Discerning the regulation and evolution of Src and Abl kinases

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Abstract

Discerning the regulation and evolution of Src and Abl kinases

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Kinases are responsible for protein phosphorylation, which regulates a myriad of cellular activities pertaining to cell proliferation, growth, and communication. The unregulated activity of kinases leads to uncontrolled cell growth and, ultimately, cancer; therefore, this has caused kinases to be critical drug targets and has elicited extensive research aimed at elucidating the details of their regulation. Src and Abl are two non-receptor tyrosine kinases that share structural similarities in their auto-inhibited states, but have distinct mechanisms by which they achieve those states. Published crystal structures have revealed essential phosphorylation sites and allosteric players that regulate activity of these enzymes. However, specifics on the quantitative effects of these regulators remain elusive.

Here, we set out to provide detailed kinetic schemes for the regulation of Src and Abl and provide insight into the evolution of their regulation. As a starting point, we determined the quantitative effect of modifying the essential tyrosine residues and later proceeded to investigate the effects of the allosteric regulators on kinase activity. Our results demonstrate that
Src and Abl are not regulated by simple ‘on’ and ‘off’ switches where they exist only in two states; instead, they exist in equilibria between various states with different activities.

In order to gain a better understanding of the evolution of Src and Abl regulation, we evaluated the effects of various regulatory players on the activity of common ancestors of Src and Abl. Interestingly, we observed a clear pattern: the effect of phosphorylation and other regulatory elements was greater in modern enzymes and gradually decreased for older ancestors.

Our kinetic experiments provided detailed quantitative information about the effect of different players on Src and Abl activity. With this information in hand, we are now proceeding to developing a single-molecule FRET assay. The main objective of this experiment is to couple the observed kinetic effects with structural information. This will shed light on the interplay between different conformational ensembles of Src and Abl, affording us a better understanding of their regulation.
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List of Abbreviations Used

Anc: Ancestor
ATP: Adenosine triphosphate
BSA: Bovine Serum Albumin
CSK: C-terminal Src kinase
FL: Full-length
IPTG: Isopropyl-Beta-D-thiogalactoside
KD: Kinase domain
LDH: Lactate dehydrogenase
MBP: Maltose binding column
MgCl2: Magnesium Chloride
NaCl: Sodium Chloride
NADH: Nicotinamide adenosine dinucleotide
p: Phosphorylated
PK: Pyruvate kinase
SDS: Sodium dodecyl sulphate
SH2: Src homology 2
SH3: Src homology 3
SHIP2: SH2 domain-containing inositol phosphatase
TCEP: Tris(2-carboxyethyl)phosphine
Tris: Trisaminomethane
Up: Unphosphorylated
WT: Wild-type
Y244F: Tyrosine 244 to phenylalanine
Y324F: Tyrosine 324 to phenylalanine
Y328F: Tyrosine 328 to phenylalanine
Y411F: Tyrosine 411 to phenylalanine
YoPH: Yersinia tyrosine phosphatase
Introduction

Without the presence of protein kinases, the transfer of phosphate groups in physiological conditions can take more than 8,000 years; whereas, kinases catalyze these reactions in a matter of milliseconds (Kerns et al. 2014). Phosphoryl-transfer serves as an ‘On-off” switch for various proteins that are involved in cellular events such as cell growth, differentiation and signaling, which attests to the significant role played by kinases. The disruption of kinase activity can therefore result in uncontrolled cell growth, eventually leading to cancer. This has rendered kinases important therapeutic targets.

In spite of great diversity within the kinase genome, the substrate binding site of kinases is highly conserved, making it difficult to attain highly selective drugs. This has inspired a more profound understanding of the differential regulation of kinases, for the advancement of intelligent drug design (Wilson et al. 2015).

Src and Abl

Src and Abl are non-receptor tyrosine kinases that have about 50% similarity at the sequence level but show notable differences on how they attain their active and inactive states. One of the similarities between the tyrosine kinase families is the kinase domain, which is known to play a crucial role in signaling and activating other proteins. Auto-inhibition, the intramolecular self-regulation of biological molecules, is a key regulatory technique employed by both Src and Abl kinases. Src and Abl have single SH2 and SH3 domains that serve as regulatory domains closely
policing the activity of the kinase domain. The regulation of Src and Abl is a complex interplay of various biochemical and structural states.

**Src**

Src is regulated by a cohort of various regulatory elements such as phosphorylation of critical tyrosine residues and inter-domain interactions that play a significant role in attaining the auto-inhibited state. Src kinases consist of a unique myristoylated N-terminal domain, used to anchor the kinases to the membrane, followed by the SH3, SH2 and kinase domains and a C-terminal tail at the end (Xu et al. 1999). Previous structural studies have revealed two tyrosine residues that are crucial for the regulation of Src: Y416 in the activation loop and Y527 on the C-terminal tail. Phosphorylation of Y416 is known to have a positive regulatory role in the regulation of Src; whereas phosphorylation of Y527 is known to play an auto-inhibitory role.
Figure 1. Open and closed conformations of Src.
a) Open and closed conformations of Src (PDB 2Src and PDB 1Src). b) Conformational changes observed in inactive Src. The C-helix of Src protrudes outward in inactive Src, forcing the activation loop to have a folded structure, hiding Y416 and blocking substrate-binding site. The inward movement of the C-helix, induced by the disruption of inter-domain interactions, results in the extension of the A-loop and exposure of Y416.

Inactive state of Src
Src is very tightly regulated in cells with about 90% of the enzyme found in its inactive, closed form (Okada 2012). The auto-inhibited state of Src is a result of various intramolecular inter-domain interactions. As seen in Figure 1, phosphorylation of Y527 at the C-terminal tail initiates an auto-inhibitory mechanism by docking into the SH2 domain via salt bridge interactions. Unlike Y416, which can be auto-phosphorylated, the phosphorylation of Y527 requires an additional kinase, CSK. CSK is a highly specific kinase that is involved in the tail phosphorylation of the Src family kinases, an essential step in their inhibitory mechanisms.

Pi-stacking interactions between proline-rich SH2-kinase linker region and the SH3 domain further stabilize this closed conformation (Parsons and Parsons 2004). These conformational changes in the SH2/SHP domains induce a change in the catalytic site of the kinase domain, forcing the C-helix to protrude outward. This in turn disrupts hydrogen bonding interactions between Glu510 and Lys254 in the ATP binding site, disrupting Mg\(^{2+}\)-ATP coordination (Shukla et al. 2014). The protrusion of the C-helix in the kinase domain buries Y416, protecting it against phosphorylation and also blocks the substrate-binding site, inactivating the kinase (Figure 2) (Xu et al. 1999).
Figure 2. Active and Inactive conformations of active site in Src.

a) Inactive conformation of Src: C-helix is protruding outwards, A-loop is folded and Y416 is buried. The folding of the A-loop blocks substrate binding. b) Active conformation of Src: C-helix I folded inwards with Glu310 forming hydrogen bonds with Lys295, allowing Mg$^{2+}$-ATP coordination. A-loop is unfolded, Y416 exposed. Substrate can now bind (picture modified from (Shukla et al. 2014)).

**Active state of Src**

Three general steps, in which Src gets activated, have been identified previously. The first step involved the interruption of the inter-domain interactions by activating proteins that bind the SH2 or SH3 domains of Src. The interruption of these inter-domain interactions destabilizes the closed conformation of Src making it possible for phosphatases such as SHIP2 to dephosphorylate Y527 on the C-terminal tail (Roskoski 2005). Dephosphorylation of Y527 is required for the phosphorylation of Y416, which is crucial for opening up of the catalytic site for substrate binding. Autophosphorylation is the mechanism by which residues are phosphorylated intramolecularly without a need for external kinases.

In the open, active conformation of Src, the C-helix in the kinase domain is pushed in. This allows Glu310 and Lys295 to form hydrogen bonding interactions that are essential for Mg$^{2+}$-ATP coordination. The inward movement of C-helix also induces conformational change
in the kinase domain, unfolding the activation loop and exposing Y416 for autophosphorylation (Figure 2). This outward movement and unfolding of the activation loop, opens up the active site and removes blockage of substrate binding (Shukla et al. 2014). Phosphorylation of Y416 provides the final step in ‘locking’ the active site in its fully active conformation (Okada 2012).

**Abl**

Similar to Src, Abl also makes use of various regulatory elements to achieve its auto-inhibited and activated states. Abl kinase constitutes of a myristoylated N-terminal domain, an SH2, an SH3 and a kinase domain. Two tyrosine residues, Y411 in the activation loop and Y244 in the SH2-kinase linker, are known to play critical roles in the regulation of Abl. However, unlike Src where one of the tyrosine residues plays an activating role and the other an inhibitory role, both tyrosine residues in Abl have an activating effect.

