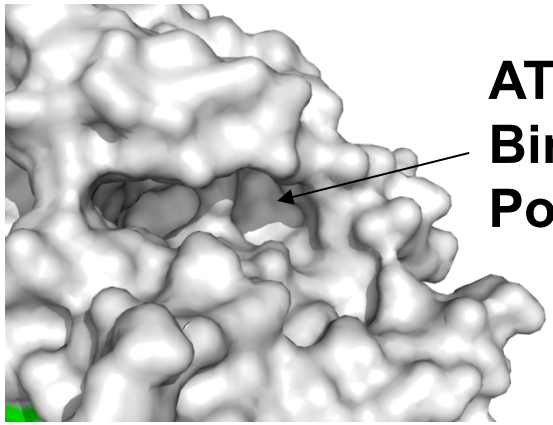
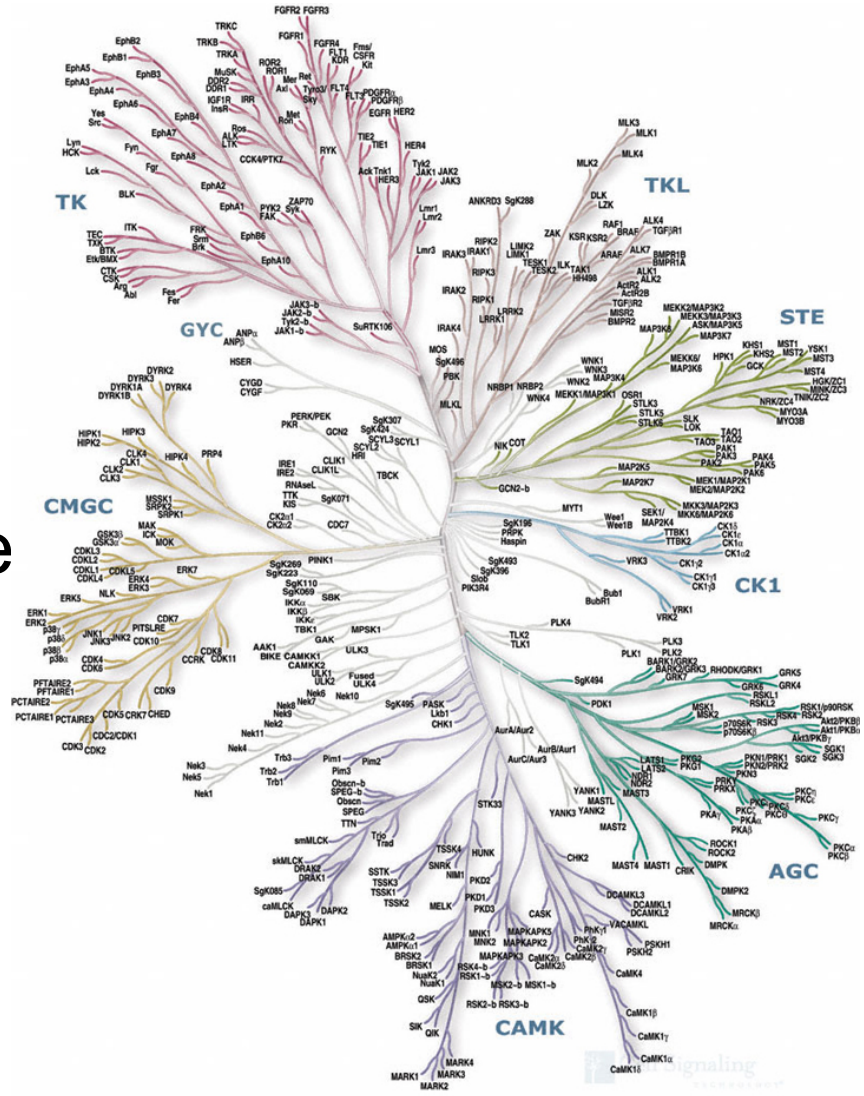
Protein Kinases

Goal: Generate a potent and selective inhibitor for every protein kinase in the human kinome.

Challenge: The ATP-binding pockets of protein kinases are highly conserved.

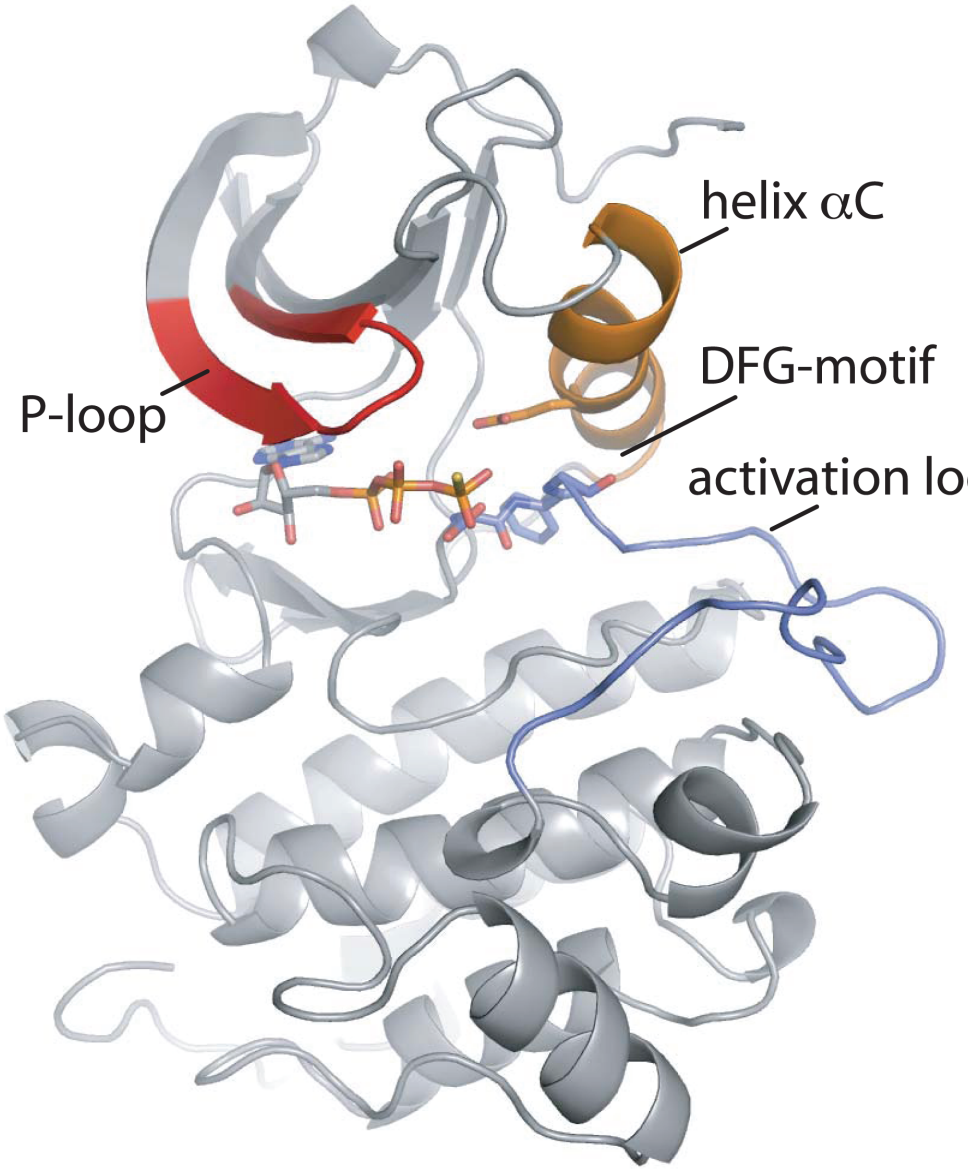


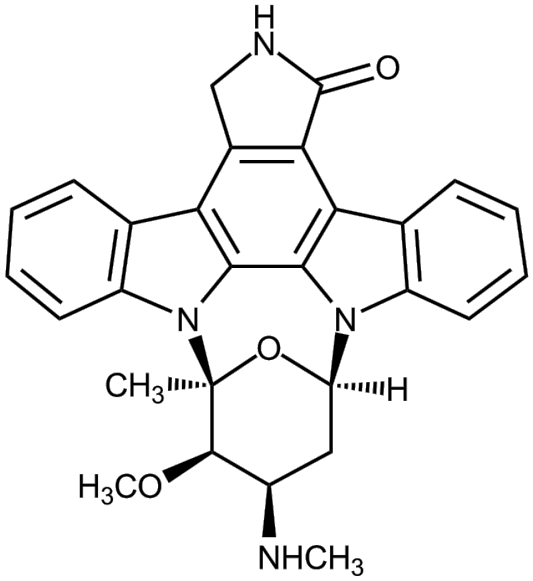
ATP-Binding Pocket



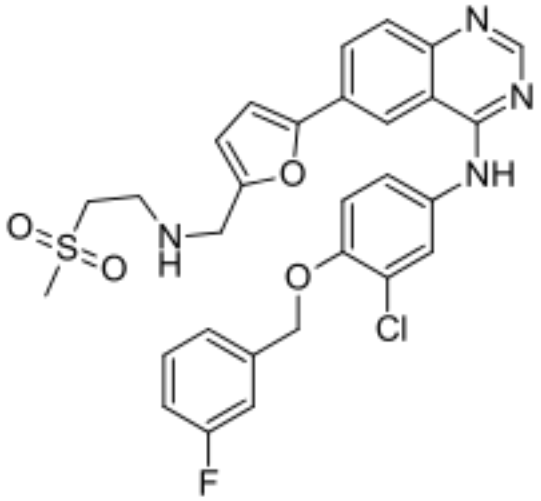
Manning, G. et al. *Science* **2002**, *298*, p.1912-34

Catalytic Domain





completely non-selective



completely selective for EGFR

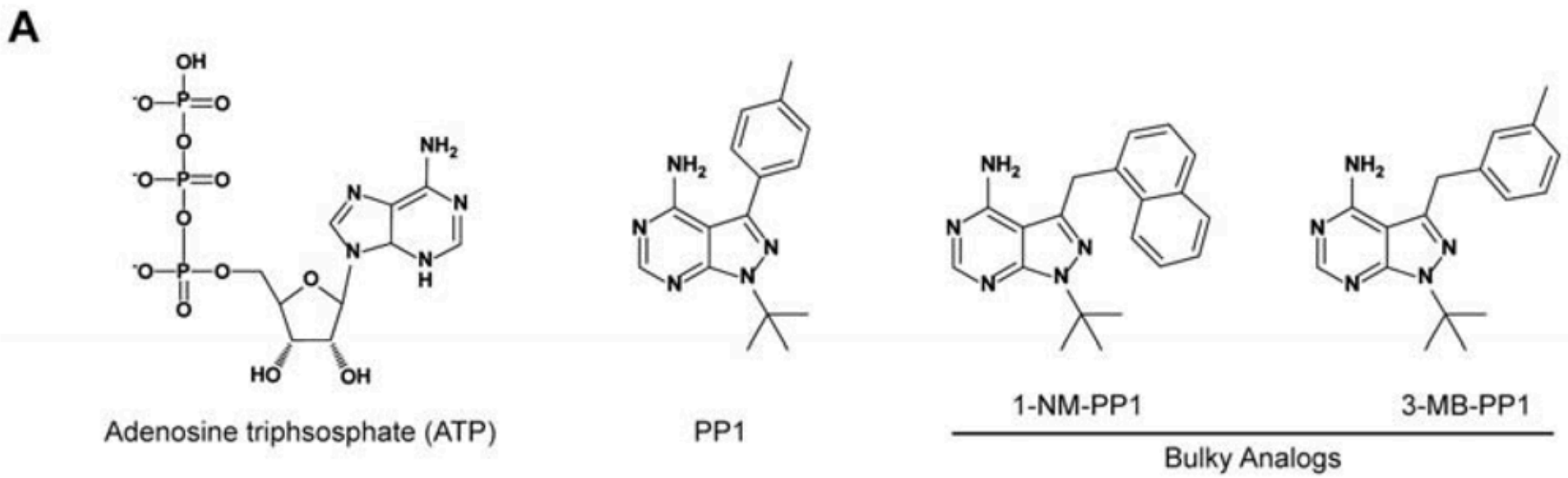
Chemical Genetic Methods for Selective Kinase Inhibition

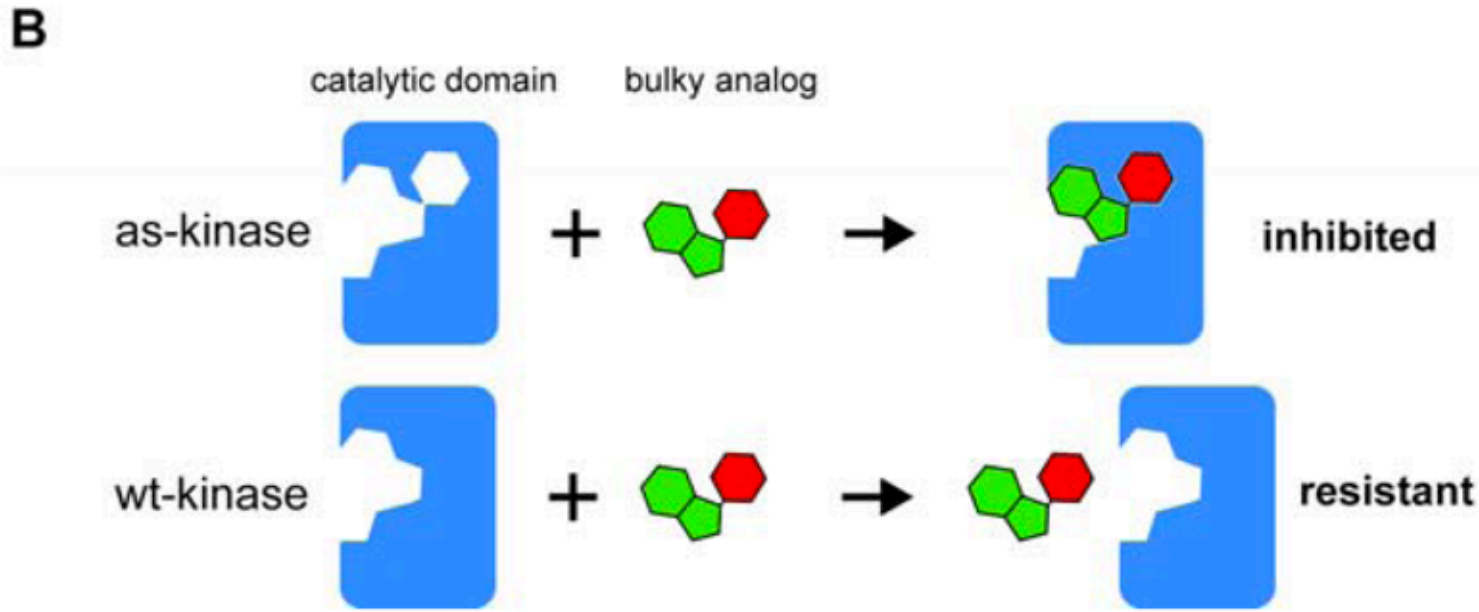
- Answer basic biological questions
- Drug target validation

Pharmacological Inhibition

1. Fast time scale
2. Only perturbs targeted sub-domains
3. graded dose response - tunability
4. Most drugs are small molecules

In many cases, RNAi and pharmacological inhibition do not phenocopy


A
Adenosine triphsosphate (ATP)
PP1
1-NM-PP1
3-MB-PP1
Bulky Analogs


B
catalytic domain
bulky analog
as-kinase
inhibited
wt-kinase
resistant

Bivalent Kinase Inhibitors

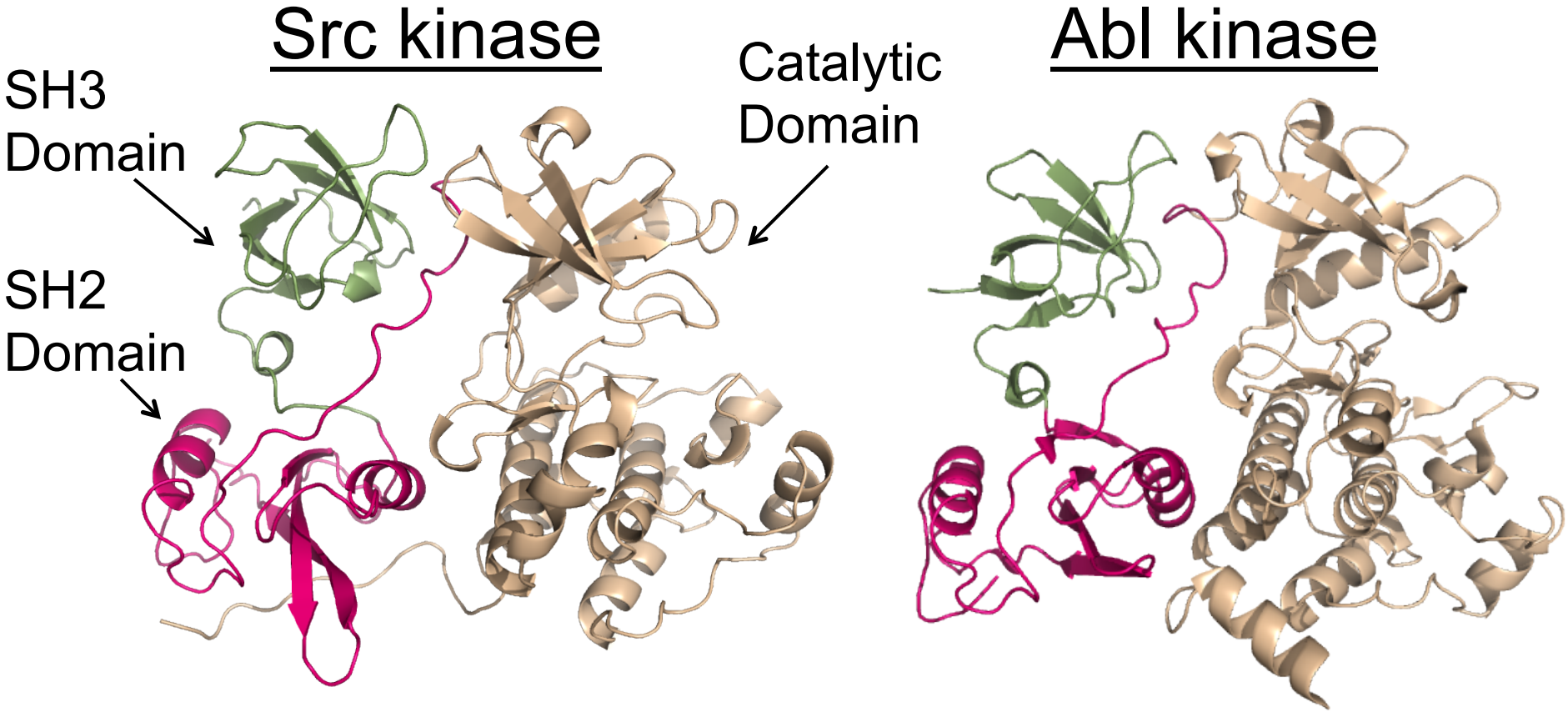
- Create a bivalent inhibitor consisting of an ATP-competitive inhibitor and a ligand for a 2° binding domain
- Capable of enhancing inhibitor selectivity and potency

$$\Delta G_{AB} = \Delta G_A + \Delta G_B + \Delta G_L$$

Goal: To develop a method that will allow the assembly of bivalent kinase inhibitors in living cells

Jencks, W. P. *Proc. Natl. Acad. Sci.* **1981**, *78*, p. 4046-50

Initial Targets

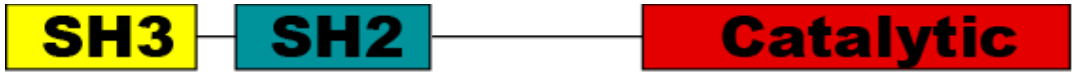


Highly homologous tyrosine kinases with a similar *N*-terminal domain architecture

