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Analysis of Src dynamics in cellular structures

Analýza dynamiky Src v buněčných strukturách

Diploma thesis

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Declaration:

I declare that I completed this master thesis independently and that all sources used are properly acknowledged and cited. Neither this thesis nor any part of it was used to obtain another or similar academic degree.

Prague, 6 August, 2021

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Abstract

Src kinase is a key element in many signaling pathways affecting cellular processes such as differentiation, proliferation, motility, or migration. Deregulation of its activity is associated with the promotion of cancer. Therefore, understanding its cellular function is vital. Src activity directly correlates with its structure; when Src is active, it adopts opened conformation, when inactive, it is in closed conformation stabilized by intramolecular interactions. Detection of the conformation can be used to analyze Src activity. In this thesis, conformation-sensitive FRET-based Src biosensor was improved using mNeonGreen as a new acceptor fluorophore in the existing design and the properties of the new biosensor were compared with the original Src biosensor. The new biosensor is able to detect changes in Src conformation and can be stably expressed in cells. Src activity in focal adhesion was analyzed and higher Src activity in these structures was confirmed. Although the new biosensor did not exhibit significantly better sensitivity to Src conformational changes, it still proved to be a useful tool to study Src activity, and mNeonGreens higher brightness makes it more suitable for microscopic experiments.

Key words: Src, FRET, biosensor, live-cell imaging, mNeonGreen

Abstrakt

Src kináza je klíčovým elementem mnoha signálních drah, které ovlivňují buněčné procesy jako diferenciaci, proliferaci, motilitu či migraci. Deregulace její aktivity je spojena s rozvojem rakoviny. Pochopení její funkce v buňce je tedy zásadní. Aktivita Src kinázy přímo koreluje s její konformací; v aktivním stavu zaujímá otevřenou konformaci, ve stavu inaktivním je v uzavřené konformaci, stabilizované intramolekulárními interakcemi. Detekce konformace Src kinázy tak může být použita ke sledování její aktivity. V této práci byl vylepšen biosenzor, detekující změnu konformace Src kinázy a založený na metodě FRET, a to použitím mNeonGreenu jako nového akceptorového fluoroforu v již existujícím designu. Vlastnosti nového senzoru byly porovnány se senzorem původním. Nový senzor je schopen detekovat konformační změny Src kinázy a lze ho stabilně exprimovat v buňkách. Analýza aktivity Src kinázy ve fokálních adhezích potvrdila zvýšenou aktivitu Src v těchto strukturách. Ačkoli nový biosenzor v porovnání s původním nevykazuje vyšší citlivost ke konformačním změnám Src, stále se jedná o validní a užitečný nástroj pro studium aktivity Src kinázy, který je díky vyššímu jasů mNeonGreenu vhodnější pro mikroskopické experimenty.

Klíčová slova: Src, FRET, biosenzor, mikroskopie živých buněk, mNeonGreen

List of abbreviations

ABL	tyrosine-protein kinase ABL
ATP	adenosine triphosphate
CD linker	linker between SH2 and KD of Src kinase
Chk	Csk-homologous kinase
c-Raf	proto-oncogene serine/threonine-protein kinase
Csk	C-terminal Src kinase
Das	dasatinib
Dock180	dedicator of cytokinesis of molecular weight 180kDa
ECM	extracellular matrix
FA	focal adhesion
FAK	focal adhesion kinase
FAT	focal adhesion targeting domain
FRET	Förster resonance energy transfer
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
Grb2	growth factor receptor-bound protein 2
KD	kinase domain
Lck	lymphocyte-specific tyrosine kinase
m-das	modified dasatinib
MELK	maternal embryonic leucine zipper kinase
p130Cas	protein of molecular weight 130kDa, Crk-associated substrate
PDB	Protein Data Bank
PEI	polyethyleneimine
PKC γ	protein kinase C gamma
Rac1	Ras-related C3 botulinum toxin substrate 1
SFK	Src family kinases
SH1	Src homology 1
SH2	Src homology 2
SH3	Src homology 3

SH4	Src homology 4
Src527F	Src FRET full-length biosensor with mutation of Tyr527
SrcFRET NG	Src FRET full-length biosensor with mNeonGreen as acceptor
SrcFRET TC	Src FRET full-length biosensor with mCitrine as acceptor
SrcNG	Src FRET biosensor control variant with mNeonGreen only
SrcT	Src FRET biosensor control variant with mTurquoise2 only
TIRF	Total Internal Reflection Fluorescence
UD	unique domain

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1. Introduction

One of the most important and developed mechanisms in cell signaling is the system of protein phosphorylation. Regulation of cellular processes depends on the balance between kinases responsible for the transfer of phosphate group to a specific substrate and their antagonists, phosphatases.

Src kinase is a non-receptor tyrosine kinase that plays a crucial role in cellular processes such as cell differentiation, proliferation, survival, adhesion, motility, invasion, morphology, and metabolism. Src represents a signaling node, part of many signaling pathways where different inputs are interpreted through Src activity. Due to its pleiotropic effects on cellular physiology, Src activity must be very strictly and precisely regulated. Deregulation or mutation of Src is associated with various pathologies like cancer, osteopetrosis, incorrect wound healing, or prion disease. Constitutive activation of Src alone can induce malignant transformation, after all, it is the very first oncogene discovered, and activation of the Src pathway has been found in about 50% of tumors from the liver, pancreas, colon, breast, and lung (Dehm and Bonham, 2004; Elsberger, 2014). Its activity in cancer is in general associated with tumor progression, malignancy, and a lower chance of patients survival (Summy and Gallick, 2003). All of this makes Src a very attractive target for potential anti-cancer therapy; indeed some Src inhibitors were already studied in clinical trials and are already used as a treatment, e.g. of chronic myelogenous leukemia (Talpaz et al., 2006).

Even though Src has been studied for over a hundred years we still do not fully understand all its functions and how Src is regulated. For this reason and because of the crucial role that Src plays, scientists need new tools to study this kinase and to fully uncover all about it. One of the methods is a FRET-based biosensor of Src activity.

2. Aims of this thesis

In this thesis, I follow up the work of my colleagues who designed and created genetically encoded FRET-based biosensor of Src activity (Koudelková et al., 2019). The overall aim of this thesis was to create a new biosensor with different FRET pair (mTurquoise2 – mNeonGreen) that should supposedly exhibit better FRET efficiency and compare its abilities and properties with the originally used FRET pair (mTurquoise2 – mCitrine).

The thesis comprises of several minor goals:

- I. Creating and testing the new FRET pair
 - a. Creation of Src biosensor with FRET pair mTurquoise2-mNeonGreen (SrcFRET NG) and all necessary controls
 - b. Preparation of stable cell lines expressing the sensor
 - c. Confirmation of correct localization and stability of the new sensor
 - d. Testing of the ability of the new sensor to detect the conformational change of Src and compare this ability to the original sensor (SrcFRET TC)
- II. Observation of Src activity in focal adhesions
- III. Testing of a new modified inhibitor of Src activity

3. Literature review

3.1. Förster resonance energy transfer

3.1.1. FRET

Förster resonance energy transfer (FRET) is a non-radiant energy transfer (dipole-dipole interaction) that occurs between two fluorophores. It was first described by Theodor Förster in 1948 (Förster, 1948a).

For this phenomenon to occur, fluorophores (called donor and acceptor) must meet three main conditions: spectral overlap, proximity, and correct orientation. These conditions are well demonstrated in various equations describing FRET, e.g. equation for transfer rate of FRET k_t created by Theodor Förster

$$k_t = \frac{1}{\tau_0} \left(\frac{R_0}{r} \right)^6$$

or equation describing FRET efficiency E

$$E = \frac{R_0^6}{R_0^6 + r^6}$$

where R_0 is Förster constant or Förster distance, that is the distance of fluorophores at which the energy transfer efficiency is 50%, τ_0 is the lifetime of the donor and r is the actual distance between donor and acceptor. The value of the Förster constant depends on the spectral overlap, quantum yield of the donor, and orientation of the fluorophores (Jares-Erijman and Jovin, 2003; Zadran et al., 2012).

Considering that FRET efficiency decreases with the sixth power of the distance, the proximity factor has the biggest impact (Edelhoch et al., 1968). Because of that, FRET occurs only when the distance between fluorophores is in interval 1–10 nm which corresponds to distances usually found in intermolecular interactions but also in intramolecular interactions such as conformational changes. Outside of this interval, no energy transfer can be observed. This spatial limitation of FRET makes this method very sensitive with very high resolution (Jares-Erijman and Jovin, 2003).

There are many approaches how to measure FRET and how to calculate FRET efficiency. In this project sensitized emission method was used – the ratio of the fluorescent intensity of acceptor and donor detected upon donor excitation.

3.1.2. FRET pairs

There are new fluorescent proteins constantly being developed to create fluorophores with better properties such as photostability, expression level, quantum yield, or extinction coefficient (Shaner et al., 2005). That also means that many fluorescent proteins can be successfully used as a FRET pair.

FRET pair currently used in Src biosensor in our lab consists of mTurquoise2 and mCitrine (Goedhart et al., 2012; Griesbeck et al., 2001). However, in 2013 there was a new fluorescent protein developed called mNeonGreen that can also serve as an acceptor for mTurquoise2. Unlike mTurquoise2 or mCitrine, mNeonGreen is not derived from *Aequorea victoria* but a different organism, *Branchiostoma lanceolatum*. Compared to other yellow/green fluorescent proteins, it has higher extinction coefficient and high quantum yield (Shaner et al., 2013).

According to fluorophore characteristics taken from www.fpbases.org and its FRET calculator, the pair mTurquoise2-mNeonGreen should theoretically exhibit better FRET efficiency compared to the pair mTurquoise2-mCitrine. R_0 for the pair mTurquoise2-mCitrine is 57.51Å, for the pair mTurquoise2-mNeonGreen 61.55Å. That means that – using the equation for FRET efficiency – at the distance $r=57.51\text{Å}$, mTurquoise2-mCitrine exhibits 50% FRET efficiency (E_1) whereas mTurquoise2-mNeonGreen 60% FRET efficiency (E_2), see calculations below. For that reason, mNeonGreen was studied in this project as a potentially better acceptor for our Src biosensor.

$$E_1 = \frac{R_0^6}{R_0^6 + r^6} = \frac{57.51^6}{57.51^6 + 57.51^6} = 0,5 = 50\%$$

$$E_2 = \frac{R_0^6}{R_0^6 + r^6} = \frac{61.55^6}{61.55^6 + 57.51^6} = 0,6 = 60\%$$

Characteristics of all three fluorescent proteins and their spectral overlap can be found in Fig.1.

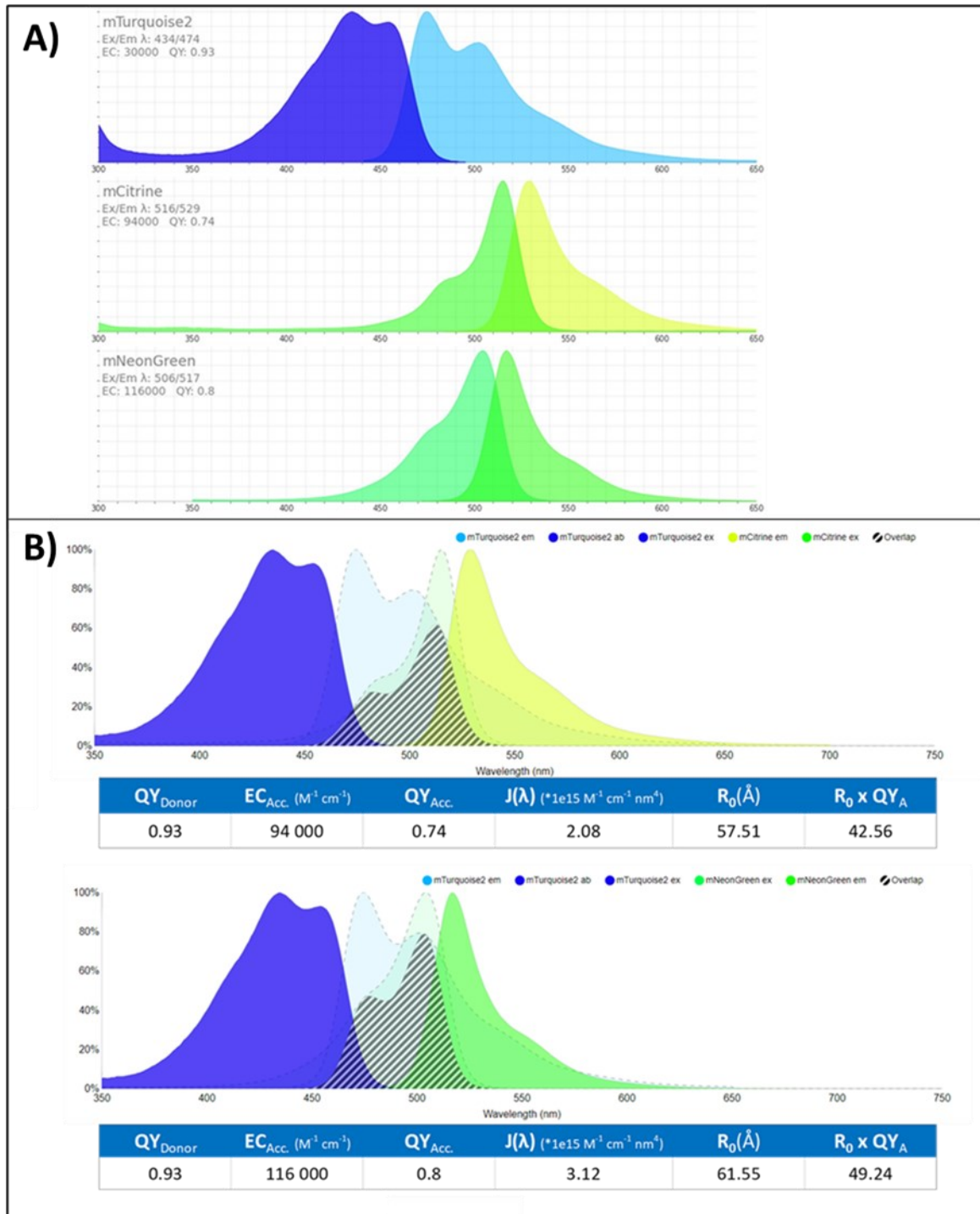


Fig.1: Properties of fluorescent proteins used in this thesis. A) Fluorescence spectrum of mTurquoise2, mCitrine, and mNeonGreen and their characteristics. **B)** Spectral overlap of two FRET pairs: mTurquoise2-mCitrine and mTurquoise2-mNeongreen. QY=quantum yield, EC=extinction coefficient, Ex=excitation wavelength, Em=emission wavelength, J=spectral overlap, R₀=Förster distance. Images were taken and modified from <https://www.fpbases.org>

3.2. Src kinase

Src kinase belongs to a group of non-receptor protein tyrosine kinases that includes ten families: FAK, Src, Jak, Syk, Csk, Frk, Abl, Fps, Tec, and Ack (Tsygankov, 2003). Src family (where Src kinase belongs) in mammals consists of eleven members: Src, Yes, Fyn, Fgr, Lck, Hck, Blk, Lyn, Srm, Brk, and Frk (Summy and Gallick, 2003). Src, Yes, and Fyn are expressed in most of the tissues although the level of expression differs, for example neurons, platelets and osteoclasts express high level of Src (Brown and Cooper, 1996; Horne et al., 1992). Expression of the remaining members of the Src family is more tissue-specific (Manning et al., 2002).

Because the tyrosine kinases are a result of divergent evolution, that results from genomic duplications and subsequent differentiation, some kinases can partially substitute each other's functions as we can see in the case of Src, Yes, and Fyn kinases (Gibson and Spring, 1998; Lowell et al., 1996; Manning et al., 2002; Oykhman et al., 2013). Thanks to that, Src kinase knock-out mice have viable phenotype only with defects in bone resorption (Lowell et al., 1996).

3.2.1. Structure of Src

Src as well as all SFKs is a multidomain protein consisting of six domains: SH4 domain, unique domain (UD), SH3 domain, SH2 domain, kinase domain (KD or SH1), and C-terminal domain. All domains (except for unique domain) are highly conserved within the SFK (Boggon and Eck, 2004a; Engen et al., 2008; Sicheri and Kuriyan, 1997; Tatosyan and Mizenina, 2000) (Fig.2). For purposes of this thesis, chicken c-Src numbering system is used.