**Figure 3. Inactive conformations of Src and Abl.**

The SH2 domain binds more closely to the kinase domain in Abl than in Src. The SH3 domain interacts with the SH2-kinase linker by forming a poly-proline type II helix, further stabilizing the inactive conformations. Both the SH2 and SH3 domains play a significant role in the inhibition of Src and Abl. Myristoyl binding potentiates the inactive state of Abl whereas phosphorylation of Y527 plays the significant role in Src auto-inhibition. Picture modified from (Hantschel 2012).
**Inactive state of Abl**

Unlike Src, Abl does not have a C-terminal tyrosine residue that triggers its auto-inhibited state. Instead, it gets inactivated by a myristoyl switch. The binding of a myristoyl group to the hydrophobic pocket of the kinase domain elicits a bend in $\alpha I$, initiating hydrogen bonding interactions between the SH2 domain and the newly formed bend between $\alpha I$ and $\alpha I'$ in the kinase domain (Figure 4).

![Figure 4. Binding of myristoyl group to Abl stabilizes the inactive conformation.](image)

The kinase domain of Abl contains a hydrophobic crevice where myristoyl binds (picture modified from Nagar et al. 2003). The binding of myristoyl induces a kink $\alpha I$ helix, forming a new I-I' loop that interacts with the SH2 domain, favoring the closed conformation. The conformational changes in the kinase domain result in a flipped conformation of Asp400, preventing ATP-Mg$^{2+}$ coordination in the active site and inactivating the kinase (Nagar et al. 2003).

The SH3 domain also plays a regulatory role in the inactivation of Abl by forming a poly-proline II helix with the SH2-kinase linker (Figure 3). In addition to these stabilizing inter-domain interactions, Y244 interacts with Lys313 and Pro315 in a hydrophobic pouch of the kinase domain contributing to the closed conformation of Abl (Nagar et al. 2003).
Active state of Abl

Phosphorylation of Y244 on the SH2-kinase linker potentiates activation of Abl by disrupting the ideal poly-proline II geometry between the linker and the SH3 domain. The SH2 and SH3 domains now partake in intermolecular interactions with other activator proteins, disrupting the inter-domain interactions that stabilize the closed, inactive state (Hantschel 2012). Furthermore, autophosphorylation of Y416 compels an electrostatic interaction between a nearby arginine residue, stabilizing the open conformation of the active site and allowing substrate binding (Panjarian et al. 2013).

Ancestral Sequence Resurrection (ASR)

Modern day Src, Abl and other similar kinases evolved from a similar ancestor over billions of years (Wilson et al. 2015). Understanding the evolution of regulation of Src and Abl will help us gain a better understanding of their mechanisms of action and how their differences came about, which in turn will shed a light on more general principles behind the evolution of kinases. Therefore, we used ancestral sequence resurrection to gain insight into the mechanistic details of Src and Abl regulation. Modern day non-receptor tyrosine kinases were used in a Bayesian phylogenetic analysis with Ser/Thr kinases as an out-group. Roman Agafonov used Bali-Phy and PAML, programs used to estimate evolutionary trees and optimize alignments, to construct the most probable sequences for ancestral kinases of Src and Abl. We resurrected a total of five ancestors, three of which are common to both Src and Abl; whereas one is an ancestor of only Abl and another one is an ancestor of only Src.
We resurrected five ancestral kinases. Anc 76, the oldest, is a common ancestor of Fer, Tec, Src and Abl family kinases; whereas, the most recent ones we resurrected, Anc 108 and Anc 125, are common ancestors of the Src family and the Abl family respectively.

Anc 76 is the oldest of the resurrected ancestors. It is the common ancestor of the Fer, Tec, Src and Abl families. Anc 86 is common to the Tec, Fer and Src families; whereas, Anc 103 is the most recent ancestor between the Src and Abl families.

Below follows a brief description of the regulation and structure of Fer and Tec family kinases.

**Fer**
Activated Fer kinase plays a role in cellular transformation and is most likely involved in cell signaling cascades (Kim and Wong 1995). Fer constitutes of an SH2 domain, a coiled-coiled domain that mediates oligomerization and autophosphorylation of Fer, a FCH domain used to bind cytoskeletal proteins and a kinase domain (Green 2002). The SH3 domain is not present in Fer.

The SH2 domain is used for binding substrate proteins and mediating phosphotyrosine-dependent interactions that are involved in the upstream regulation of Fer. The flexibility of the
SH2-kinase linker has made it difficult to analyze the SH2-kinase interactions in Fer kinase (Green 2002).

Three important tyrosine residues, one of which is in the kinase domain activation loop, have been identified as important sites of autophosphorylation. However, autophosphorylation does not have any effect on the activation of Fer. Instead, trans-phosphorylation -the inter-molecular phosphorylation of residues by other kinases- of a different tyrosine residue is used to activate Fer. Binding of receptor proteins and growth factors to coiled-coiled domain and SH2 domain of Fer results in trans-phosphorylation, which in turn activates the enzyme. For example, PDGF has been shown to increase phosphorylation and activity of Fer (Kim and Wong 1995).

**Tec**

Similar to Src and Abl, the Tec family kinases are also involved in cell signaling, proliferation and differentiation. Structurally speaking, Tec kinases resemble Src family kinases. They both contain a unique N-terminal domain, single SH2 and SH3 domains and a kinase domain that is crucial for activity. Their differences lie in the presence of a myristoylation site and a C-terminal tail in Src. The myristoylated N-terminal sites of Src are replaced by a pleckstrin homology (PH) domain which serves a similar purpose of anchoring the kinases to the cell membrane. The proline reach Tec homology (TH) domain binds intramolecularly to a binding site in the SH3 domain, stabilizing the inactive conformation (Mano 1999).

Two tyrosine residues, Y223 in the SH3-TH interface and Y519 in the activation loop, are found to play essential roles in the regulation of Tec. Activation of Tec first requires the phosphorylation of Y519 by Src in order to open up the catalytic site and stabilize he active
conformation. This in turn leads to the autophosphorylation of Y223 in the SH3 domain, which disrupts the SH3-TH interface, achieving the fully activated state (Mano 1999).

The main goal of resurrecting common ancestors between the Fer, Tec, Src and Abl family of kinases was to observe the evolution of regulatory techniques employed by both Src and Abl, such as autophosphorylation. Whereas, Anc 108, from the Src family branch, and Anc 125 from the Abl family branch, were resurrected with an attempt to investigate the evolution of regulatory techniques that are specific to only Src and only Abl, respectively.
Results and Discussion

There are two distinct parts to my project on the regulation and evolution of Src and Abl. The first part is where we used kinetic assays, point mutations and ancestral sequence resurrection to get a detailed kinetic scheme of Src and Abl regulations and to get a better understanding of their evolution. The second part of the project is where we proceed to use single-molecule Fluorescence Resonance Energy Transfer (FRET) to couple the detailed kinetic information we obtained with structural information on the various conformational ensembles of Src and Abl.

Part 1- Regulation and evolution of Src and Abl

Regulation of Src

Effect of phosphorylation on Src regulation

Two important residues for the regulation of Src are Y416 in the activation loop and Y527 on the C-terminal tail. Phosphorylation of Y416 is known to activate Src; whereas, phosphorylation of Y527 reduces activity by favoring the inactive, closed state. We are interested in quantifying the effect phosphorylation of these two residues has on the regulation of Src. In order to do so, we first investigated Src’s ability to autophosphorylate using western blots and primary antibodies specific to pY416 and pY527.
Figure 6. Autophosphorylation of Src at Y416 and Y527.
Time points noted below the western blots are in minutes. Std stands for standard used as protein ladder. Conditions for autophosphorylation: 20µM of Src, 20 units/mL of PK, 30 units/mL of LDH, 150mM PEP, 20mM MgCl₂, 5mM ATP, 50mM Tris and 500mM NaCl at pH 8 and 25°C. a) Specific antibody against Y416 was used to observe the phosphorylation at Y416. b) Specific antibody against Y527 was used to observe autophosphorylation at Y527. c) Quantification of the autophosphorylation at Y416, used to determine the amount of time it requires to complete autophosphorylation. Src mainly autophosphorylates at Y416, with residual phosphorylation on Y527. It is completely phosphorylated within two hours.

We co-expressed YopH to make sure that Src was at its unphosphorylated state in the beginning of the autophosphorylation reactions, which can be demonstrated by the lack of an intensity band at the zero time point. We quantified the western blots to determine how long it takes for Src to be completely autophosphorylated. The intensity of the bands stops changing and the graph plateaus at four hours, indicating complete autophosphorylation of Src.
Src autophosphorylates readily at Y416 but, autophosphorylates marginally at Y527. Previous studies also suggest that Src needs another kinase, CSK, to phosphorylate at Y527 and attain an inactive confirmation.

CSK is a highly specific kinase that is found constitutively active in cells but only targets the Src family kinases (Levinson et al. 2008). Here, we utilized CSK to phosphorylate the tail tyrosine of Src. It is important to use a small concentration of Src (1µM) to get a clean phosphorylation of Y527 by CSK without interference of autophosphorylation of Y416. Figure 6(b) that shows no autophosphorylation occurred on the activation loop tyrosine at the noted concentration of Src in the presence of CSK. This shows we had a clean phosphorylation state.

