations of *in vitro* data for biological problems and highlight the importance of cell-based assays in drug development.

Notably, no drugs induced complete closure of c-Src^{XTC}. Both PP1 and PP2 reduced percent of high FIAsh cells from over 80% to just under 70% (Figure 4), whereas the closed mutant had approximately 30% high FIAsh (Figure 2b). The drugs are likely to alter the equilibrium between open and closed conformations; how this occurs remains to be determined. One possibility is that drug-induced changes in the active site conformation further toggle the open and closed states. Another consideration is that the high expression level of the SFKs in our assays may lead to hyperphosphorylation of the activation loop, which conceivably influences the potency of the drugs for conformational change.

In summary, we have developed a novel genetically encoded biosensor to study conformation of three SFKs, c-Src, Lck, and Lyn, with just 4 point mutations. Thus, the XTC biosensor strategy provides a new capability to study modulation of SFK conformation in living cells and is in principle adaptable to other proteins, especially those to which detailed structural knowledge is available for modeling.

METHODS

DNA Constructs. c-Src (NP_005408), Lyn (NP_002341), and Lck (NP_005347) cDNA appended C-terminally with linkers encoding amino acids GSGSDPPVAT, GSGSGSDPPVAT, and AGSGSGSDPPVAT respectively, as well as XTC variants thereof (R159C, E160C, R163C, L164C), (K137C, D138C, R141C, Q142C), and (K135C, D136C, R139C, Q140C), respectively, were commercially synthesized using human-optimized codons and cloned into pT-Rex (Life Technologies). Cerulean, Emerald,²⁵ and the C-terminal amino acid sequence CCPGCC (for c-Src^{CTC}) were introduced via PCR-mediated cloning. Open (Src: Y530F; Lyn: Y508F; Lck: Y505F) and closed (Src: Q531E, P532E, G533I; Lyn: Q509E, Q510E, Q511I; Lck: Q506E, P507E, Q508I) mutations were introduced using QuikChange mutagenesis (Stratagene).

Cloning and Expression of the SH2 Domain. SH2 sequences (residues 145–249) were cloned via PCR from c-Src^{XTC} and c-Src^{NTC} into pET21(d). A 6 $\times$ His-tag was added to the N-terminus of the SH2^{XTC} by PCR-mediated cloning. Proteins were expressed in

BL21(DE3) *E. coli* and purified by phosphotyrosine affinity chromatography (SH2^{NTC}) or Ni²⁺-affinity chromatography (SH2^{XTC}) to >95% purity. Correct masses were confirmed using a 6510 Q-TOF LC–MS (Agilent Technologies).

Mammalian Cell Culture. AD293 and COS-1 cells were maintained in DMEM supplemented with 10% FCS, 1 mM glutamine, 100 U/mL penicillin, and 100 U/mL streptomycin in a humidified incubator with 5% CO₂.

ReAsH Staining and Imaging. AD293 cells (8×10^4) were plated into 8-well microslides (Ibidi) precoated with 0.01% (w/v) poly-L-lysine and transfected the following day with 0.5 μ g of DNA using Lipofectamine 2000. After 24 h, cells were labeled for 30 min with 10 μ M 1,2-ethanedithiol (EDT) and 2.5 μ M ReAsH (Life Technologies) in HBSS and then washed for 15 min in 250 μ M 2,3-dimercaptopropanol (BAL) in HBSS. Cells were fixed with 3.2% (w/v) paraformaldehyde for 15 min at RT and then imaged by a TCS SP2 confocal microscope (Leica). Emerald and ReAsH fluorescence were collected using 458 and 543 nm excitation lasers and emission wavelengths of 510–530 and 580–640 nm, respectively.

Western Blotting. Western blotting was performed as described previously.¹³ Blots were matched for total STAT3, and then equivalent blots were probed for phospho-STAT3 or GFP (for SFK levels). Antibodies used were 1:10,000 anti-GFP from Life Technologies, 1:1,000 anti-STAT3 from BD Biosciences, and 1:1,000 anti-phospho STAT3 from Cell Signaling.