Fig.2: Schematic representation of Src kinase structure. From N-terminus to C-terminus: SH4 domain with myristyl, unique domain (UD), SH3 domain, SH2 domain, CD linker, kinase domain (KD), and regulatory C-terminus.

SH4 domain

SFKs have the ability to bind to cellular membranes thanks to their modification by fatty acyl: myristate and except for Src and Blk also palmitate (Koegl et al., 1994; Resh, 1999). Src ability to bind to the membrane is necessary for Src-induced cell transformation (Pellman et al., 1985).

The N-terminus of the Src molecule contains 7 amino acids forming the myristoylation signal sequence. During Src translation, N-terminal methionine is removed and myristic acid is subsequently added to the N-terminal glycine by N-myristoyl transferase (Buss and Sefton, 1985; Garber et al., 1985; Kaplan et al., 1988). Src occurs also in cytosolic form and there are also other proteins with this modification that exist only in a cytosolic form indicating that myristoylation alone is not enough to account for Src binding to the membrane (Buss and Sefton, 1985; Garber et al., 1985; Olson et al., 1985). Src interaction with the membrane is further mediated by basic residues following the myristoylation signal sequence that interact with negatively charged phospholipids of the membrane enhancing the stability of Src association with the membrane (McLaughlin and Aderem, 1995; Resh, 1999; Sigal et al., 1994; Silverman and Resh, 1992).

Unique domain

Unique domain (amino acids 18-81) is a long unstructured region that varies between members of the SFK (Roskoski, 2004; Tatosyan and Mizenina, 2000). Interaction with the SH3 domain stabilizes the UD and enhances its binding to the membrane. UD also plays role in the specificity of protein-protein interaction and regulation of Src activity though its function has not been fully uncovered (Amata et al., 2014; Brown and Cooper, 1996; Hoey et al., 2000; Maffei et al., 2015; Pérez et al., 2013; Summy et al., 2003).

SH3 domain

SH3 domain (amino acids 81-142) is a small globular β -sandwich module consisting of 5 β -strands forming two β -sheets found in many proteins (Cohen et al., 1995; Pawson, 1995; Roskoski, 2004; Weng et al., 1994; Xu et al., 1997). It binds proline-rich sequences with the PxxP motif that upon SH3-binding acquire type II polyproline helix conformation (Feng et al.,

1994; Pawson, 1995; Sicheri and Kuriyan, 1997; Tatosyan and Mizenina, 2000). SH3 can bind ligand in both orientations - orientation is determined by anchoring residue (usually arginine) localized either at the C- or N-terminus of the pseudo-symmetrical PxxP motif. Based on the arginine position, we can distinguish two classes of substrates, Class I with arginine before and Class II with arginine after the PxxP motif (Keenan et al., 2015; Lim et al., 1994; Pawson, 1995; Yu et al., 1994).

SH3 domain is known not just to mediate the interaction between Src kinase and other proteins, but also to directly regulate Src activity. It is necessary for SH2 domain-induced inhibition of Src, and even SH3 domain itself regulates Src activity by direct inhibitory interactions of RT and nSrc loop with the kinase domain (Brábek et al., 2002; Feng et al., 1994; Groveman et al., 2011; Okada et al., 1993; S.W. et al., 2003; Superti-Furga et al., 1993; Weng et al., 1993, 1994; Yadav and Miller, 2007).

SH2 domain

SH2 domain (amino acids 148-245) is a globular module that consists of two helices each on one side of a four-stranded β -sheet (Roskoski, 2004; Tatosyan and Mizenina, 2000; Xu et al., 1997). It can be found in many proteins involved in the signal transduction process (Cohen et al., 1995). Its ligand specificity and affinity are determined by residues around phosphotyrosine contained in the recognized short sequence (Koch et al., 1991; Zhou et al., 1993). Optimal binding sequence for Src and Lck SH2 domain contains pYEEI motif where phosphotyrosine interacts with conserved Arg175 and isoleucine can interact with the deep hydrophobic pocket of the domain. However, not every ligand contains this optimal binding sequence (Brown and Cooper, 1996; Eck et al., 1993; Eckert et al., 2011; Waksman, 1993; Xu et al., 1997).

Besides mediating interaction with proteins such as FAK, paxillin, or p130 Cas (Cobb et al., 1994; Kanner et al., 1991; Weng et al., 1993), the most significant role of the SH2 domain is regulation of Src activity. Through the interaction of the SH2 domain and phosphorylated Tyr527 at the C-terminus, a closed conformation of Src is induced, which inactivates Src activity (mechanism of Src regulation is more thoroughly discussed below) (Roskoski, 2005).

CD linker

CD linker (catalytic domain linker, residues 246-266) is a long extended structure connecting SH2 and kinase domain. Together with SH2 and SH3 domain, it participates in the regulation of KD activity – CD linker interacts with SH3 domain and the small lobe of kinase domain affecting the position of the α C-helix. Highly conserved residue Trp260 plays a crucial role in this interaction (Gonfloni et al., 1997; Sicheri et al., 1997; Xu et al., 1997).

Kinase domain

The structure of the kinase domain is highly homologous to other protein kinases. Kinase domain (KD, amino acid residues 267-520) has its characteristic two-lobe structure of smaller N-lobe and larger C-lobe. N-lobe constitutes of 5 stranded β -sheet and one helix (α C helix), the C-lobe is formed by 7 α -helices and 2 β -strands. At the interface of these two lobes, the active site of the kinase domain is located (Xu et al., 1997).

The active site contains many conserved residues crucial for kinase domain activity. Namely, N-lobe contains a glycine-rich loop also called G-loop (Gly274, Gly276, Gly279) responsible for the coordination of γ -phosphate of ATP and set of residues responsible for the coordination of α - and β -phosphate (Lys295, Gly310). C-lobe includes activation loop (A-loop) containing Tyr416 and Mg^{2+} -binding Asp404 (part of DFG motif that coordinates ATP in the active site) and catalytic loop with conserved residue Asp386 responsible for correct orientation of the substrate tyrosine. In the inactive state, the activation loop forms α -helix hiding Tyr416 in its structure so that it cannot be phosphorylated (Breitenlechner et al., 2005; Cowan-Jacob, 2006; Roskoski, 2004; Xu et al., 1997).

The linker between N-lobe and C-lobe is flexible enabling opening and closing of the active site. Altering mutual movement of the lobes alters kinase domain activity (Roskoski, 2004).

C-terminal domain

C-terminal domain (residues 521-533) is a short sequence containing regulatory Tyr527 present in all vertebrate SFKs. Tyr527 phosphorylated by Csk kinase binds intramolecularly to SH2 inducing the autoinhibitory conformation of Src. Interaction between phosphorylated Tyr527 (pTyr527) and SH2 domain is relatively weak and can be disrupted by other interacting

proteins, for the C-terminal domain does not contain the optimal binding sequence pYEEI. However, it is sufficient for maintaining the inhibited conformation; under basal conditions, 90-95% of Src is phosphorylated at Tyr527, thus in its inactive state (Brown and Cooper, 1996; Lowell et al., 1996; Roskoski, 2004; Shenoy et al., 1992; Yadav and Miller, 2007).

Mutation of Tyr527 disrupts the regulatory function of the C-terminal domain leading to increased activity of Src. The oncogenic variant of Src (v-Src) even lacks the entire C-terminal regulatory domain and therefore is constitutively active (Hunter, 1987; Jove and Hanafusa, 1987; Zheng et al., 2000).

3.2.3. Regulation of Src

Src plays a crucial role in many signaling pathways affecting cell processes such as proliferation, migration, differentiation, etc., and defects in its regulation lead to various pathologies. Therefore, Src activity is very strictly regulated by several mechanisms, most importantly by intramolecular interactions, phosphorylation, or binding of Src interacting partners. All the mechanisms, in the end, result in a conformational change in the active site of the kinase domain and subsequently to a modification of the kinase activity (Thomas and Brugge, 1997; Xu et al., 1999).

Regulatory elements in the kinase domain

Several main components form the active site of the kinase domain. ATP is in the active site coordinated and held by cooperation of the α C helix and DFG motif of the activation loop (A-loop). Glu310 (part of the α C helix) forms a salt bridge with Lys295 and together they coordinate α - and β -phosphate of ATP; γ -phosphate of ATP is coordinated by G-rich loop containing Gly274, Gly276, Gly279. ATP is further coordinated by the interaction of Asp404 (part of the DFG motif) that binds Mg^{2+} cation (Cowan-Jacob et al., 2005; Hubbard et al., 1998).

A-loop also contains Tyr416. When the kinase is active, Tyr416 is phosphorylated as a result of Src trans-autophosphorylation (Boczek et al., 2019). A-loop forms a strand without any secondary structures and does not block the active site. This loose structure is achieved by the interaction of pTyr416 and Arg409, Arg385 and Ala418 stabilizing the A-loop in an active

position and locking the kinase domain in catalytically competent conformation (Meng and Roux, 2014).

Upon transition of Src to its inactive state, the geometry of the active site changes. The α C helix moves from its active “in” position to its “out” position withdrawing the key residue Glu310. Lys295 is no longer able to bind to Glu310 and instead forms a salt bridge with Asp404. Asp404 then moves from its “in” to “out” position and subsequently cannot bind Mg^{2+} and coordinate position of ATP in the active site. A-loop also forms a short α -helix that blocks the active site of the kinase domain and buries the non-phosphorylated Tyr416 in its structure making it inaccessible to phosphorylation (Fig.3) (Koudelková, 2020; Meng et al., 2017; Xu et al., 1999).

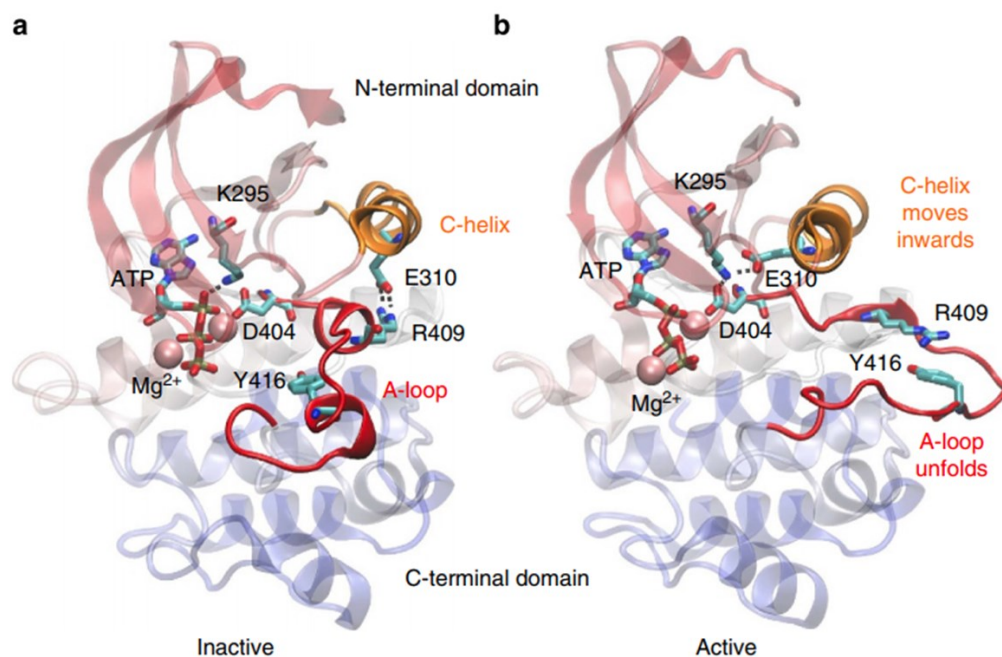


Fig.3: Conformational changes in the active site. A) In the inactive conformation, Tyr416 is dephosphorylated and hidden in the structure of A-loop (red), A-loop forms α -helix blocking the active site. C-helix (yellow) is in the inactive “out” position. B) In the active conformation α C helix is moved to “in” position, A-loop is unfolded, Tyr416 is accessible for phosphorylation. Image taken from (Shukla et al., 2014).

SH2 and SH3 domains in the regulation of Src activity and structure

The activity of Src kinase depends on its conformation; in its inactive state, Src is in closed conformation and transitions into a loose opened structure upon activation. The inactivation process is regulated by a complex mechanism of intramolecular interactions resulting in stabilizing the inactive conformation of Src. Disruption of these interactions leads to opening of the structure, therefore activation of kinase activity (Xu et al., 1997).

In the inactive state, Tyr527 of the C-terminus is phosphorylated - this phosphorylation is mediated by Csk and Chk kinases. Csk exhibits high specificity for Src kinase; C-lobe of its kinase domain associates with C-lobe of Src kinase domain bringing its active site to the position of Tyr527 enabling transfer of the phosphate. Chk kinase, beside phosphorylation of the Tyr527, is also able to bind and by that inhibit the active form of Src, although the exact process and structural aspects of this non-catalytic inhibitory mechanism are not yet fully discovered (Advani et al., 2017; Chong et al., 2004, 2006; Ia et al., 2010; Levinson et al., 2008; Okada, 2012; Okada et al., 1993). Tyr527 dephosphorylation (leading to Src activation) is mediated by various phosphatases: Shp1, Shp2, PTP1B, CD45, PTP α (Roskoski, 2005; Thomas and Brugge, 1997; Zheng et al., 2000)

SH2 domain binds the phosphorylated pTyr527 in its binding pocket. This interaction is quite weak – the SH2 domain preferentially binds motif pYEEI but at the C-terminal domain there is glycine at the pY+3 position. Besides interaction with pTyr527, the SH2 domain also electrostatically interacts with the C-lobe of the kinase domain (Xu et al., 1997).

SH3 domain interacts with the CD linker. Similar to SH2-pTyr527, this interaction is relatively weak because the CD linker does not contain the optimal binding motif PxxP. Instead of proline, glutamine is present at the position of the second proline. SH3 domain also associates with N-lobe of the kinase domain directly through RT and nSrc loop. (Hubbard et al., 1998; Sicheri et al., 1997; Xu et al., 1997)

CD linker is a crucial player in Src activity regulation responsible for direct inhibition of the kinase domain. SH2 and SH3 domains stabilize the CD linker and restrain it in its inhibitory conformation. CD linker directly interacts with both the SH3 domain (with RT loop) and the kinase domain (C-lobe) acting as a glue connecting these three structures. Due to the interaction with the SH3 domain, the CD linker is packed between the kinase domain and the

SH3 domain. CD linker binds to C-lobe close to α C helix and pushes it by highly conserved Trp260 out of the catalytically active position. For activation of Src kinase, change of CD linker conformation is necessary: Trp260 rotates out of the C-lobe allowing rotation of the α C helix to its active position (Fig.4) (Fajer et al., 2017; Gonfloni et al., 1997; Hubbard et al., 1998; Xu et al., 1997).

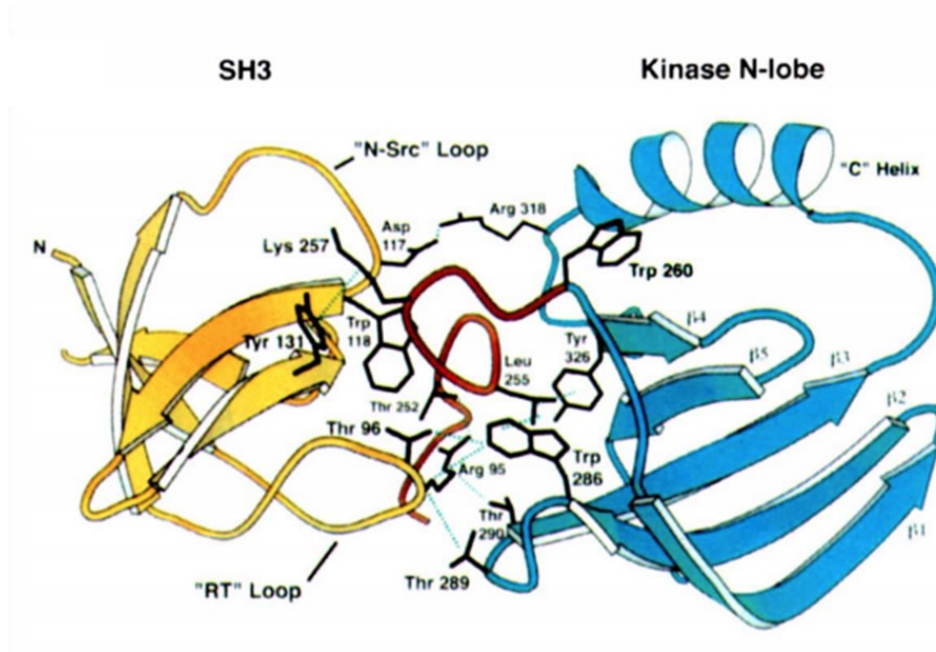


Fig.4: Interaction between SH3 domain, kinase domain, and the CD linker. SH3 domain (yellow) associates with the kinase domain (blue) by RT and nSrc loop. CD linker (red) interacts with both SH3 and kinase domains. Trp260 stabilizes the α C helix in a catalytically incompetent position. The image was taken from (Xu et al., 1999).