![Image](image.png)

a) Y416 does not get phosphorylated by CSK  b) CSK phosphorylates Y527  c) Quantification for CSK phosphorylation of Src at Y527

**Figure 7. Phosphorylation of Src using CSK**

Time points noted below the western blots are in minutes. Std stands for standard used as protein ladder. Conditions for phosphorylation: 1µM of Src, 10µM of CSK, 20 units/mL of PK, 30 units/mL of LDH, 150mM PEP, 20mM MgCl₂, 5mM ATP, 50mM Tris and 500mM NaCl at pH 8 and 25°C. a) Phosphorylation of Y416 is undetectable b) CSK phosphorylates Y527. The western blots indicate CSK phosphorylates only Y527. c) Quantification of the western blot shows that phosphorylation by CSK on Y527 is completed within an hour.
After we established the amount of time required for Src to get completely phosphorylated, we used this information to phosphorylate Src at the two different residues separately in order to measure the effect of each phosphorylation on its activity.

Figure 8. Kinetic assays on full-length Src.
upSrc represents unphosphorylated Src, pSrc$^{Y416}$ represents Src phosphorylated at Y416 and pSrc$^{Y527}$ represents Src phosphorylated at Y527. 50nM of upSrc was used whereas pSrc$^{Y416}$ and pSrc$^{Y527}$ were at 3nM and 1uM respectively. Conditions for kinetic assays: 20 units/mL of PK, 30 units/mL of LDH, 150mM PEP, 20mM MgCl$_2$, 5mM ATP, 1mM TCEP, 50mM Tris, 0.3mg/mL BSA and 500mM NaCl at pH 8 and 25°C. A 60 fold difference in activity is observed between Src phosphorylated at Y416 and Src phosphorylated at Y527.

Src achieves its most inactive form, 0.5±0.1s$^{-1}$, when it is phosphorylated at Y527 on the C-terminal tail. Phosphorylation of Y527 ‘locks’ Src in its inactive state. This disrupts essential interactions in the catalytic site and ‘hides’ Y416, preventing autophosphorylation at this residue (Shukla et al. 2014).

For this reason, we were not able to get a doubly phosphorylated Src by phosphorylating Y416 after phosphorylating Y527. Instead, we had to phosphorylate Y416 first and then phosphorylate Y527 in order to get the doubly phosphorylated Src kinase. Although it is interesting to see what happens when both tyrosine residues are phosphorylated, it is worth noting that this doubly phosphorylated form of Src does not occur in vivo (Xu et al. 1999). Src phosphorylated at both Y416 and Y527 has a rate of 1.0± 0.2s$^{-1}$, which is twice more active than...
Src phosphorlated at Y527. On the other hand, phosphorylation of Src only at the Y416 gives about a threefold increase in activity as compared to the unphosphorylated Src.

Phosphorylation at Y416 has a 2-3 fold effect on the activity of Src (Figure 8). We wanted to explore further whether this 2-3 fold effect of was a result of shifting the equilibrium between open and closed conformations of Src. Or, if instead, phosphorylation of Y416 was only intrinsically affecting the kinase domain to increase activity. To test this, we expressed and purified the kinase domain alone and performed activity assays. The kinase domain of Src is active on its own and able to autophosphorylate.

![Graph showing the effect of activation loop phosphorylation on activity of Src kinase domain.](image)

**Figure 9. Effect of activation loop phosphorylation on activity of Src kinase domain.**

upSrc-KD represents unphosphorylated kinase domain Src, pSrc-KD represents Src kinase domain phosphorylated at Y416. 3nM of Src-KD was used for the kinetic assays. Conditions for kinetic assays: 20 units/mL of PK, 30 units/mL of LDH, 150mM PEP, 0.3mg/mL NADH, 20mM MgCl₂, 5mM ATP, 1mM TCEP, 50mM Tris, 0.3mg/mL BSA and 500mM NaCl at pH 8 and 25°C. Phosphorylation of Y416 increases the activity of the kinase domain of Src by about twofold.

Unphosphorylated kinase domain of Src is more active than full-length Src. This suggests that the SH2/SH3 domains play a significant role to down regulate Src even in its unphosphorylated state. Autophosphorylation of Y416 increases the activity of Src-KD about two folds, which is consistent with its effect on full-length Src. This suggests that phosphorylation at Y416 plays a positive regulatory role in Src by intrinsically affecting the
kinase domain. This indicates the existence of another equilibrium between active and inactive forms of the kinase domain in addition to the equilibrium that exists between open and closed states of Src.

**Effect of regulatory elements on activation of Src**

Various other SH2 and SH3 binding proteins are involved in the regulation of Src in vivo. About 90% of Src is found in its inactive state in the cells where it is phosphorylated at Y527. Thus, SH2/SH3 binding proteins and phosphatases play an essential role in activating Src via dephosphorylating Y527 and interfering with SH2/SH3-kinase domain interactions that keep the enzyme in its inactive form (Roskoski 2005). We mimicked these SH2/SH3 binding proteins by using SH2/SH3 binding peptides for our kinetic assays and measured their effect on Src activation.

**Figure 10. Effect of regulatory elements on the activity of phosphorylated Src.**

pSrc\textsuperscript{Y416} represents Src phosphorylated at Y416 and pSrc\textsuperscript{Y527} represents Src phosphorylated at Y527. 3nM of pSrc\textsuperscript{Y416} was used for the kinetic assays whereas, pSrc\textsuperscript{Y527} was at 1µM. 2mM of SH2 and SH3 peptides were used. Conditions for kinetic assays: 20 units/mL of PK, 30 units/mL of LDH, 150mM PEP, 0.3mg/mL NADH, 20mM MgCl\textsubscript{2}, 5mM ATP, 1mM TCEP, 50mM Tris, 0.3mg/mL BSA and 500mM NaCl at pH 8 and 25\textdegree C. a) Src phosphorylated at Y416 shows an increase in activity in the presence of SH3 binding peptide. The most active form of Src is attained when both SH2/SH3 peptides are present and Src is phosphorylated at Y416. b) Src phosphorylated at Y527 shows a 2-fold increase in activity in the presence of SH3 binding peptide.

The SH3 domain forms pi-stacking interactions with a PXXP motif in the SH2-kinase linker, while the SH2 domain provides a docking site for pY527 to stabilize the closed conformation of Src. (Parsons and Parsons 2004). Addition of SH2/SH3 binding peptides
destabilizes the closed state by disrupting these interactions and favoring a more open conformation. This can be seen in that the most active form of Src is achieved by phosphorylation at Y416 and the presence of both SH2 and SH3 binding peptides. Similarly, addition of both SH2 and SH3 binding peptides gives a twofold increase in the activity of Src phosphorylated at Y527.

Overall, Src, when phosphorylated at Y527, exhibits its most inactive form with an activity of $0.5 \pm 0.1 \text{s}^{-1}$. On the other hand, the most active form of Src has an activity of $45 \pm 3 \text{s}^{-1}$ and it is acquired by the phosphorylation of Y416 and presence of SH2/SH3 binding peptides.

**Regulation of Abl**

**Effect of phosphorylation on the regulation of Abl**

Similar to Src, Abl also has two tyrosine residues that are essential for regulation. Unlike Src, where phosphorylation of the tail tyrosine residue down-regulates the enzyme, both tyrosine residues in Abl are known to play positive regulatory roles. Here, we are interested in studying the ability of Abl to autophosphorylate at these two residues and in quantifying the effect of phosphorylating each tyrosine residue on the activation of Abl.

**Figure 11. Autophosphorylation of Abl at Y411 and Y244.**

Time points noted below the western blots are in minutes. Conditions for autophosphorylation: 50µM of Abl, 20 units/mL of PK, 30 units/mL of LDH, 150mM PEP, 20mM MgCl$_2$, 5mM ATP, 1mM TCEP, 50mM Tris, 1mM TCEP and 500mM NaCl at pH 8 and 25°C. a,c) Specific antibody against Y411 was used to observe the
phosphorylation at Y411. b,d) Specific antibody against Y244 was used to observe autophosphorylation at Y244. The ability of both Abl-Y244F and Abl-Y411F mutants to get autophosphorylated shows that phosphorylation of one residue is not dependent on the other. Abl phosphorylates more readily at the activation loop tyrosine, Y411.

Another important distinction between Src and Abl is that Src requires CSK for phosphorylation at Y527, making it easier to get clean phosphorylated states while Abl autophosphorylates at both Y244 and Y411. In order to study the effect phosphorylation of each tyrosine residue has on activity without interference from phosphorylating the other, we designed a Y244F mutant and a Y411F mutant. We were then able to phosphorylate Y244 and Y411 separately (Figure 11) and measure their individual effects on activation of Abl.

Figure 12. Kinetic assays on full-length Abl.
upAbl represents unphosphorylated Abl, pAblY411 represents Abl phosphorylated at Y411 and pAblY244 represents Abl phosphorylated at Y244. 100nM of upAbl was used whereas pSrcY411 and pSrcY244 were at 50nM. Conditions for kinetic assays: 20 units/mL of PK, 30 units/mL of LDH, 150mM PEP, 20mM MgCl2, 0.3mg/mL NADH, 5mM ATP, 1mM TCEP, 50mM Tris, 0.3mg/mL BSA and 500mM NaCl at pH 8 and 25°C. A 2-3 fold increase is observed when Abl is phosphorylated at both Y411 and Y244. Phosphorylation of Y411 and Y244 have an additive effect on the activity of Abl.