Flow Cytometry and Analysis. Preparation of cells for flow cytometry, FIAsh labeling, and the cytometer configuration were as described previously.²⁶ Cerulean and FIAsh fluorescence were collected using the Pacific blue (405 nm laser, 450/50 nm bandpass filter) and FITC (488 nm laser, 530/30 nm bandpass filter) channels, respectively.

Data were analyzed using FlowJo (TreeStar, Inc.) by gating for high expression (Cerulean fluorescence above 500 AFU) and then for high or low FIAsh reactivity. The high FIAsh gate was defined as a diagonal line in a logarithmic FIAsh to Cerulean cytogram that was adjusted by translation so as to contain 80% cells expressing c-Src^{XTC}. The percent of high expressing cells with high FIAsh was calculated.

For drug screening, 6.6×10^4 COS-1 cells were transfected in solution with 0.32 μ g of c-Src^{XTC}-Cerulean DNA using 0.4 μ L of Lipofectamine 2000 and plated in 96-well tissue culture plates. The following day, cells were treated for 2 h with 5 μ M drug or DMSO vehicle in HBSS. PP1 and PP2 were from Sigma, and all other drug screen compounds were supplied by the Walter and Eliza Hall HTS group. FIAsh staining was performed as described previously.³ Cell suspension (100 μ L) was analyzed using an LSRFortessa flow cytometer equipped with a high-throughput sampler at 2.5 μ L/s. Three independent experiments were conducted with randomized compound locations. Concentration–response curves were performed under the same conditions with 8 replicates. Half-maximal effective concentrations (EC_{50} values) were estimated by fitting to a four-parameter sigmoidal equation using Sigmaplot 12 (Systat).

Statistics. For the drug screen, percent high FIAsh values were normalized to plate means and standard deviations using the z-score method.²⁷ z-Scores across replicates were compared to DMSO vehicle controls, and hits were identified via a two-tailed *t* test with respect to the DMSO control, with a cutoff of $p < 0.05$.

Circular Dichroism Spectroscopy. Circular dichroism (CD) spectra of 0.2 mg/mL protein were measured in a 1 mm path length cuvette in 10 mM phosphate pH 7, 1 mM DTT at 20 $^{\circ}$ C using a model 410 SF CD spectrometer (Aviv) at 100 nm/min in continuous scanning mode with a 0.5 nm data-pitch. Final spectra were averages of 3 scans with buffer subtraction.

SH2 Binding Assay. The phosphotyrosyl peptide corresponded to residues 321–331 of the hamster polyomavirus middle T antigen (NP_056731) phosphorylated at Tyr342 (pY) was synthesized commercially (GL Biochem) with FITC attached to the N-terminus via aminocaproic acid (Acp) (FITC-Acp-EPQpYEEIPIYL). Dilution titrations were measured in 50 mM Tris pH 7.4, 150 mM NaCl, 12.5 mM imidazole, 1 mM TCEP using a Cary Eclipse fluorescence spectrophotometer with automated polarizers (Agilent Technologies).

Equilibrium dissociation constant (K_d) values were calculated as described previously.²⁸

■ ASSOCIATED CONTENT

■ Supporting Information

Supplementary Tables 1 and 2. This material is available free of charge via the Internet at <http://pubs.acs.org>.

■ AUTHOR INFORMATION

Corresponding Authors

*E-mail: tmulhern@unimelb.edu.au.

*E-mail: dhatters@unimelb.edu.au.

Author Contributions

^{||}These authors contributed equally to this work.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

The work was funded in part by grants to D.M.H. and T.D.M. from the Australian National Health and Medical Research Council and the Australian Research Council. D.M.H. was a Grimwade Fellow, supported by the Miegunyah Trust and currently an ARC Future Fellow. The Huang lab is supported by the Australian National Health and Medical Research Council (Program Grant 1016701; Independent Research Institutes Infrastructure Support Scheme grant 361646), the Leukemia and Lymphoma Society (SCOR grants) and a Victorian State Government Operational Infrastructure Support (OIS) Grant.