In the assembled inactive state, SH3 and SH2 domains exhibit strongly correlated dynamics meaning their combined structure is more rigid in comparison to the active state of the kinase. Therefore, SH3 and SH2 domains do not act independently when in the inactive conformation. On the other hand, when Tyr527 is not phosphorylated (and Src is in the active state), SH3 and SH2 domains move independently on each other. This ability to increase the rigidity of their combined structure is necessary for the correct regulation of Src. 6-amino acid linker connecting SH3 and SH2 domain (SH3-SH2 linker) was shown to play an essential role in this process. Substitution of three residues in this linker by glycine results in higher flexibility of this linker and subsequently leads to constitutive activation of Src (Young et al., 2001). The importance of SH3-SH2 linker for the overall structure was also studied and confirmed in Hck kinase (Meiselbach and Sticht, 2011).

To summarize, SH2 and SH3 domains form a clamp that clutches the two lobes of the kinase domain and stabilizes the position of the CD linker. The structures of SH2 and SH3 domains are transmitted and projected through the CD linker into conformation (therefore activity) of the kinase domain. Modifying interactions and mutual movement of the regulatory domains impairs the strength of the clamp and its ability to inhibit the kinase domain. Such modifications can be mutations in the CD linker, binding pocket of the SH3 domain, RT and nSrc loop of the SH3 domain, SH3-SH2 linker, SH2 binding pocket, or C-terminal domain (Tyr527). In general, mutations inducing higher flexibility (disabling the clamp) lead to higher activity of the kinase (Brábek et al., 2002; Cowan-Jacob et al., 2005; Fajer et al., 2017; Gonfloni et al., 1997; Harrison, 2003; Xu et al., 1997; Young et al., 2001). Disrupting the clamp is also the principal of physiological regulation of Src kinase – either through phosphorylation of the Tyr527 or by binding SH2 and SH3 domain by other interacting partners (Alexandropoulos and Baltimore, 1996; Boggon and Eck, 2004b; Roskoski, 2005; Yadav and Miller, 2007). (Fig.5)

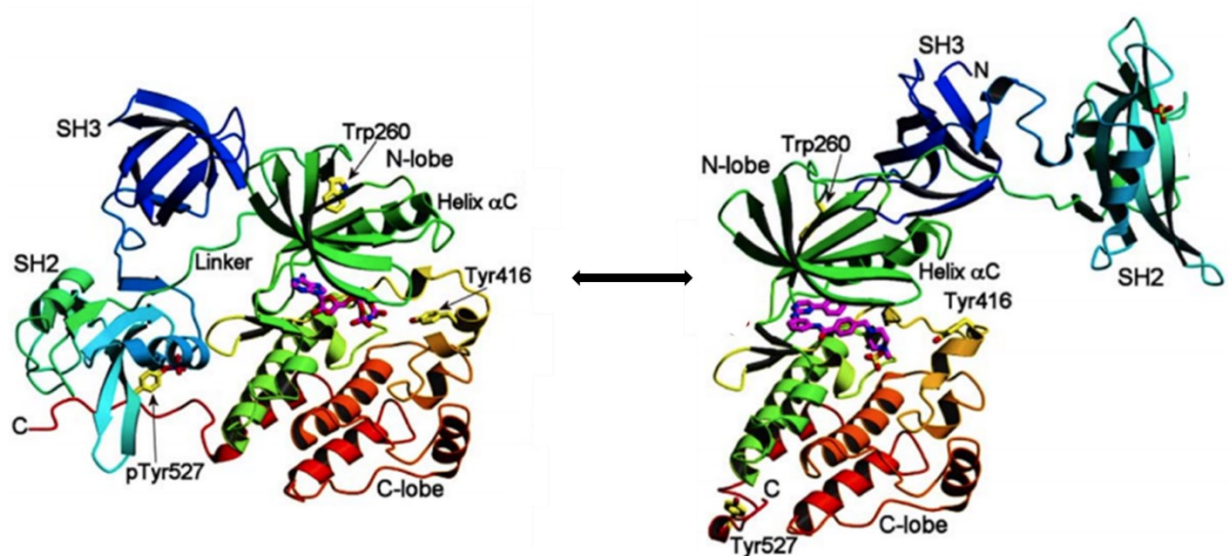


Fig.5: Overall conformational changes upon Src activation. pTyr527 binds to the SH2 domain and induces compact structure of the Src kinase, where SH3 and SH2 domains stabilize the CD linker's inhibitory conformation. When Tyr527 is dephosphorylated, the SH2-SH3 clamp loosens and the Src molecule opens. SH2 domain (light blue), SH3 domain (dark blue), N-lobe of the kinase domain (green), C-lobe of the kinase domain (red). The image was taken and modified from (Cowan-Jacob et al., 2005).

3.3. Src biosensor design

In my thesis, I use a biosensor design that was developed and published in 2019 by my colleagues (Koudelková et al., 2019). As I described, FRET is highly dependable on the distance between fluorophores, therefore FRET-based biosensors can detect changes in the distance between two molecules or two parts of the same molecule. Src kinase rapidly changes its conformation between active and inactive states. During the process of activation and inhibition, domains of Src change their proximity to each other - the greatest difference being between the SH2 domain and C-terminus. Therefore, it was decided to put fluorophores at these two places, the donor in the structure of the SH2 domain and the acceptor at the very C-terminus. To avoid any disruption of the normal regulatory function of these parts, the donor fluorophore was flanked by flexible linkers 8 and 11 amino acids long and the acceptor fluorophore was connected to the C-terminus by 7 amino acid long linker (Koudelková et al., 2019). (Fig.6)

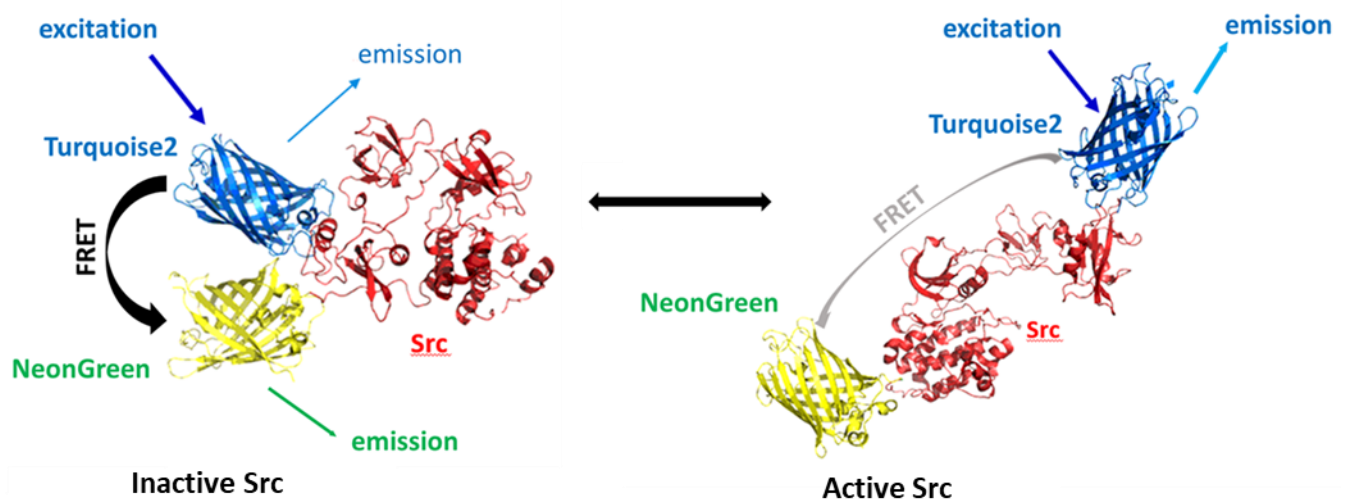


Fig.6: Model of the Src biosensor. In the inactive state, the Src molecule is in the closed conformation, fluorophores are close to each other, and high FRET can be detected. In the active state, the Src molecule is opened, fluorophores further from each other, and FRET is decreased. Image taken and modified from (Koudelková et al., 2019).

3.4. Src role in focal adhesions and integrin signaling

Focal adhesions (FA) are large multiprotein complexes mediating cell interaction with extracellular matrix (ECM). Their function is not only in mechanical anchoring of the cell to ECM but also in signaling role associated with mechanotransduction, cell survival, migration,

etc. When integrins bind their extracellular ligands, they form large clusters and recruit other proteins resulting in the assembly of focal adhesion. Src kinase plays role in the processes, especially in regulating FAs dynamics (Ciobanasu et al., 2013; Playford and Schaller, 2004).

When Src activity is not induced, Src is preferentially localized in the perinuclear area where it associates for example with endosomal membranes. Upon Src activation, Src is recruited to focal adhesions. Interestingly, for its localization to FAs, kinase activity is not essential. Opening of the Src structure (either by activation or for example treatment by dasatinib inducing opened conformation of Src) is sufficient for its recruitment to focal adhesion (Fincham and Frame, 1998; Hamadi et al., 2009; Koudelková et al., 2019). Src activation as a response to cell adhesion is probably mediated by phosphatases PTP α or PTP1B that dephosphorylate Tyr527 of Src. This induces the opening of Src structure, and therefore recruitment to FAs, where it can phosphorylate its substrates (Playford and Schaller, 2004).

When integrins are bound to ECM, associated focal adhesion kinase (FAK) is activated, FAK undergoes autophosphorylation of Tyr397 which creates a binding site for the Src SH2 domain (Cobb et al., 1994). Src subsequently binds to FAK and phosphorylates it at other residues (Tyr407, 576, 577, 861, and 925). Src-mediated phosphorylation leads to the full activation of FAK signaling in two different ways. First, Tyr576 and Tyr577 in the activation loop are phosphorylated by Src and their phosphorylation leads to full catalytic activation of FAK. Second, phosphorylation of Tyr861 and Tyr925 creates binding sites for FAK interacting proteins which activate the FAK signaling pathway, e.g. for the SH2 domain of Grb2 resulting in the activation of the Ras/MAPK pathway (Brunton et al., 2005; Calalb et al., 1995, 1996; Jones et al., 2000; Playford and Schaller, 2004; Schlaepfer and Hunter, 1996; Westhoff et al., 2004). Mutation of Tyr397 of FAK is not able to bind Src and subsequently cannot induce FA disassembly, resulting in more and enlarged FAs. The same phenotype can be observed in FAK or Src knock-out cells. That indicates that Src and FAK activity is not necessary for FA assembly itself, but rather for regulation of its dynamics (Fincham and Frame, 1998; Ilić et al., 1995; Sieg et al., 1999).

Another Src target associated with focal adhesions is p130Cas. This protein is also localized to focal adhesions and is phosphorylated by both Src and FAK. Its phosphorylation leads to activation of the Crk/Dock180/Rac1 pathway that is responsible for the regulation of cell migration (Cary et al., 1998; Fonseca et al., 2004; Hsia et al., 2003; Sakai et al., 1994; Wang et

al., 2010). Phosphorylation of SH3 domain of p130Cas by Src also leads to inhibition of p130Cas binding to focal adhesions which results in destabilization of FAs and decreased cell migration (Janoštiak et al., 2011).

Besides FAK and p130Cas, there are other Src substrates such as paxillin or vinculin, their phosphorylation is also associated with higher focal adhesions dynamics (Ishizawa and Parsons, 2004; Sefton et al., 1981; Webb et al., 2004; Zaidel-Bar et al., 2007).

3.5. Dasatinib

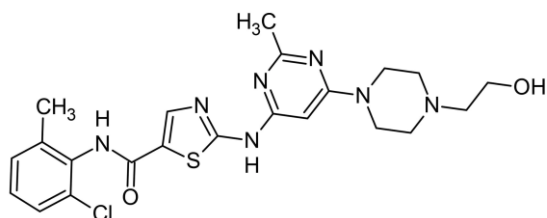


Fig.7: The structure of **dasatinib**. The image was taken from <https://www.abcam.com/>

Dasatinib is a small ATP-competitive inhibitor of tyrosine kinases (Fig.7). It belongs to the class of Type I inhibitors. This class is characterized by the binding of the adenine pocket of the kinase in its active conformation, that is with the DFG motif as well as α C helix “in” (Roskoski, 2016). Interestingly, when dasatinib and its effect on Src kinase were studied, data indicated that mere stabilization of this active conformation of the kinase domain (α C helix “in”) was sufficient for inducing opened structure of the whole Src molecule. That means that although dasatinib inhibits Src activity by blocking the ATP-binding site, it also stabilizes opened conformation of Src (characteristic for active Src) and for this stabilization, active conformation of the active site is sufficient (Koudelková et al., 2019). Opening of the Src structure by dasatinib subsequently leads to Src recruitment to focal adhesions as opened Src is preferentially localized to FAs (Fincham and Frame, 1998; Koudelkova et al., 2018). A similar effect on Src was observed with other α C helix “in” Src inhibitors - bosutinib and UM-164 (Koudelková et al., 2019).

For its ability to bind and inhibit ABL kinase, dasatinib is used as a treatment of chronic myeloid leukemia and Philadelphia chromosome-positive acute lymphoblastic leukemia (Talpaz et al.,

2006). Besides ABL kinase, dasatinib inhibits other kinases such as Src family kinase including Src kinase itself, and therefore influences many processes like cell invasion and proliferation (Johnson et al., 2005).

4. Material and methods

4.1. Cell cultures

4.1.1. Cell lines

U2OS – human bone osteosarcoma epithelial cells

3T3 – mouse embryonal fibroblasts

SYF – mouse embryonal fibroblasts with a triple knock-out of kinases Src, Yes, and Fyn

Phoenix Ampho – packaging cell line for retroviral transfection

4.1.2. Culturing cells

All cell lines were cultured in humidified atmosphere with 5% CO₂ under standard conditions in 37 °C. Cells were cultivated in plastic cell dishes (see Table 1 in Attachments). Using a light microscope, the confluence of the cells was regularly determined and cells split accordingly so that overgrown cultures would be avoided.

4.1.3. Splitting cells

Cells were split every 2-3 days to a final confluence between 20-80%. The ratio of the splitting depended on the cell type and its proliferation rate.

- I. Pre-heat culture medium (37 °C)
- II. Aspirate culture medium using a vacuum pump
- III. Wash cells with PBS (~1/5 of the original medium volume)
- IV. Aspirate PBS using a vacuum pump
- V. Add trypsin solution and by moving the cell dish from side to side cover the whole surface
- VI. Leave cells with trypsin to detach from the plate (~3-8 min, depends on the cell line and confluence)
- VII. Add cell culture medium (double the volume of trypsin solution)
- VIII. Re-suspend the cells
- IX. Accordingly to desired splitting ratio, remove the given volume of suspension

- X. Add culture medium to the remaining cells (the volume depends on the size of the cell dish)
- XI. Move the cell dish from side to side to ensure even distribution

4.1.4. Stock cells

Cells were temporarily placed in a freezer (-80 °C) and then moved to liquid nitrogen (-196 °C) for long-term storage.