Unlike Src where the Cα-helix protrudes outward in the active site, blocking substrate binding and causing the activation loop to twist inwards, Abl blocks substrate binding by mimicking the substrate with its unphosphorylated activation loop. Phosphorylation on Y411 plays a role in destabilizing this inactive conformation due to repulsion between the negatively
charged phosphate group and negatively charged residues present in the substrate-binding site (Schindler et al. 2000). Thus, we expect to see activation of Abl upon phosphorylation on Y416. Interestingly, phosphorylation of Y411 gives only a 1.5-fold increase in activity as compared to unphosphorylated Abl.

Another interaction that plays an important role in keeping Abl in its inactive state is the interaction between the SH3 domain and Y244 in the SH2-kinase linker. Abl has Y244 instead of the second proline in the conserved PXXP motif that is required for binding to the SH3 domain. Y244 takes part in pi-stacking interactions with residues of the SH3 domain to further stabilize the closed state (Panjarian et al. 2013). Therefore, phosphorylation of Y244 interferes with the interaction between SH2-kinase linker and the SH3 domain, favoring the open, more active state of the kinase. Phosphorylation of Y244 increases activity about 1.5 fold.

Individually, phosphorylation of Abl at Y411 and at Y244 give about a 1.5-fold increase. Our data shows that phosphorylating these residues has a cumulative effect, because phosphorylation of both residues has a higher effect on activity of Abl as compared to phosphorylation of Y411 and Y244 individually. This indicates that the effects are independent of each other. Fully autoprophosphorylated Abl shows a 2-3 fold increase in activity from the unphosphorylated Abl.

Similar to what we did with Src, we were also interested in seeing if the effect of phosphorylating Y411 had a global effect on the opening and closing of the kinase or if it was only affecting the kinase domain, making it more active. Thus, we expressed the kinase domain of Abl and measured the effect of autoprophosphorylation on its activity.
Figure 13. Effect of activation loop phosphorylation on activity of Abl-kinase domain.

upAbl-KD represents unphosphorylated kinase domain Abl, pAbl-KD represents Abl kinase domain phosphorylated at Y411. 50nM of uAbl-KD and 20nM of pAbl-KD were used for the kinetic assays. Conditions for kinetic assays: 20 units/mL of PK, 30 units/mL of LDH, 150mM PEP, 0.3mg/mL NADH, 20mM MgCl$_2$, 5mM ATP, 1mM TCEP, 50mM Tris, 0.3mg/mL BSA and 500mM NaCl at pH 8 and 25°C. Phosphorylation of Y411 increases the activity of the kinase domain of Abl by about twofold.

In a similar manner to Src, the free kinase domain of Abl is more active than the full-length and phosphorylation of Y411 activates the kinase domain (Figure 13). This 2-fold increase in activity is close to the 1.5-fold increase seen in full-length Abl due to phosphorylation of Y411. Therefore, we can say that phosphorylation of Y411 does not affect the global open-closed states of Abl but rather increases activity by favoring the more active state of the kinase domain.

Effect of allosteric regulatory elements on activation and inhibition of Abl

One of intramolecular interactions that play a crucial role in the inactivation of Abl is the SH2-kinase domain interaction. The C-lobe of the kinase domain forms several hydrogen bonding interactions with residues of the SH2 domain, stabilizing the closed conformation. Thus, the SH2 domain is much closer to the kinase domain in the closed, inactive conformation of Abl than it is in Src (Hantschel 2012). In addition to the SH2-kinase interaction, the SH3 domain interacts with the SH2-kinase linker through pi-stacking interactions. For these reasons, we expect SH2 and SH3 binding proteins to have an activating effect on the regulation of Abl.
Similar to the experiments we conducted on Src, we used SH2/SH3 binding peptides to mimic SH2/SH3 binding proteins that interact with Abl in the cell. We then measured their effects on the activity of Abl.

**Figure 14. Effect of SH2/SH3 binding peptides and presence of myristoyl group on the activity of Abl.**

upAbl represents unphosphorylated Abl and pAbl$^{Y411,Y244}$ represents Abl phosphorylated at both Y411 and Y244. 50nM of upAbl was used for the kinetic assays whereas, pAbl was at 20nM. SH2/SH3 peptides were both at 2mM. Myristoylated peptide was added in trans at 2mM. Conditions for kinetic assays: 20 units/mL of PK, 30 units/mL of LDH, 150mM PEP, 0.3mg/mL NADH, 20mM MgCl$_2$, 5mM ATP, 1mM TCEP, 50mM Tris, 0.3mg/mL BSA and 500mM NaCl at pH 8 and 25°C. a) Myristoylation decreases kinase activity of upAbl by about 6 folds b) The most active form of Abl is attained when both Y411 and Y244 are phosphorylated and SH2/SH3 peptides are present. There is about a 15-fold difference in activity between the most active and inactive forms of Abl.

The presence of SH2 peptide activates unphosphorylated Abl about two folds; whereas, SH3 peptide has a slightly less effect with a 1.5-fold activation. This agrees well with the structure of Abl that demonstrates the especially close interactions between SH2 and kinase domains that are essential in stabilizing the closed conformation. This is also a plausible explanation as for why SH2 binding peptide has a higher effect on Abl than it does on Src.
Effect of myristoylation on regulation of Abl

As mentioned earlier, Abl does not have a C-terminal tail tyrosine residue that gets phosphorylated in order to elicit the auto-inhibited form. Instead, Abl uses a myristoyl switch that plays a crucial role in inactivating the kinase and keeping it in its inactive, assembled state (Nagar et al. 2003). Abl has a hydrophobic pocket in the kinase domain that serves as a myristoyl binding socket. The binding of myristoyl in this pocket triggers a change in the configuration of the C-terminal helix (αI) of Abl, pushing the SH2 and kinase domains together where they can stabilize the closed conformation through hydrogen bonding interactions (Figure 4 of the myristoyl-SH2 binding site).

![Graph showing kinetic assays on full-length Abl and Abl kinase domain in the presence of myristoyl](image)

**Figure 15. Effect of myristoyl binding on the activity of full-length Abl and Abl kinase domain.** uAbl represents unphosphorylated full-length Abl, pAbl represents full-length Abl phosphorylated at both Y411 and Y244 and Abl-KD represents Abl kinase domain only. 100nM of uAbl was used for the kinetic assays whereas, pAbl was at 20nM. Myristoylated peptide was added in trans at 2mM to observe effect of myristoylation. SH2 and SH3 binding peptides were also at 2mM. Conditions for kinetic assays: 20 units/mL of PK, 30 units/mL of LDH, 150mM PEP, 0.3mg/mL NADH, 20mM MgCl2, 5mM ATP, 1mM TCEP, 50mM Tris, 0.3mg/mL BSA and 500mM NaCl at pH 8 and 25°C. Myristoylation decreases kinase activity of both full-length and kinase domain Abl.

There is a significant 15-fold decrease in the activity of unphosphorylated Abl in the presence of a myristoyl group. On the other hand, we see only a twofold decrease in activity for phosphorylated Abl. As mentioned previously, phosphorylation of Y416 and Y244 disrupt the inactive form of Abl by inducing electrostatic repulsion in the active site of the kinase domain.
and by interfering with the interaction of SH3 domain with SH2-kinase linker, respectively. It is possible that these interactions make it more difficult for the myristoyl to bind in the kinase domain and elicit changes in the c-lobe.

Addition of SH2/SH3 peptides increases the activity of Abl in both unphosphorylated and phosphorylated states. This is agreement with structural information that suggests the close SH2-kinase interface that is elicited by the binding of myristoyl to the kinase pocket (Nagar et al. 2003). The peptides disrupt this interface giving, favoring the more open, active state of Abl.

We wanted to see if myristoylation would have an effect on the kinase domain alone. Thus, we measured the activity of Abl-KD in the presence of myristoyl-peptide. Figure 15(b) shows a significant decrease in the activity of Abl-kinase domain in the presence of myristoyl, even more so than the one we observed in full-length. Thus, we now know that myristoyl group does not need the SH2/SH3 domains to reduce kinase activity.

Summary of Src and Abl regulation

Both Src and Abl are able to autophosphorylate on the activation loop with autophosphorylation having a 2-3 fold activation effect on Src and 1.5-2 fold activation effect on Abl. About a 90-fold difference in activity was observed between the most inactive state of Src, with a rate of $0.5\pm0.2s^{-1}$, and its most active state, with a rate of $45\pm4 s^{-1}$ while a 15-fold difference was seen between the most inactive and most active states of Abl, with rates of $0.5\pm0.1s^{-1}$ and $7.8\pm0.3s^{-1}$ respectively.

Phosphorylation of Y527 in the C-terminal tail of Src was the strongest inhibitory effect, with a 30-fold decrease in activity as compared to the unphosphorylated state. Abl employed a ‘myristoyl switch’ that was observed to cause a 15-fold decrease of activity upon myristoyl binding. After identifying these critical players in the regulation of Src and Abl, we repeated the
experiments on the ancestral proteins we resurrected with an attempt to better understand the evolution of their regulation.