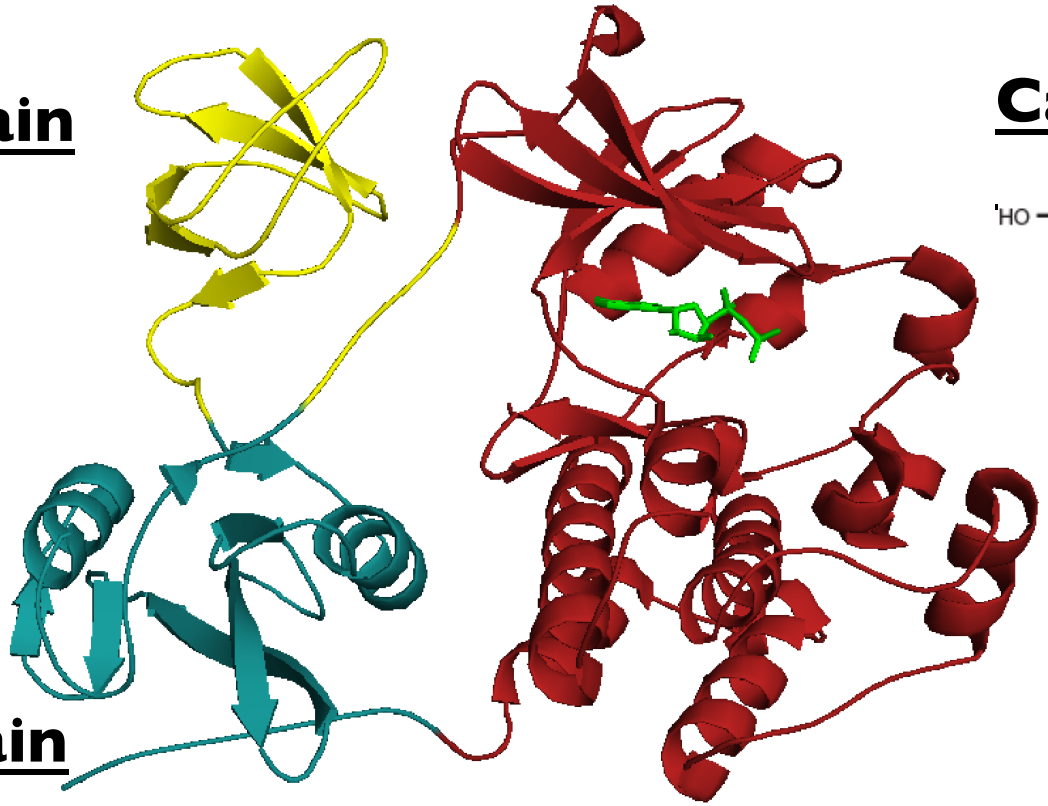
Generating selective inhibitors is challenging

PDB ID: 2H8H
PDB ID: 1OPL

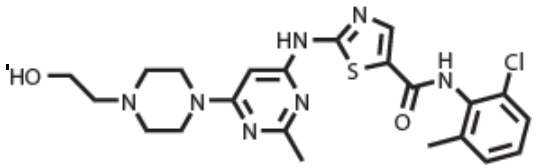
Domain Ligands

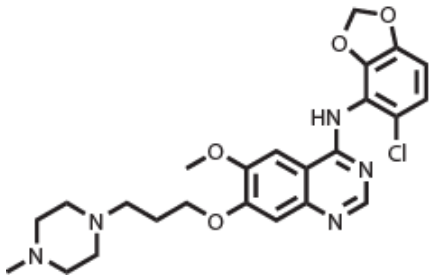


SH3 Domain
Proline-rich peptides



Catalytic

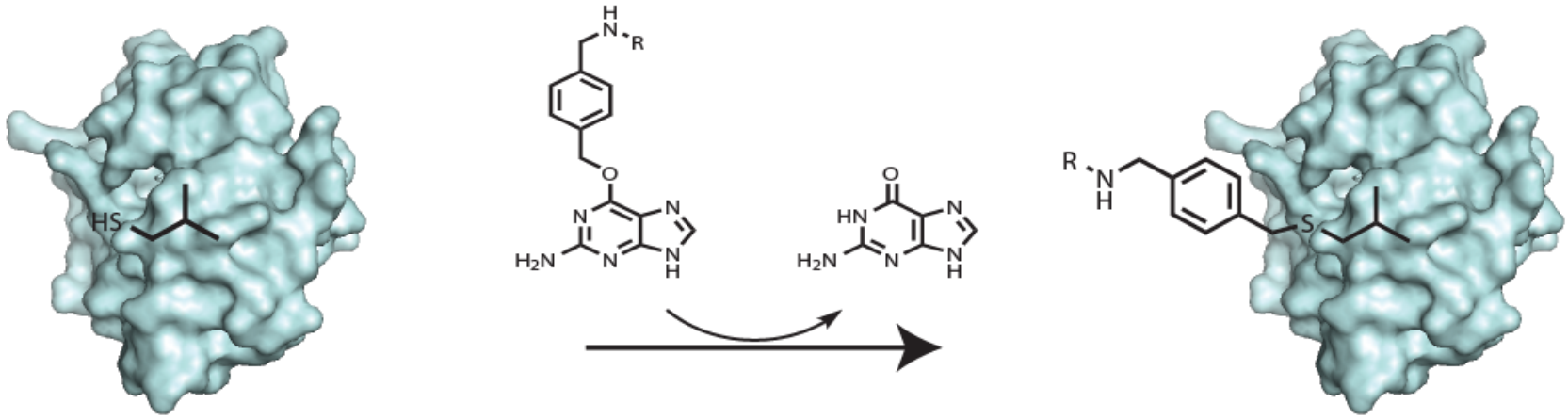




SH2 Domain
Proteins with pYEEI motif

Almost all ATP-competitive inhibitors are equipotent for Src and Abl

SNAPtag Labeling Method

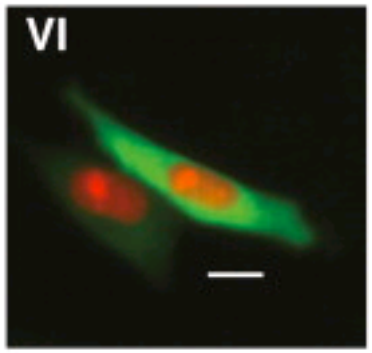


Human O^6-alkylguanine DNA alkyltransferase (SNAPtag)

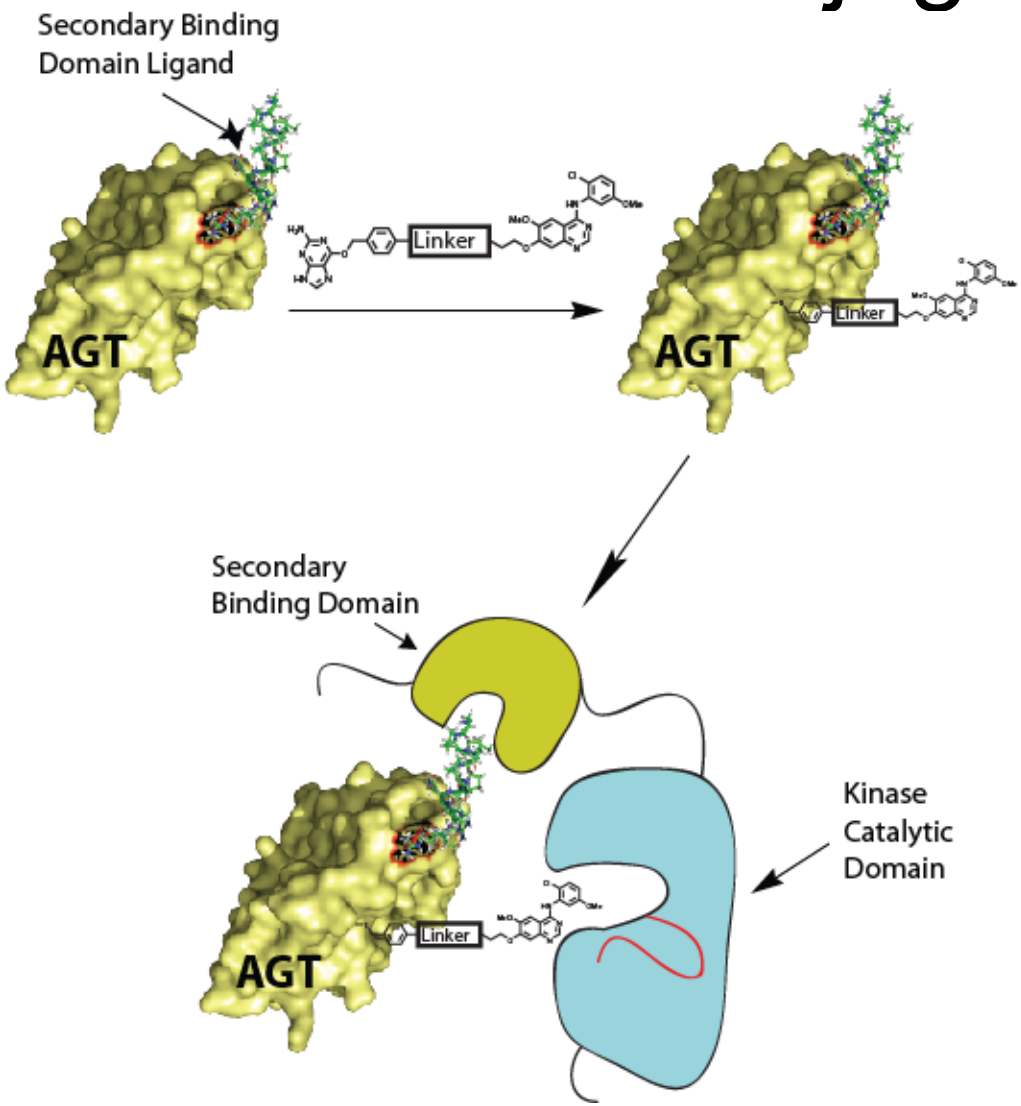
Small, self-labeling protein

Engineered to selectively and rapidly react with O^6-benzylguanine derivatives

Allows proteins to be rapidly labeled with fluorophores in living cells



SNAPtag-Small Molecule Conjugates



Strategy

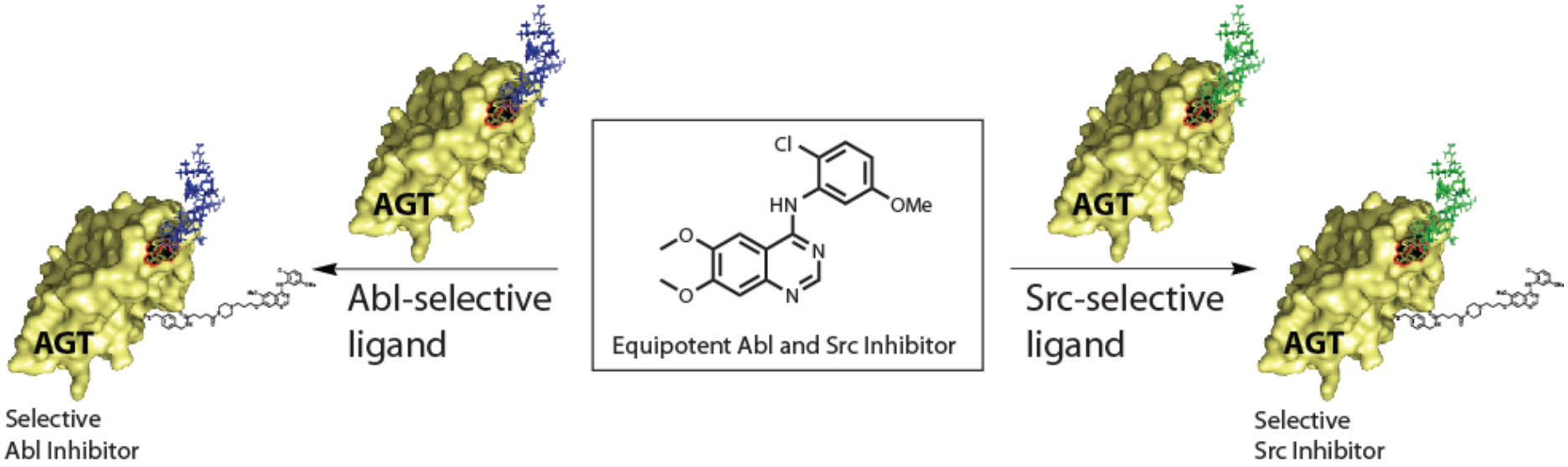
Use the SNAPtag labeling technique to generate selective kinase inhibitors

Requirements

1. SNAPtag fusion proteins that contain a ligand that targets a secondary binding domain

2. An ATP-competitive inhibitor that is linked to *O*^{6}-benzylguanine

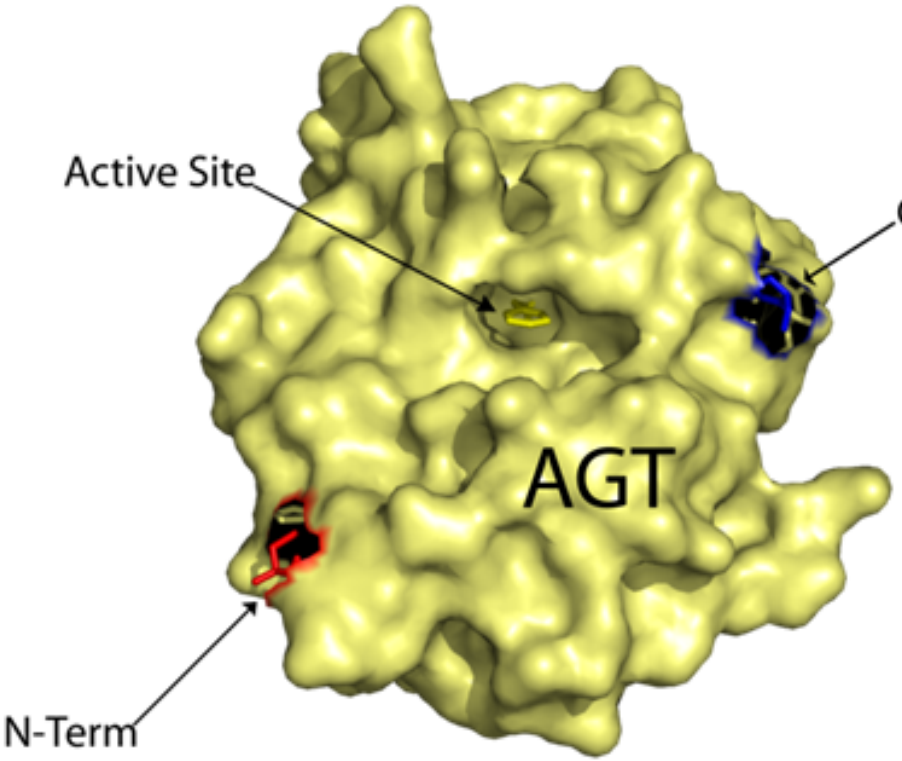
SNAPtag-Small Molecule Conjugates



SNAPtag fusion proteins provide kinase selectivity

Selective polyproline motifs have been identified for many SH3 domains

SNAPtag Fusion Proteins



AGT(PP1) = APPLPPRNRPRL-$(SG)_2$-AGT
AGT(PP2) = APPLPPRNRPRL-$(SG)_5$-AGT
AGT(PP3) = AGT-$(GS)_2$-APPLPPRNRPRL
AGT(PP4) = AGT-$(GS)_5$-APPLPPRNRPRL

Src-Family Selective

AGT(PP5) = APTYSPPPPP-$(SG)_2$-AGT
AGT(PP6) = APTYSPPPPP-$(SG)_5$-AGT
AGT(PP7) = AGT-$(GS)_2$-APTYSPPPPP
AGT(PP8) = AGT-$(GS)_5$-APTYSPPPPP

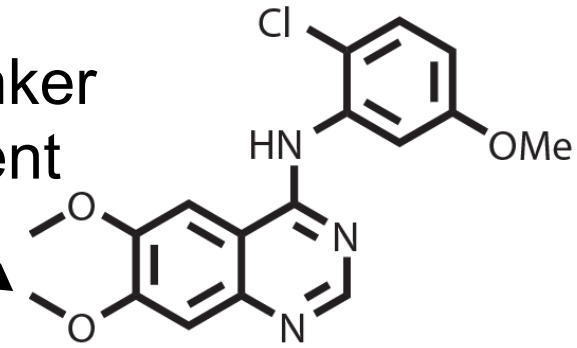
Abl Selective

N- and *C*-terminal fusions with variable linker lengths were generated for each polyproline ligand

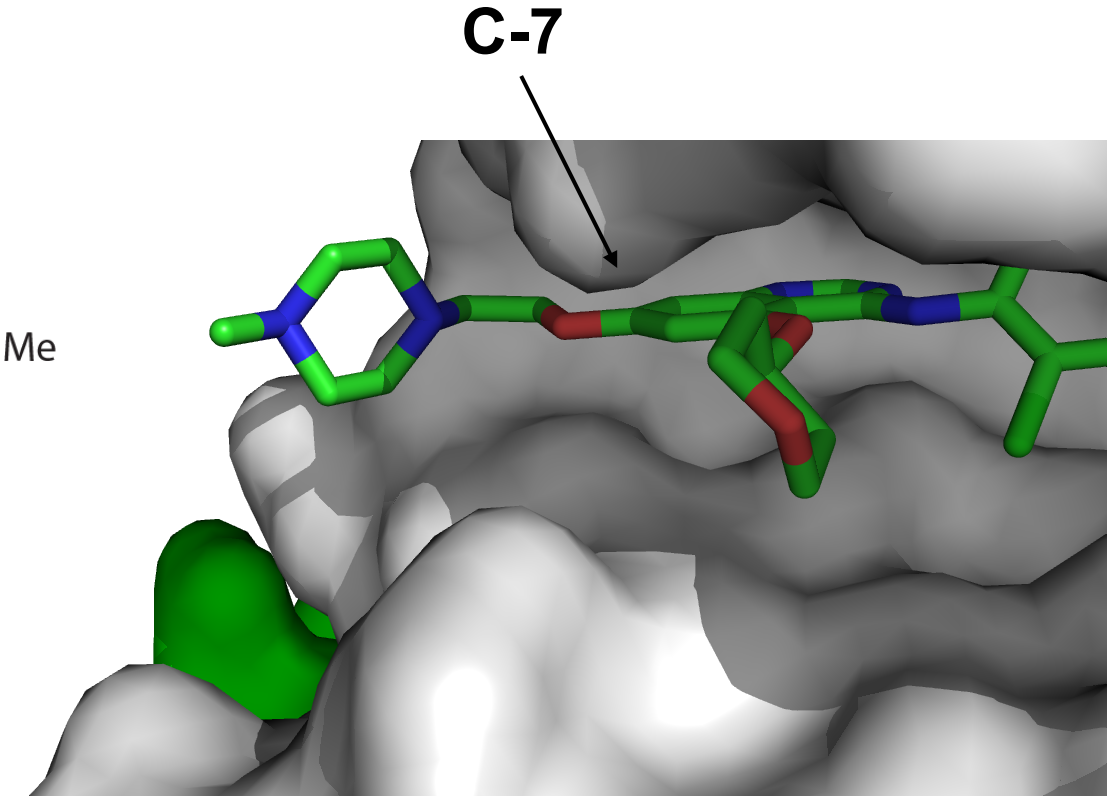
All fusion proteins have the same catalytic activity as SNAPtag

ATP-Binding Site Ligand

Site of linker attachment



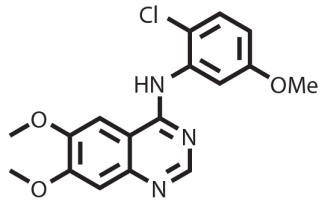
IC_{50} (Src) = 640 nM
IC_{50} (Abl) = 710 nM



Structure of a 4-anilinoquinazoline derivative bound to Src kinase (PDB ID: 2H8H)

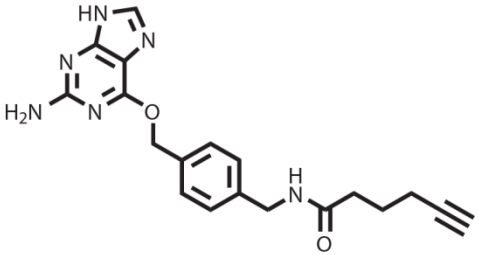
Several 4-anilinoquinazolines are potent inhibitors of Src and Abl