- I. Prepare the freezing medium (~1 ml per aliquot)
- II. Prepare 15ml spinning tube with 4 ml of medium per cell line
- III. Aspirate culture medium from cells using a vacuum pump
- IV. Wash cells with PBS (~1/5 of the original medium volume)
- V. Aspire PBS using a vacuum pump
- VI. Add trypsin solution
- VII. Leave cells with trypsin to detach from the plate (~3-8 min, depends on the cell line and confluence)
- VIII. Add cell culture medium (double the volume of trypsin solution)
- IX. Re-suspend the cells and transfer them to the 15ml spinning tube
- X. Centrifuge the cells for 4 min at room temperature, 1000 rpm (180 rcf)
- XI. Re-suspend pellet in freezing medium
- XII. Add 1 ml of the cell suspension to each cryotube
- XIII. Place the cryotubes in a freezing container
- XIV. Place the freezing container in a freezer (-80 °C)
- XV. After 2-3 days, move the cryotubes to liquid nitrogen

Thawing frozen cells

- I. Pre-heat culture medium to 37 °C
- II. Prepare 15ml spinning tube with 4 ml of medium per cryotube
- III. Remove cryotube from liquid nitrogen and place it on ice
- IV. Thaw cells in 37 °C water bath
- V. Transfer cell suspension from cryotube to 15ml spinning tube

- VI. Centrifuge the cells for 4 min at room temperature, 1000 rpm (180 rcf)
- VII. Aspirate the supernatant using a vacuum pump
- VIII. Re-suspend pellet in cell culture medium
- IX. Transfer the suspension to a new cell dish with a fresh culture medium (the volume depends on the size of the dish)

4.1.5. Transient cell transfection

Cells were transfected at the confluence between 20-50%. As a transfection reagent, polyethyleneimine (PEI) was used. PEI is a polymer with polycationic character forming complexes with DNA that undergo endocytosis (Boussif et al., 1995).

- I. Prepare two 0.5ml tubes per every transfected construct
- II. Add serum-free DMEM medium to every tube (the volume depends on the size of the dish – see Table 2 in Attachments)
- III. To every tube add a given amount of either DNA or PEI
- IV. Thoroughly mix the content of PEI-containing and DNA-containing tube
- V. Incubate at room temperature for 15 min
- VI. Drop-wise, add the transfection mixture to cells
- VII. Move the cell dish from side to side to ensure even distribution
- VIII. After 24 hours, change culture medium
- IX. Wait another 24 hours before conducting any experiment

4.1.6. Retroviral transfection

Retroviral transfection was used to prepare both SYF and 3T3 stable cell lines expressing Src biosensor. For transfection, the Phoenix Amfo system was used.

- I. Transfect Phoenix Ampho cells with pMSCV vector carrying our protein of interest (Src biosensor) according to the Cell transfection protocol
- II. Collect the conditioned medium from Phoenix Ampho cells and replace it with a fresh culture medium
- III. Filter the conditioned medium using 0.45µm filter

- IV. Replace culture medium from the dish with SYF or 3T3 cells with the filtered condition medium (size of the dish with SYF or 3T3 should be the same as of the dish with Phoenix Ampho)
- V. Repeat steps every II and III every 24 hours for total of three times
- VI. Sort positive cells by flow cytometry

4.1.7 Flow cytometry

Flow cytometry was used to sort positive cells when preparing stable cell lines. Positive cells expressed Src biosensor or one of the controls that all contain fluorescent protein, therefore no additional staining was necessary.

Cell sorting was performed at the IMCF facility in BIOCEV by MSc. Marziyya Askarzade and Mgr. Ondřej Honc.

- I. Prepare one 15ml tube with 10 ml of culture medium per cell line to be sorted (plus control cells)
- II. Aspirate culture medium using a vacuum pump
- III. Wash cells with PBS (~1/5 of the original medium volume)
- IV. Aspirate PBS using a vacuum pump
- V. Add trypsin solution and by moving the cell dish from side to side cover the whole surface
- VI. Leave cells with trypsin to detach from the plate (~3-8 min, depends on the cell line and confluence)
- VII. After the cells detach, add cell culture medium (double the volume of trypsin solution) and transfer the cells to a 15ml tube
- VIII. Centrifuge at 180 rcf for 4 min
- IX. Aspirate the supernatant
- X. Re-suspend the pellet in flow cytometry medium (the volume depends on the number of cells; 0.5-2 ml; ideal final concentration is about $5 \cdot 10^6$ cells per 1 ml)
- XI. Sort cells on the flow cytometer

4.1.8. Preparation of cells for microscopy

- I. Coat 35mm glass-bottom cell dish (MatTek) with 10 $\mu\text{g/ml}$ fibronectin for 2 h at room temperature
- II. Aspirate surplus of fibronectin solution from the glass-bottom dish
- III. Add 2 ml of culture medium to the 35mm dish
- IV. Aspirate culture medium using a vacuum pump
- V. Wash cells with PBS ($\sim 1/5$ of the original medium volume)
- VI. Aspirate PBS using a vacuum pump
- VII. Add trypsin solution and by moving the cell dish from side to side cover the whole surface
- VIII. Leave cells with trypsin to detach from the plate ($\sim 3-8$ min, depends on the cell line and confluence)
- IX. Add cell culture medium (double the volume of trypsin solution)
- X. Transfer appropriate volume of the cell suspension to the glass-bottom dish (the volume depends on the desired confluence)
- XI. Add 2 ml of cell culture medium
- XII. After 24 hours (or after the cells attach to the dish), observe the cells under a microscope. Beforehand, replace the culture medium with a medium for microscopy.

4.1.9. Calculation of FRET sensitized emission from microscopic images

Images were obtained by Nikon N-SIM & N-STORM microscope equipped with EM CCD Andor iXon Ultra DU897 camera and Nikon CFI HP Apo TIRF 100x Oil, NA 1.49 objective. In the light path before the camera, an image splitter (DualView DV2, Photometrics, AZ) with filter cube splitting the laser beam at 505 nm was inserted. Excitation lasers 445nm and 488nm were used. Obtained dual images were processed using ImageJ software and analyzed in ImageJ using the PixFRET plugin (Feige et al., 2005). FRET normalized to mTurquoise2 (donor) emission and with correction for bleed-through was calculated as nF/I_{CFP} according to (Xia and Liu, 2001).

4.1.10. Statistical analysis

Data for the statistical analysis were acquired from at least three independent replicates. Statistical analysis was conducted in Excel using unpaired T-Test.

4.1.11. Solutions and chemicals

Cell culture medium

- Dulbecco's Modified Eagle's Medium (DMEM, Sigma, D2429-100ML)
- 10% Fetal Bovine Serum (FBS)
- ciprofloxacin 10 µg/ml

Cell culture medium for microscopy

- FluoroBrite™ DMEM (Thermofisher, A18967-01)
- 10% FBS

Cell culture medium for cell sorting

- Dulbecco's Modified Eagle's Medium (Thermofisher, 21063029)
- 1% FBS

Ciprofloxacin (Sigma, 17850)

Dasatinib (LC Laboratories, D3307), working concentration 1 µM – Src inhibitor

Dimethyl sulfoxide (DMSO, Sigma, D2650-100ML)

Dulbecco's Modified Eagle's Medium (DMEM, Sigma, D2429-100ML)

Fetal Bovine Serum (FBS, Sigma, F7524)

Freezing medium

- 90% FBS
- 10% DMSO

Trypsin-EDTA solution 0,25% (Sigma, T4049-100ML)

4.1.12. Equipment

BD FACS Aria Fusion

Centrifuge 5804 R

Leica Thunder Imaging System

Nikon-Eclipse TES100

Nikon N-SIM & N-STORM

4.2. Biochemical and molecular methods

4.2.1. Preparation of protein lysates

- I. Place cell plates on ice
- II. Aspirate culture medium using a vacuum pump
- III. 2x wash cells with cold PBS
- IV. Add lysis buffer
- V. Incubate in the fridge for 15 min
- VI. Scrape the cells using a cell scraper
- VII. Transfer lysate to 1.5ml tube
- VIII. Centrifuge tubes at 4 °C and 15000 rcf for 15 min
- IX. Transfer supernatant to clean 1.5ml tubes and place them on ice
- X. Fluorescence spectrometry measurement

- XI. Add 6x Laemmli buffer and 50 μ M DTT (Dithiothreitol)
- XII. Freeze the lysates / continue with SDS-PAGE or other experiments

4.2.2. Western blotting

SDS-PAGE electrophoresis

Gel preparation

- I. Wash the glass plates
- II. Assemble the glasses in the casting frame

- III. Place the casting frame in casting stand
- IV. Prepare 10% non-gradient running gel (see Table 3 in Attachments)
- V. Pour running gel between the glass plates to a height of about two thirds
- VI. Layer the top of the gel with distilled water
- VII. Let the gel at room temperature to polymerize (about 30 min)
- VIII. Prepare stacking gel (see Table 3 in Attachments)
- IX. Pour out the water overlaying running gel
- X. Pour stacking gel between the glass plates on top of the running gel
- XI. Add combs to create wells
- XII. Let the gel to polymerize at room temperature (about 15 min)

Running the gel

- I. Assemble the running apparatus
- II. Remove combs
- III. Add running buffer (SDS buffer) in both chambers of the apparatus
- IV. Load the samples in wells
- V. Run the gel at 10 mA per gel until the samples are stacked on the running gel
- VI. Increase to 20 mA per gel and continue the run until the loading dye reaches the bottom of the gel
- VII. Disassemble the SDS-PAGE apparatus
- VIII. Remove stacking gel from the running gel

Protein transfer

- I. Prepare nitrocellulose membrane of the same size as gel
- II. Soak the membrane and sponges in transfer buffer
- III. Assemble the transfer sandwich in the transfer cassette in the following order:
sponge, membrane, running gel, sponge
- IV. Carefully remove any air bubbles with a roller
- V. Perform transfer (9 min, 2.5 A)

- VI. Disassemble the cassette and carefully take out the membrane for further processing (e.g. Western blotting)

Protein staining

- I. Place nitrocellulose membrane with transferred proteins in a bath with TBS
- II. Replace TBS with a 5% solution of non-fat dry milk in TBS
- III. Incubate for 1 h at room temperature
- IV. Place the membrane in 1% bovine serum albumin solution in TBS with primary antibody (see Table 4 in Attachments)
- V. Incubate at 5 °C overnight on a tilting laboratory shaker
- VI. Remove the primary antibody solution by pipette and stock it for recycle (for stocking add sodium azide to final concentration 0.02%)
- VII. 3x wash membrane by placing it in fresh TTBS for 10 min on a tilting laboratory shaker
- VIII. Place the membrane in a bath with a solution of secondary antibodies conjugated with horseradish peroxidase in 2% non-fat dry milk in TBS (see Table 4 in Attachments)
- IX. 1x wash membrane by placing it in fresh TBS for 10 min on a tilting laboratory shaker
- X. Place the membrane in foil
- XI. Mix the horseradish peroxidase substrate (Wester Bright™ ECL)
- XII. Transfer the mixture equally to the membrane
- XIII. Visualize proteins by Amersham Imager

Optionally, it is possible to strip the membrane of the antibody and detect other proteins on the same membrane, for example, GAPDH as control of the equal loading amount of the sample

- I. Place the membrane in 200 nM NaOH solution
- II. Incubate for 10 min at 42 °C on shaker or tilting shaker
- III. Stain protein according to protein staining protocol

4.2.3. Agarose electrophoresis

Gel preparation – 10% agarose gel

- I. Dissolve 1 g of agarose in 100 ml TBE buffer in Erlenmeyer flask
- II. Melt agarose by heating the solution in a microwave
- III. Prepare a gel tray
- IV. Let the melted agarose cool down to 40-50 °C
- V. Add 1 µl of DNA stain
- VI. Pour agarose in gel tray
- VII. Place combs to create wells
- VIII. Let the gel solidify at room temperature

Running agarose gel

- I. Place the gel tray in the electrophoresis apparatus
- II. Fill the apparatus with 0.5% TBE
- III. Remove the combs
- IV. Load 4 µl of 1 kb DNA marker
- V. Load mixture of 0.2-1 µg of DNA and 6x loading dye
- VI. Run the gel at 150 V until the loading dye reaches the end of the gel
- VII. Take out the gel visualize fragments by UV transilluminator

4.2.4. DNA extraction from agarose gel

DNA was extracted from agarose gel using NucleoSpin® Gel and PCR Clean-up kit according to the protocol provided by the manufacturer.

4.2.5. Bacterial heat shock transformation

- I. Place competent E. Coli from the freezer (-80 °C) on ice
- II. Let competent cells thaw on ice
- III. Add ~ 10 pg of DNA into 30-50 µl of competent cells
- IV. Incubate on ice for 20 min

- V. Place the tubes for 30 s in a thermoblock heated to 42 °C
- VI. Immediately place on ice and add 200 µl of SOC medium
- VII. Plate 50-250 µl of the transformation on 10 cm LB agar plate containing ampicillin
- VIII. Incubate the LB agar plate at 37 °C overnight

4.2.6. Bacterial culture inoculation

- I. Prepare cultivation tubes with KB medium with antibiotics (ampicillin)
- II. Using pipette tip scoop up either a single colony from LB agar plate or frozen stock bacteria culture and swirl it in the tube with KB medium
- III. Place the tube in a shaking incubator
- IV. Incubate at 37 °C overnight (~18 h)

4.2.7. Plasmid DNA isolation from bacterial suspension culture

- I. Centrifuge 2 ml of bacterial culture in 2ml tube for 1 min at 18000 rcf
- II. Aspirate supernatant
- III. Add 100 µl of TEG solution
- IV. Vortex tubes so all the pellet is re-suspended
- V. Prepare lysis buffer
- VI. Add 280 µl of lysis buffer to each tube simultaneously with vortexing tube for 1 s
- VII. After 60 s, neutralize the lysis buffer by adding 420 µl of 10 M NH₄Ac (ammonium acetate) and simultaneously vortexing the tube for 5 s
- VIII. Add 280 µl of phenol/chloroform solution
- IX. Centrifuge tubes for 5 min at 14000 rcf
- X. Transfer upper colorless fraction to clean 1.5ml tube
- XI. Add 450-200 µl of isopropanol (about 0.7 of the volume of the colorless fraction)
- XII. Vortex vigorously for 10 s
- XIII. Incubate for 10 min at room temperature
- XIV. Centrifuge for 15 min at 14000 rcf
- XV. Discard supernatant
- XVI. Add 600 µl of 80% ethanol
- XVII. Tilt tubes until pellet detaches

- XVIII. Centrifuge for 1 min at 14000 rcf
- XIX. Carefully aspirate supernatant
- XX. Let the pellet dry at room temperature
- XXI. Dissolve dried pellet in RNase solution (0.2 mg/ml)
- XXII. Incubate for 30 min at 37 °C
- XXIII. Stock DNA at -20 °C or process it further (restriction enzyme digestion, purification, agarose electrophoresis, etc.)

4.2.8. DNA purification and concentration measurement

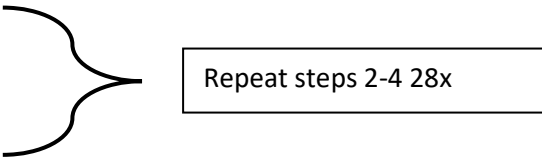
- I. Add magnetic beads to unpurified DNA in 1.5ml tube (2.5 ml of the original volume)
- II. Incubate for 5 min at room temperature
- III. Place tube in a magnetic stand
- IV. After 5 min aspirate supernatant
- V. Add 300 µl of 70% EtOH
- VI. After 1 min aspirate supernatant
- VII. Add another 300 µl of 70% EtOH
- VIII. After 1 min aspirate EtOH
- IX. Place the tube outside the magnetic stand and let magnetic beads dry at room temperature
- X. Resuspend magnetic beads in distilled water (the volume depends on the original volume of unpurified DNA and expected yield)
- XI. Incubate for 5 min at room temperature
- XII. Place tube back in the magnetic stand
- XIII. After 5min transfer supernatant to clean 1.5ml tube
- XIV. Measure DNA concentration on Nanodrop UV/VIS spectrophotometer

4.2.9. Polymerase chain reaction (PCR)

- I. Prepare reaction mix (see Table 5 in Attachments)
- II. Mix on vortex
- III. Run PCR

IV. Purify the product

1	95 °C	2 min
2	95 °C	30 s
3	X °C	30 s
4	72 °C	1 min/kb
5	4 °C	unlimited



Repeat steps 2-4 28x

X... The annealing temperature of primers

For a full list of primers, see Table 6 in Attachments.