*Regulation of the ancestral kinases*

After manipulating various regulatory elements and quantifying their effect on the activation and inhibition of Src and Abl, we wanted to get a better understanding of how the regulation evolved. We therefore resurrected five ancestral proteins of Src and Abl, three of which are common to both whereas two are ancestors of either Src or Abl. Afterwards, we extended the experiments we carried out on the modern kinases to the ancestors. We measured 1) the ability of the ancestors to autophosphorylate, 2) the effect of autophosphorylation on their activity, 3) their ability to be phosphorylated by CSK and if it has any effect on their activity and 4) the effect of myristoylation on the activity of the ancestors.

*Effect of autophosphorylation on regulation of the ancestors*

a) Western blot for autophosphorylation of Anc76 WT and Y328F mutant

b) Western blot for autophosphorylation of Anc86 WT and Y324F mutant

c) Western blot for autophosphorylation of Anc103 WT and Y328F mutant
Figure 16. Autophosphorylation of Anc76, 86 and 103.

Conditions for autophosphorylation: 5μM of enzyme, 20 units/mL of PK, 30 units/mL of LDH, 150mM PEP, 0.3mg/mL NADH, 20mM MgCl₂, 5mM ATP, 50mM Tris and 500mM NaCl at pH 8 and 25°C. a-c) All ancestral kinases were able to autophosphorylate and their activation loop tyrosine residues are major autophosphorylation sites, as can be seen by the western blots of the Y-F mutants. d) Quantification of western blot for Anc 103. All the ancestral kinases completed phosphorylation within an hour of the start of the reaction.

The ancestral proteins were all able to autophosphorylate. To check whether the activation loop is the major site of autophosphorylation for ancestors, we made single point Y to F mutations in Anc 76, Anc 86 and Anc 103. We saw that all three mutants of the ancestors portrayed reduced autophosphorylation as compared to wild-type Anc 76, 86 and 103 (Figure 16). This demonstrates that the activation loop tyrosine is autophosphorylated and it makes up a considerable amount of total autophosphorylation. After we established the amount of time it takes for the ancestors to autophosphorylate, we used this information to measure the effect of autophosphorylation on the activity of the ancestors.
Figure 17. Kinetic assays on Anc76, 86 and 103 and their mutants.

upAnc-WT represents unphosphorylated wild-type ancestor 76, 86 or 103, pAnc-WT represents wild-type autophosphorylated ancestors. upAnc -Y→F represents unphosphorylated ancestor with a mutation of the activation loop tyrosine. 20nM of upAnc76 and pAnc76 were used in the kinetic assays; whereas different phosphorylation states of Anc86 and Anc103 were at 100nM. Conditions for kinetic assays: 20 units/mL of PK, 30 units/mL of LDH, 150mM PEP, 20mM MgCl2, 0.3mg/mL NADH, 5mM ATP, 1mM TCEP, 50mM Tris, 0.3mg/mL BSA and 500mM NaCl at pH 8 and 25°C. Autophosphorylation had no activity on Anc76 while Anc86 and Anc103 both showed an increase of 1.5-2 folds in activity upon activation loop phosphorylation.

The oldest of the ancestors, Anc 76, is the least affected by autophosphorylation. Both the wild-type and the Y328F mutant of Anc 76 show no increase in activity upon phosphorylation.
This indicates that phosphorylation of the activation loop tyrosine has no effect on activity of Anc76.

Anc 86 is the next oldest ancestor, which is common between the Tec, Abl and Src families, all of which are known to be regulated by activation loop phosphorylation. Wild-type Anc 86 shows about a twofold increase upon autophosphorylation; whereas Anc 86 Y→F mutant shows no change in activity upon phosphorylation. This shows that phosphorylation at the activation loop tyrosine, Y324, is what leads to the twofold increase in activity of Anc 86.

The most recent ancestor between Src and Abl, Anc 103, also shows a slight increase in activity upon phosphorylation. There is a 1.5-fold increase in activity of wild-type Anc 103 whereas, there is no effect observed upon phosphorylation of Anc 86 Y→F mutant. Again, this indicates that phosphorylation of Y328 in the activation loop is essential for the increase in activity we observed upon autophosphorylation of wild-type Anc 103. If other residues were responsible for the increase in activity we observed in autophosphorylated Anc 86-WT and Anc 103-WT, then we would have observed the same effects upon phosphorylation of their Y→F mutants. Hence, we can conclude that Anc 86 and Anc 103 show a 1.5-2 fold increase in activity upon phosphorylation of their activation loop tyrosine residues.
Phosphorylation by CSK

Phosphorylation of the C-terminal residue of Src by CSK has a large inactivating effect on Src. Although the docking of a C-terminal tyrosine residue is not used to auto-inhibit Abl, Abl still displays a closed state where the SH2 and SH3 domains play a crucial role in stabilizing the inactive state. Similarly, the SH2 and SH3 domains of Tec family kinases play significant regulatory roles by stabilizing the inactive, closed conformation of the kinase (Mano 1999). In addition to serving as a phospho-tyrosine docking site for other proteins, the SH2 domain also provides an interface for hydrogen bonding interactions with the kinase domain in Abl.

All three kinase families exhibit a similarity in how they are inactivated by inter-domain interactions involving the SH2 domain. We asked if the SH2 domain had already evolved its regulatory role in the common ancestors of the tyrosine kinases, before each of the families evolved their own inhibitory mechanism to make use of this regulatory role being played by the SH2 domains (such as the mechanism of docking Y527 into the SH2 domain used Src).

In order to help us answer this question, we designed mutants of Anc 86 and Anc 103 by adding tyrosine residues to their C-terminal tails. Our goal was to phosphorylate the newly introduced C-terminal tyrosine residues using CSK and to check whether phosphorylation on the tail affected their activity. However, we later found that CSK was not able to phosphorylate the ancestor mutants due to its high specificity for Src family kinases.

Based on certain studies, the conserved QYQ sequences of the C-terminal tails among Src family kinases is likely an important factor in determining the specificity of CSK (Okada 2012). However, other studies have suggested that three residues found at the C-terminal end of the kinase domain are the essential players for the recognition and binding of Src to CSK (Figure 18) (Levinson et al. 2008).
Figure 18. Src-CSK interface.

a-c) Three arginine residues, Arg\textsuperscript{384}, Arg\textsuperscript{389} and Arg\textsuperscript{279}, in CSK form hydrogen bonding interactions with Glu\textsuperscript{510}, Tyr\textsuperscript{511}, Tyr\textsuperscript{518} and Lys\textsuperscript{442}. Based on studies done by Kuriyan and collaborators, these residues are essential for the binding and recognition of Src whereas the sequence of the remaining parts of the C-terminal tail do not play a significant role in the Src-CSK interface (Levinson et al. 2008).

The inability of Anc\textsuperscript{86}+Src tail and Anc\textsuperscript{103}+Src tail to get phosphorylated by CSK, regardless of having the conserved C-terminal tail sequence, supports that there are other residues at play that make binding to CSK possible.

We expressed and purified another ancestor, Anc\textsuperscript{108}, on Src’s branch of the phylogenetic tree (Figure 5), to check whether or not it can be phosphorylated by CSK; and, if it can be phosphorylated, to analyze the effect of tail phosphorylation on its activity.

Anc\textsuperscript{108} is the first ancestor on the Src branch where the C-terminal tyrosine appeared. It also had the three residues that were identified as essential players for binding of CSK to Src. However, Anc\textsuperscript{108} does not have the conserved QYQ tail sequence of the Src family kinases. CSK was not able to phosphorylate Anc\textsuperscript{108}.

By closely analyzing Anc\textsuperscript{108} at the sequence level, we found close resemblance between Anc\textsuperscript{108} and a Src-like kinase found in Amphimedon Queenslantica (sea sponges) which also happen to have their own homologue of CSK. The conserved QYQ sequence of Src family
kinases is replaced by EAG in both Anc 108 and the Src-like kinase in C. Sponges. The existence of both a Src-like kinase and a CSK homologue in these species of sea sponges suggests that the Src-like kinase is most likely the substrate of the CSK homologue. In return, the resemblance between Anc 108 and the Src-like kinase in the more primitive sea sponges indicates the possibility of Anc 108 being able to get phosphorylated by the CSK homologue.

The existence of a CSK homologue in the more primitive eukaryotes, that could possibly phosphorylate Anc 108, hints on the possibility of co-evolution between Src and CSK. This is worth looking further into.

It would also be interesting to use more promiscuous kinases to phosphorylate the tail tyrosine residues of Anc 103+Src tail in order to observe whether or not phosphorylation at this site induces inter-domain interactions between the SH2 and kinase domains. Doing so would provide us better insight into how the regulatory function of the SH2 domain evolved.