In vitro Activities of BG Derivatives



IC_{50} (Src) = 640 nM

IC_{50} (Abl) = 710 nM

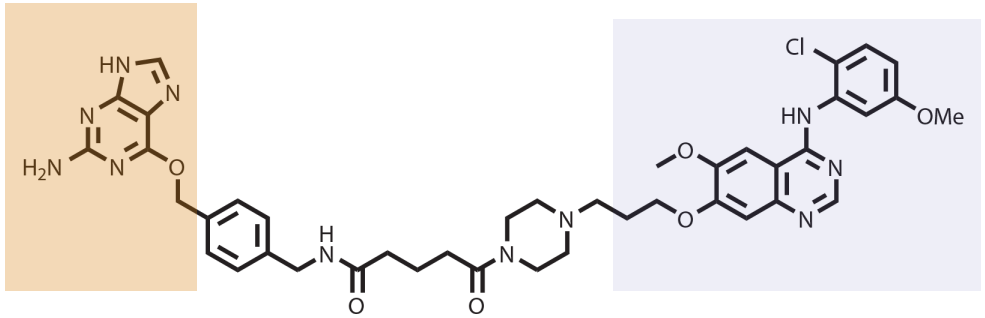


IC_{50} (Src) = >10,000 nM

IC_{50} (Abl) = >10,000 nM

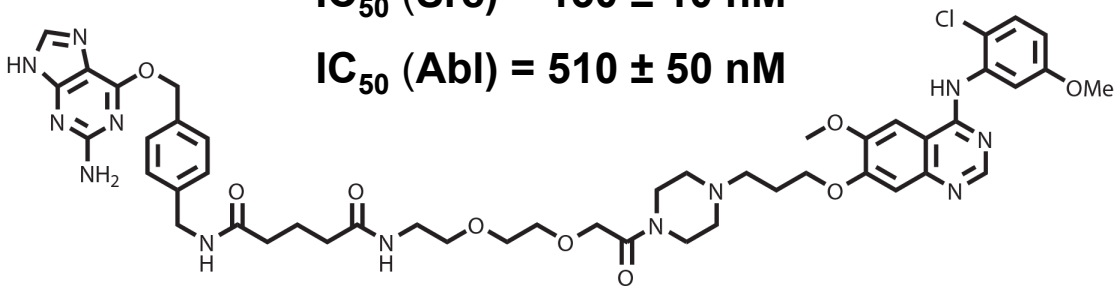
AGT-labeling group

ATP-Competitive Inhibitor



IC_{50} (Src) = 300 ± 20 nM

IC_{50} (Abl) = 400 ± 50 nM

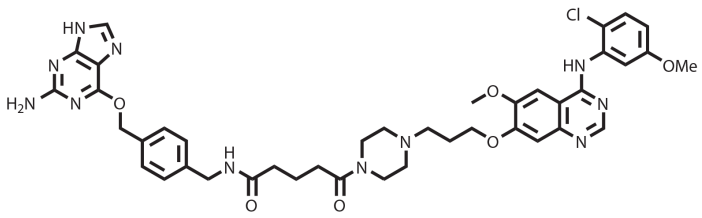


IC_{50} (Src) = 130 ± 10 nM

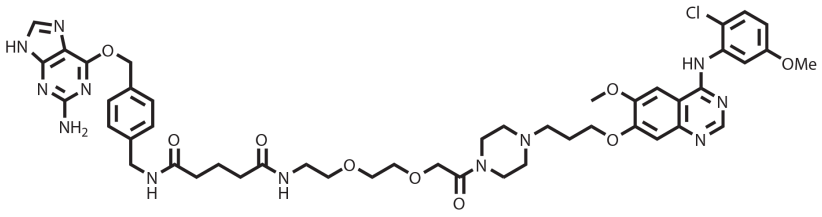
IC_{50} (Abl) = 510 ± 50 nM

Attaching an AGT-labeling group does not affect inhibitor potency

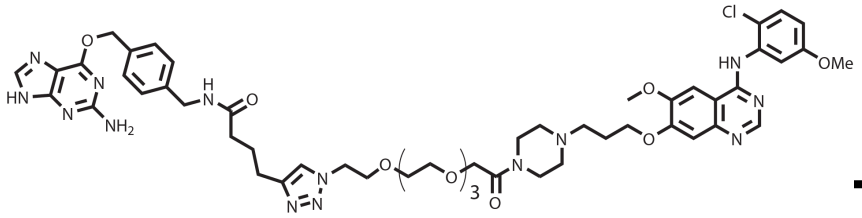
Wild Type Conjugates



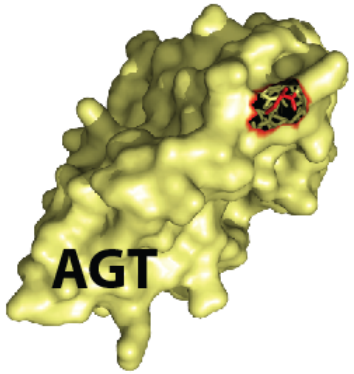
A



B



C



Src IC_{50} (nM)

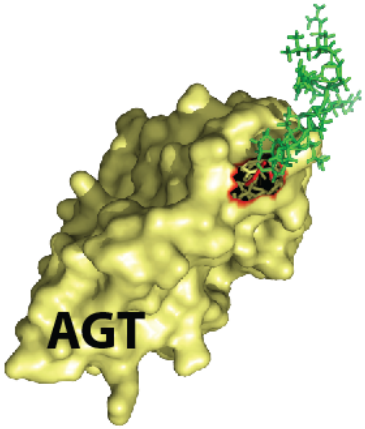
	A	B	C
AGT(wt)	> 5000	2000 ± 300	240 ± 30

Abl IC_{50} (nM)

	A	B	C
AGT(wt)	3200 ± 100	1300 ± 200	1200 ± 100

Conjugation to SNAPtag causes a modest drop in potency for the 4-anilinoquinazoline inhibitor

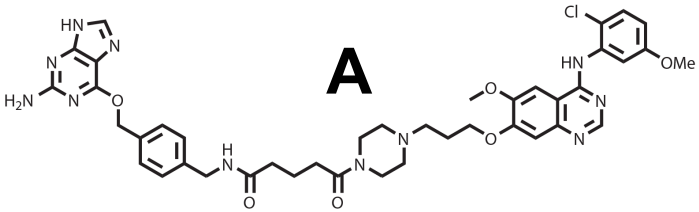
Src-Selective Conjugates



Src-selective
SH3 ligand
APPLPPRNRPRL

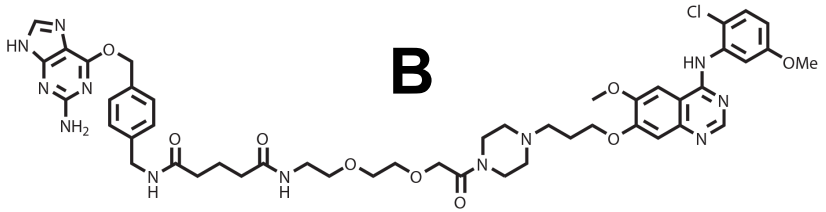
PP1 and PP2 are *N*-terminal fusions

PP3 and PP4 are *C*-terminal fusions



IC_{50} (nM)

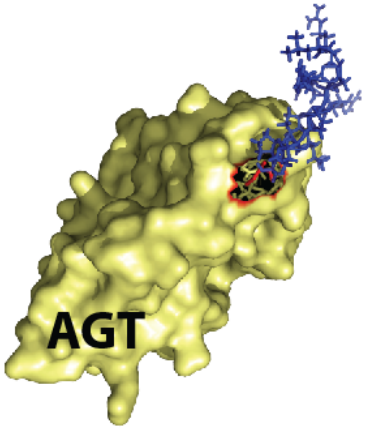
	Src
AGT(wt)-**A**	**>5000**
AGT(PP1)-**A**	**13 ± 3**
AGT(PP2)-**A**	**25 ± 6**
AGT(PP3)-**A**	**72 ± 10**
AGT(PP4)-**A**	**34 ± 3**



IC_{50} (nM)

	Src
AGT(wt)-**B**	**2000 ± 300**
AGT(PP1)-**B**	**12 ± 1**
AGT(PP2)-**B**	**15 ± 1**
AGT(PP3)-**B**	**66 ± 10**
AGT(PP4)-**B**	**16 ± 1**

Abl-Selective Conjugates

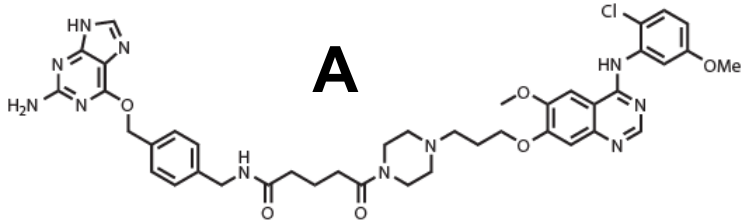


Abl-selective
SH3 ligand
APTYSPPPPP

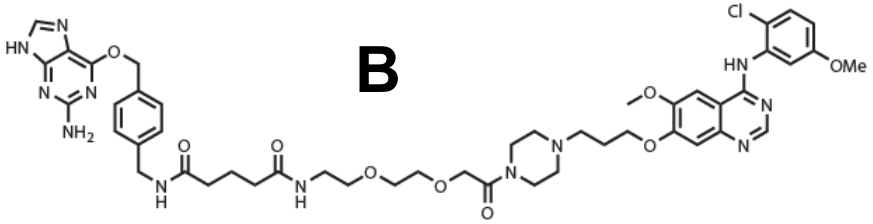
PP5 and PP6 are *N*-terminal fusions

PP7 and PP8 are *C*-terminal fusions

A



B



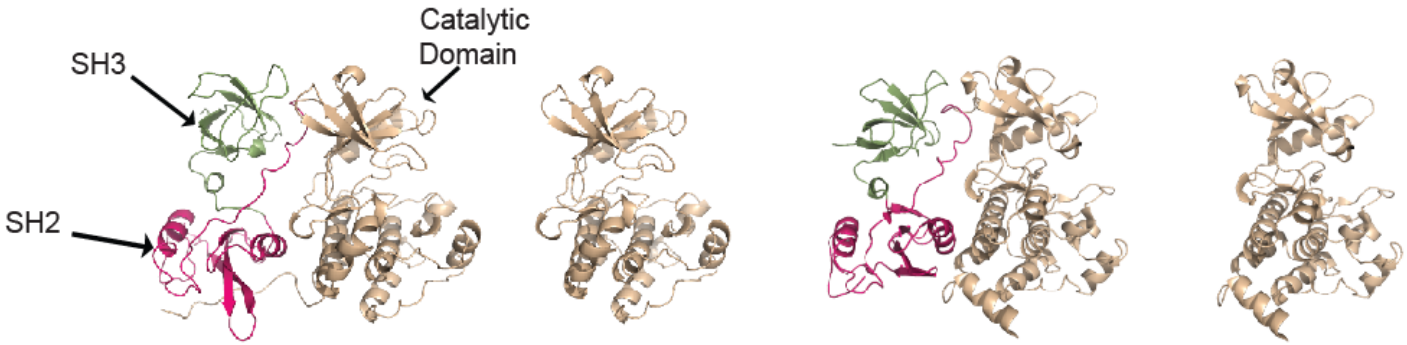
IC_{50} (nM)

	Abl
AGT(wt)-**A**	**3200 ± 100**
AGT(PP5)-**A**	**18 ± 6**
AGT(PP6)-**A**	**15 ± 4**
AGT(PP7)-**A**	**18 ± 7**
AGT(PP8)-**A**	**15 ± 3**

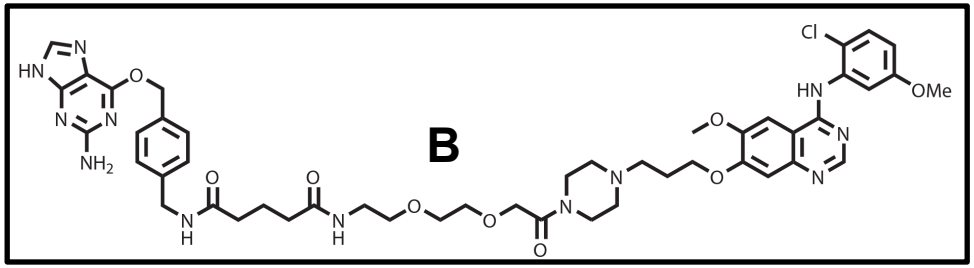
IC_{50} (nM)

	Abl
AGT(wt)-**B**	**1300 ± 200**
AGT(PP5)-**B**	**< 6**
AGT(PP6)-**B**	**< 6**
AGT(PP7)-**B**	**< 6**
AGT(PP8)-**B**	**< 6**

SH3 Domain Contribution



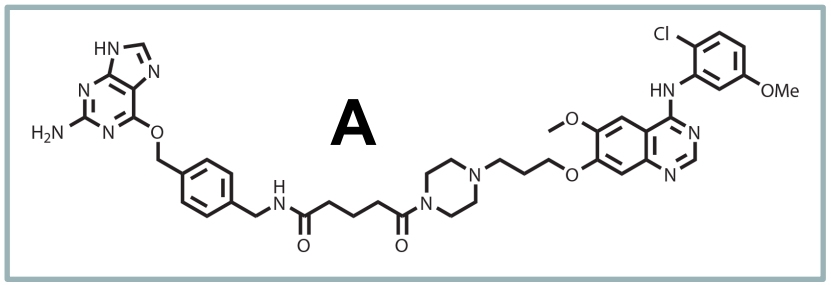
	Src-3D	Src-KD	Abl-3D	Abl-KD
AGT(Src-selective)-**B**	12 ± 1	2600 ± 300	N/T	3200 ± 300
AGT(Abl-selective)-**B**	N/T	4200 ± 500	< 6	3100 ± 400
AGT(wt)-**B**	2000 ± 300	3700 ± 700	1300 ± 200	3600 ± 200



All conjugates are equipotent inhibitors of Src KD and Abl KD

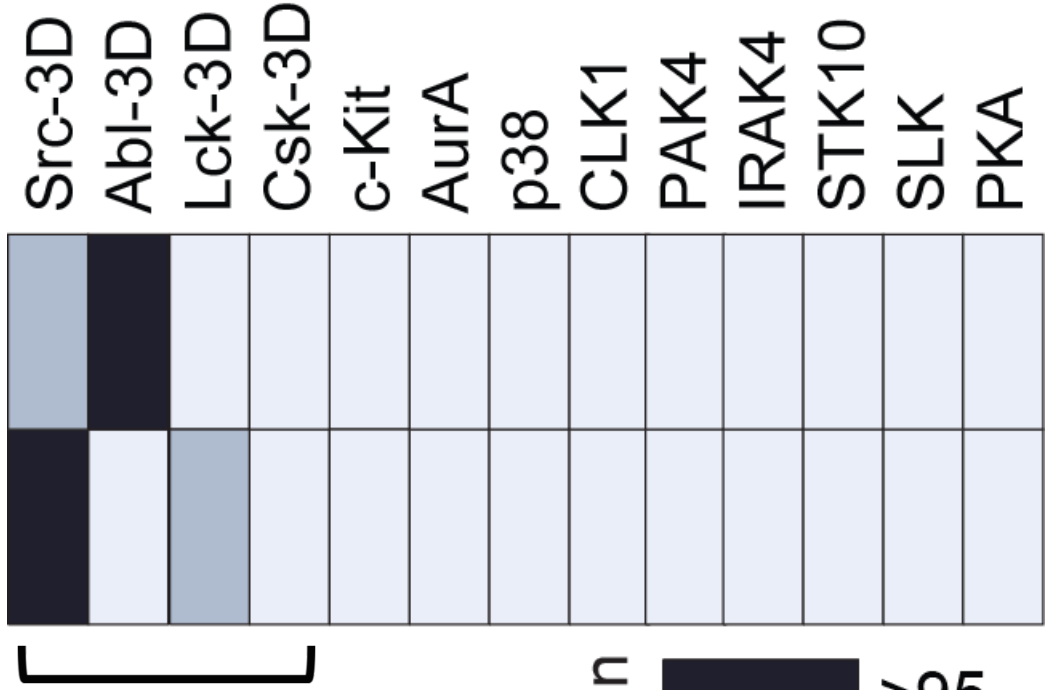
Interaction with the SH3 domain is the main contributor to increased potency

Kinase Selectivity of Conjugates



2.5 μM

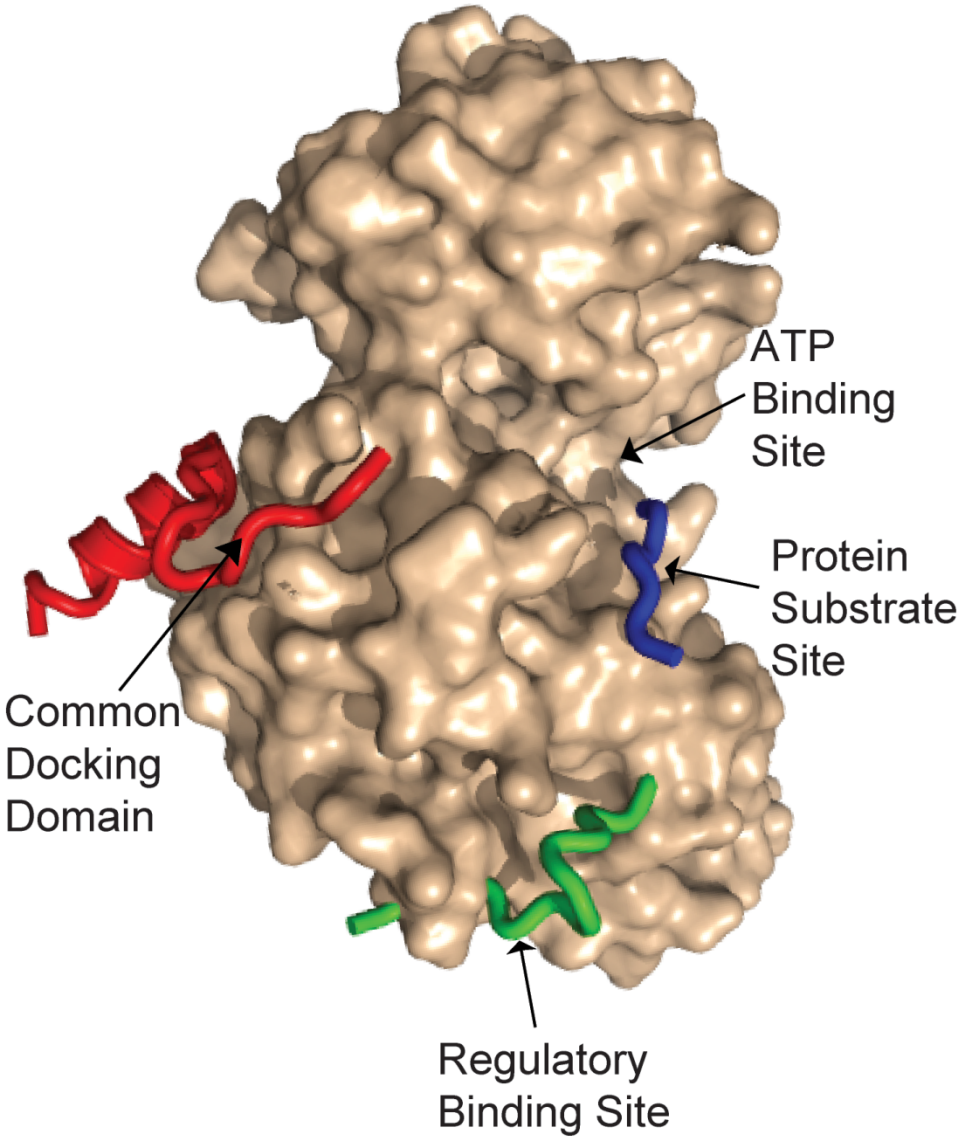
- SNAP(Abl selective)-**A**
- SNAP(Src selective)-**A**



AGT conjugates are selective for kinases that contain SH3 domains

No inhibition of 20 additional kinases

Method Generality



The catalytic domains of protein kinases contain diverse ligand binding sites

Signaling specificity is achieved through these sites

These binding sites can be effectively targeted with bivalent inhibitors

Summary

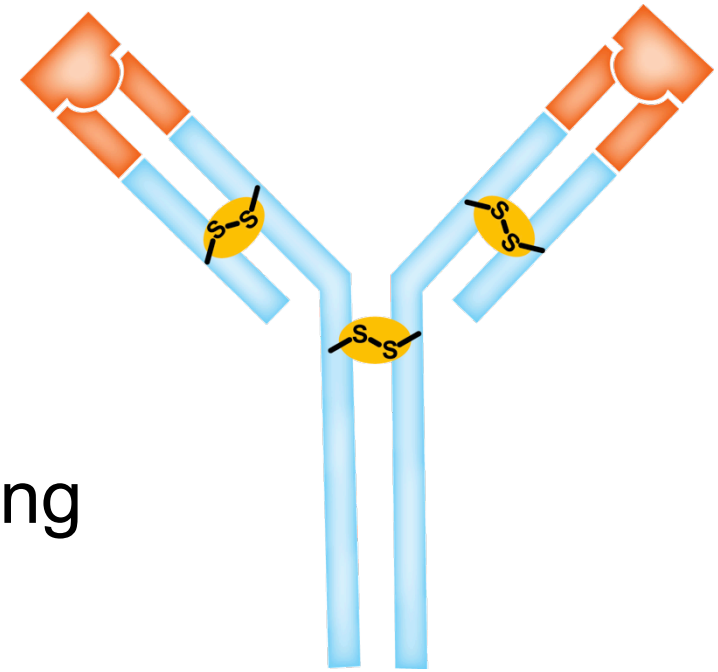
1. SNAPtag is an efficient scaffold for displaying bivalent kinase inhibitors

2. Any interaction site for which a suitable ligand has been identified (K_D < 10 μM) can be used to construct bivalent inhibitors