4.2.10. Restriction enzyme digestion

- I. Prepare reaction in 0.5ml tube (see Table 7 in Attachments)
- II. Incubate at 37 °C for 1 h
- III. Verify on agarose gel

4.2.11. DNA ligation

- I. Prepare reaction in 0.5ml tube (see Table 8 in Attachments)
- II. Incubate at room temperature for 1 h
- III. Proceed to bacterial transformation

4.2.11. Gibson Assembly

For GeneArt® Seamless Cloning and Assembly, the protocol provided by the manufacturer was used.

- I. In a microcentrifuge tube, set up the seamless cloning and assembly reaction. It is crucial that you add the GeneArt® 2X Enzyme Mix as the last component (see Step 2)

Insert(s) (200 ng each)	x µL
Linear cloning vector (50 ng)	1 µL
Deionized water	to 5 µL

- II. Quickly thaw the GeneArt® 2X Enzyme Mix on ice and pipette up and down to mix thoroughly. Add 5 µL of the thawed GeneArt® 2X Enzyme Mix to each reaction mixture, and immediately return it back to –80 °C
- III. Mix the reaction components completely by pipetting them up and down 3 times and then gently tapping the sides of the tube 3–5 times
- IV. Briefly centrifuge (<500 rpm for <5 seconds) to collect the reaction components to the bottom of the microcentrifuge tube
- V. Incubate the reaction mix at room temperature for 15–30 minutes. Do not incubate longer than 30 minutes
- VI. After incubation is complete, place the reaction mix on ice for 2–5 minutes before proceeding to the transformation step. Do not let the samples stay on ice for more

4.2.12. Solutions and chemicals

6x loading dye (BioLabs New England, B7024S)

Acrylamide/Bis-acrylamide, 30% solution (Sigma, A3699-100ML)

Bacteriological agar (VWR Chemicals, 97064-336)

Bovine serum albumin (Sigma, 9048-46-8)

Chloroform (Lach-Ner, 30450)

Dasatinib (LC Laboratories, D3307), working concentration 1 µM – Src inhibitor

DNA extraction from gel – kit

Ethanol 96% (Lach-Ner, 20025-A96)

Gene Ruler 1kb DNA Ladder (Thermo Scientific, SM0311)

HEPES (Carl Roth, 9105.2)

Isopropyl alcohol (Lach-Ner, 20037-AT0)

KAPA pure beads (KAPA Biosystems, 07983298001)

KB medium

- 20 g/l LB Broth low salt
- 0,5% glucose
- 100 mM phosphates

LB agar plates

- 20 g/l LB Broth low salt
- 20 g/l bacteriological agar

LB Broth low salt (Biochemie, L1703.0500)

Lysis buffer for DNA isolation

- 2% NaOH
- 88% H₂O
- 10% SDS

Lysis buffer for protein isolation

- 50 mM HEPES (pH=7.4)
- 1% Triton X-100
- 1 µM DTT
- Protease and phosphatase inhibitors

NucleoSpin® Gel and PCR Clean-up kit (MACHERY-NAGEL, 740609.50)

Phenol (Sigma, P1037-500G)

Running buffer (SDS buffer, pH=8.3)

- 3.02 g/l Tris
- 14.4 g/l Glycine
- 1 g/l SDS

Sodium chloride (NaCl, Sigma, S3014-500G)

Sodium dodecyl sulfate (SDS, Sigma, L3771-1KG)

TBE buffer (pH=8,3)

- 5.4 g/l Tris

- 2.75 g/l boric acid
- 0.29 g/l EDTA

TBS (Tris Buffer Saline)

- 50 mM Tris (pH=7.5)
- 150 mM NaCl

TTBS (Tween Tris Buffer Saline)

- 50 mM Tris (pH=7.5)
- 150 mM NaCl
- Tween-20 0.05% (0.5 g/l)

Transfer buffer

- 20% EtOH
- 0.025% SDS
- 300 mM Tris
- 300 mM glycine

Tris-EDTA-Glucose (TEG)

- 50 mM glucose
- 25 mM Tris-HCl (pH = 8.0)
- 10 mM EDTA (pH = 8.0)

Triton® X-100 Molecular biology grade (AppliChem, A4975)

Trypsin–EDTA Solution (Sigma, T4049-100ML)

Tween-20 (Carl Roth, 9127.1)

UltraPure™ Agarose (Invitrogen, 16500-500)

Western Bright™ ECL (Advansta, K-12045-D50)

4.2.13. Enzymes and enzyme buffers

Pfu X7 polymerase (Thermofisher)

EcoRV HF (NEB)

EcoRI HF (NEB)

GeneArt® Seamless Cloning and Assembly Enzyme Mix (A14606)

Deoxynucleotide Set, 100 mM (Sigma, DNTP-100-1KT)

CutSmart® Buffer (NEB)

4.2.14. Equipment

Biosan Environmental Shaker Incubator ES-20

Eppendorf Centrifuge 5415 D

Eppendorf Centrifuge 5417 R

Trans illuminator Syngene InGenius3

Spectrophotometer Shimadzu UV-1650PC

Thermoblock Major Science Dry Bath Incubator

Thermocycler BIOER-Gene Pro

Nanodrop 2000 (Thermo scientific)

Amersham™ Imager 600 (GE Healthcare)

Trans-Blot® Turbo™ Transfer System (Bio-Rad)

5. Results

5.1. Construct preparation

For this project, seven constructs were designed for both new and original biosensors: full-length biosensors (SrcFRET NG and SrcFRET TC), mutated constitutively active biosensors (Src527F, Src527F TC), and single-donor controls (SrcT only, SrcCit only, SrcNG only) (Fig.8). Two types of vectors containing these constructs were needed: with pIRESpuro3 vector backbone for transient transfections and with retroviral pMSCV vector backbone for creating the stable cell lines.

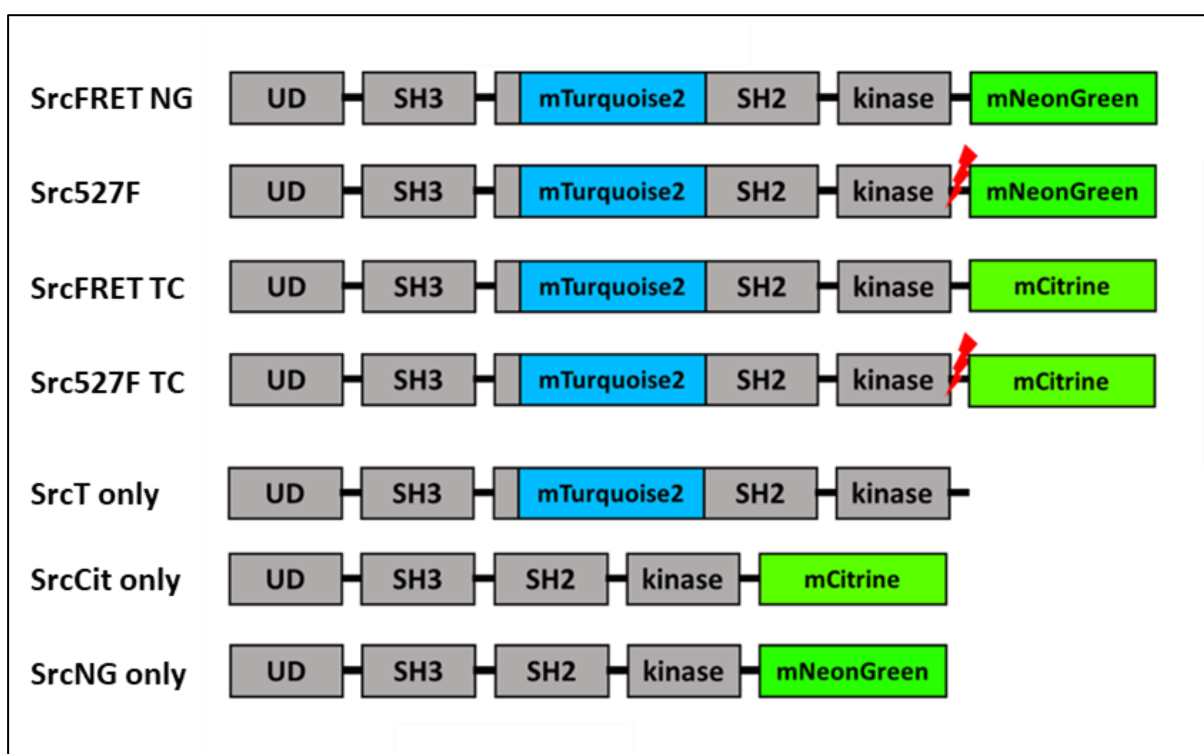


Fig.8: Biosensor constructs. Schematic representation of the design of all constructs used in this project. Src527F and Src527F TC represent full-length biosensors with point mutation leading to the replacement of Tyr527 with phenylalanine disabling the inhibitory phosphorylation of Tyr527. The colors of the fluorophores correspond to their emission wavelength.

The original biosensor and all its versions SrcFRET TC, Src527F TC, SrcT only, and SrcCit only were previously prepared and cloned to both pIRESpuro3 and pMSCV vectors by Mgr. Lenka Koudelková, Ph.D. To create the rest of the constructs needed, mCitrine in the existing pMSCV constructs was swapped for PCR-amplified mNeonGreen creating SrcFRET NG and SrcNG only constructs in the pMSCV vector. First, mNeonGreen insert (kindly provided by Veronika Kolomazníková) was amplified with added restriction site EcoRI at both its ends.

Then, using HF-EcoRI restriction enzyme digestion, mCitrine was cut out of the vector and swapped for PCR-amplified mNeonGreen cut with the same restriction enzyme. pMSCV vector with Src527F was created by PCR mutagenesis – pMSCV vector containing SrcFRET NG was amplified using primers with single nucleotide mutation leading to substitution of Tyr527 with Phe527. The resulting construct was verified by sequencing.

To clone the new biosensor and necessary control constructs to IRES vector, the same method could not be used for the restriction site used to create pMSCV constructs are not unique in the pIRESpuro3 vector. Therefore, Gibson Assembly Method was used. SrcFRET NG, Src527F and SrcNG only were PCR amplified from pMSCV vectors with overhangs homologous to ends of empty IRES vector cut by EcoRV (creating insert for the Gibson assembly reaction). The empty IRES vector was cut by EcoRV creating the vector for the reaction. Assembly itself was conducted according to the protocol described in chapter 4.2.11. All constructs were verified by sequencing.

5.2. Stable cell lines preparation

For stable cell lines, 3T3 and SYF cells were used. Both cell lines represent mouse embryonic fibroblasts. SYF cell line has a triple knock-out for Src, Yes, and Fyn kinases (Klinghoffer et al., 1999). Therefore, SYF stable cell lines contain no endogenous Src, only the Src biosensor constructs introduced by the transfection.

Stable cell lines were prepared according to retroviral transfection protocol (see chapter 4.1.6.) and positive cells were selected using Flow cytometry-based cell sorting (see chapter 4.1.7).

5.3. SrcFRET NG biosensor localization

In uninduced cells, Src is relatively homogenously localized in the cytoplasm with significant enrichment in the perinuclear area where it associates e.g. with endosomal vesicles. This localization is due to the closed conformation of Src – at basal conditions, about 95% of the Src is phosphorylated at Tyr527. Upon opening of the Src (either by its activation or treatment by dasatinib), Src re-localizes to focal adhesions (Hamadi et al., 2009; Koudelková

et al., 2019; Shenoy et al., 1992). This effect was used to test correct SrcFRET NG localization in stable cell lines.

Localization was checked microscopically in 3T3 as well as SYF cell line stably expressing SrcFRET NGbiosensor. Cells were plated on fibronectin-coated glass-bottom plates (See chapter 4.1.8.). Pictures were taken before and after treatment by 100 nM dasatinib for 10 min.

In both cell lines, the SrcFRET NG biosensor exhibited expected localization, that is enrichment in the perinuclear region when not treated and in FAs when treated by dasatinib for 10 min. (Fig.9)

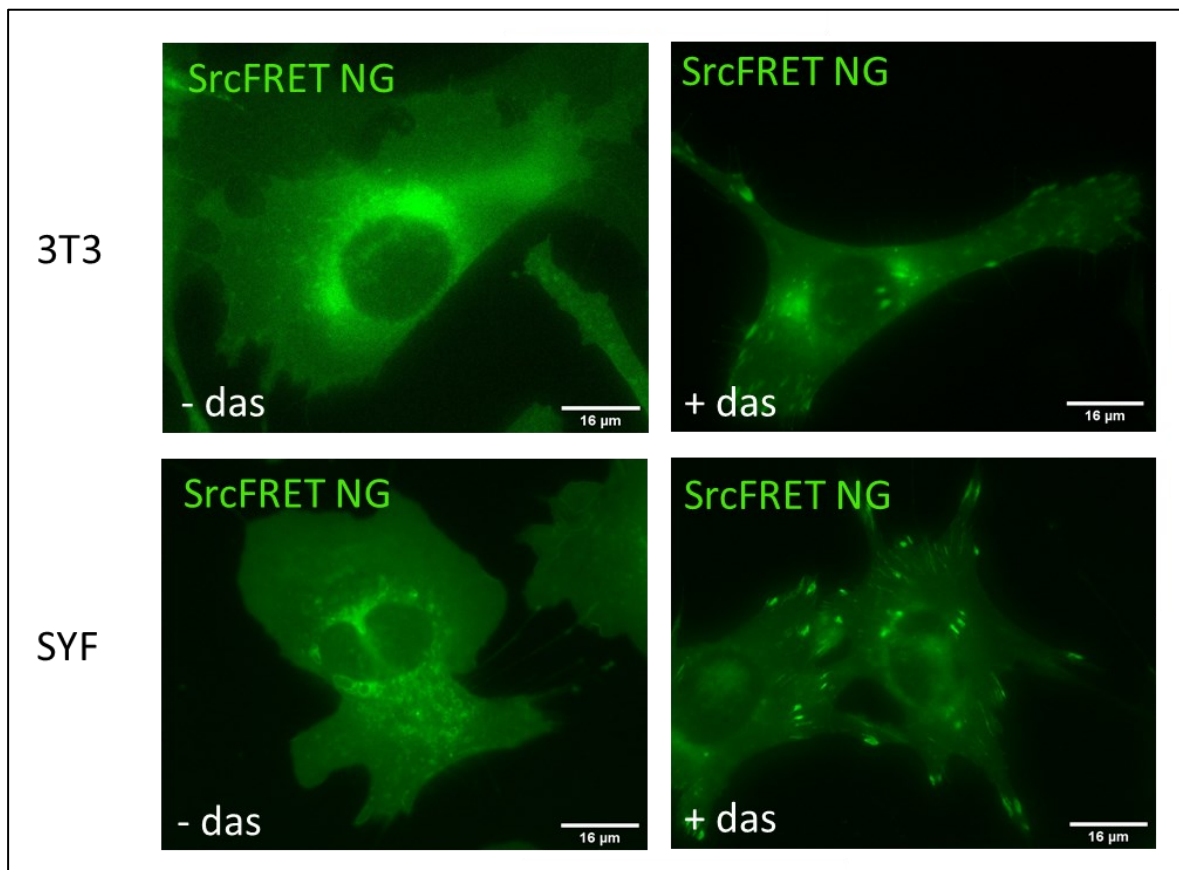


Fig.9: Localization of SrcFRET NG biosensor. Except for the perinuclear area, Src is localized homogenously in SYF and 3T3 stable cell lines. Upon 100 nM dasatinib treatment, Src changes its localization to FAs. mNeonGreen signal is detected and visualized by wide-field Leica Thunder microscopy system.

5.4. SrcFRET NG stability

To further verify SrcFRET NG potential use as a functional biosensor of Src activity, its stability in cells had to be examined. For that, western blotting was used (see chapter 4.2.2). Protein lysates were collected from SYF stable cell line after 4 weeks in cell culture, proteins were detected by anti-Src, anti-GFP (detects mTurquoise2), and anti-mNeonGreen antibody. As a loading control, GAPDH detection was used.

Src molecular weight is 60 kDa, GFP 27 kDa, and mNeonGreen 26.6 kDa. Therefore, the expected height of the bands for individual constructs would be:

SrcFRET NG and Src527F: 113,6 kDa

SrcT only: 87 kDa

SrcNG only: 86,6 kDa

Results showed that all the constructs are expressed in the stable cell lines, Src527F exhibiting the lowest expression. The biosensor is stable (no smaller bands were observed), and all the constructs exhibit the expected molecular weight (Fig.10). Together, it is possible to conclude that the biosensor and its variants are effectively expressed in the stable cell lines and do not undergo any apparent degradation.