Effect of myristoylation on activity of ancestors

The binding of a myristoyl group to the kinase domain of Abl results in conformational changes that stabilize the inactive conformation. Although, Src gets myristoylated on the N-terminus, in vivo, the presence of a myristoyl group does not affect its activity. Here, we are interested in investigating whether myristoylation down-regulates the ancestral proteins of Src and Abl.
Figure 19. Kinetic assays on Anc76, 86, 103 and 125 in the presence of myristoyl group.

upAnc represents unphosphorylated ancestral kinase while upAnc+Myr represents activity of unphosphorylated kinase in the presence of myristoyl peptide. 20nM of upAnc76 was used in the kinetic assays; whereas Anc86, 103 and 125 were at 100nM. Conditions for kinetic assays: 20 units/mL of PK, 30 units/mL of LDH, 150mM PEP, 20mM MgCl₂, 0.3mg/mL NADH, 5mM ATP, 1mM TCEP, 50mM Tris, 0.3mg/mL BSA and 500mM NaCl at pH 8 and 25°C. a) The presence of myristoyl group had no effect on the activity of the ancestral kinases. b) Titration of Abl and Anc125 with different concentrations of myristoyl peptide shows that Anc125 is not inhibited by myristoyl.

None of the ancestors exhibited a significant decrease in activity upon myristoylation (Figure 19a), indicating that regulation via a ‘myristoyl switch’ evolved at a later time. To further investigate the evolution of myristoylation as an inhibitory mechanism and to trace the residues that played a key role in this process, we resurrected Anc 125 from the Abl branch of the phylogenetic tree and measured its activity both in the presence and absence of myristoyl. Similar to the older ancestors, Anc 125 was not significantly affected by the addition of myristoyl group (figure 19b).

To get better insight into why myristoylation does not inhibit the ancestors, we took a closer look at the structure of Abl at the myristoyl binding pocket and compared it to the sequences we have for the ancestors.

The myristoyl binding pocket of Abl is lined by small hydrophobic residues such as Ala452, Gly482 and Val487; whereas in Src, these residues are replaced with bulkier polar residues like threonine and glutamate (Nagar et al. 2003). This makes it difficult for the
myristoyl group fit into the pocket to interact with the kinase domain of Src. Similarly in the ancestors, several of these residues lining the hydrophobic myristoyl binding pocket are replaced with bulkier residues that hinder binding of the myristoyl group.

**Figure 20. Differences between Anc 125 and Abl plotted (PDB 1opj).**
KD stands for kinase domain. We speculate that the residues in pink are crucial differences between Anc 103 and Abl. Ala451 in Abl, was F451 in Anc 103 and earlier ancestors, which shows the possibility of a steric clash between the myristoyl group and phenylalanine side chain, making it difficult for the binding of myristoyl. Anc 125 is the first ancestor that has Ala451 in the myristoyl binding pocket, which hints the possibility of myristoyl binding. Replacement of Gln517 and Glu523 by alanine residues in Anc 103 disrupts hydrogen bonding interactions that exist between SH2 domain and kinase domain of Abl.

There are notable differences between the residues lining the myristoyl binding pocket in Abl, and the residues that are present in Anc125 (Figure 20). The bulkier size of these residues lining the inside of the pocket makes it more difficult for the myristoyl group to bind. Residue Ala451 in Abl is a phenylalanine in Anc 103, which completely blocks the wider opening of the myristoyl binding pocket. However, this is not the case in Anc125, where we see the first appearance of Ala451 instead of a bulky residue. This suggests the possibility of myristoyl binding to Anc125, without any effect on its activity. This would mean that the inhibition
mechanism of myristoyl binding succeeds the ability of myristoyl to bind; that is they did not both happen at the same time.

Another significant mismatch between Anc 103 and Abl is the absence of Asp523 and the replacement of Gln517 by alanine in Anc 103. Asp523 and Gln517 are involved in hydrogen bonding interactions with Arg189 and Arg153 respectively (Figure 4). These interactions make up the SH2-kinase interface and stabilize the inactive form of full-length Abl (Nagar et al. 2003). The lack of these interactions in Anc 103 suggests that the SH2-kinase interface had not evolved yet.

However, there are changes observed in Anc 125 as compared to Anc 103 that make these SH2-kinase interactions more favorable. Gln517 is present in Anc 125 and Asp523 is replaced by a glutamate, which makes the hydrogen bonding observed in Abl between Arg189 and Asp523 and between Arg153 and Gln517 possible for Anc125. From this, we can speculate that the SH2-kinase interface might have evolved before the evolving of myristoyl binding and inhibition.

Since our check of the ancestors’ ability to bind myristoyl is solely based on the lack of change in activity of the kinases, we cannot be certain that the ancestors do not bind myristoyl at all. This is because it is possible that they do bind myristoyl in the pocket but their activities are not affected by this binding. Thus, an interesting experiment would be to measure the binding of myristoyl to Anc 125 using ITC or other binding assays.
**Part 2- FRET Project**

Quantifying the effect of various regulatory elements on the activation and inhibition of Src and Abl offers a great deal of information on the kinetic effect of each regulatory on the equilibrium between the closed and open states. With this kinetic information at hand, we are now proceeding to developing single-molecule Fluorescent Resonance Energy Transfer (FRET) assays in order to get a better understanding of the interplay between open and closed states of Src and Abl. The ultimate goal of conducting the FRET experiments is to couple our kinetic observations with structural and conformational information.

![Fluorescent labeling of Src for FRET.](image)

**Figure 21. Fluorescent labeling of Src for FRET.**
When Src is in the closed state, the fluorescent dyes are close enough for FRET to happen. However, when Src attains an open state, the dyes are far away and FRET is not observed. This difference in FRET will help us gain information on conformational transitions and their time scale.

FRET works in such a way that the donor chromophore absorbs energy at a certain wavelength and emits at a longer wavelength, transferring energy onto an adjacent acceptor.
chromophore, which then emits at a different wavelength. We designed our FRET assay so that the donor fluorophore is attached to a cysteine residue in the SH2 domain and the acceptor is attached to the kinase domain. The inactive, closed form of the kinase will bring the two fluorophores together and allow transfer of energy, which can then be measured. Whereas, the active open conformation increases the distance between donor and acceptor dyes, prohibiting energy transfer.

We used maleimide-conjugated Cy3B fluorescent dyes to tag Cys162 on the SH2 domain of Src. Since we want to selectively tag Cys162, we engineered a single-Cys construct of the SH2 and SH3 domains in which other Cys residues were replaced with Ser residues. We then purified the new SH2/SH3 domain construct without the kinase domain and incubated it with the fluorescent dye.

Next, we achieved site-specific tagging of the kinase domain through enzymatic labeling by transglutaminase. We introduced a loop, which is recognized by transglutaminase, to the kinase domain of Src. After purifying the kinase domain separate from the SH2/SH3 domains, we attached a fluorescent dye, cadaverin-Alexa 647, to the loop using transglutaminase. Once we were done labeling, and excess of free dye was removed with size-exclusion columns, we used Sortase-A enzyme to ligate the tagged SH2/SH3 domains with the tagged kinase domain. In order to attach the protein to the microscope slides, we appended a biotin tag to the C-terminus of the ligated Src using enzyme BirA, which we had purified prior. We were able to achieve 100% labeling on the kinase domain via enzymatic labeling using transglutaminase. However, we only achieved about 50% yield for labeling by Cy3b on the SH2 domain.
Figure 22. Fluorescence of labeled Src shows success in double labeling.
a) Green laser (532nm) and Red laser (645nm) were used to excite Cy3B and Alexa-647 respectively. b) Emission-excitation spectra of Cy3B and Alea-647 (picture modified from fluorophores.org). The emission of the dyes was observed using 540nm and 670nm filters. The kinase domain alone fluoresced when excited at 532nm and the SH2/SH3 label fluoresced when excited at 640nm. The first sample of ligated Src is clean whereas the last two have contamination with Src-KD. We observed fluorescence in ligated, full-length Src at both excitations, indicating that it was indeed doubly labeled.

Although the relatively low yield of our fully ligated Src construct was fairly low, we were still able to detect it using SDS page and observe emission after exciting the dyes at different wavelengths (Figure 22). This was done in order to check for complete labeling of the ligated construct, as seen in Figure 22. Once we achieved complete labeling of full-length Src, we used the clean sample to do preliminary analyses and detect if there was any FRET happening between the two dyes.
**Preliminary Analysis of fluorescent traces**

*Figure 23. Fluorescent traces indicate occurrence of FRET in unphosphorylated Src and pSrc\(^{Y527}\).*

The microscope is set up so we can observe the fluorescent emission, of the donor (Cy3B in the SH2 domain) through the green field lens and simultaneously observe the emission of the acceptor (Alexa-647) through the red field lens. We observed anti-correlated fluorescent traces for the donor and acceptor. This suggests the occurrence of FRET between the two dyes. It also tells us about conformational transitions that determine the adjacency of the dyes in order for FRET to happen. Preliminary analysis of fluorescent traces for the two dyes was done by Roman Agafonov.

We used unphosphorylated Src and Src phosphorylated at Y527 to investigate FRET happening between the two fluorescent labels. The anti-correlation observed in the fluorescent traces proves that there is FRET occurring and also, that there is conformational interchange between open and closed conformations of Src (Figure 23).