■ REFERENCES

- (1) Bermejo, C.; Ewald, J. C.; Lanquar, V.; Jones, A. M.; and Frommer, W. B. (2011) *In vivo* biochemistry: quantifying ion and metabolite levels in individual cells or cultures of yeast. *Biochem. J.* 438, 1–10.
- (2) Griffin, B. A.; Adams, S. R.; and Tsien, R. Y. (1998) Specific covalent labeling of recombinant protein molecules inside live cells. *Science* 281, 269–272.
- (3) Ramdzan, Y. M.; Nisbet, R. M.; Miller, J.; Finkbeiner, S.; Hill, A. F.; and Hatters, D. M. (2010) Conformation sensors that distinguish monomeric proteins from oligomers in live cells. *Chem. Biol.* 17, 371–379.
- (4) Luedtke, N. W.; Dexter, R. J.; Fried, D. B.; and Schepartz, A. (2007) Surveying polypeptide and protein domain conformation and association with FLAsH and ReAsH. *Nat. Chem. Biol.* 3, 779–784.
- (5) Ignatova, Z.; and Gierasch, L. M. (2004) Monitoring protein stability and aggregation *in vivo* by real-time fluorescent labeling. *Proc. Natl. Acad. Sci. U.S.A.* 101, 523–528.
- (6) Playford, M. P.; and Schaller, M. D. (2004) The interplay between Src and integrins in normal and tumor biology. *Oncogene* 23, 7928–7946.
- (7) Cowan-Jacob, S. W.; Fendrich, G.; Manley, P. W.; Jahnke, W.; Fabbro, D.; Liebetanz, J.; and Meyer, T. (2005) The crystal structure of a c-Src complex in an active conformation suggests possible steps in c-Src activation. *Structure* 13, 861–871.
- (8) Xu, W.; Harrison, S.; and Eck, M. (1997) Three-dimensional structure of the tyrosine kinase c-Src. *Nature* 385, 595–602.
- (9) Wang, Y.; Botvinick, E.; Zhao, Y.; Berns, M.; Usami, S.; Tsien, R.; and Chien, S. (2005) Visualizing the mechanical activation of Src. *Nature* 434, 1040–1045.
- (10) Gulyani, A.; Vitriol, E.; Allen, R.; Wu, J.; Gremyachinskiy, D.; Lewis, S.; Dewar, B.; Graves, L. M.; Kay, B. K.; and Kuhlman, B. (2011) A biosensor generated via high-throughput screening quantifies cell edge Src dynamics. *Nat. Chem. Biol.* 7, 437–444.
- (11) Karatan, E.; Merguerian, M.; Han, Z.; Scholle, M. D.; Koide, S.; and Kay, B. K. (2004) Molecular recognition properties of FN3 monobodies that bind the Src SH3 domain. *Chem. Biol.* 11, 835–844.
- (12) Adams, S. R.; Campbell, R. E.; Gross, L. A.; Martin, B. R.; Walkup, G. K.; Yao, Y.; Llopis, J.; and Tsien, R. Y. (2002) New biarsenical ligands and tetracysteine motifs for protein labeling *in vitro* and *in vivo*: Synthesis and biological applications. *J. Am. Chem. Soc.* 124, 6063–6076.
- (13) Irtegun, S.; Wood, R. J.; Ormsby, A. R.; Mulhern, T. D.; and Hatters, D. M. (2013) Tyrosine 416 is phosphorylated in the closed, repressed conformation of c-Src. *PLoS One* 8, e71035.
- (14) Sandilands, E.; Cans, C.; Fincham, V. J.; Brunton, V. G.; Mellor, H.; Prendergast, G. C.; Norman, J. C.; Superti-Furga, G.; and Frame, M. C. (2004) RhoB and actin polymerization coordinate Src activation with endosome-mediated delivery to the membrane. *Dev. Cell* 7, 855–869.
- (15) Cooper, J. A.; Gould, K. L.; Cartwright, C. A.; and Hunter, T. (1986) Tyr527 is phosphorylated in pp60c-src: implications for regulation. *Science* 231, 1431–1434.
