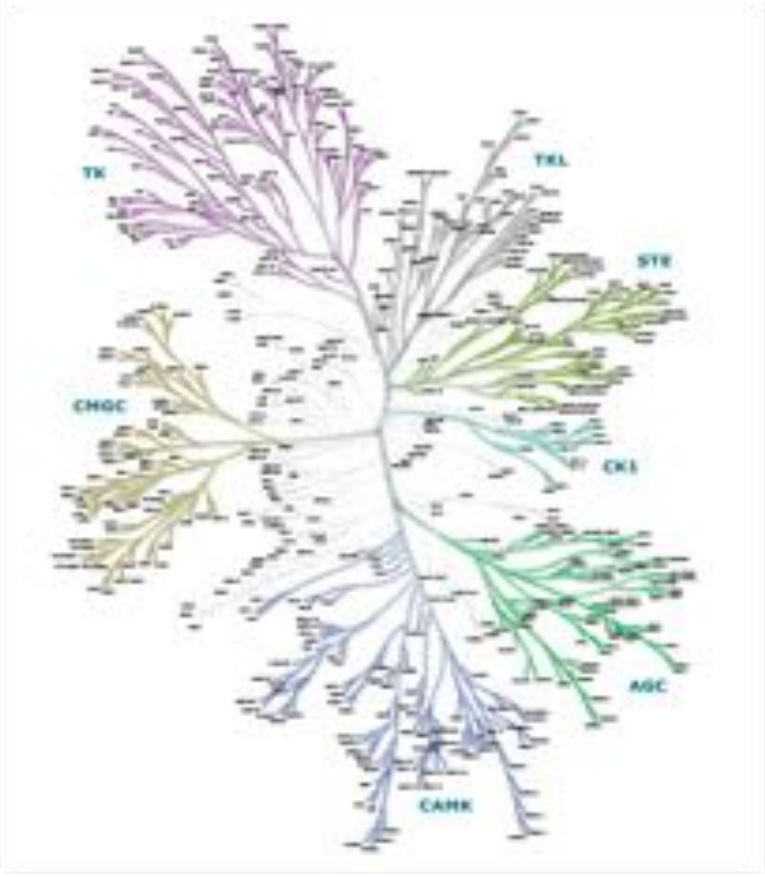
The Career of Kevan Shokat



Michael Pirnot
MacMillan Group Meeting
02.20.2013

Biography

- Reed College, B.A., 1986, Chemistry

- UC Berkeley, Ph.D., 1991, Organic Chemistry

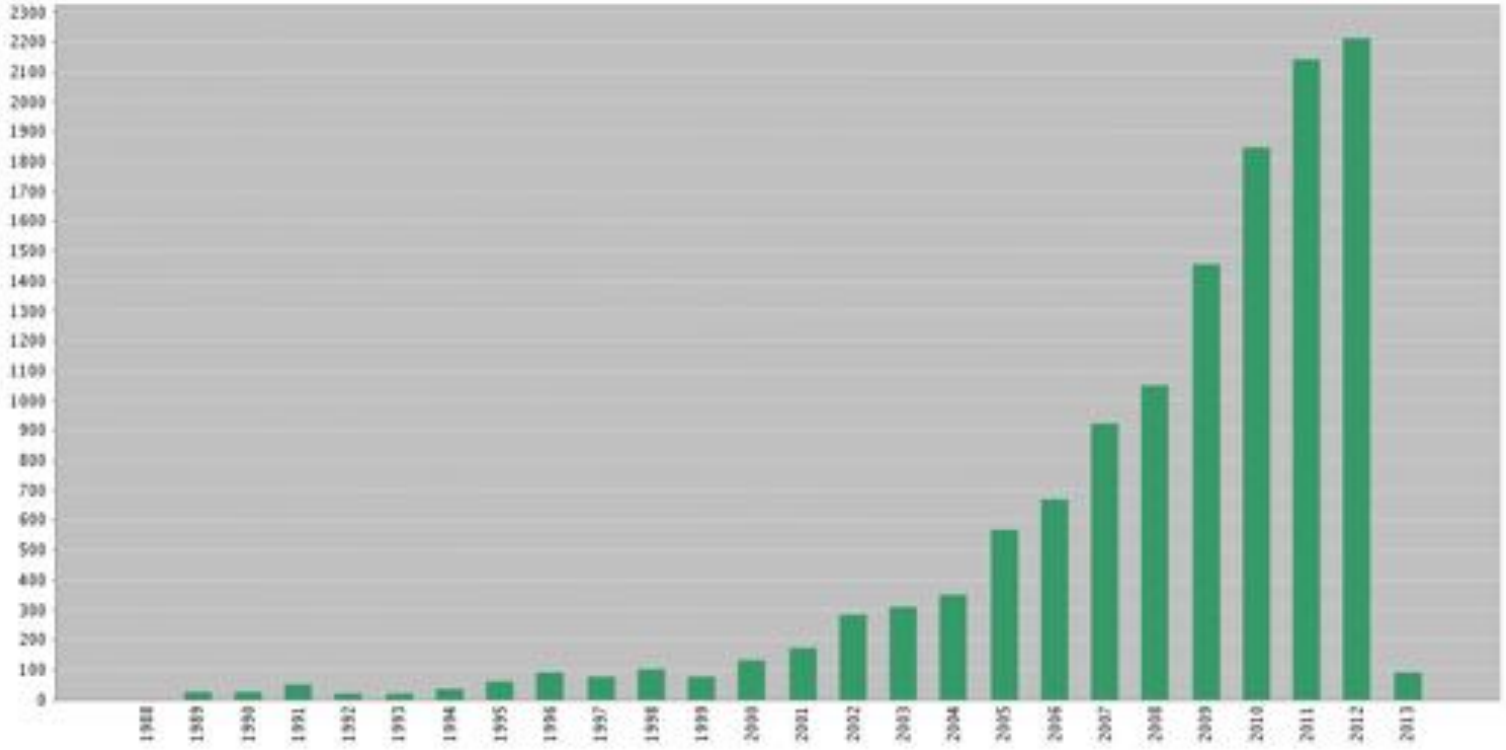
  Advisor: Prof. Peter G. Schultz
  Design and synthesis of haptens for the generation of catalytic antibodies.

- Stanford University, Post-doc, 1992-1994

  Advisor: Prof. Christopher C. Goodnow.
  Investigation of mechanisms of immune self-tolerance in transgenic mice.

- Assistant Professor of Chemistry and Molecular Biology, Princeton University, 1994-1998.
- Associate Professor of Chemistry and Molecular Biology, Princeton University, 1998-1999.