Challenge

There are not well defined interaction sites for most kinases

Solution: Intracellular Antibodies

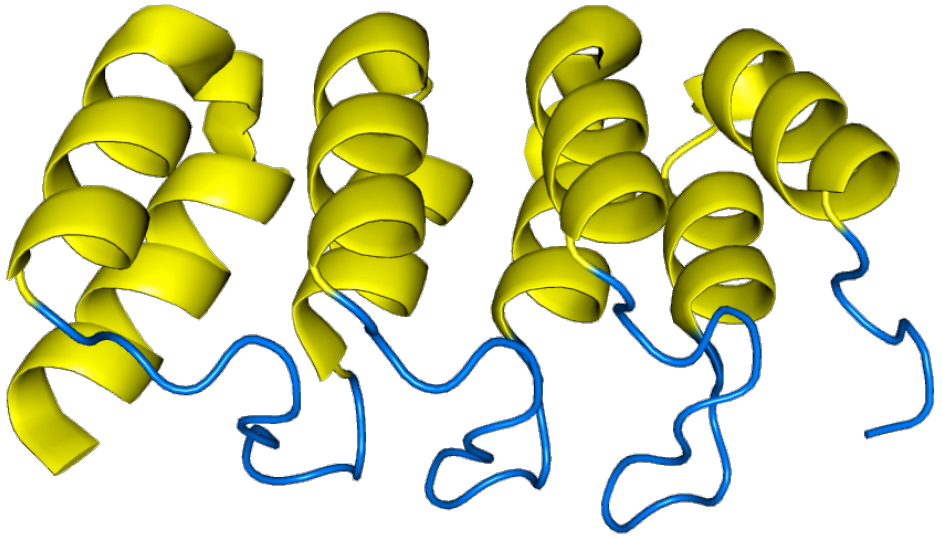


- Antibodies can be developed to recognize a single antigen
  - Not functional in the reducing cellular environment
- Potent and selective protein binders have been designed
  - Termed “intracellular antibodies”
  - Stable structure not dependent on disulfide

A potential general solution to finding secondary binding ligands

DARPins

- Antibody-like protein binding molecules
- Stable tertiary structure allows extensive mutation of the variable loop regions
- Libraries are panned using ribosome display



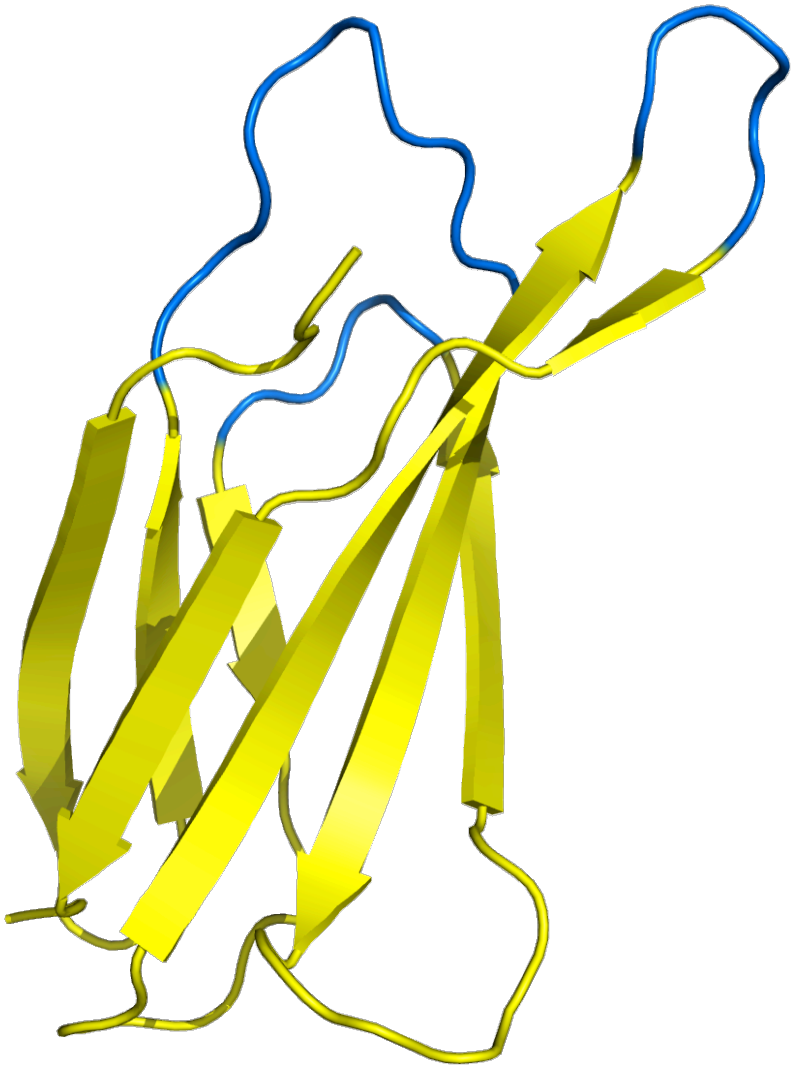
Amstutz, P. et. al.. *Protein Eng. Des. Sel.* **2006**, *19*, 219-229.

Monobodies

Very similar to DARPins

-Stable β-sandwich structure

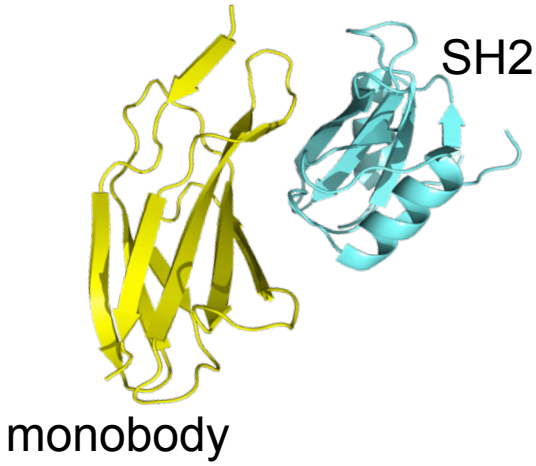
-Loops can be extensively mutated to select for binding interactions (phage display)

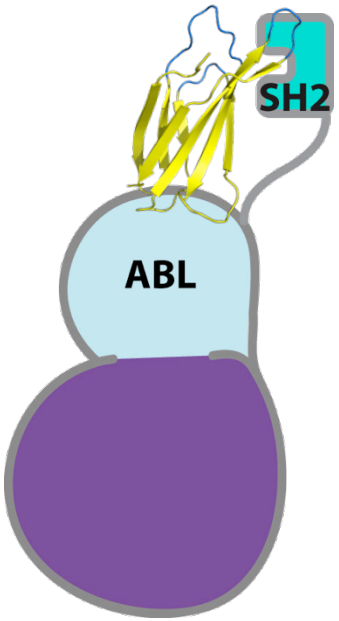


Wojcik, J. et. al. *Nat. Struct. Mol. Biol.* **2010**, *17*, 519–527.

Bivalent Inhibition with Monobodies and DARPins

- Selective monobody has been developed to bind SH2 domain of Abl
- Monobody does not affect non-activated Abl





Bivalent SNAPtag fusions containing Monobodies or DARPins are highly selective

Bivalent Inhibitors Can Be Assembled in Cells

Cell Permeability Assay

Transfect cells with AGT

↓

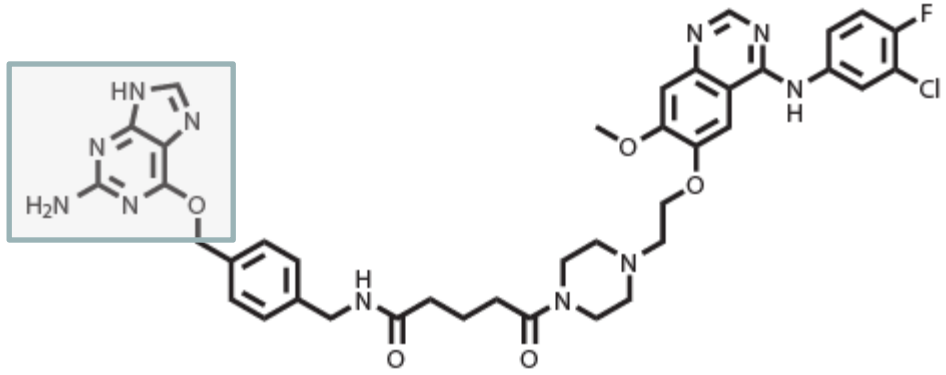
Incubate cells with linkable inhibitor (or DMSO)

↓

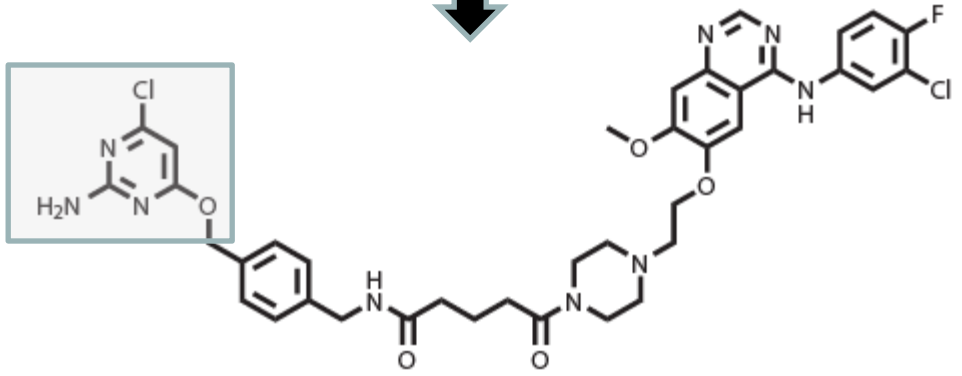
Incubate cells with linkable Rhodamine

↓

Lyse cells, SDS-PAGE, and determine labeling efficiency

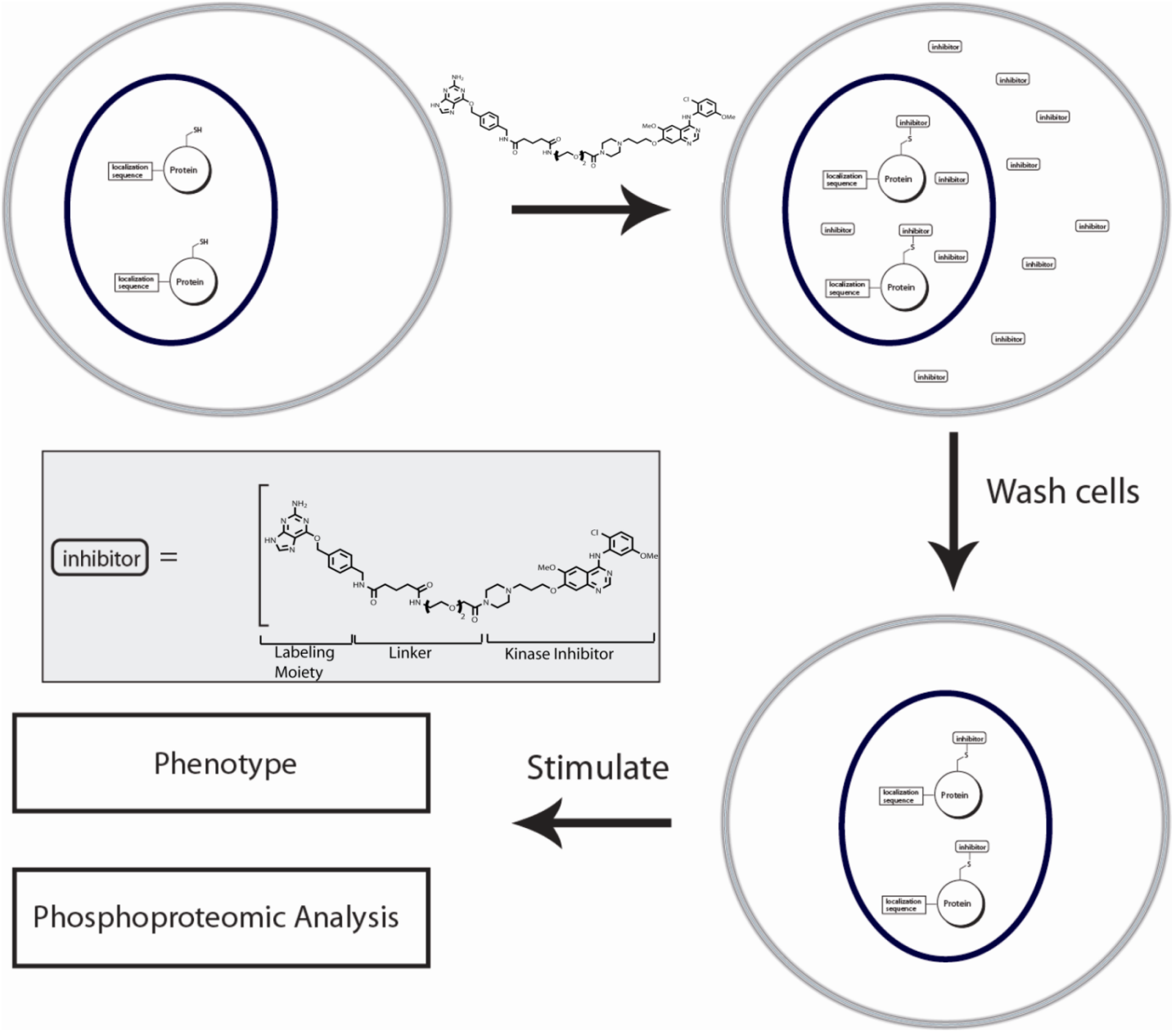


Low cell permeability (Cos-7, HeLa, HEK293, K562, NIH-3T3)



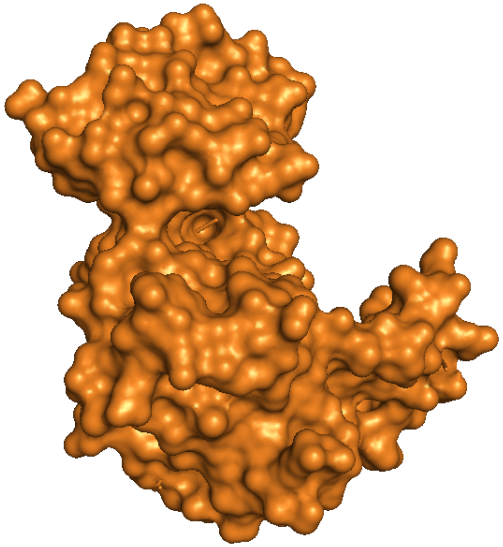
High cell permeability (Cos-7, HeLa, HEK293, K562, NIH-3T3)

Sub-Cellular Pharmacology



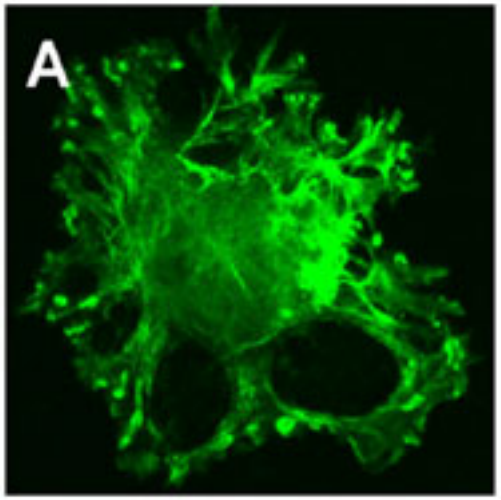
Methods for Studying Signaling Enzyme Function

Loss-of-function studies:



Protein of Interest

RNAi or
gene knock-out

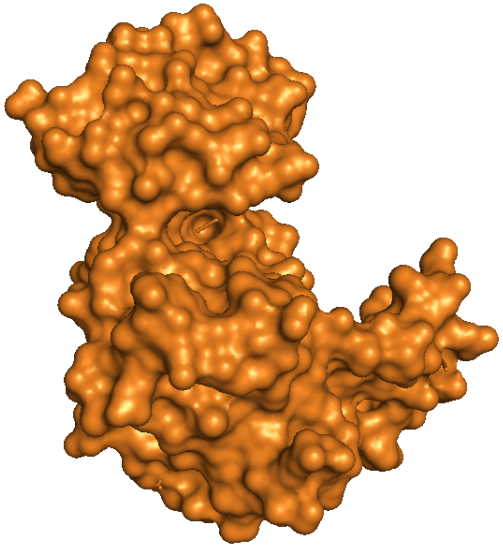


phenotype

Small-molecule inhibitors provide rapid, reversible, and dose-dependent control over protein function

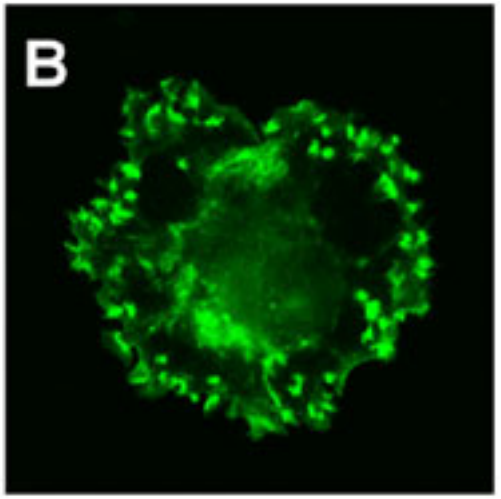
Methods for Studying Signaling Enzyme Function

Gain-of-function studies:



Protein of Interest

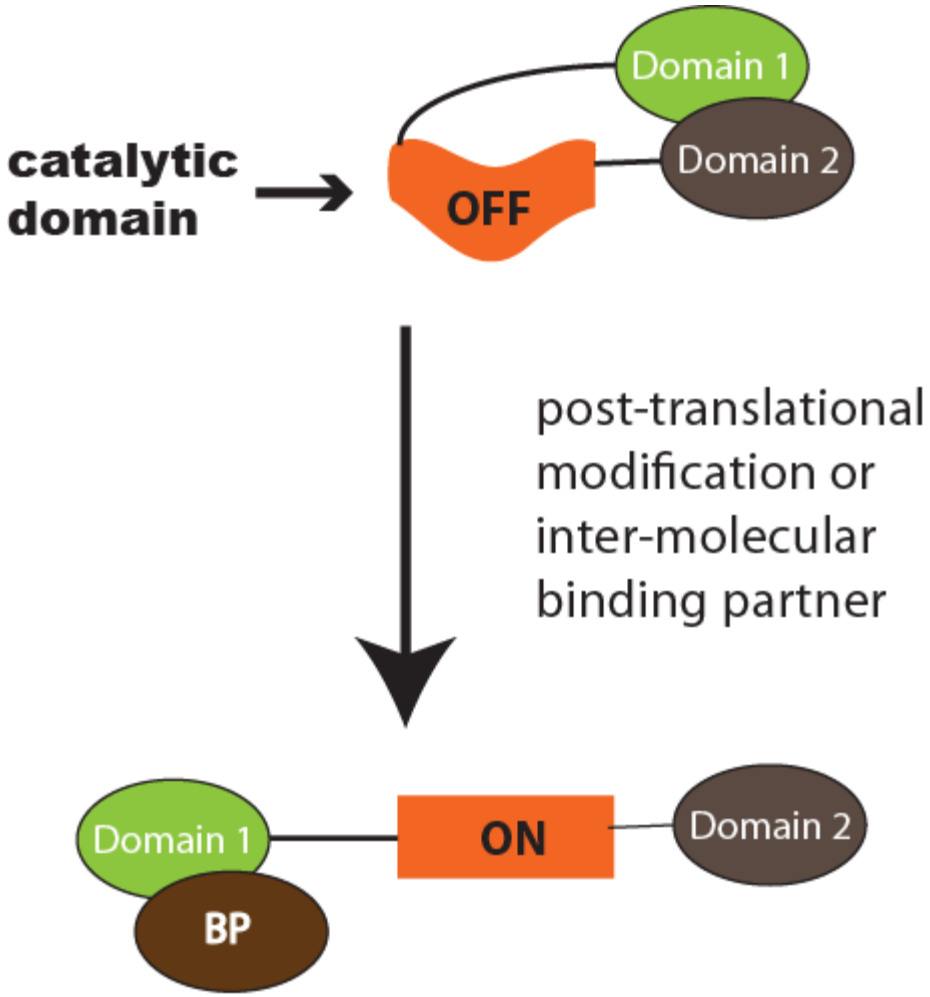
over-expression
or constitutively active
mutant



phenotype

GOAL: Develop a general method for activating signaling enzymes with a small molecule probe