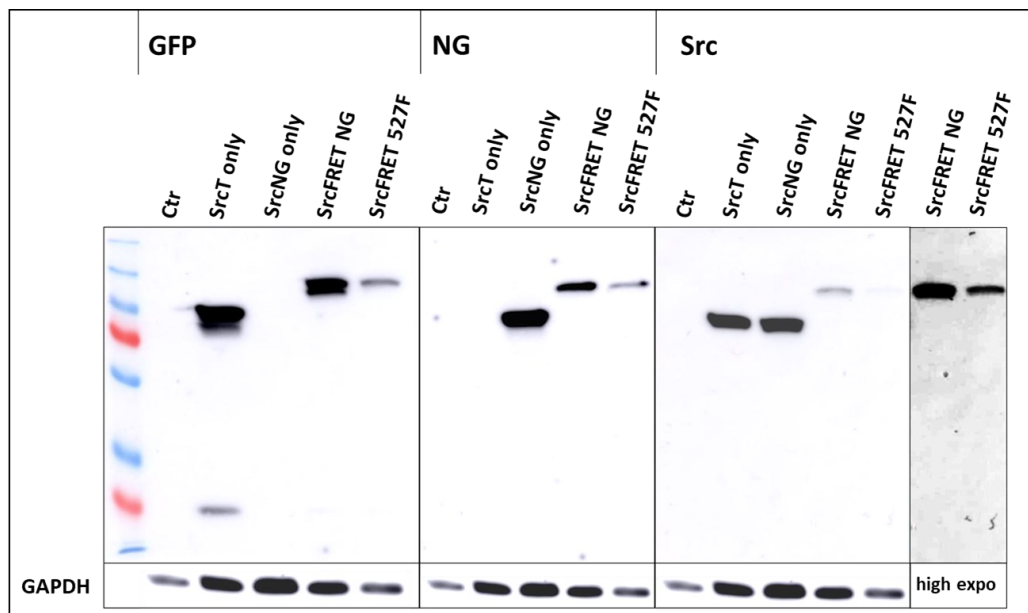


Fig.10: SrcFRET NG biosensor is stable in cells. Representative immunoblots show full-length biosensor SrcFRET NG, its mutated version Src527F, and single-fluorophore controls SrcT and SrcNG detected by anti-GFP, anti-mNeonGreen, and anti-Src antibody. GAPDH detection was used as a loading control. Src biosensor and its versions are expressed and stay stable in cells. Ctr=empty SYF cells (with no endogenous Src), High expo=high exposition.

5.5. FRET comparison of SrcFRET NG and SrcFRET TC

The FRET pair mTurquoise2-mNeonGreen should theoretically exhibit better FRET efficiency than the originally used pair mTurquoise2-mCitrine (See chapter 3.1.1.), therefore (theoretically) should be able to detect the conformational change of Src in the biosensor upon Src opening more sensitively. To test this hypothesis, FRET measurements from cell lysates and subsequent calculation of FRET ratios were used.

Using the ratiometric measurement of FRET, it is impossible to directly compare two different biosensors. Therefore, we decided to compare their ability to change the level of FRET after treatment by dasatinib, that is upon opening of Src conformation.

SYF as well as 3T3 stable cell lines exhibit a quite low expression level of biosensor constructs. Also, we had no stable cell lines expressing SrcFRET TC available. To effectively detect FRET signal in cell lysates for all variants of SrcFRET NG and SrcFRET TC biosensor, instead of SrcFRET NG stable cell lines transient transfection (which exhibits a much higher expression level) was used for both the original and the new biosensor.

U2OS cell line was transiently transfected by SrcFRET NG and SrcFRET TC, respectively. Before lysing, one plate of the cells with SrcFRET NG and SrcFRET TC was treated by 100nM dasatinib for 30min. Protein lysates (prepared according to the protocol in chapter 4.2.1.) were then placed in spectral scanning fluorescence reader Tecan 200Pro and the FRET fluorescence spectra were recorded (466-650nm with 2nm steps), that is the fluorescence spectra after excitation of mTurquoise2 by 433nm excitation wavelength. The background signal (from empty non-transfected U2OS cells) was subtracted and the FRET ratio for both biosensors was calculated as the ratio of mCitrine (526nm emission wavelength) and mTurquoise2 (486nm emission wavelength) or mNeonGreen (518nm emission wavelength) and mTurquoise2 (486nm emission wavelength), respectively. In all replicates, the sample with the highest fluorescence intensity was used to calculate gating for the whole experimental cohort so that the set value would be the same for all samples in the cohort.

Calculated ratios were then compared to determine if there is a significant difference between the two biosensors. Results showed that the difference in FRET ratio between dasatinibtreated and untreated cells is significantly higher for the new SrcFRET NG biosensor. However, the percentage of FRET ratio decrease doesn't significantly differ between the two

biosensors. We were, therefore, unable to confirm our hypothesis that the new SrcFRET NG biosensor is more sensitive to Src conformational change compared to the original (Fig.11).

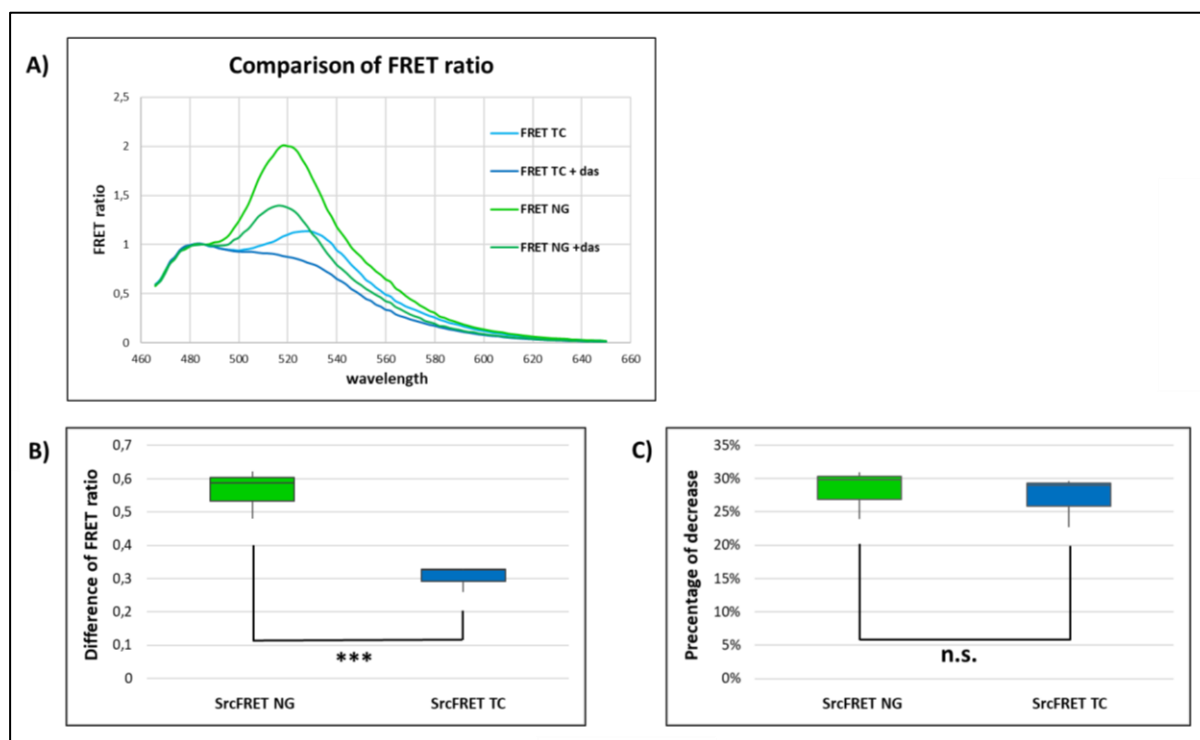


Fig.11: Comparison of FRET of SrcFRET NG and SrcFRET TC biosensor. A) Representative emission spectra normalized to the emission of mTurquoise2 at 486 nm. B) Comparison of the difference between FRET ratio of by 100 nM dasatinib treated or untreated U2OS cells expressing SrcFRET NG and SrcFRET TC biosensor, respectively. SrcFRET NG biosensor exhibits a significantly higher difference in FRET ratio after the treatment (unpaired T-Test). C) Comparison of the percentage of FRET decrease after treating U2OS cells expressing SrcFRET NG and SrcFRET TC biosensor by 100 nM dasatinib. The percentage of decrease does not significantly differ between SrcFRET NG and SrcFRET TC biosensor (unpaired T-Test). Data were collected from three independent replicates.

5.6. Src in focal adhesions

Using the original biosensor, Src activity in focal adhesions was studied. It was shown, that Src activity in FAs is higher compared to the rest of the membrane (Koudelkova et al., 2018) (Fig.12A). We tried to repeat the experiment and by that prove that it is also possible to use the new biosensor for life-cell FRET measurement.

For visualization of the FRET, TIRF microscopy was used. TIRF microscopy (Total Internal Reflection Fluorescence Microscopy) allows excitation and detection of fluorescence near the surface of the cell dish or microscopical slide detecting signal from about 100 nm area above the surface. That results in almost no out-of-focus signal detection, and it is also live-cell-friendly as the rest of the cell is not exposed to the laser. In this 100nm range, cell

membranes can be observed, therefore also Src activity in FAs (Axelrod, 2016; Steyer and Almers, 2001).

3T3 stable cell lines expressing either SrcFRET NG, SrcT, or SrcNG were plated on 35mm fibronectin-coated glass-bottom plates. Images of cells expressing SrcFRET NG were taken before and after 10min of 100nM dasatinib treatment. Single-fluorophore controls were not treated by dasatinib. FRET sensitized emission analysis of the obtained images was conducted as described in chapter 4.1.9.

FRET analysis and resulting Src activity map show, that Src is active in areas of focal adhesions. That is in agreement with results obtained using the original biosensor (Fig.12).

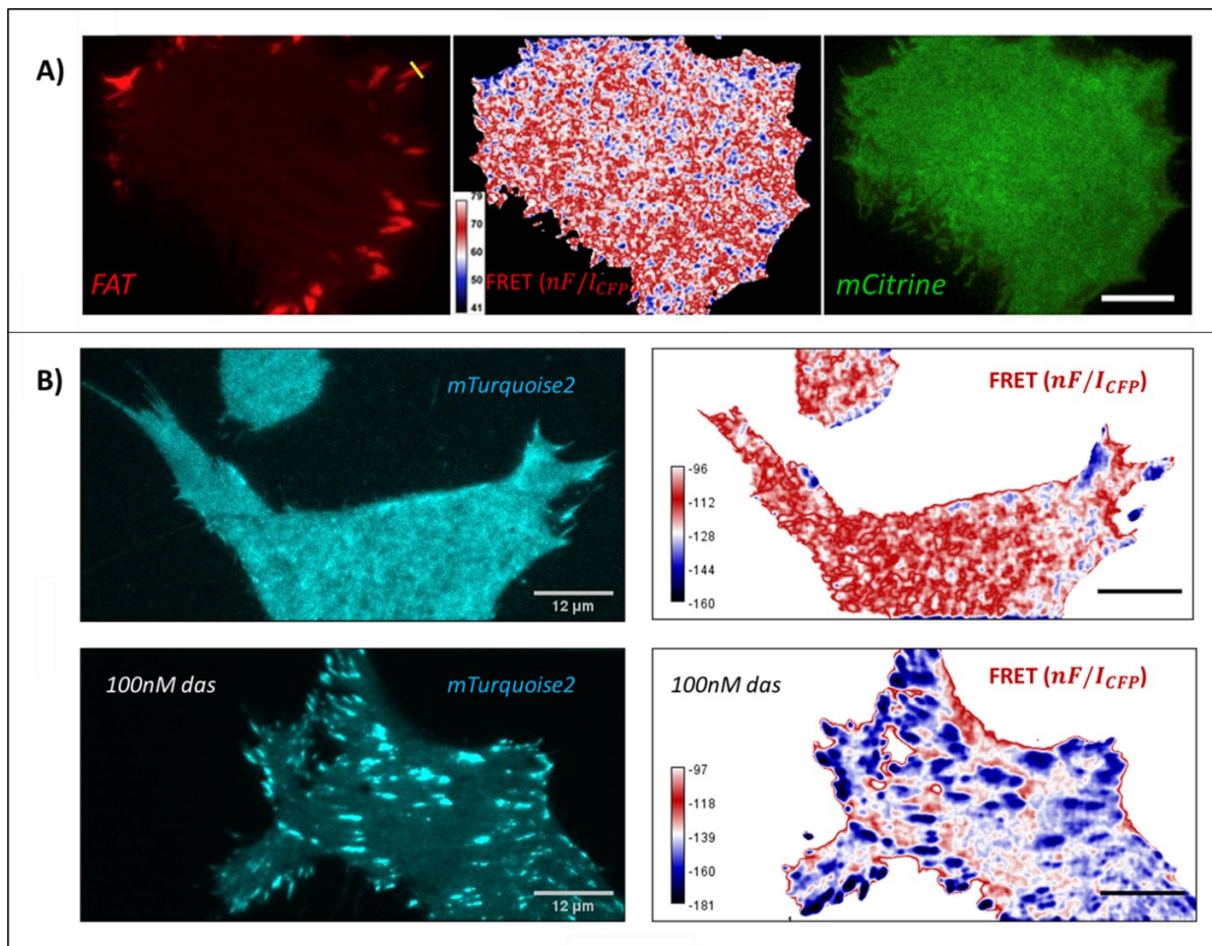


Fig.12: Src is active in FAs. A) U2OS transiently expressing the original biosensor SrcFRET TC and mCherry-FAT. From left: FAs (FAT-mCherry), Src activity map (FRET), Src localization (mCitrine). Scale bar, 10 μ m. Images were taken and modified from (Koudelkova et al., 2018) B) 3T3 stable cell line expressing SrcFRET NG biosensor. Representative images show Src localization in cells (left, mTurquoise2 signal) and Src conformation map (right, FRET, the blue color indicates low FRET/opened conformation). Cells treated by 100 nM dasatinib for 10min (bottom row) exhibit enhanced Src localization to FAs and low FRET.

5.7. Modified dasatinib

Dasatinib can inhibit more kinases than just the Src kinase. For example, its ability to inhibit Abl kinase is used therapeutically to treat chronic myelogenous leukemia (Johnson et al., 2005). Src is a potential therapeutic target for cancer treatment, therefore new inhibitors of Src are being developed. One way to develop a new inhibitor with potentially different features is through modifying an already existing inhibitor. That is the case of new modified dasatinib (in this project referred to as m-dasatinib), which ability to inhibit Src we also studied in this project.

To analyze the effect of m-dasatinib on Src we used the newly prepared SrcFRET NG biosensor. First, the effect of m-dasatinib on localization of the biosensor. SYF cell line expressing the SrcFRET NG biosensor was plated on a fibronectin-coated 35mm glass-bottom cell dish. Images were taken before and after 20min treatment by 100nM dasatinib and 100nM m dasatinib, respectively.

As results show, the effect of m-dasatinib was the same as the effect of dasatinib, that is upon inhibitor treatment, Src preferentially localizes to FAs (Fig.13).