Fluorescent traces for unphosphorylated Src demonstrate longer lifetimes of both open and closed states as compared to the lifetime of the open state in tail-phosphorylated Src. (figure
23a). On the contrary, traces for Src phosphorylated on Y527 depict much shorter life times of the open state as compared to the closed state (Figure 23b). Although these traces are preliminary, the shorter time spent in the open state when there is tail phosphorylation suggests that phosphorylation of Y527 favors the closed conformation. This shows that upon phosphorylation of Y527, the two states of Src are still in equilibrium. That is, although there is a bias towards one state, this is not a complete, rigid structural change upon phosphorylation.

We are now in the process of optimizing labeling conditions in order to get better yield of ligated Src construct. In addition, we will also be adjusting bin times and laser intensities to help us follow the conformational changes happening in the faster time scale and reduce background noise in the fluorescent signals.

The ultimate goal of this project is to observe changes in fluorescent signals of the dyes depending on the interactions and proximity of SH2 and catalytic domains and use this information to determine the conformational ensembles of Src and the time-scales of the transitions.
Conclusion

Src and Abl show great structural similarity in their active and inactive conformations. However, the mechanism in which they achieve these conformations is quite different. Although there have been previous studies and crystal structures that identified have various essential regulatory elements that dictate the regulations of Src and Abl, there is still a lack of quantitative information on the regulatory schemes of the kinases. In order to better understand regulation of Src and Abl and investigate their evolution, we manipulated various regulatory elements and attained quantitative information. We identified autophosphorylation on the activation loop tyrosines to have a similar effect of 2-3 folds on both Src and Abl; whereas, we observed significant differences on the inhibitory mechanisms of the enzymes. This same effect of activation loop phosphorylation was observed in the kinase domains alone, indicating that 1) there is another active-inactive equilibrium within the kinase domains of Src and Abl that is separate from the open and closed states of the enzymes and 2) the phosphorylation of activation loop tyrosine residues only affect the active-inactive equilibrium of the kinase domain and not the global open-closed equilibrium of the enzymes.

Phosphorylation on the C-terminal tyrosine played a crucial role in deactivating Src with a 25-fold effect; while addition of myristoyl group played the inhibitory role in Abl with a 15-fold decrease in activity. There appears to be a wider variation between the most active and
inactive forms of Src, with about a 90-fold difference in activity, as compared to a 40-fold difference that was observed in Abl.

Figure 24. Equilibria between active and inactive states of Src and Abl.
Src and Abl show various biochemical states and do not have a simple on and off switch. The kinase domains also have an intrinsic equilibrium where they are activated by phosphorylation on the activation loop.

We extended our kinetic experiments to the ancestors of Src and Abl and found that Anc 76, the oldest ancestor we resurrected, is constitutively active, showing no significant increase or decrease in activity upon phosphorylation or myristoylation. This agrees with what is known about the Fer family kinases, which are one of the modern-day tyrosine kinases that share Anc 76 with the Tec, Src and Abl families. As mentioned earlier, Fer can get autophosphorylated but is not activated by autophosphorylation. Anc 86 and Anc 103 showed a 1.5-2 fold increase in activity upon autophosphorylation on the activation loop tyrosine. This attests that the ability to autophosphorylate preceded the effect of phosphorylation on the activation loop.

None of the ancestors we resurrected, including Anc 125 from Abl’s branch of the phylogenetic tree, showed a decrease in activity upon myristoylation. However, certain bulky residues that are expected to block the myristoyl binding pocket have evolved to smaller
hydrophobic residues in Anc 125, which suggests that the ancestor might be able to bind myristoyl without being inactivated by it.

Residues crucial for the SH2-kinase domain interface in the inhibited form of Abl have also fully evolved in Anc 125, which infers the evolution of the SH2-kinase interface before the evolution of the ‘myristoyl switch’ as a deactivator.

Our results on the regulation and evolution of Src and Abl confirm that the kinases are not controlled by a simple on and off switch. Instead, they employ various regulatory elements to attain different levels of activity. Additionally, we conclude that the modern kinases are more tightly regulated than their ancestors.
**Future Directions**

Moving forward, we will further investigate the binding of myristoyl to the ancestral proteins using ITC. This will give us information on whether the binding of myristoyl preceded the evolution of myristoyl inhibition. We are also currently working towards crystallizing the ancestral kinases to help us get a better understanding of their structure and to observe possible inter-domain interactions.

Additionally, we are looking further into the inability of CSK to phosphorylate the ancestral kinases. In order to do so, we resurrected another ancestor on the Src branch that contains both the conserved QYQ sequence of the Src family kinases and the three residues identified as crucial components of the Src-CSK interface. Investigating CSK-phosphorylation of this ancestor will help us decide whether or not the conserved QYQ sequence is crucial for recognition by CSK or if there are other essential components that play a role in the recognition of Src by CSK. In addition, if the newly resurrected ancestor does get phosphorylated by CSK, we will be able to test the presence of the auto-inhibitory mechanism involving the tail tyrosine residue and SH2 docking site has also evolved. This will shed light on the evolution of the tail tyrosine and its role in the auto-inhibited Src kinases.

With regards to the FRET project, we will be optimizing labeling conditions to achieve better yield of fully labeled, ligated Src. We will simultaneously work towards improving FRET measurement conditions, such as bin time and laser intensity in order to decrease background noise and to observe fast conformational inter-conversions, which we were not able to catch in
the longer time frame. Once we test our system with Src, we will move forward to using similar methods to observe conformational interplays in Abl.
Materials and Methods

Protein purification

Genes for Abl, Src and ancestral proteins were transfected into BL21 cells for cloning. The vectors are sub-cloned with His tags, Maltose Binding Protein (MBP) tags and TEV cleavage sites for specific column binding and for increased solubility. We co-transformed the cells with YopH phosphatase to make sure the kinases are dephosphorylated and to reduce cell toxicity during overexpression (Agafonov et al. 2014). The cells were grown at 37°C in the presence of 0.1µg/mL Kanamycin and 50µg/mL Streptomycin until they reached an OD of 0.6-0.8. We then induced the cells with 200µg/mL IPTG and let them grow overnight at 20°C. After harvesting the cells and separating the lysate, we started purification process with Talon Beads which have high affinity for His tagged proteins. Our next step was to run our sample through an MBP column to separate our kinase from YopH. Then, we cleaved the His and MBP tags overnight using 1mg/mL of TEV protease and used Ni columns to separate the tag from our protein (Figure 25). Our final step was to use size exclusion column to separate any unnecessary contaminations from our proteins.

Figure 25. Depiction of tags and cleavage site used for purification.
The His-tag is followed by an MBP tag, followed by the TEV recognition sequence. After that starts the N-terminal of the proteins.
### Table 1. Sequence of full-length Src and Abl constructs.

The construct for full-length Src and Abl includes 80 residues on the N-terminal tail, followed by SH2, SH3 and kinase domains and lastly a C-terminal tail. Residues colored in purple are the 80 N-terminal residues, residues colored in blue are the SH2 and SH3 domains and residues colored in brown are residues of the kinase domain. The ancestral kinases include all the aforementioned domains with an exception of the 80 residues on the N-terminal.

To attain the different Src, Abl and ancestral mutants we used in our experiments, we did site directed mutagenesis using the sequence of the WT proteins as templates. We designed the different primers and used Quick Change Lighting kit to introduce the mutations using polymerase chain reaction. We transformed the mutated genes to DH5-α cells, did mini-prep to extract the DNA from the cells and confirmed the sequences via the Genewiz sequencing facility.

**Autophosphorylation and western blots**

To autophosphorylate the ancestral proteins, we prepared a mix containing 500mM NaCl, 1mM TCEP, 50mM Tris, 20mM MgCl2, 1mM TCEP and 5µM of the ancestral protein. We used variable enzyme concentrations for Src and Abl depending on their rate of
autophosphorylation. Since Src and Abl hydrolyze ATP unproductively, we used 7.5 units/mL of PK/LDH as recycling enzymes along with PEP to make sure the ATP in our mixture is regenerated. We initiated the autophosphorylation reaction by adding 5 mM ATP and carried out all reactions at 25°C. We then took samples at different time points, quenching them using 1:3 ratio of protein to 4x protein denaturing buffer. We loaded each of the samples into NuPAGE Bis-Tris gels and ran the gels at 200V/200A for 35 minutes.

The gel was transferred to nitrocellulose membranes using eblot pads. We used an unspecific antibody against phosho-tyrosines (p1000) to stain the ancestors because there are no specific antibodies that have been developed for ancestral proteins. Site specific antibodies against Y527 and Y416 were used to target tyrosine residues at the C-terminal tail and the activation loop of Src respectively; whereas antibodies against Y244 and Y411 were used to target tyrosine residues in the SH2/Kinase linker and in the activation loop of Abl respectively.

After incubating the membranes with their respective primary antibodies for an hour, we then stained the membranes using a fluorescent secondary antibody, Alexa-567 and analyzed them using their fluorescence.