Regulation of Signaling Enzyme Activity

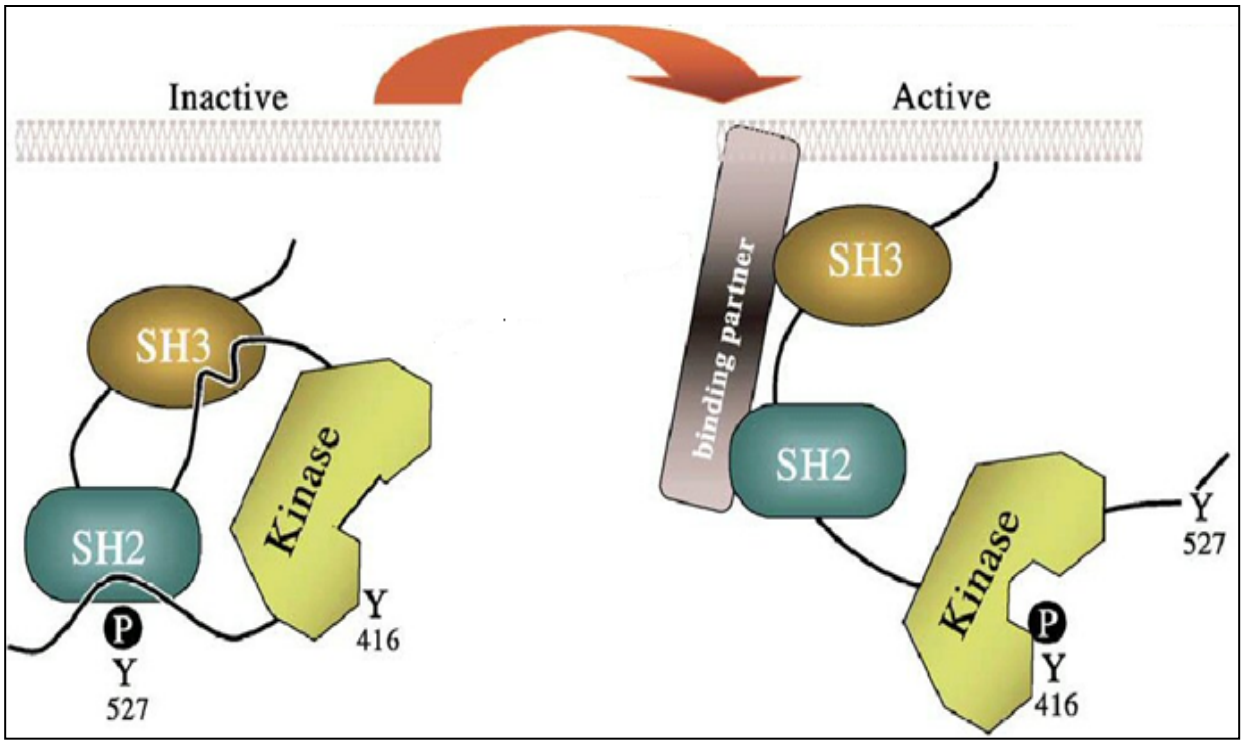


Release of intra-molecular auto-inhibition is a common mechanism of enzyme activation

Allows tight control over enzymatic activity coupled with rapid activation kinetics

Kuriyan and Eisenberg *Nature* **2007**, *450*, 983-90

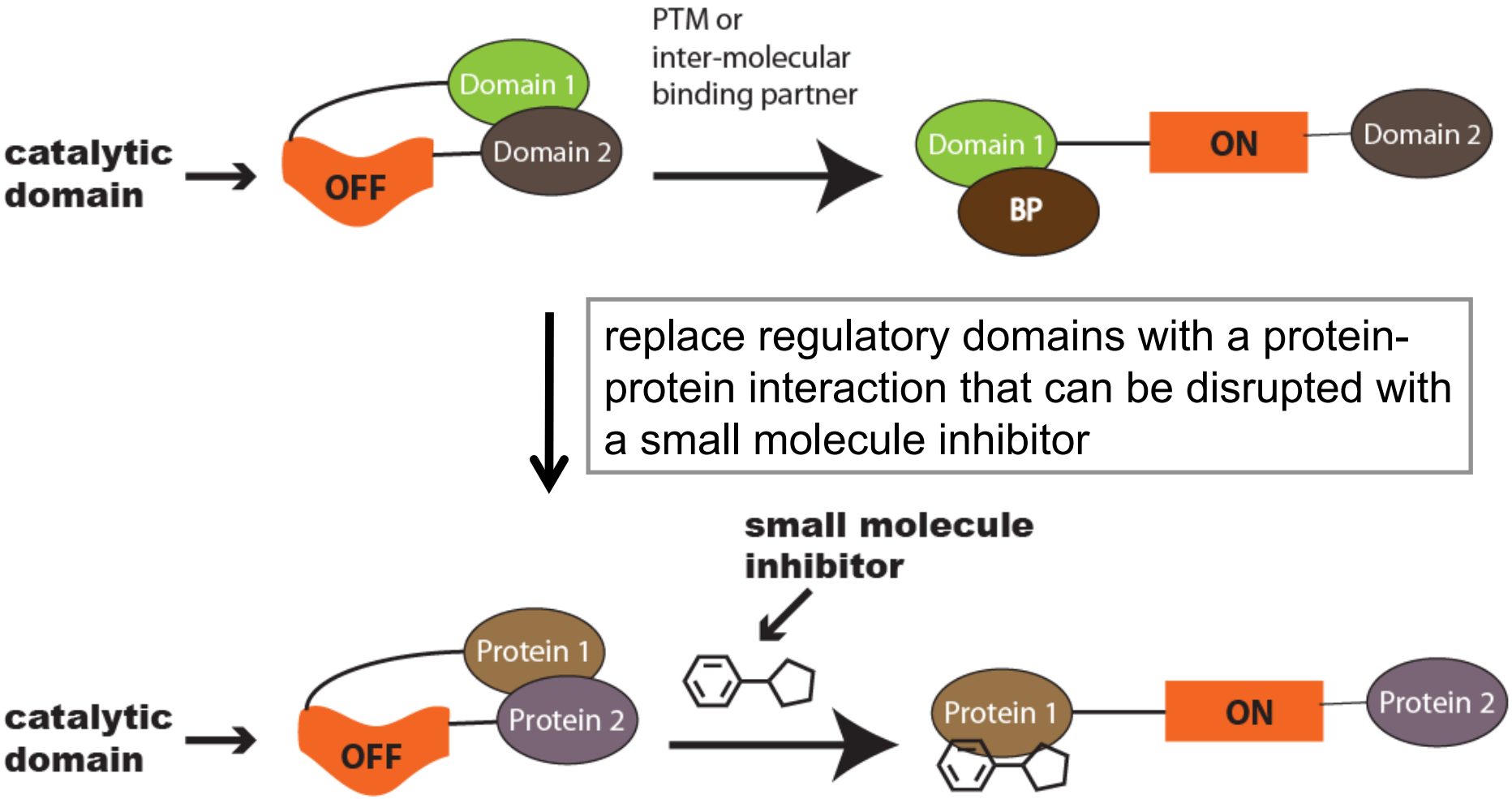
Regulation of Signaling Enzyme Activity



Example: Src-family kinases

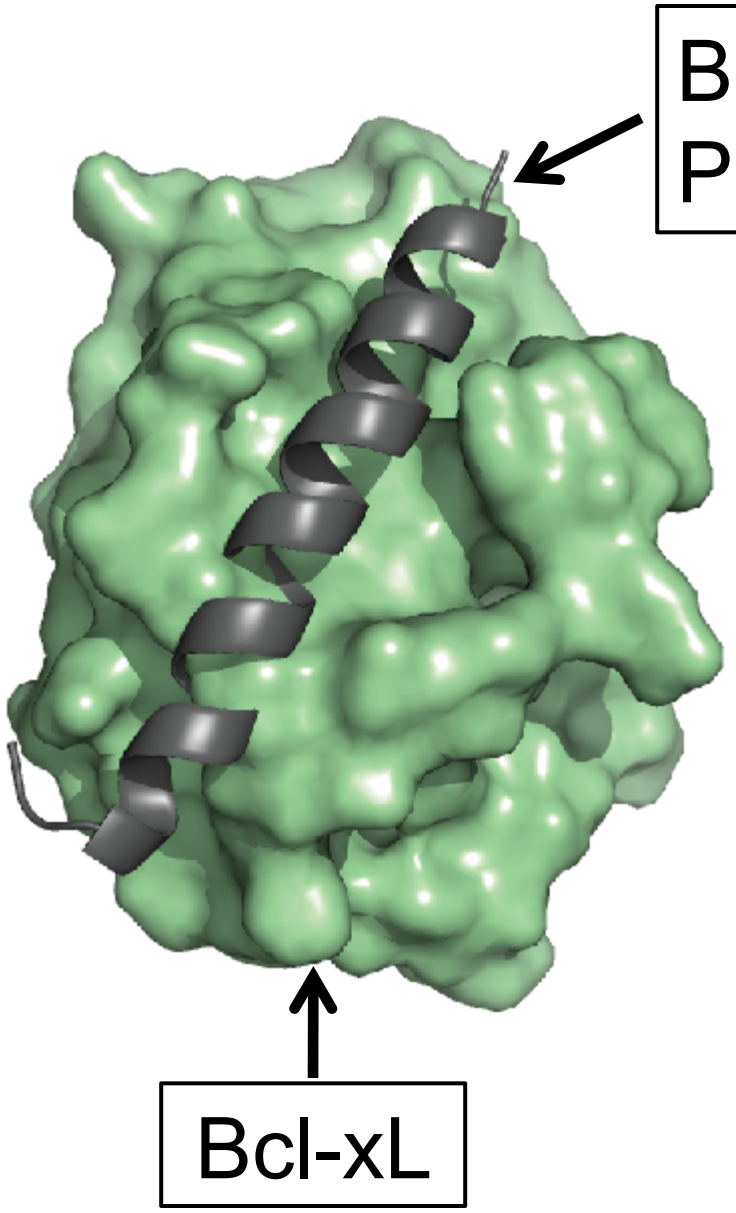
An inter-molecular binding event releases intra-molecular auto-inhibition

Proposed Method



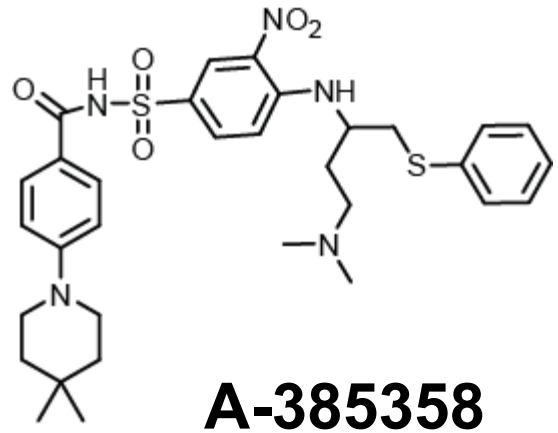
The same small molecule inhibitor can be used to control multiple enzymes

Bcl-xL/BH3 Peptide Interaction

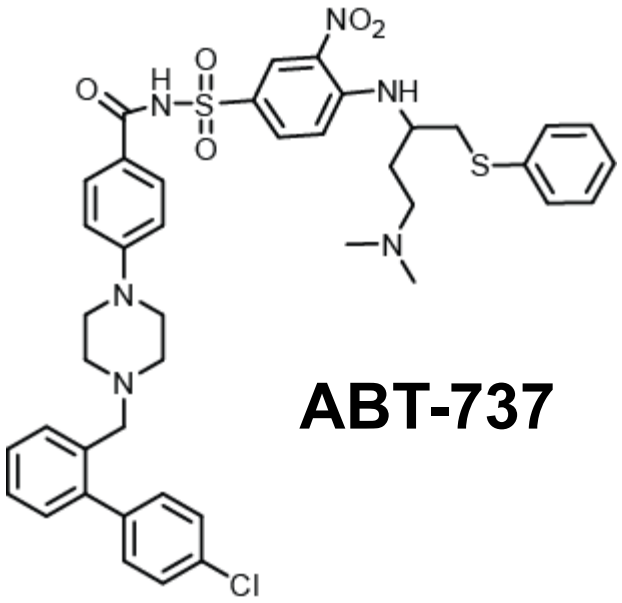


- Bcl-xL is an anti-apoptotic protein that can be minimized to a functional domain of 20-25 kDa
- 20-30 amino acid peptides from BH3-only proteins (BH3 peptides) bind to Bcl-xL
- Bcl-xL has a high affinity for BH3 peptides (K_D = 1-20 nM)

Bcl-xL/BH3 Peptide Interaction Inhibitors



A-385358

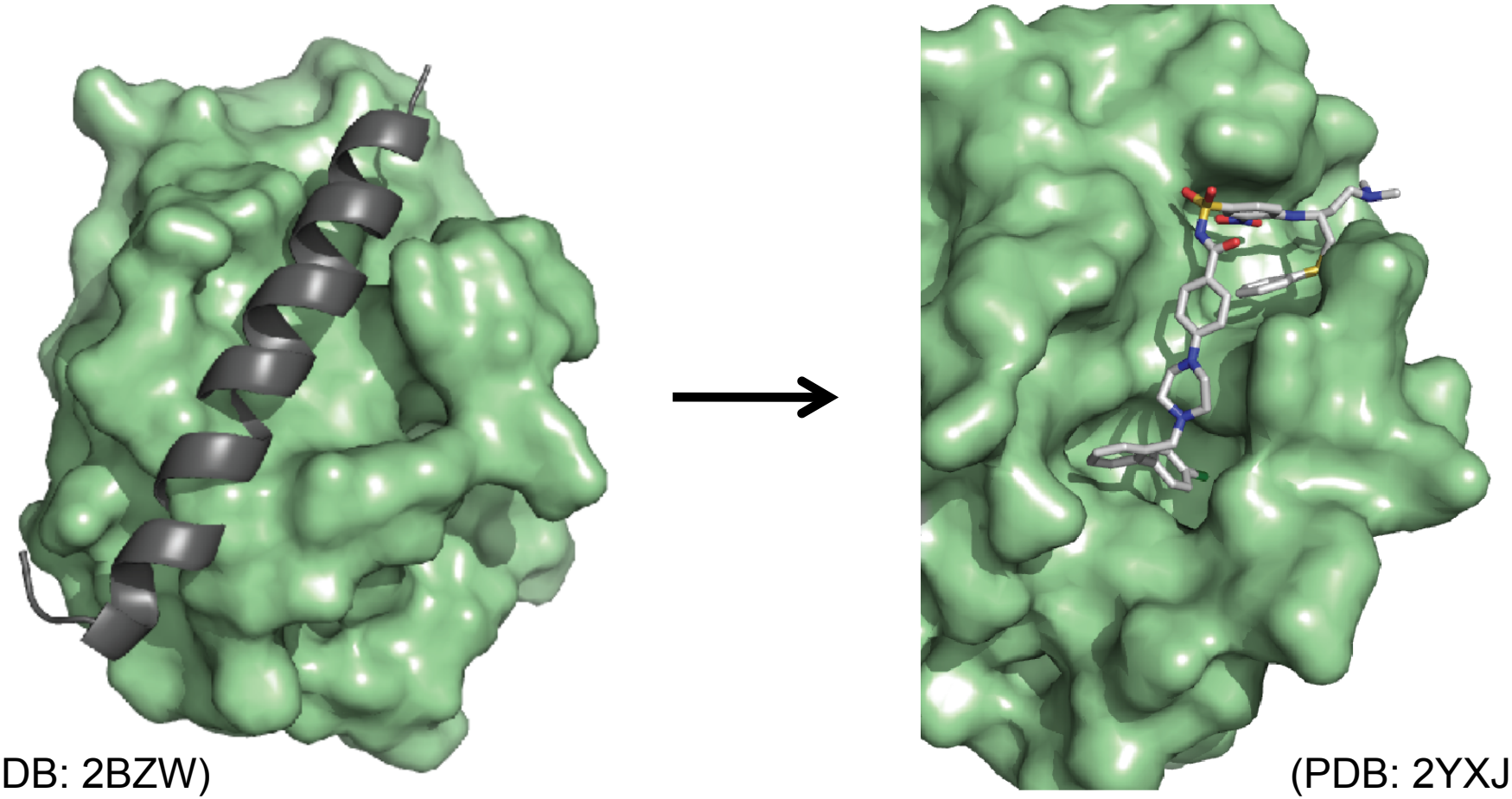


ABT-737

ABT-737 and **A-385358** disrupt the interaction between Bcl-xL and BH3 peptides

Both inhibitors bind tightly to Bcl-xL (K_D = 0.1 – 5 nM)

Bcl-xL/BH3 Peptide Interaction Inhibitors

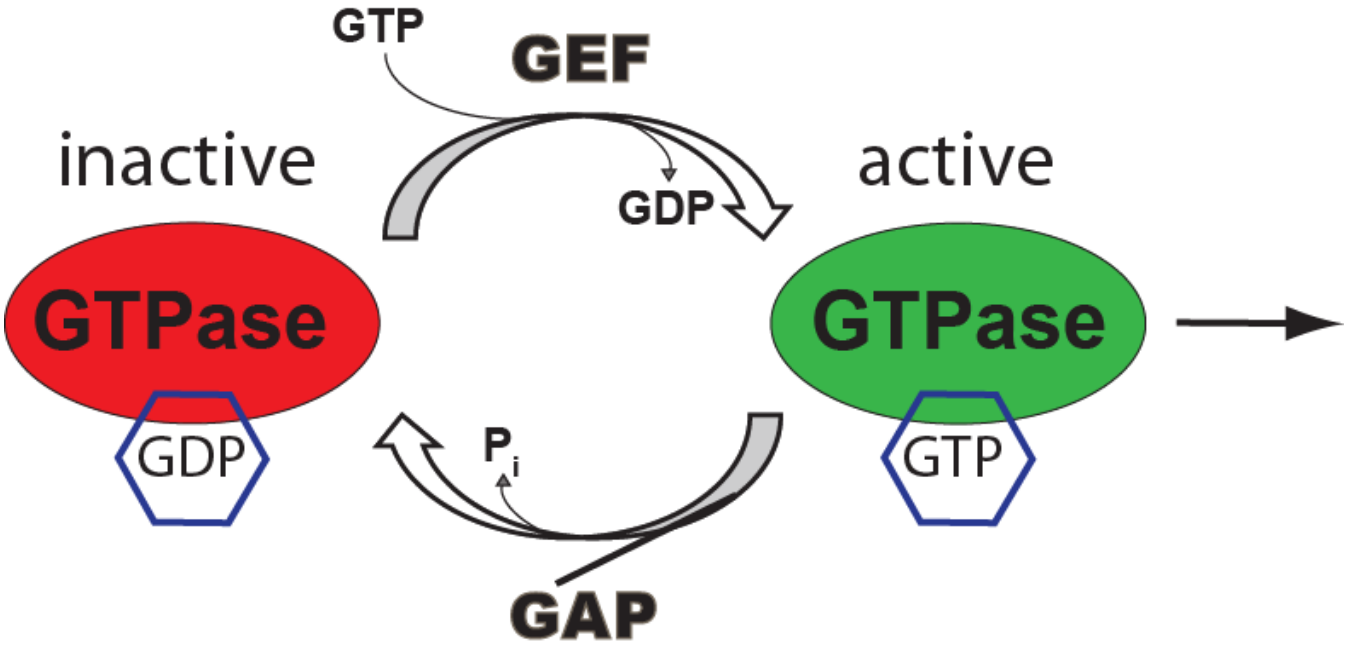


(PDB: 2BZW)

(PDB: 2YXJ)

ABT-737 and **A-385358** bind to the same hydrophobic groove as BH3 peptides

Target = Small GTPases

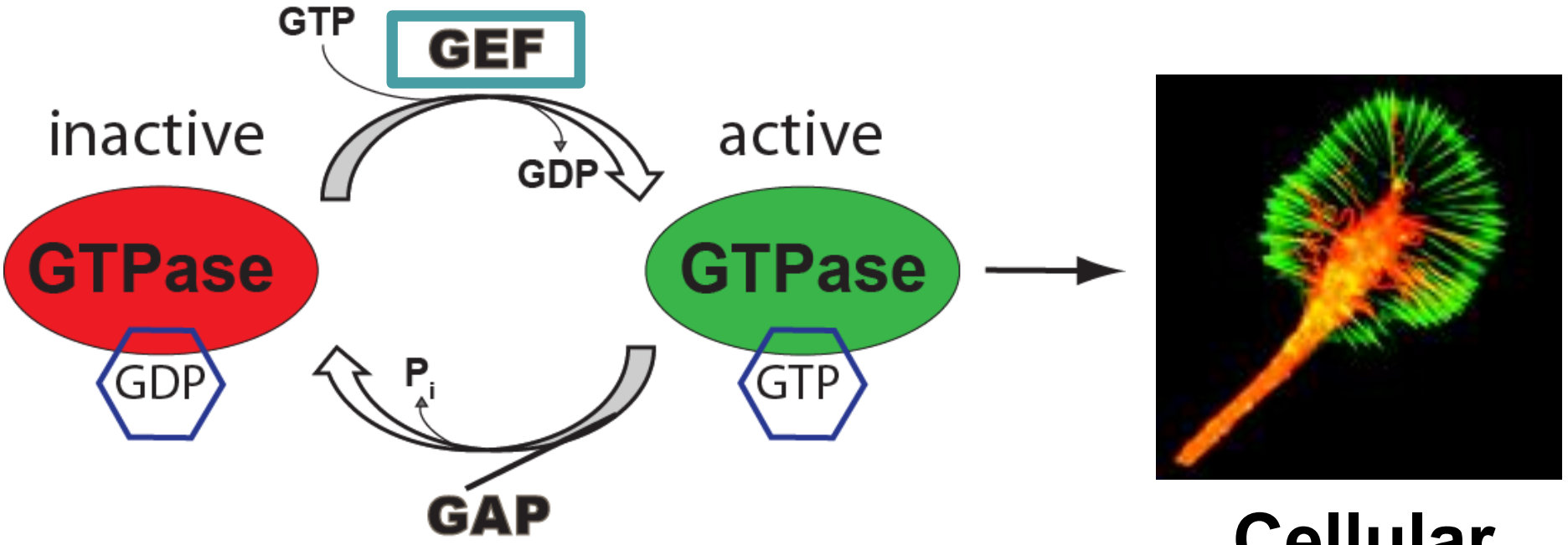


Cellular Function

Small GTPases act as molecular switches in cell signaling

22 Rho-family GTPases in humans

Rho GEF Engineering



Cellular Function

Small GTPases act as molecular switches in cell signaling

22 Rho-family GTPases in humans

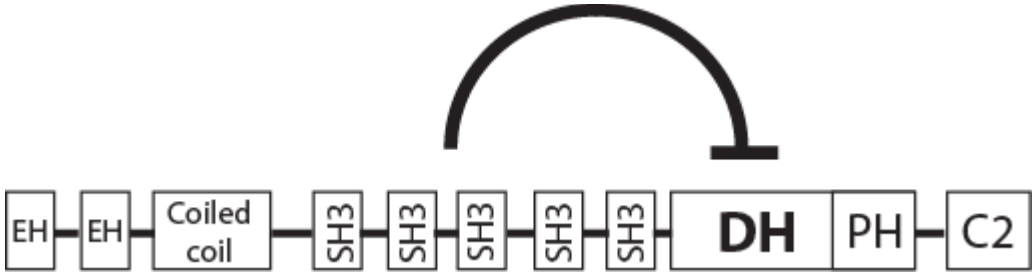
Dbl-Family Guanine Nucleotide Exchange Factors (GEFs)