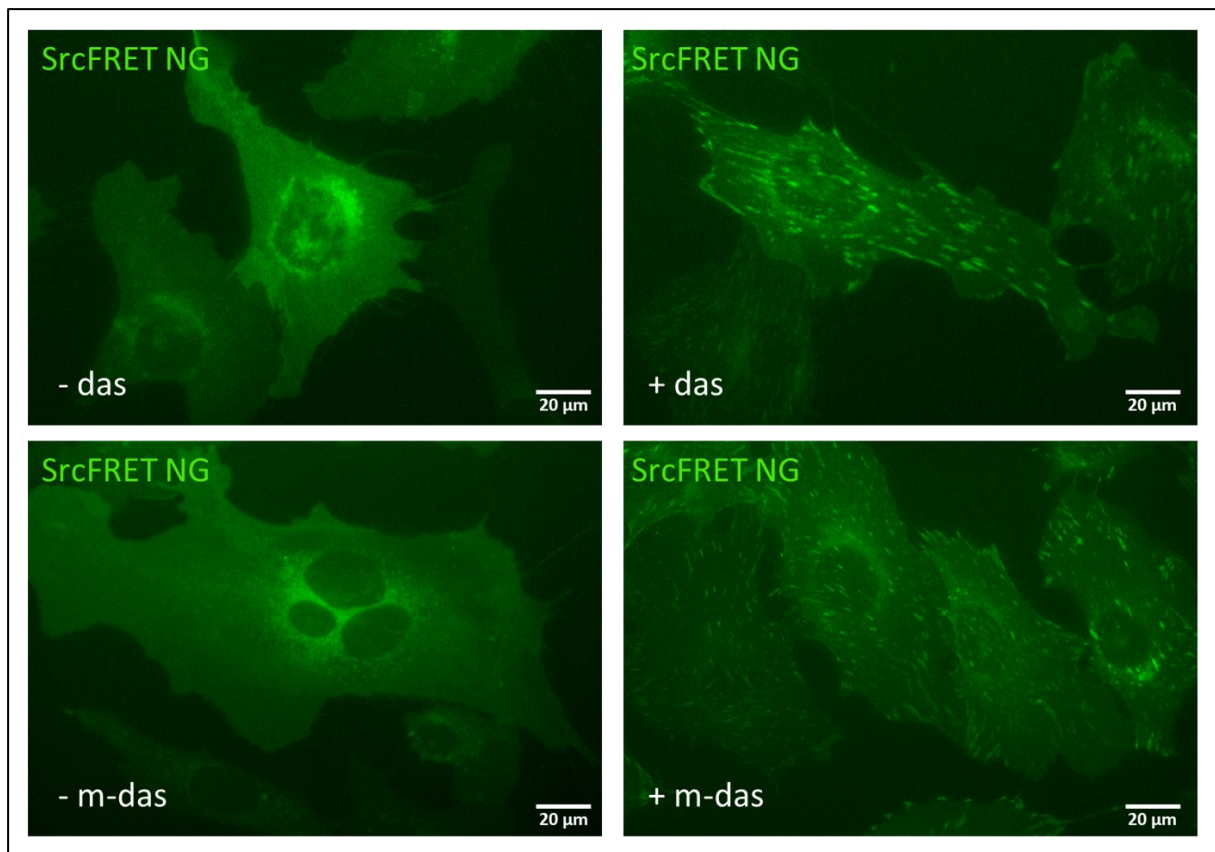


Fig.13: Biosensor localization before and after dasatinib and m-dasatinib treatment. Without treatment, Src is preferentially localized homogenously (except for the perinuclear area). Upon 100 nM dasatinib and 100 nM m-das treatment, Src changes its localization to FAs. There is no apparent difference between the effect of dasatinib (das) and m-dasatinib (m-das). mNeonGreen signal is detected and visualized by wide-field Leica Thunder microscopy system

Next, the effect of m-dasatinib on the FRET of the biosensor was analyzed. Protein lysates were collected from SYF stable cell line expressing SrcFRET NG treated or untreated by 100nM dasatinib and m-dasatinib, respectively. FRET spectra (that is the fluorescence intensity after mTurquoise2 excitation by 433nm laser) were recorded using Tecan 200Pro plate reader and the FRET ratio was calculated.

The results showed that treatment by m-dasatinib has a similar effect on FRET of the biosensor as dasatinib (Fig.14). That suggests that m-dasatinib has the same mode of binding to Src as dasatinib, that is in the α C helix “in” position.

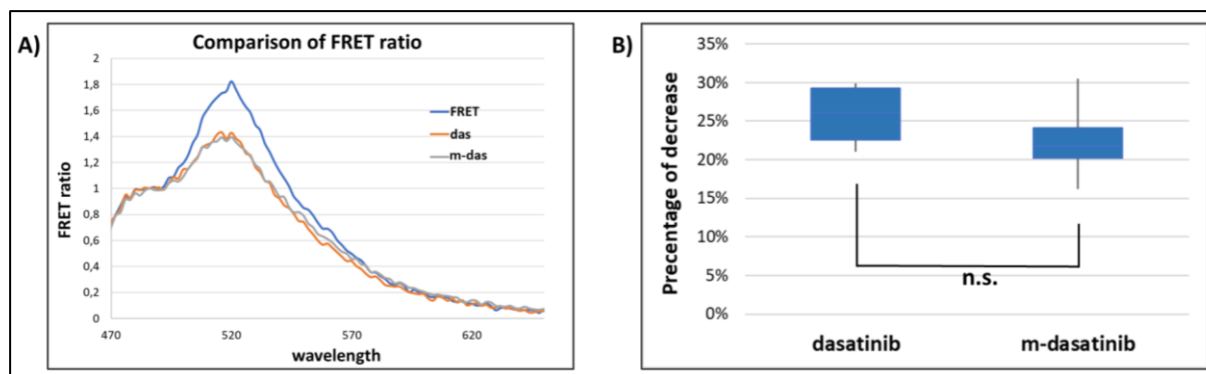


Fig.14: Effect of dasatinib and m-dasatinib on FRET. A) Representative emission spectra normalized to mTurquoise2 emission at 486nm. B) Comparison of the percentage of FRET decrease after treating SYF cells expressing SrcFRET NG by 100 nM dasatinib (das) and 100nM m-dasatinib (m-das), respectively. The percentage of decrease does not significantly differ between dasatinib and m-dasatinib treatment (unpaired T-Test). Data were collected from three independent replicates.

6. Discussion

6.1. mNeonGreen as FRET acceptor - Spectrometry measurements

To compare the sensitivity of the original and new sensors, their ability to detect the change of Src conformation upon dasatinib treatment was compared. As about only 5% of the Src is active in a cell at basal condition, these 5% were deemed negligible and no inhibitor closing the Src structure was used; Src from untreated cells was regarded to as Src in the closed conformation, therefore exhibiting the highest FRET, Src from cells treated by dasatinib as Src in opened conformation, exhibiting the lowest FRET.

According to a theoretical mathematical model, FRET pair mTurquoise2-mNeonGreen should exhibit better FRET efficiency than mTurquoise2-mCitrine (discussed previously in chapter 3.1.2.). We were unable to experimentally confirm this hypothesis – we did not measure a significant difference in the relative FRET decrease after dasatinib treatment between the original and new Src biosensor. There are various possible reasons for these results.

6.1.1. Distance between fluorophores

One explanation for this result is the actual distance between donor and acceptor in our biosensor. Calculations predict 10% better FRET efficiency of mTurquoise2-mNeonGreen pair compared with the original when the distance is 57.51Å, that is the Förster radius for mTurquoise2-mCitrine. However, we do not know the actual distance between fluorophores in our biosensor. If the distance is shorter, the difference between the FRET efficiencies decreases and vice versa. For example, if the actual distance between fluorophores would be 36 Å (distance measured in PyMOL in the theoretical model of inactivated SrcFRET biosensor (see Fig.6)), the difference in FRET efficiencies would be only 1.83%. The same happens when the distance is too long. If the distance would be 130 Å (that is the distance between fluorophores measured in PyMOL on the theoretical model of activated SrcFRET biosensor (see Fig.6)), the difference in FRET efficiency would be 0.37% (see calculations below). Our theoretical model of Src biosensor and distance measurements are based on the known structure of opened and closed Src kinase available at Protein Data Bank (PDB) website <https://www.rcsb.org/> under accession codes 2PTK and 1Y57.

Therefore, it is simply possible that the difference in FRET efficiency measured at these distances (because we measure the difference between opened and closed Src) is under the detection limit of our method or that the small difference is hidden by orientation and environmental factors (described below).

$$E_{1\ mTurquoise2-mCitrine} = \frac{R_0^6}{R_0^6 + r^6} = \frac{57.51^6}{57.51^6 + 36^6} = 0.9432 = 94.32\%$$

$$E_{1\ mTurquoise2-mNeonGreen} = \frac{R_0^6}{R_0^6 + r^6} = \frac{61.55^6}{61.55^6 + 36^6} = 0.9615 = 95.15\%$$

$$E_{2\ mTurquoise2-mCitrine} = \frac{R_0^6}{R_0^6 + r^6} = \frac{57.51^6}{57.51^6 + 130^6} = 0.0074 = 0.74\%$$

$$E_{2\ mTurquoise2-mNeonGreen} = \frac{R_0^6}{R_0^6 + r^6} = \frac{61.55^6}{61.55^6 + 130^6} = 0.0111 = 1.11\%$$

6.1.2. Environmental factors

Fluorophores and their abilities are influenced by their environment, and mNeonGreen is no exception. For example, higher solvent polarity leads to a shift of the emission spectrum of various fluorophores to longer wavelengths (Lakowicz, 1983). Other factors affecting the fluorescence of the fluorophore are chloride ions concentration or acidity of the environment, which can be used to study secretory vesicles or differences in mitochondrial and cytosolic pH (Matsuyama et al., 2000; Miesenböck et al., 1998). On the other hand, the pH sensitivity of fluorophores can also be a potential problem and when choosing fluorophore for experiments, its characteristics including the pH resistance should be kept in mind (Shaner et al., 2005). Therefore, scientists try to develop new fluorophores that are pH and chloride-resistant (Griesbeck et al., 2001; Nagai et al., 2002).

In the original article where mNeonGreen was first introduced, authors reported its pKa=5.7 which is the same as of mCitrine, but they also claimed that mNeonGreen did not show any sensitivity to chloride ions (Shaner et al., 2013). That was later disproved (Tutol et al., 2019). The binding of chloride to mNeonGreen leads to lower pKa and results in much higher fluorescence intensity in a pH-dependent manner. This effect was observed at lower pH

around 4.5. Comparing the properties of our lysis buffer (pH=7.4) with the study, our buffer should not significantly influence mNeonGreen's fluorescence properties (Tutol et al., 2019). However, we cannot rule out the possibility that there is some influence of the buffer.

6.1.3. FRET pair orientation factor

As described in chapter 3.1., the proximity factor has the biggest influence on the FRET. But there is also the orientation factor k^2 to be considered that was first theorized by Theodor Förster and later experimentally proven (Förster, 1948b; Iqbal et al., 2008; Lewis et al., 2005). The orientation factor depends on the transition dipole moments of donor and acceptor and normalized vector connecting the dyes (fluorophores). It is very complicated to precisely evaluate and measure the orientation factor. Therefore, scientists often settle for the simplified approximation. If the fluorophores move freely and independently, the value of k^2 is estimated to be 2/3 and the estimation is in this case justified (Demchenko, 2015; Khrenova et al., 2015). However, if the presumption of free movement is wrong and fluorophores are somehow fixed in a certain mutual orientation, this simplification can be misleading and result in incorrect calculations. We based our calculation of theoretical FRET efficiency of the FRET pair mTurquoise2-mNeonGreen on values obtained from the fluorescent protein database (<https://www.fpdbase.org/fret/>), where the orientation factor k^2 is presented to be 0.6667=2/3, that is the simplified estimate for freely moving fluorophores.

To ensure free movement of the fluorophores and prevent any disruption of the SH2 domain and C-terminus functions (where donor and acceptor were placed), in the original biosensor design (as well as in the new one), fluorophores were flanked with flexible linkers as described in chapter 3.3. Therefore, we do not expect the mTurquoise2-mNeonGreen pair to adopt a different orientation in the Src biosensor than the mTurquoise2-mCitrine pair. However, it is possible that the orientation of the fluorescence centrum of mNeonGreen slightly differs from mCitrine, which would also be hidden in the simplified estimate of k^2 . Unfortunately, it is very difficult to distinguish the potential effect of fluorophore orientation from other factors like environmental or proximity factors.

It is interesting to note, that some FRET experiments are designed based on the orientation of fluorophores rather than the more commonly used proximity – such experiments are used e.g. to study DNA and its structural changes (Kashida et al., 2017).

6.1.4. mNeonGreen advantages

Although mNeonGreen as an acceptor did not improve biosensors sensitivity to Src conformational changes, it still has advantages over the mCitrine. mNeonGreen itself exhibits a very fast maturation time and high quantum yield surpassing the mCitrine. Thanks to the higher quantum yield, mNeonGreen is brighter, and therefore in microscopic experiments, its signal is higher above the background compared to mCitrine. That can also be observed in the spectrometry measurement, where the mNeonGreen signal normalized to mTurquoise2 exhibits a much higher fluorescence signal compared to mCitrine (see Fig.11).

6.1.5. Possible better donor for mNeonGreen

According to www.fpbases.org, mNeonGreen is currently the best existing FRET acceptor for donors from the CFP spectrum. There are however other fluorophores than mTurquoise2 that should exhibit even better properties as donors for mNeonGreen - Aquamarine and mTFP1. Mathematically, both these FRET pairs (Aquamarine-mNeonGreen and mTFP-mNeonGreen) should perform slightly better in FRET efficiency than mTurquoise2-mNeonGreen. Therefore, using Aquamarine or mTFP as a donor could also improve the biosensor. But, considering the speed by which new fluorophores are being developed, it is only a matter of time before there is a new fluorophore surpassing mNeonGreen as acceptor.

6.2. Sensors of Src activity

In this thesis, a genetically encoded FRET-based conformational biosensor was used. Throughout the history of Src research, there have been developed more types of sensors to study Src activity. In general, we could divide them into three groups: substrate sensors, conformational ligand-binding sensors, and conformational single-molecule FRET biosensors.

6.2.1. Substrate sensors

Substrate sensors detect the activity of kinase through the detection of phosphorylation of its substrates. Their general design consists of substrate peptide, SH2 domain, and FRET pair flanking the reporter molecule. When the substrate peptide is phosphorylated, it is bound to the SH2 domain resulting in changed conformation with fluorophores further from each other, which can be detected as lower FRET. Dephosphorylation of the reporter leads to a reverse process, that is release of the SH2 domain and substrate peptide binding detected as higher FRET (Fig.15). The first such reporter was designed for three tyrosine kinases Src, Abl, and epidermal growth factor receptor (EGFR) (Ting et al., 2001). This design was later optimized by using new fluorophores, different substrate peptides, or by mutation of the SH2 domain. However, these improvements still did not include the fatty acyl anchor that Src physiologically has. The sensor was localized only in the cytoplasm and could not bind to the membrane (which does not correspond to physiological Src), where Src plays role in various signaling pathways. Later on, other changes were added to the original sensor design: N-terminal peptide of Lyn kinase containing the myristoylation and palmitoylation signal to study Src activity at membranes or FAT domain to study Src activity in FAs (Li et al., 2018; Ting et al., 2001; Wang and Chien, 2007; Wang et al., 2018; Wu et al., 2016).

Other issues besides nonoptimal localization have arisen from using this type of sensor. In the original study, the sensor phosphorylation was shown not to be Src-specific, but rather a result of phosphorylation by other tyrosine kinases (Ting et al., 2001). Therefore, the specificity of the substrate sensor is crucial.

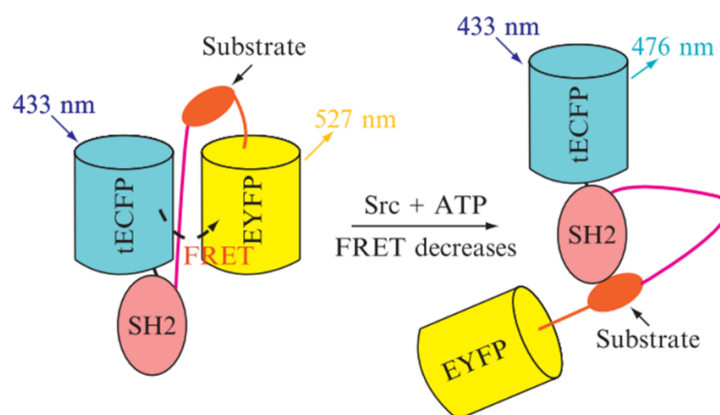


Fig.15: Schematic representation of Src substrate sensor. When the sensor is phosphorylated, substrate peptide binds to the SH2 domain and lower FRET can be detected. The image was taken from (Wang and Chien, 2007).

6.2.2. Conformation sensitive ligand-binding sensors

Like our biosensor, these sensors use the correlation of conformation of kinase and its activity and detect the activity based on the accessibility of specific binding surfaces that are sterically hidden in the inactive/closed state of Src. Two approaches for designing these biosensors have been described and used, based on the steric availability SH3 or SH2 domain. In closed conformation, some epitopes of the SH2 and SH3 domain are hidden in the closed structure and become accessible only when the structure is opened. Detection of such epitopes can be used to detect the opened conformation, thus active kinase. Therefore, the basic principle of the design is selecting protein with the ability to specifically bind such epitopes and provided with means to visualize them (e.g., by tagging them by dye) (Fig.16).