We used the above information on the enzymes’ ability to autophosphorylate to determine the minimum time required to complete phosphorylation. Once we established the time required for each kinase to completely phosphorylate, we repeated the autophosphorylation procedure for each kinase. Similar to our autophosphorylation experiments, the mixtures were composed of 500 mM NaCl, 5 mM ATP, 20 mM MgCl2, 1 mM TCEP, 50 mM Tris, 1 mM TCEP, 0.3 mg/mL BSA and variable enzyme concentrations. We incubated the mixtures at 25°C for the respective amount of time they require for complete phosphorylation. We then separated the nucleotides from the phosphorylated proteins using 4-5 size exclusion Zeba columns.
**CSK phosphorylation of Src**

We repeated the procedures mentioned under *Autophosphorylation and Western Blots*, now using 1:10 ratio of Src to CSK. We found that Src gets completely phosphorylated at the tail within an hour of the start of the reaction. We repeated the same procedures to check if CSK was able to phosphorylate Anc 86/103 + Src tails.

We used these Western Blots to obtain the incubation time required in order to get completely phosphorylated pSrc\(^{Y27}\) samples for our kinetic assays. We incubated 1\(\mu\)M Src and 10\(\mu\)M CSK with 20mM MgCl\(_2\), 20mM Tris, 1mM TCEP. We also included 20 units/mL of PK, 30 units/mL of LDH and PEP for recycling hydrolyzed ATP. We started the reaction by adding 5mM ATP to the mix and incubated it at 25°C for one hour.

**ATP-NADH Coupled Assay**

We used a kinase assay buffer containing the following: 500mM NaCl, 5mM ATP, 20mM MgCl\(_2\), 1mM TCEP, 50mM Tris, 1mM TCEP, 0.3mg/mL BSA, 7.5 units/mL PK/LDH, 4mM PEP, and 0.5mg/mL NADH. We use varying concentrations of enzyme and carried out the assay at pH 8 and 25°C.

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**Figure 26. Coupled ATP-NADH Assay.**

In this assay, the kinases phosphorylate the target peptide and convert ATP to ADP. The ADP produced is then recycled back to ATP by PK, which also converts the PEP in the mixture to Pyruvate. LDH in turn changes pyruvate to lactate and in the process produces NAD\(^+\) by dehydrogenating NADH. NADH has an absorbance at 344nm, whereas NAD\(^+\) does not; thus, we observe a drop in absorbance at 344nm.
We used the drop in absorbance at 344nm to analyze how much NAD$^+$ is produced, which indirectly tells us about how much ADP is produced. We could then calculate the $k_{cat}$ of our enzymes using this information.

We manipulated various regulatory elements such as SH2 binding peptide and myristoylated peptide to quantify the effect they have on regulation of Src and Abl. The sequences of the peptides are provided below. Myristoyl group was added in trans with a peptide group attached to it for increased binding and solubility.

<table>
<thead>
<tr>
<th>Peptide sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Src peptide</td>
</tr>
<tr>
<td>SH2 binding peptide</td>
</tr>
<tr>
<td>SH3 binding peptide</td>
</tr>
<tr>
<td>Myristoylated peptide</td>
</tr>
</tbody>
</table>

Table 2. Sequences of peptides used for the kinase assays.
Src peptide was used as substrate in all kinetic assays done on both modern and ancestral kinases.
Sample preparation for FRET

Tagging the SH2/SH3 domains with Cy3B

We introduced two point mutations to the SH2/SH3 domains of Src, getting rid of two of their three Cys residues. The vectors were sub-cloned with His tags. We expressed the SH2/SH3 domains of Src along with His tag and separate from the kinase domain in order to tag the remaining Cys residue (Cys 238) of the SH2/SH3 domains without interference from other Cys residues from the kinase domain. We purified the SH2/SH3 domains using Ni columns that have affinity for the His tag and used size exclusion to separate any remaining contaminations.

In order to tag the Cys residue with a maleimide-linked fluorescent dye, Cy3b, we first reaffirmed that all the cysteine residues were reduced by adding 10mM TCEP and incubated the mix for an hour. After doing buffer exchange to wash out the extra TCEP so that it does not interfere with the tagging process, we mixed Cy3b to various samples of SH2/SH3 domains, in dye concentration to enzyme concentration ratios ranging from 3:1 to 20:1. We varied the concentration of the enzyme from 10µM to 50µM, while concentration of the dye was kept between 50µM and 1mM to avoid precipitation at higher concentrations. After incubating the samples at different temperatures for different time length, we separated our protein from excess dye using size-exclusion Zeba columns. We then measured percent yield of tagged protein by comparing the ratio of absorbance at 559nm (which is the absorbance peak for Cy3b) to the absorbance at 280nm. Based on our calculations for yield of tagged protein, we found that 10:1 ratio of Cy3B concentration to enzyme concentration, incubated at 4°C overnight, is the most optimal labeling with a final yield of about 50% tagged SH2/SH3 domain.
Tagging the kinase domain using cadaverin-Alexa-647

In order to enzymatically label the kinase domain, we introduced a QQQ sequence after residue Gly465 of Src. This QQQ insertion is recognized by the enzyme transglutaminase, which we later used to tag the kinase domain using the fluorescent dye, cadaverin-Alexa 647.

We purified the kinase domain following the same procedures mentioned under the Protein Purification section. Then, we incubated 100µM Alexa-647 dye and 10µM Src-KD with 0.5µM transglutaminase, at different temperatures and for different time periods. We found that a 100% yield of labeled Src-KD is achieved after incubation at room temperature, for two hours.

We simultaneously tagged the kinase domain to a biotin tag using 10µM enzyme BirA, which we have purified previously, 10mM ATP, 15mM MgCl₂, and 100µM biotin. To purify BirA, we sub-cloned the vector with His tag and used Ni affinity column to separate the protein in the lysate. We then used size exclusion column to separate remaining contaminations.

Next, we got rid of excess dye and biotin using size exclusion Zeba columns and used Talon beads to remove BirA from our mix.

<table>
<thead>
<tr>
<th>Fluorescent dye</th>
<th>Excitation peak</th>
<th>Emission</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cy3b</td>
<td>559nm</td>
<td>570nm</td>
</tr>
<tr>
<td>Alexa 647</td>
<td>650nm</td>
<td>667nm</td>
</tr>
</tbody>
</table>

Table 3. Excitation and emission peak wavelengths for Cy3b and Alexa 647.

Ligation of tagged SH2/SH3 domain with tagged kinase domain

Our next step was to ligate the separately tagged kinase and SH2/SH3 domains. We added Sortase enzyme, kinase domain and SH2/SH3 domains in a ratio of 10:1:1. We used dialysis to remove the His tag that was cleaved off from the SH2/SH3 domain by the Sortase enzyme. We let the reaction sit overnight at 4°C, without the presence of light.
Figure 27. Sortase ligation of labeled SH2/SH3 domain and labeled kinase domain.
Sortase-A cleaves after the threonine residue of the SH2/SH3 domain, forming a thioester with carboxyl carbon of Thr. The N-terminal end of the glycine on the kinase domain then attacks the thioester, forming a peptide bond with the Thr residue. The His-tag used to purify the SH2/SH3 domain was cleaved by the Sortase.

Once the reaction was done, we ran SDS-page gels of the following samples and analyzed them based on their fluorescence: Cy3b tagged SH2/SH3 domain, Alexa-647 tagged kinase domain, ligated full-length Src with both dyes. The main goal of this step was to make sure the both the regulatory domains and the kinase domains were tagged properly and to check if there is any FRET that was happening between the two.

We then used the total internal reflection fluorescence (TIRF) microscope in the Gelles lab to take preliminary data and to optimize conditions to observe FRET happening between the two dyes placed at the SH2 domain and the kinase domain of Src. In order to do so, we flowed the protein on fluorescent slides covered by streptavidin, which has a high affinity for biotin. The binding of biotin to streptavidin is one of the strongest non-covalent interactions with a Kd of around $10^{-15}$ M (Boer et al. 2003). This indicates the efficiency of using biotin tag to attach the ligated Src enzymes to the streptavidin coated fluorescent-microscope slides.
The microscope is set up in such a way that we can excite the donor dye at a certain wavelength and observe the fluorescent emission, of the donor (Cy3B in the SH2 domain) through the green-field lens and simultaneously observe the emission of the acceptor (Alexa-647) through the red-field lens. We were also able to observe fluorescent ‘spots’ that appear in the red field but not in the green field when the green and red lasers were used for excitation respectively. This tells us the region of ‘spots’ were energy transfer is happening, because the green excited Cy3B is not observed because it was transferred to Alexa-647 and hence, we observe the emission in the red field (Figure 28). Once we identified the spots were FRET was happening using the images from the microscope, we then measured their fluorescent intensities using 100s bin times and green laser excitations as can be seen in Figure 23.

Figure 28. Spots observed under the microscope corresponding to fluorescence of ligated Src.
a) Excitation with green laser (532nm) and fluorescence was observed. Spots observed in the green field are those in which Cy3B emitted without any FRET happening between Cy3B and Alexa-647. b) Excitation with red laser (633nm). Spots observed in the red field are fluorescent emission of Alexa-647 from fully ligated and labeled Src. c) Spots that were observed in the red but not green represent fluorescent emission of Alexa-647 but not Cy3B, which indicates the transfer of energy from Cy3B to Alexa-647. Thus, these spots were further analyzed, to observe the FRET that was happening and to do preliminary analysis on the fluorescent traces.
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