69 Dbl-family members in humans

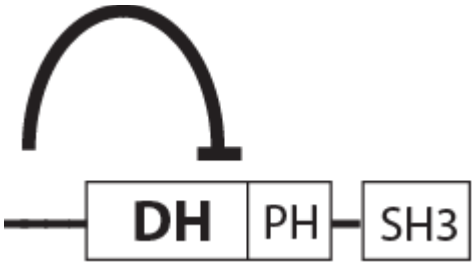
Multi-domain proteins that activate Rho-family GTPases

All members contain a Dbl homology (**DH**) domain that catalyzes nucleotide exchange in Rho-family GTPases

Truncated mutants are constitutively active – regulated by intra-molecular auto-inhibition



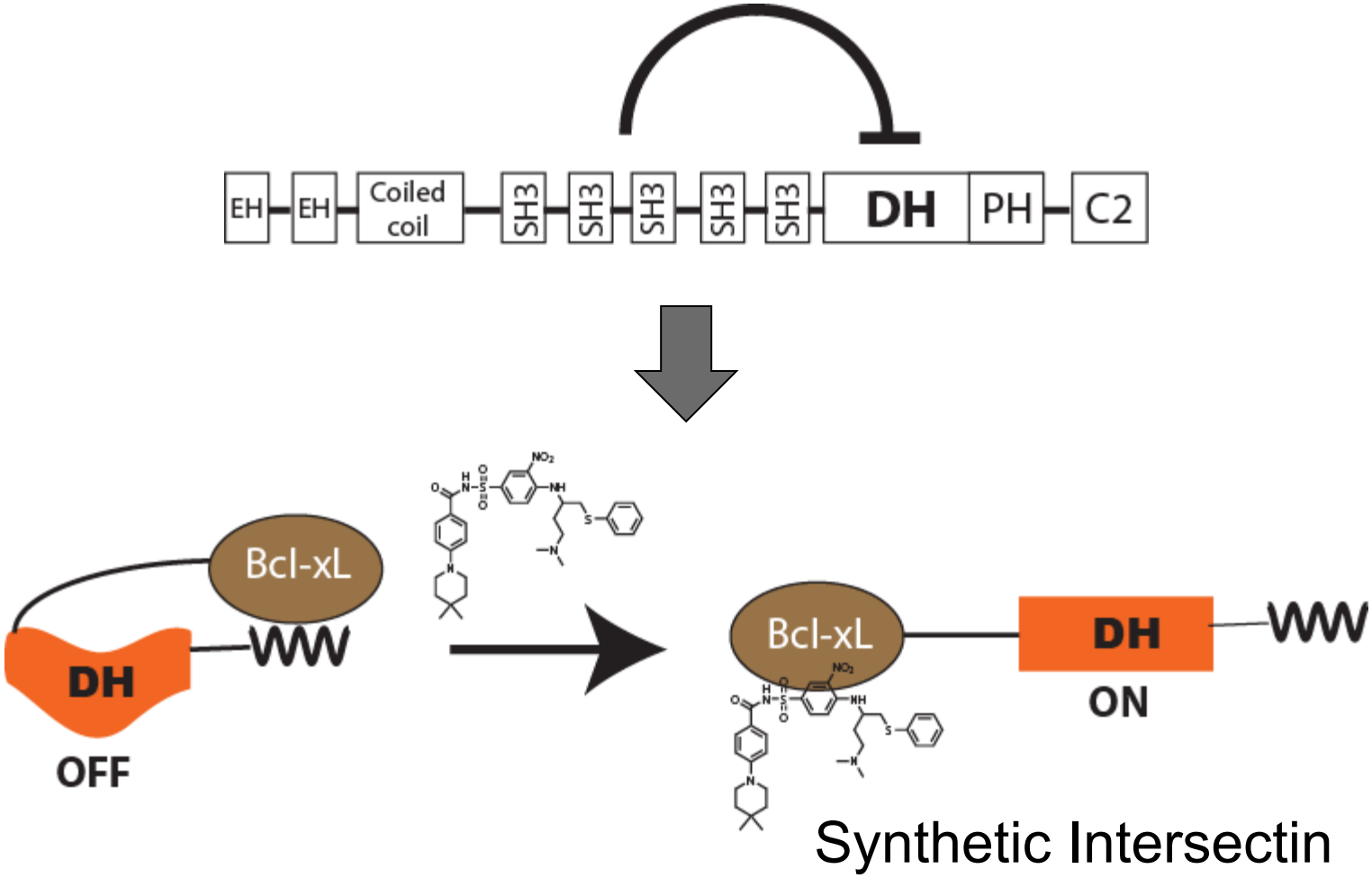
Intersectin – activates Cdc42



Tim – activates RhoA

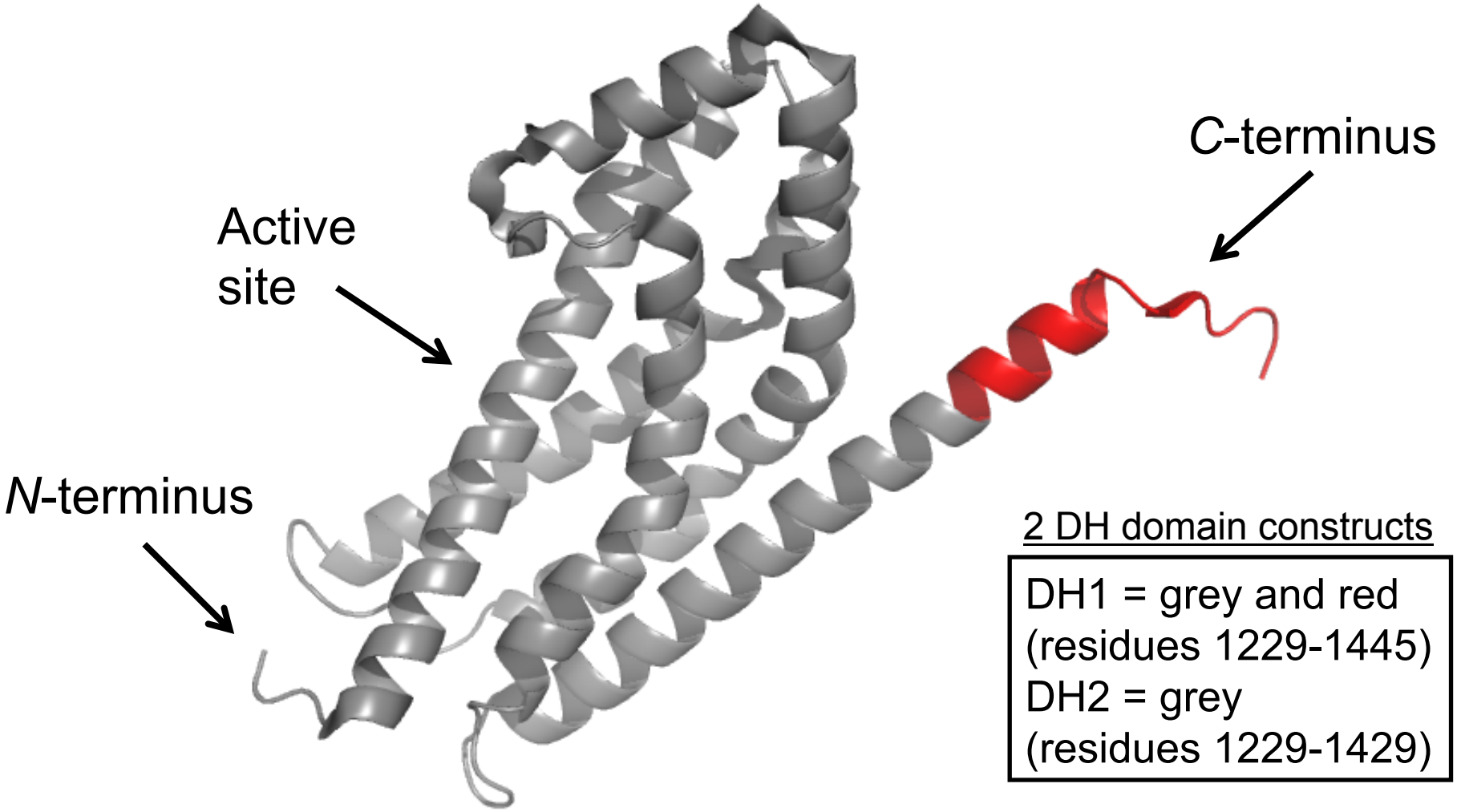
Yeh et al. *Nature* **2007**, *447*, 596-600

Small Molecule-Regulated Intersectin



Synthetic Intersectins can activate Cdc42 (GTPase) in a small molecule-dependent manner

DH Domain of Intersectin



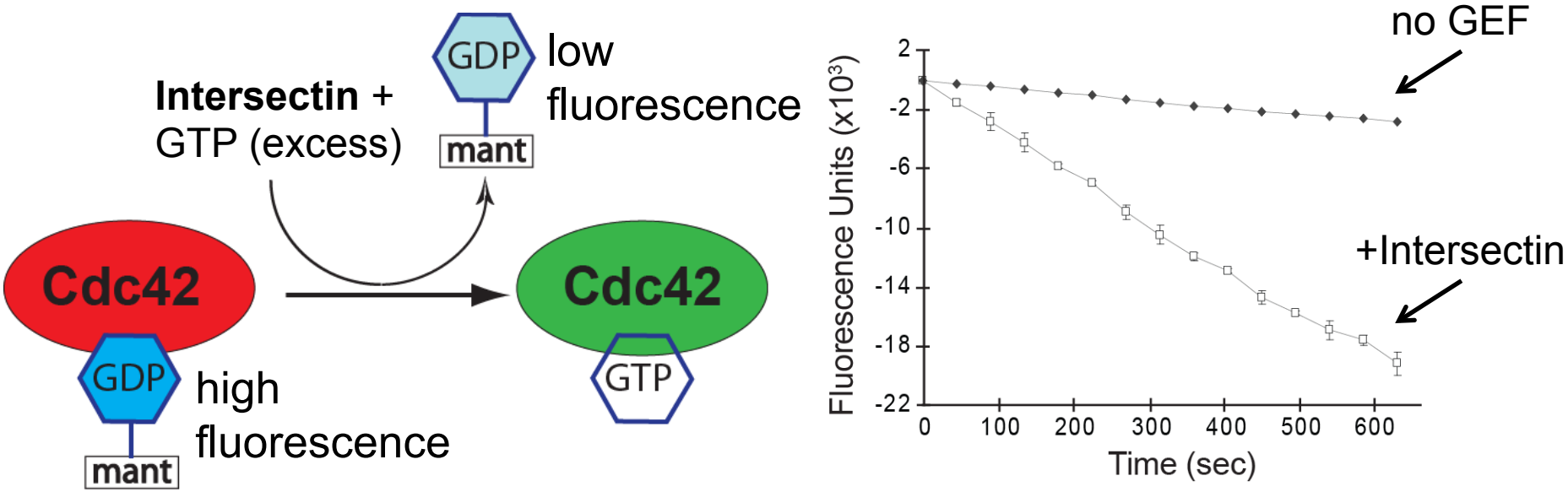
The DH domain of Intersectin catalyzes nucleotide exchange for the GTPase Cdc42

Synthetic Intersectin (sITSN) Constructs

All constructs are monomeric (confirmed by size exclusion chromatography)

Four synthetic Intersectin (sITSN) constructs containing artificial regulatory domains were generated

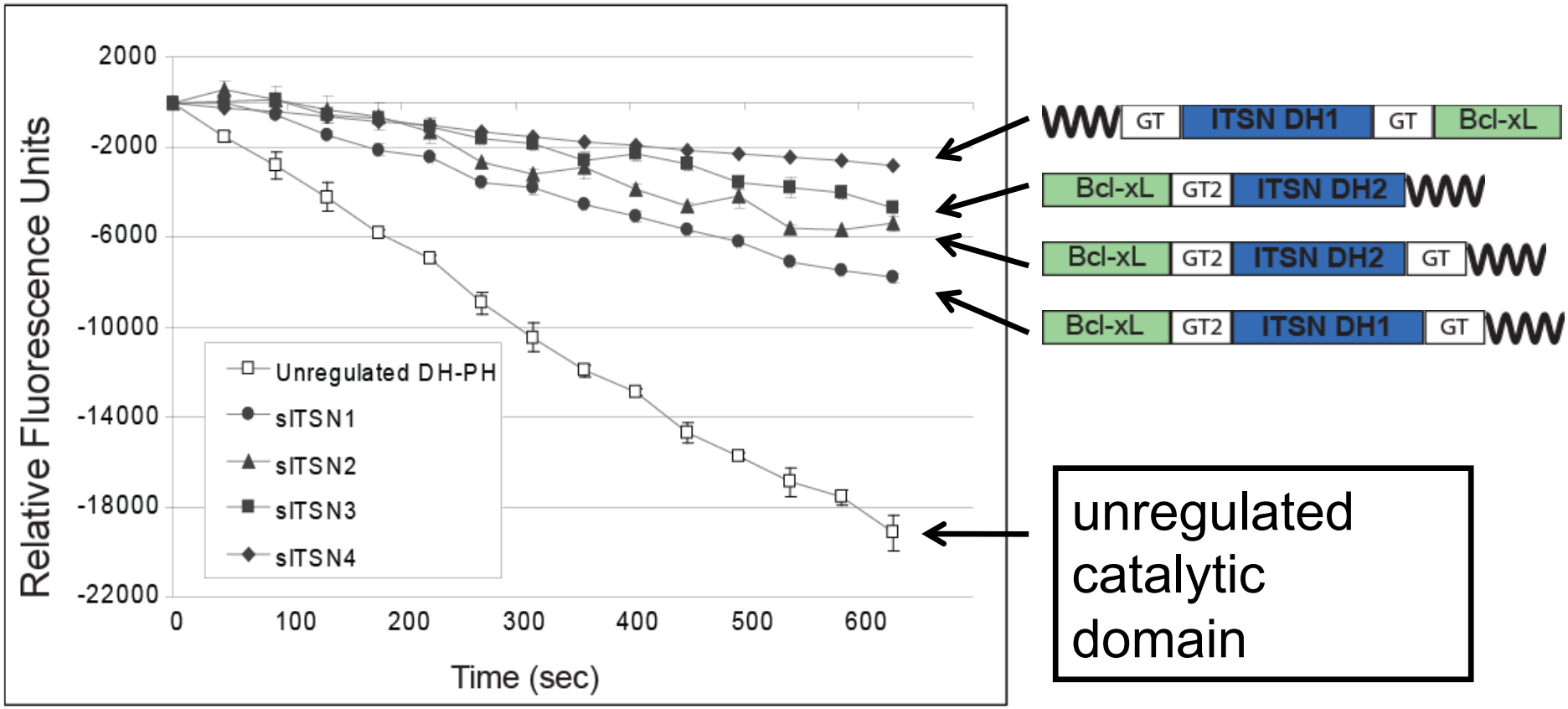
Nucleotide Exchange Assay



A fluorescent nucleotide exchange assay was used to determine the catalytic activity of the sITSN constructs

Nucleotide exchange activity is determined by fluorescence loss (release of mant-GDP from Cdc42)

sITSN Constructs Show Reduced GEF Activity

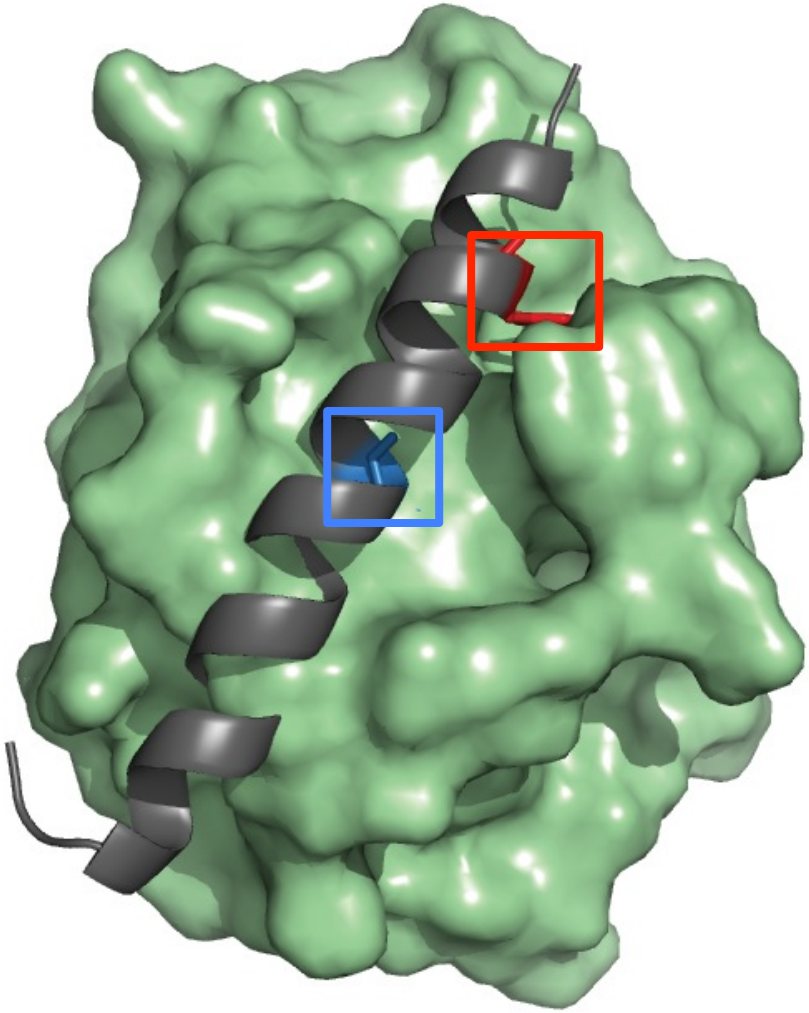


All four sITSN constructs are highly auto-inhibited

Constructs showed little activation in the presence of competitor (5 μM Bad peptide (K_D (Bcl-xL) = 5 nM)

BH3 Peptides with Reduced Affinity for Bcl-xL

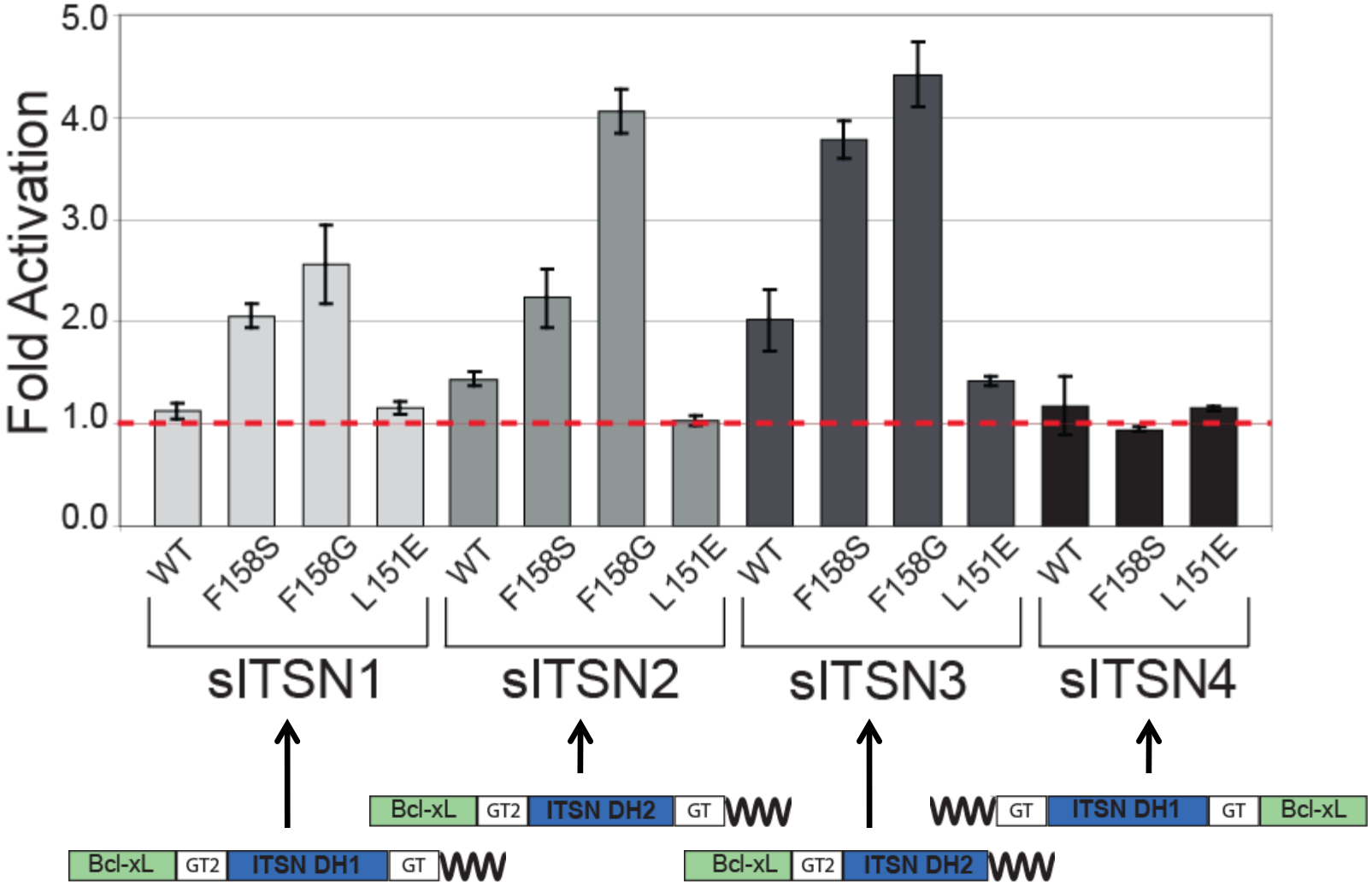
BH3 Peptide: N L W A A Q R Y G R E **L** R R M S D E **F** V D S F K K G