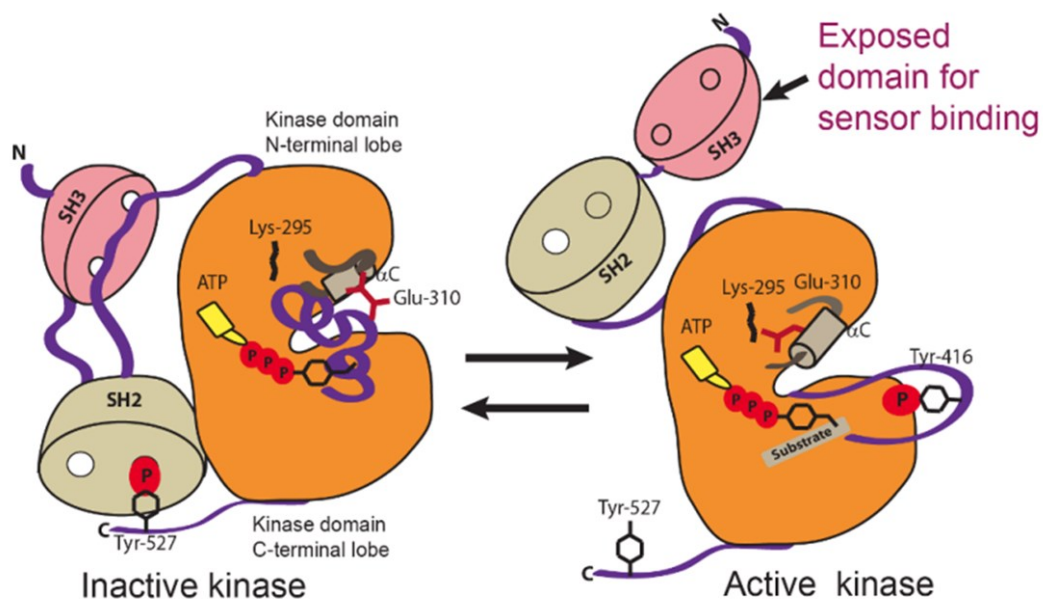


Fig.16: Representation of the principle of conformation-sensitive ligand-binding sensors (Fyn biosensor). In the inactive state, the binding surface of either SH2 or SH domain is hidden. Upon activation, the conformation loosens, and the domain becomes exposed for sensor binding. The image was taken and modified from (Mukherjee et al., 2020).

An example of the SH3 domain-based sensor is the merobody biosensor of SFKs. The biosensor is a small protein (fibronectin monobody) specifically binding the SH3 domain when Src is in the active conformation, selected from monobody library, and tagged by merocyanine dye. Upon activation of the kinase, the SH3 epitope recognized by the merobody biosensor becomes accessible to the binding. The biosensor binds to the SH3 domain and as a result, the merocyanine dye gets in a hydrophobic environment which results in a detectable higher fluorescence signal of this dye. When the kinase is in the inactive closed conformation, the epitope is hidden, the biosensor cannot bind and the fluorescence signal can not be detected (Gulyani et al., 2011) (Fig.17).

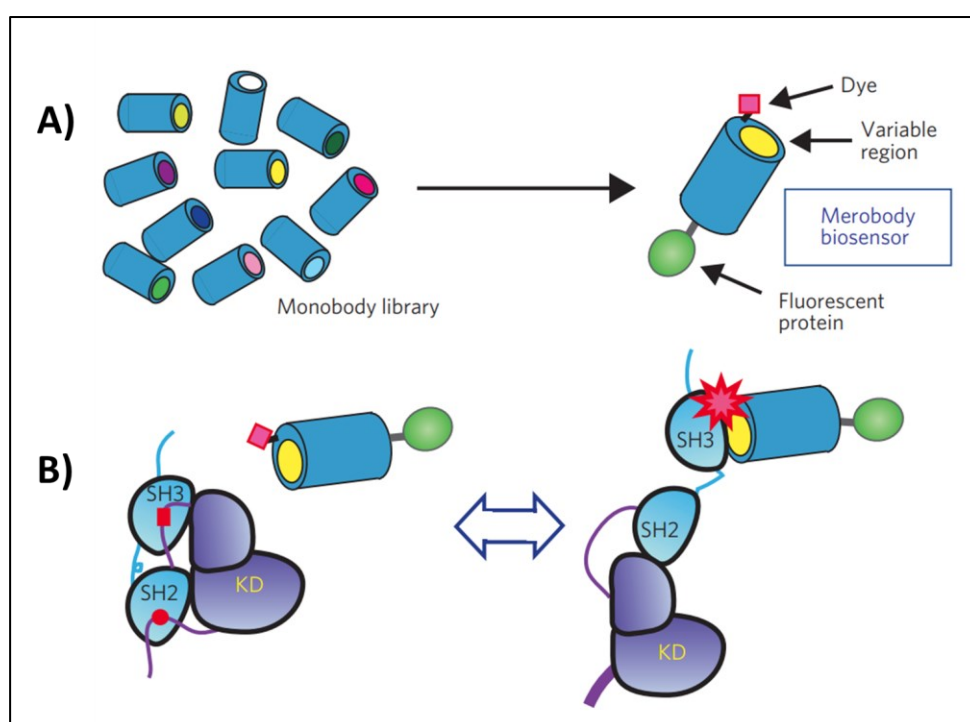


Fig.17: Schematic representation of the merobody biosensor. A) From monobody library, a protein with specificity to the desired epitope must be selected and provided with detection dye. B) When the kinase is in the inactive state, the biosensor cannot bind to the kinase. Upon activation, the SH3 domain becomes available, the biosensor can bind, and fluorescence is detected. Image was taken from (Gulyani et al., 2011).

An example of the SH2 domain-based design with different detection method is the tetracysteine biosensor. This biosensor is using the tetracysteine system based on the ability of tetracysteine to bind biarsenical fluorophores that emit light only upon binding to the tetracysteine motif. Four residues in the SH2 domain are swapped for the tetracysteine sequence. When the kinase is activated, the tetracysteine is available for binding of biarsenical

fluorophore which results in detection of fluorescence signal. However, the introduction of the tetracysteine motif in the SH2 domain resulted in aberrant activation of Src kinase. Therefore, this system is not suited for studying the physiological function of Src in cells (Irtegun et al., 2011, 2014).

The newly developed Fyn sensor uses FRET detection. The FRET donor mCerulean was inserted between the UD and SH3 domain of Fyn kinase. Acceptor fluorophore mVenus was fused with selected protein able to specifically bind the SH3 domain of Fyn. Upon binding of this protein, mVenus got close mCerulean resulting in higher FRET (Mukherjee et al., 2020).

6.2.3. Conformation sensitive intramolecular FRET biosensors

This type of sensor also uses the correlation between kinase conformation and activity. It directly measures conformational changes within the molecule of kinase without the need for a specific ligand. Two fluorophores (FRET pair) are inserted into the structure of kinase. Activation-dependent changes in the conformation result in a change in the distance of these fluorophores that is detectable as a change in FRET. Several biosensors belong to this group: c-Raf, MELK, PKC γ , FAK, Lck, or our Src biosensors (Cai et al., 2014, 2008; Le Page et al., 2011; Paster et al., 2009; Terai and Matsuda, 2005; Verbeek et al., 2008). Besides kinases, FRET-based conformational biosensors are used to study other proteins such as vinculin (Case et al., 2015). For us, the Lck kinase biosensor design is the most interesting (Paster et al., 2009).

The mechanism of Lck autoinhibition and general conformational changes upon Lck activation are very similar to those of Src. Tyr505 phosphorylation (the equivalent of Tyr527 in Src) leads to the interaction of the C-terminal end with the SH2 domain inducing the compact inhibitory conformation of Lck. When Lck is activated, the structure loosens. Also, other aspects of Lck regulation, such as the importance of the CD linker, are similar to Src (Boggon and Eck, 2004a; Harrison, 2003). In the Lck biosensor, FRET acceptor fluorophore EYFP was inserted on the C-terminus of the molecule (same as mNeonGreen in our biosensor). However, the position of donor differs between the biosensors. In the Lck biosensor, ECFP was inserted between the SH3 and SH2 domain into the SH3-SH2 linker. When Lck was in the inactive conformation, both fluorophores were close to each other, and high FRET could be detected. When the kinase

was active, ECFP and EYFP were further from each other resulting in lower FRET (Paster et al., 2009).

In our Src biosensor, the donor was placed in the structure of the SH2 domain. We believe that our placement of the donor is better. As described in chapter 3.2.3., SH3-SH2 linker plays a crucial role in the regulation of Src and its mutation can lead to constitutive activation of Src (Young et al., 2001). Therefore, placing the donor in the SH2 domain seems like a safer choice.

Later, the Lck biosensor design was modified, and a new sensor was developed. In the new design of the Lck biosensor, the donor is inserted between the UD and SH3 domain of Lck kinase (Stirnweiss et al., 2013).

6.2.4. Src FRET biosensor advantages

Compared with FRET-based biosensors, substrate sensors are relatively easy to design. However, there are some risks and limitations of their use. The main problem is the specificity as substrate sensor phosphorylation can be a result of the activity of other kinases (Ting et al., 2001). Another problem is localization. Unless myristoyl or palmitoyl anchor is added, the sensor has only cytoplasmatic localization which does not correspond to reality, because Src is primarily active at the membrane. There is also a delay in the effect of the kinase on the substrate sensor that depends on the kinetics of the reaction. And lastly, the reporter cannot detect quick changes of kinase activity – it takes time to dephosphorylate the reporter and therefore to detect a decrease in kinase activity. Rather than kinase activity itself, substrate sensors detect the phosphorylation status of the substrate.

The conformation-sensitive ligand-binding sensors detect the conformation of kinase, therefore indirectly its activity. However, their disadvantage lies in the risk of influencing the physiological function of the kinase. Altering the SH2 domain such as in the tetracysteine biosensor can lead to aberrant activation of kinase through destabilization of the closed conformation (Irtegun et al., 2014). Similarly, it is conceivable that the binding of the SH3 domain by SH3-binding protein can also disrupt the physiological protein-protein interaction with other interacting partners.

The conformation-sensitive intramolecular FRET-based biosensors are relatively difficult to design. However, they can directly and in real-time detect conformation (therefore activity)

of kinase and in addition also the localization of the kinase. Also, it is possible to create mutant organisms endogenously expressing such sensors that would allow studying the role of kinases in living animals.

Taken into consideration that SFKs have similar aspects of their conformational changes, the design of the SrcFRET biosensor could be used to develop sensors of other kinases of Src or related kinase families as well.

6.3. Analysis of Src inhibitors using the SrcFRET biosensor

One of the characteristics of an inhibitor is the mechanism by which it inhibits the target. FRET biosensors can help reveal the mechanism. Using the original SrcFRET TC biosensor, the effect of a few Src inhibitors was analyzed. Bosutinib, dasatinib, and UM-164 induced opened conformation of Src. Saracatinib as well as SK-1 did not induce the opened conformation, SK-1 is hypothesized to force the closed conformation of Src (Koudelková et al., 2019). Interestingly, that was not the first time that the effect of bosutinib on Src conformation was described. The tetracysteine sensor (described previously) was not suited for physiological experiments, however, it was used to screen 72 inhibitors of SFKs including bosutinib, and to determine their effect on the tetracysteine sensor. The different effects of various inhibitors were observed (Irtegun et al., 2014).

Conformational-based biosensors allow determining the mode of binding of inhibitors without the complicated process of resolving the structure of the kinase-inhibitor complex. As α C helix “in” position induces opened conformation of Src, inhibitors inducing the opened conformation can be expected to belong to the group of α C helix “in” inhibitors. That is especially useful in the development of new drugs considering that inhibitors of SFKs can be used to treat various diseases.

7. Summary

In this project, I prepared and tested a new Src biosensor, which uses a new combination of fluorophores in an already existing design. I created all necessary constructs and prepared cell lines stably expressing the biosensor and control constructs.

I was able to confirm the expression and correct localization of the biosensor and all its variants in cells stably expressing the sensor. The results show that the biosensor remains stable and does not undergo any extensive degradation.

Using the new biosensor, I replicated the experiments conducted by the original biosensor and confirmed that Src has higher activity in FAs. That proved the functionality of the new biosensor as a tool to study Src activity in cells.

I was not able to confirm our hypothesis that the new combination of fluorophores would exhibit better sensitivity to conformational changes of Src. The results did not show any significant difference between the original and new biosensor. However, the new combination of fluorophores has the advantage of the higher fluorescence signal of mNeonGreen that is apparent in microscopic experiments.

Using the new biosensor, we also tested modified dasatinib and its effect on the Src biosensor. Results showed that the modified dasatinib exhibits the same effect as dasatinib suggesting that the modified dasatinib inhibits Src in the same manner.

8. Literature

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9. Attachments

9.1. Tables

Table 1: Different types of cell dishes.

Cell dish size	S [cm ²]	V _{cell culture medium} [ml]	Number of cell (confluence 100%)
10 cm	55	10	8.8×10^6
6 cm	21	5	3.2×10^6

Table 2: Reaction mixture for cell transfection.

Cell dish size	S [cm ²]	V _{serum-free medium} [μl]	DNA [μg]	PEI [μl]
10 cm	55	2x100	15	15
6 cm	21	2x50	5	5

Table 3: Composition of running and stacking gel for SDS-PAGE electrophoresis.

Stacking gel	Volume	Running gel	Volume
H ₂ O	3.1 ml	H ₂ O	4.17 ml
Protogel (30% AA / 0.8% BIS)	0.65 ml	Protogel (30% AA / 0.8% BIS)	3.33 ml
4xTris, SDS (pH=6.8)	1.25 ml	4xTris, SDS (pH=8.8)	2.5 ml
TEMED	5 μl	TEMED	6.7 μl
APS	25 μl	APS	33 μl

Table 4: List of primary and secondary antibodies.

Primary antibodies	Company	Catalogue number	Dilution
v-Src	Calbiochem	OP07	1:500
mNeonGreen	Cell Signaling	53061S	1:1000
GFP	abcam	ab290	1:5000
GAPDH	Invitrogen	MA5-15738	1:5000
Secondary antibodies	Company	Catalogue number	Dilution
Anti-mouse HRP	Cell Signaling	7076	1:15000
Anti-rabbit HRP	Cell Signaling	7074	1:15000

Table 5: Reaction mixture for PCR amplification.

H ₂ O	15.75 µl
Pfu X7 buffer 10x	2.5 µl
DNA 10ng/µl	1 µl
Primer 2 10µM	2.5 µl
Primer 1 10µM	2.5 µl
dNTP	0.5 µl
Pfu X7	0.25 µl
Final volume	25 µl

Table 6: List of primers for PCR.

Primer	Purpose	Sequence
5NeG-SFRET	Forward, mNeonGreen amplification	GTTGAATTCGGTGGCAGTGGAGGGATGGTGAGCAAGGGAG
3NeG-SFRET	Reverse, mNeonGreen amplification	TTAGAATTCCTACTTGTAGAGCTCGTCCATTC
F527F	Forward, Src Y527F mutagenesis	CGACAGAGCCCCAGTTCAGCCTGGAGAGAAC
R527F	Reverse, Src Y527F mutagenesis	GTTCTCTCCAGGCTGGAAGTGGGGCTCTGTCG
F-GibAss-Src	Forward, Gibson Assembly	ACCGAGCTCGGATCGATGGATCCATGGGTAGTAGCAAG
R-GibAss-Src	Reverse, Gibson Assembly	CTAGCTAGGCCGCAGATCTCGAGGAATTCCTACTTGTAGAGCTCG

Table 7: Reaction mix for restriction enzyme digestion.

DNA	5 µg
Restriction enzyme	5 U
10xbuffer	2 µl
H ₂ O	to 20 µl
Final volume	20 µl

Table 8: Reaction mix for DNA ligation. X and Y – volumes of insert and vector, the molar ratio of insert and vector should be 3:1.

Insert	X µl
Vector	Y µl
10x T4 DNA ligase buffer	1µl
T4 DNA ligase	0.5 µl
H ₂ O	to 10 µl
Final volume	10 µl

9.2. Vectors