- (16) Yi, L.; and Gray, N. S. (2006) Rational design of inhibitors that bind to inactive kinase conformations. *Nat. Chem. Biol.* 2, 358–364.
- (17) Levinson, N. M.; and Boxer, S. G. (2013) A conserved water-mediated hydrogen bond network defines bosutinib's kinase selectivity. *Nat. Chem. Biol.* 10, 127–132.
- (18) Brave, S. R.; Odedra, R.; James, N. H.; Smith, N. R.; Marshall, G. B.; Acheson, K. L.; Baker, D.; Howard, Z.; Jackson, L.; and Ratcliffe, K. (2011) Vandetanib inhibits both VEGFR-2 and EGFR signalling at clinically relevant drug levels in preclinical models of human cancer. *Int. J. Oncol.* 39, 271–278.
- (19) Jia, H.-Y.; Wu, J.-X.; Zhu, X.-F.; Chen, J.-M.; Yang, S.-P.; Yan, H.-J.; Tan, L.; Zeng, Y.-X.; and Huang, W. (2009) ZD6474 inhibits Src kinase leading to apoptosis of imatinib-resistant K562 cells. *Leuk. Res.* 33, 1512–1519.
- (20) Harrington, E. A.; Bebbington, D.; Moore, J.; Rasmussen, R. K.; Ajose-Adeogun, A. O.; Nakayama, T.; Graham, J. A.; Demur, C.; Hercend, T.; Diu-Hercend, A.; Su, M.; Golec, J. M.; and Miller, K. M. (2004) VX-680, a potent and selective small-molecule inhibitor of the Aurora kinases, suppresses tumor growth *in vivo*. *Nat. Med.* 10, 262–267.
- (21) Wang, S.; Midgley, C. A.; Scaërou, F.; Grabarek, J. B.; Griffiths, G.; Jackson, W.; Kontopidis, G.; McClue, S. J.; McInnes, C.; and Meades, C. (2010) Discovery of N-phenyl-4-(thiazol-5-yl) pyrimidin-2-amine aurora kinase inhibitors. *J. Med. Chem.* 53, 4367–4378.
- (22) Bain, J.; Plater, L.; Elliott, M.; Shpiro, N.; Hastie, C.; Mclauchlan, H.; Klevernic, I.; Arthur, J.; Alessi, D.; and Cohen, P. (2007) The selectivity of protein kinase inhibitors: a further update. *Biochem. J.* 408, 297–315.
- (23) Karni, R.; Mizrahi, S.; Reiss-Sklan, E.; Gazit, A.; Livnah, O.; and Levitzki, A. (2003) The pp60(c-Src) inhibitor PP1 is non-competitive against ATP. *FEBS Lett.* 537, 47–52.
- (24) Han, X.; Yamanouchi, G.; Mori, T.; Kang, J.-H.; Niidome, T.; and Katayama, Y. (2009) Monitoring protein kinase activity in cell lysates using a high-density peptide microarray. *J. Biomol. Screening* 14, 256–262.
- (25) Olshina, M. A.; Angley, L. M.; Ramdzan, Y. M.; Tang, J.; Bailey, M. F.; Hill, A. F.; and Hatters, D. M. (2010) Tracking mutant huntingtin aggregation kinetics in cells reveals three major populations that include an invariant oligomer pool. *J. Biol. Chem.* 285, 21807–21816.
- (26) Ramdzan, Y. M.; Polling, S.; Chia, C. P.; Ng, I. H.; Ormsby, A. R.; Croft, N. P.; Purcell, A. W.; Bogoyevitch, M. A.; Ng, D. C.; Gleeson, P. A.; and Hatters, D. M. (2012) Tracking protein aggregation and mislocalization in cells with flow cytometry. *Nat. Methods* 9, 467–470.
- (27) Brideau, C.; Gunter, B.; Pikounis, B.; and Liaw, A. (2003) Improved statistical methods for hit selection in high-throughput screening. *J. Biomol. Screening* 8, 634–647.
- (28) Mills, R. D.; Trewella, J.; Qiu, T. W.; Welte, T.; Ryan, T. M.; Hanley, T.; Knott, R. B.; Lithgow, T.; and Mulhern, T. D. (2009)

Domain organization of the monomeric form of the Tom70 mitochondrial import receptor. *J. Mol. Biol.* 388, 1043–1058.