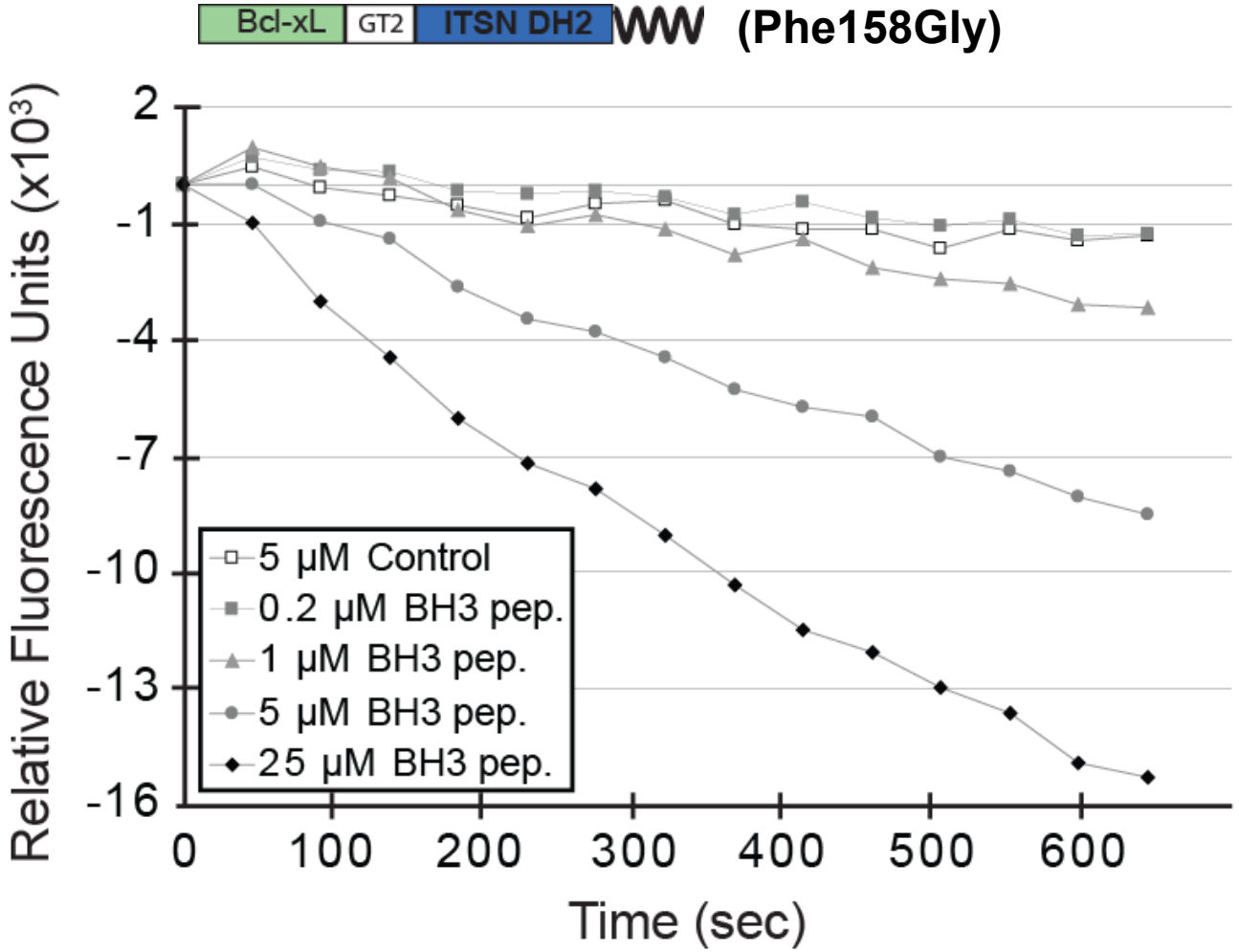
Two hydrophobic residues (Leu151 and Phe158) make a major contribution to the binding affinity of the BH3 peptide

3 mutations:
Leu151Glu – no affinity
Phe158Ser – medium affinity
Phe158Gly – low affinity

Characterization of sITSN Constructs

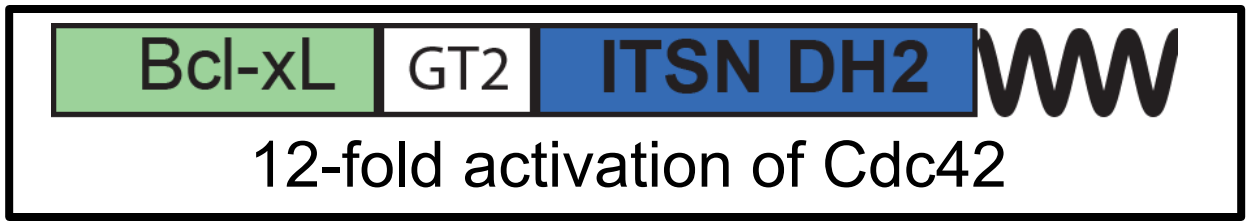


Dose-Dependent Activity

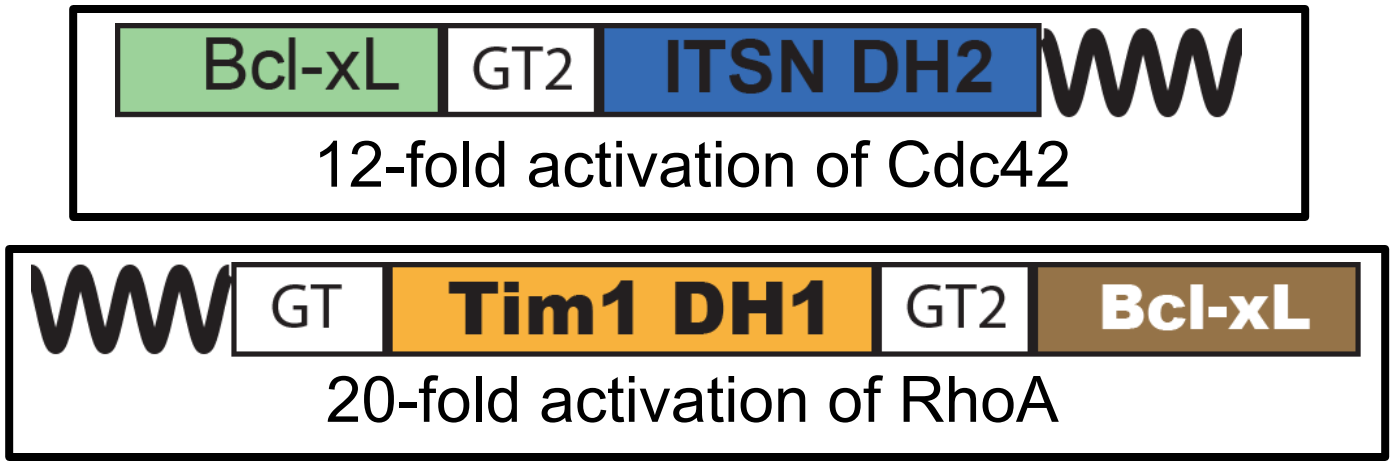


Higher concentrations of the BH3 competitor increase the catalytic activity of the sITSN constructs (12-fold activation)

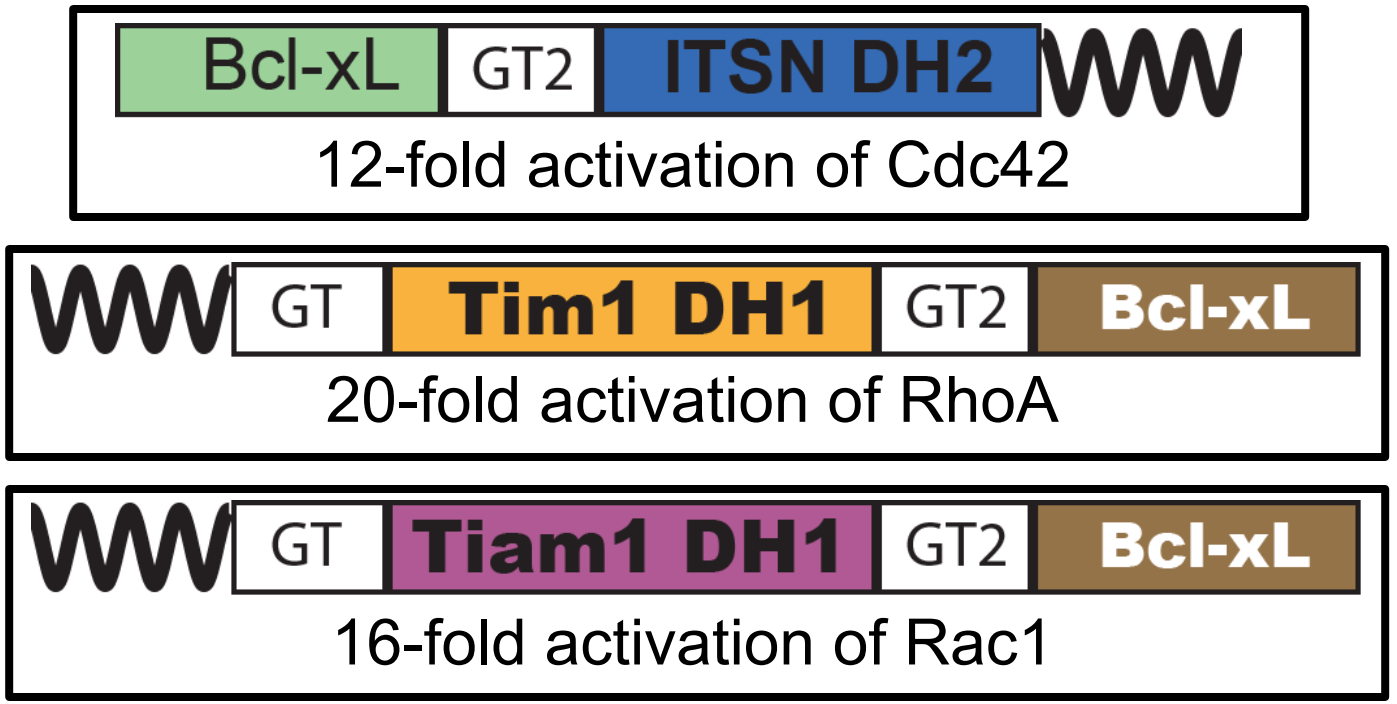
Generality of Strategy



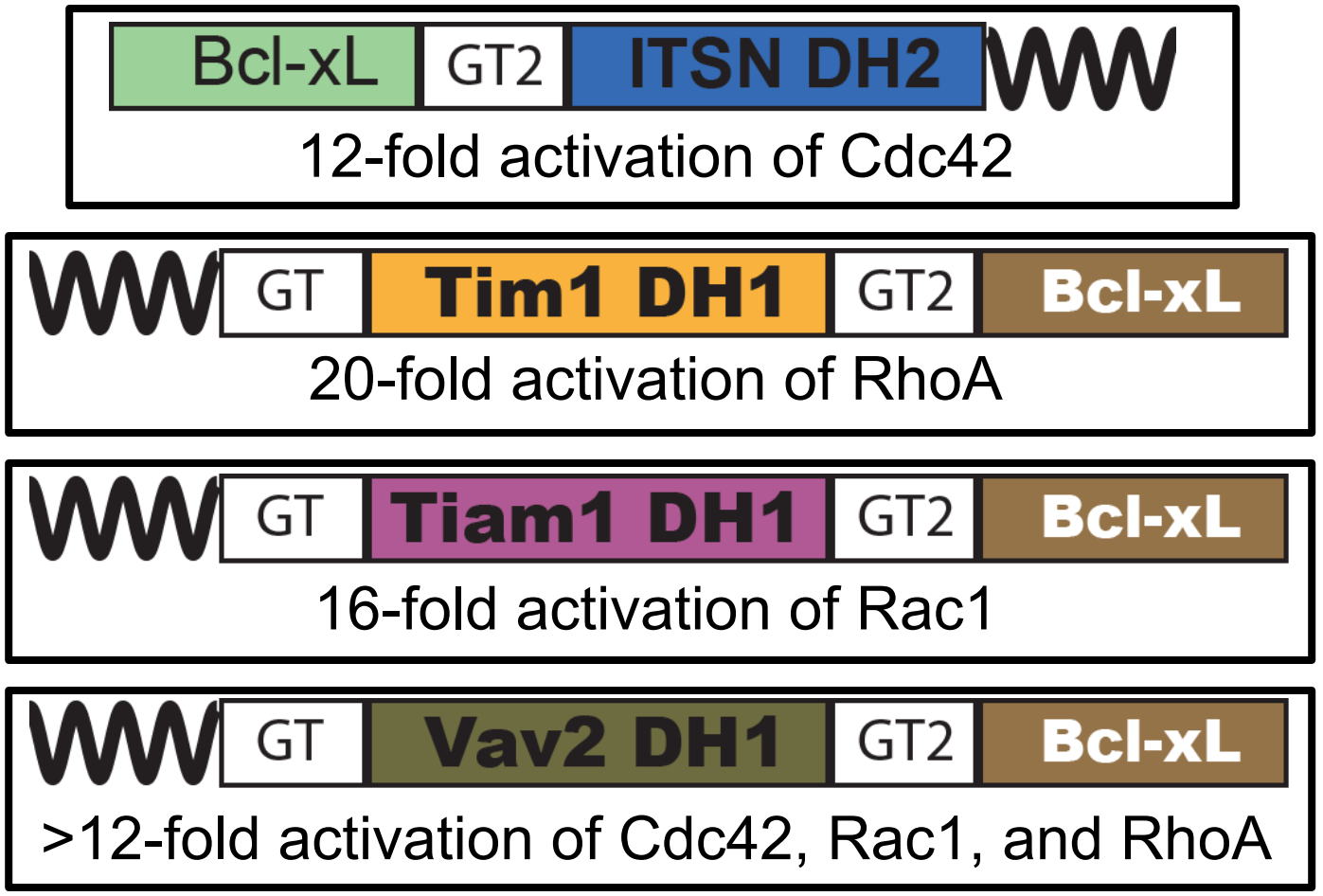
Generality of Strategy



Generality of Strategy

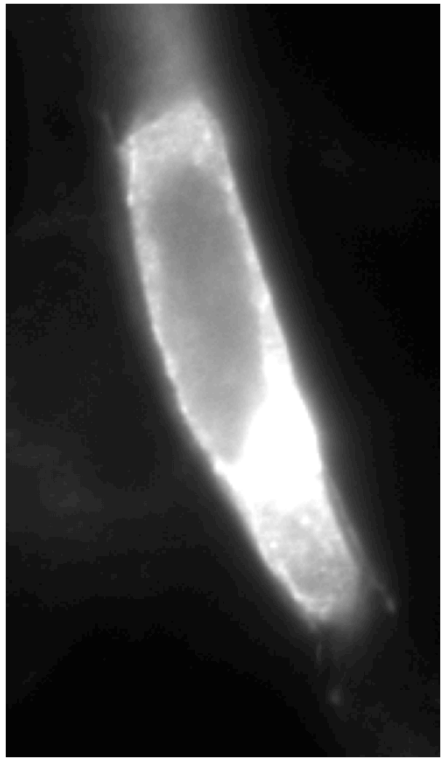


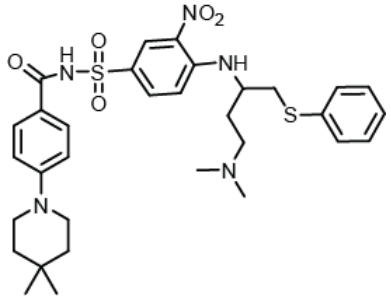
Generality of Strategy



Engineering of GEFs allows small molecule activation of the Rho GTPases Cdc42, RhoA, and Rac1

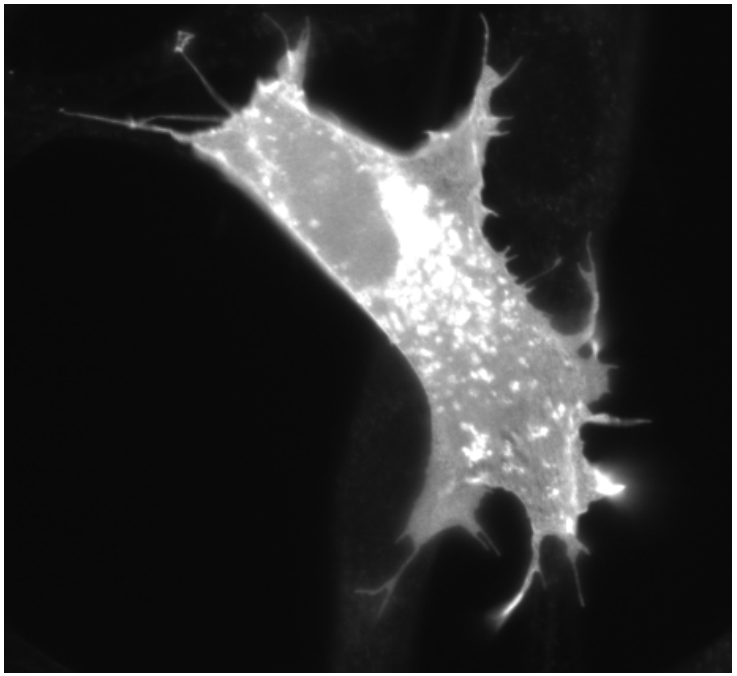
Activation of GTPases (Cdc42) in Cells





A-385358

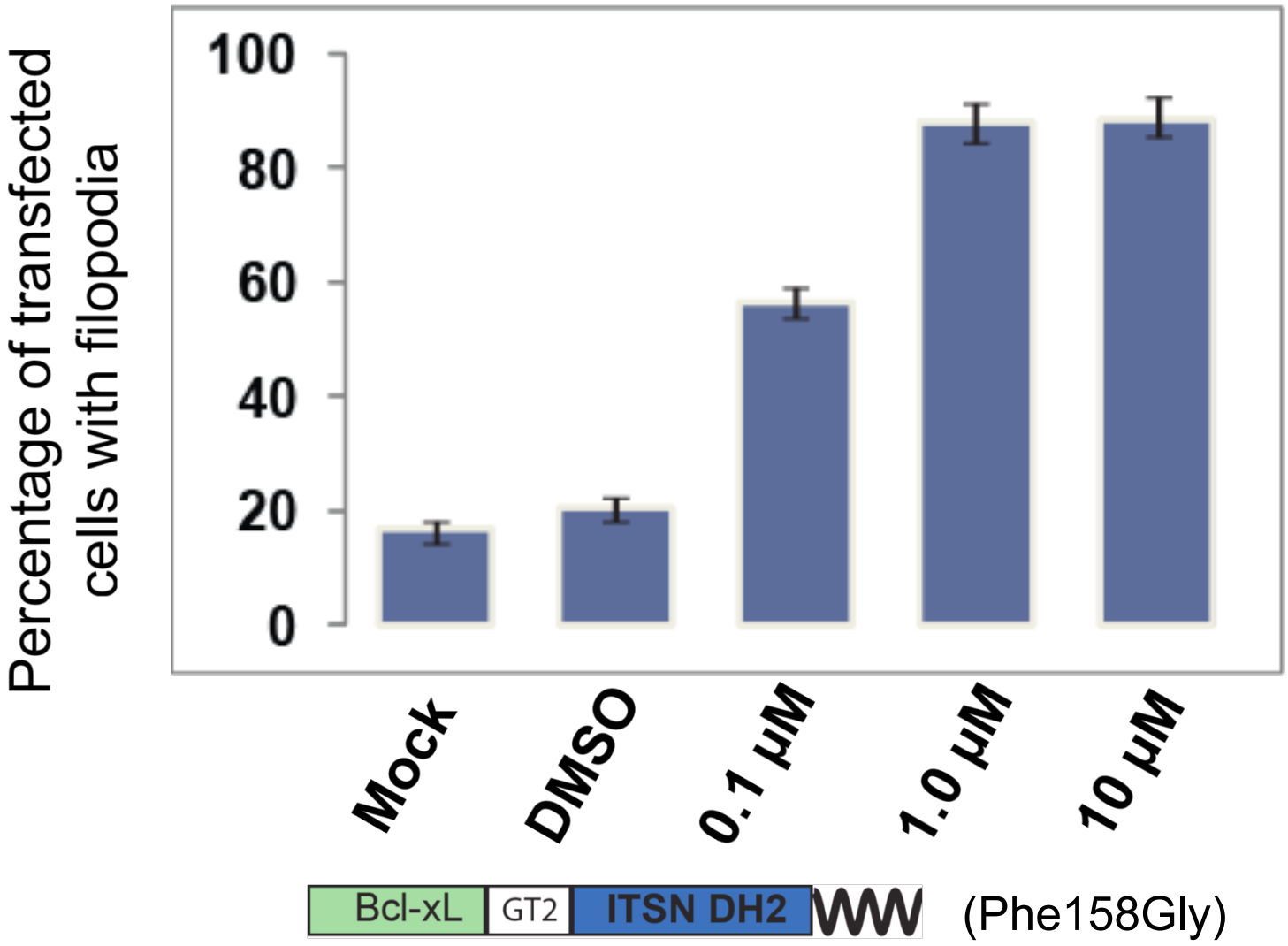
15 minutes



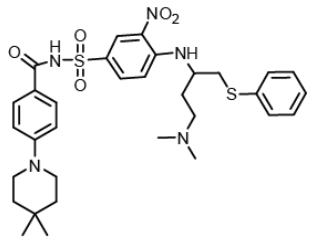
Cells were transfected with sITSN Phe158Gly (or control) and treated with **A-385358** (or DMSO)

NIH 3T3

Dose-Dependent Activation of Cdc42



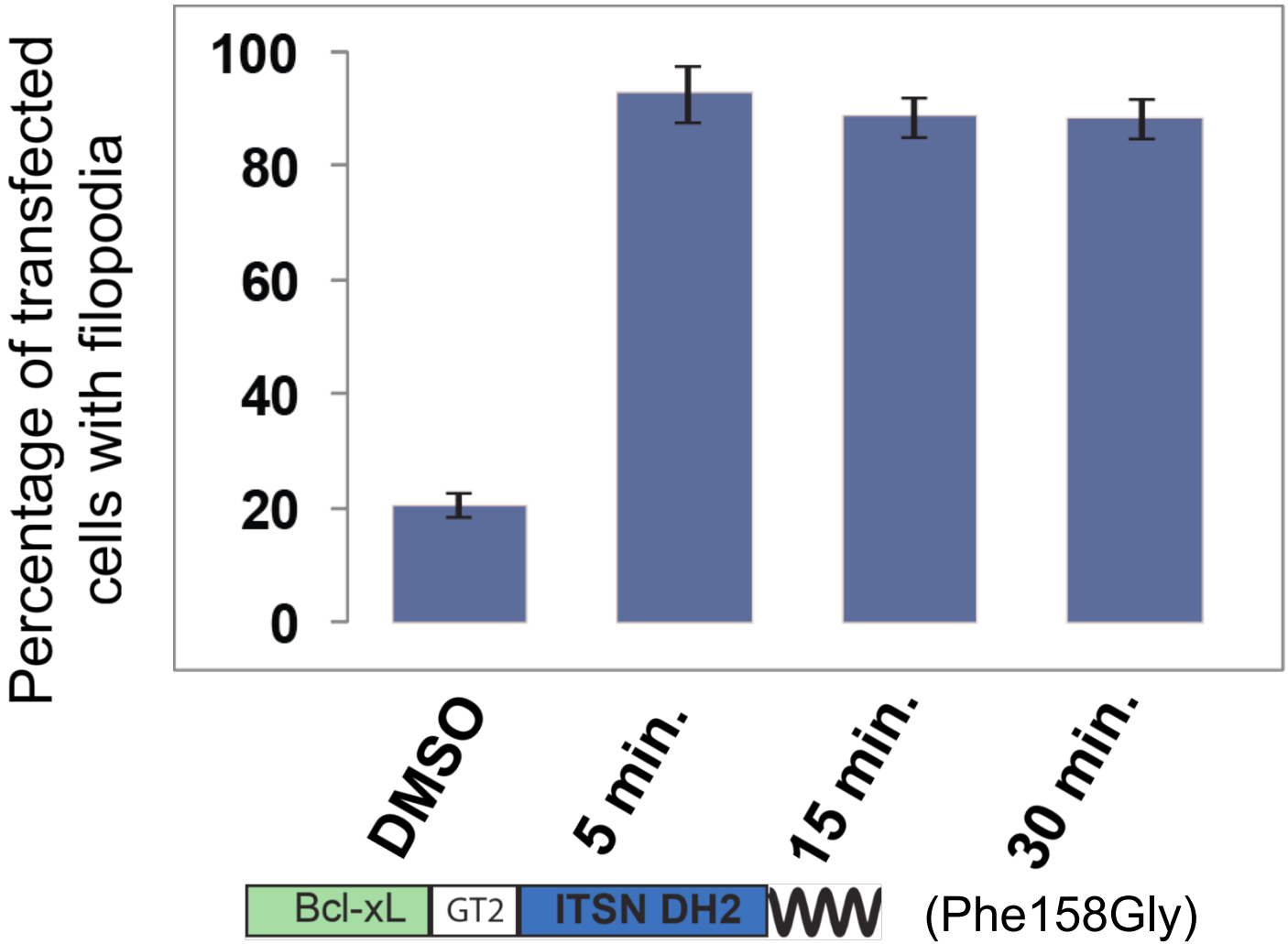
NIH 3T3



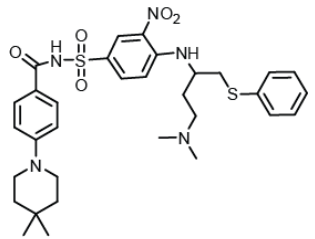
[A-385358]

Cells were treated with A-385358 for 30 minutes

Rapid Activation of Cdc42



NIH 3T3

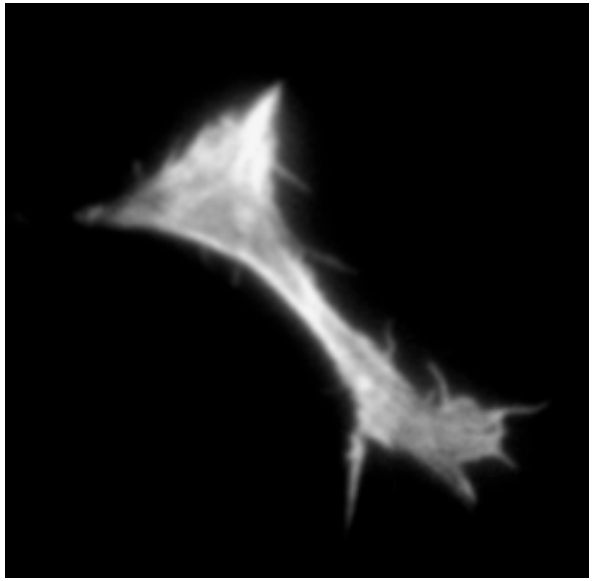


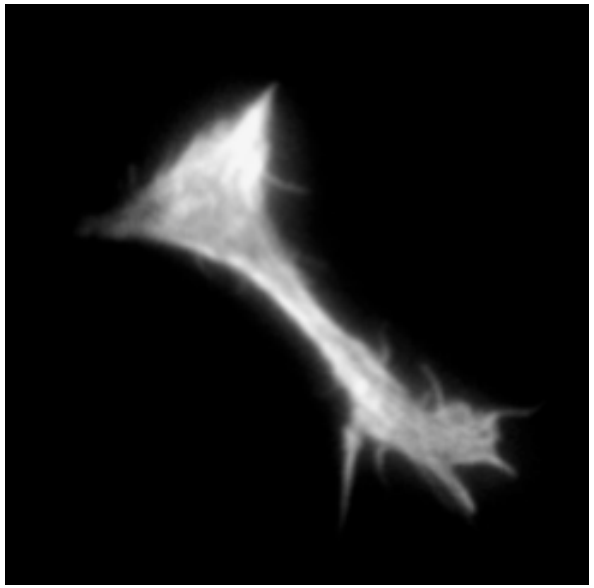
[A-385358] = 10 µM

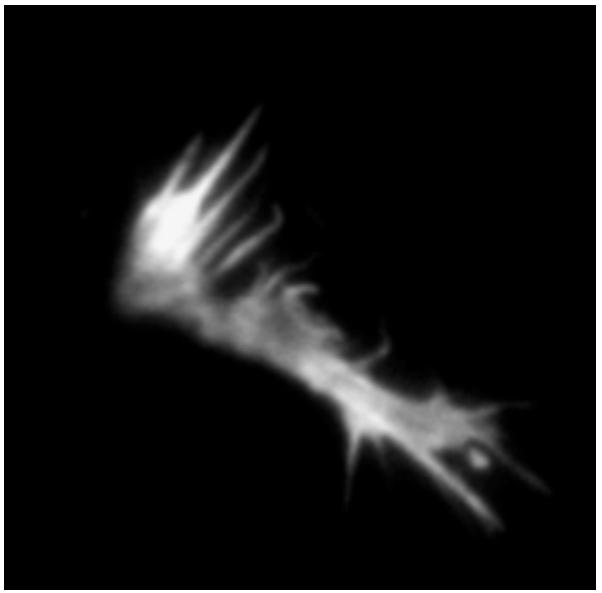
Similar activation kinetics are observed for RhoA and Rac1
Live cell imaging confirms activation kinetics

Live Cell Imaging of Cdc42 Activation

(Phe158Gly) MEFs





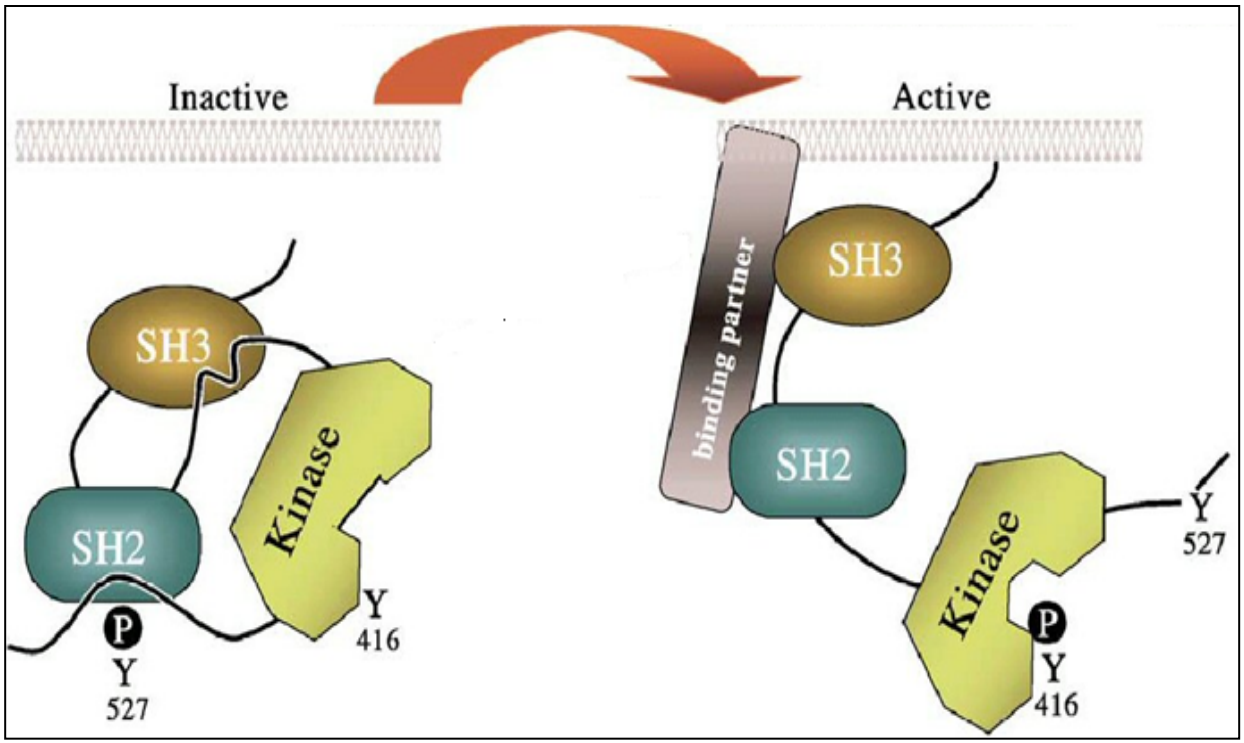


-15 Minutes **0 Minutes** **10 Minutes**

TIRF imaging of MEFs expressing GFP-LifeAct (Actin marker)

Confirms rapid activation kinetics

Location-Specific Activation

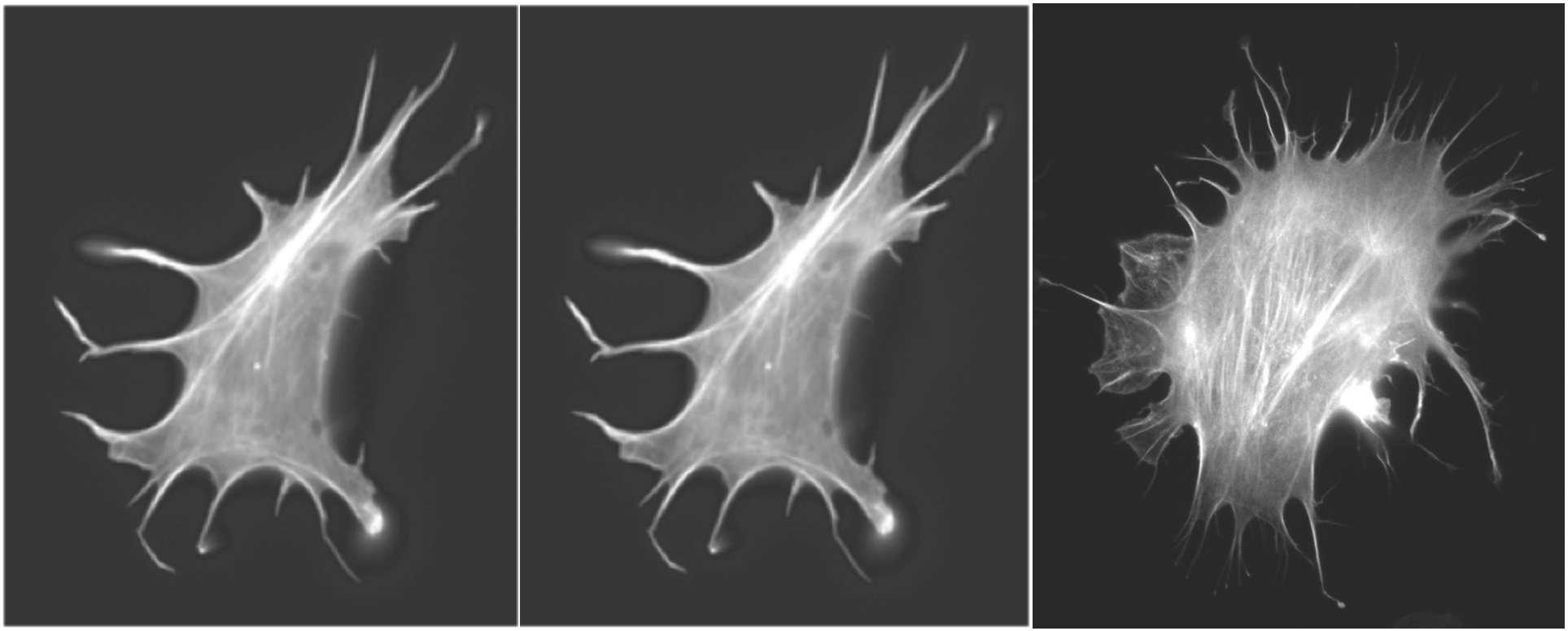


Example: Src-family kinases

Regulatory domains often provide proper localization for cellular function

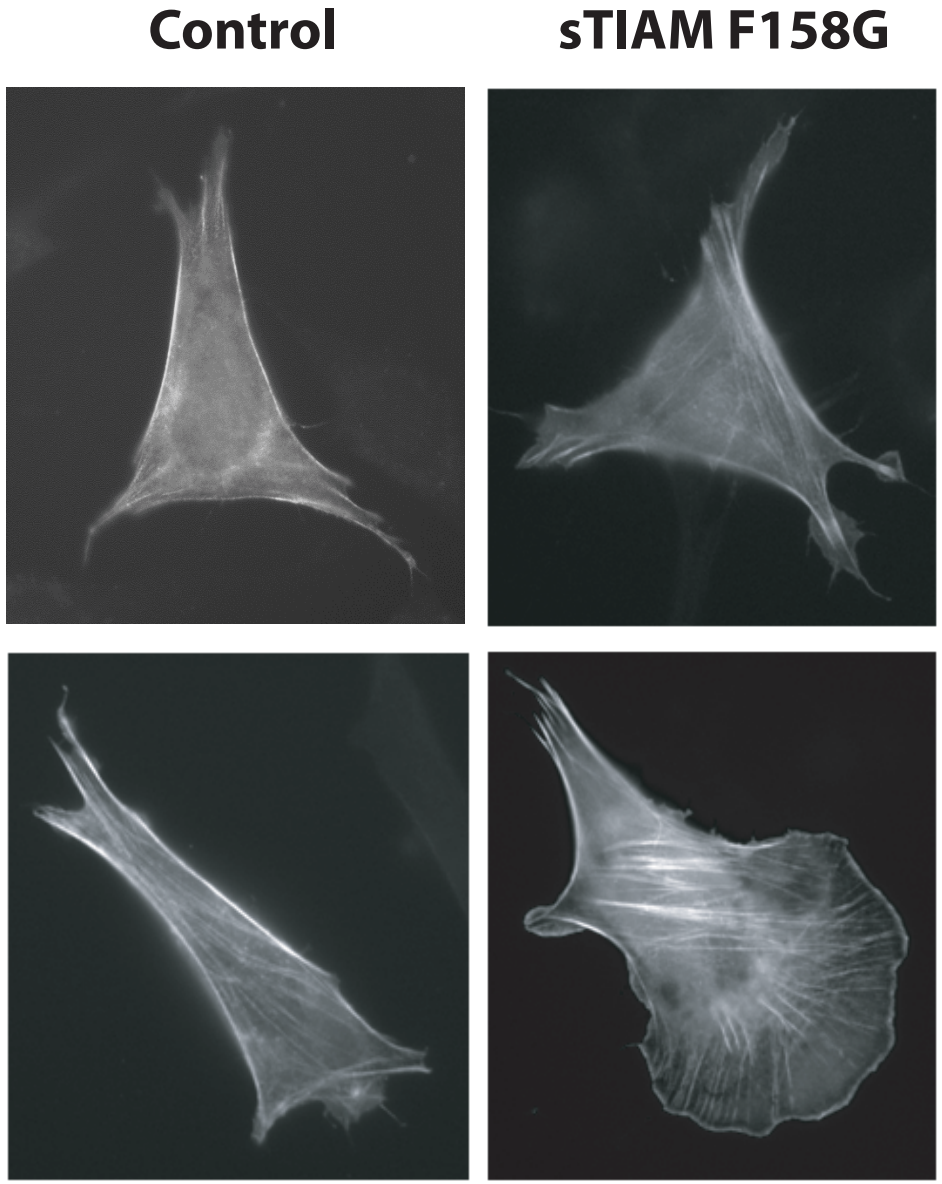
Location-Specific Activation of Cdc42

MEFs



Activating different populations of Cdc42 leads to varying phenotypes

Activation of Other GTPases



Additional GEFs allow selective and rapid activation of other GTPases (Rac and Rho) in cells

The ssTIAM construc activates Rac1 (lamellipodia)

Summary

The interaction between Bcl-xL and a BH3 peptide can be used as artificial regulatory domains for GEFs

Small molecule disruptors of the Bcl-xL/BH3 peptide interaction can be used to activate synthetic GEFs

Tuning the affinity between Bcl-xL and the BH3 peptide is essential for generating synthetic GEFs with a dynamic range of activities

Synthetic GEFs can be used to activate Cdc42, RhoA, and Rac1 (GTPases) in cells

Cellular activation is rapid and dose dependent