- Joint-professorship, Associate Professor, UCSF and UCB, 1999-2002.
- Promoted to Professor, UCSF and UCB, 2002-present.
- Vice-Chairman of Cellular and Molecular Pharmacology, UCSF, 2004-present.
- Investigator, Howard Hughes Medical Institute, 2004-present.

Citations



citations per year

- Papers published - **284**
- h-index - **65**
- Cited **>12,000** times
- Average paper is cited **>45** times

ISI Web of Knowledge

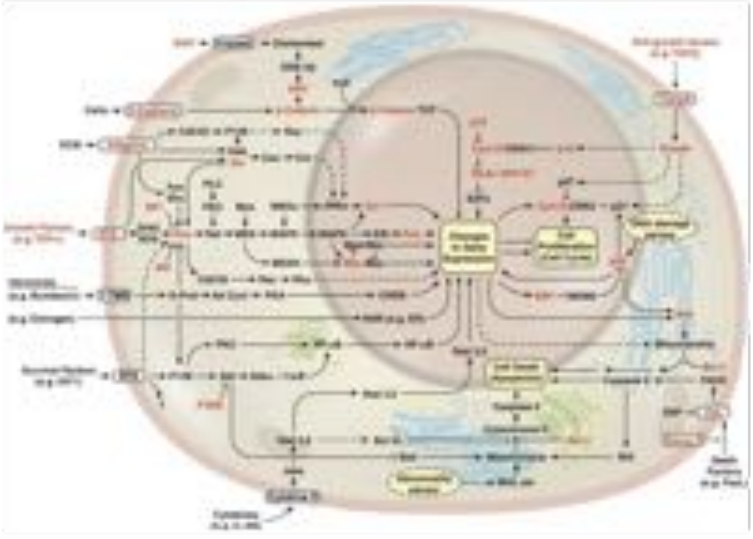
Research Areas

"Chemical Approaches to Deciphering and Controlling Signal Transduction Pathways"

- Developing chemical tools for studying cellular **kinase** function and mapping signal networks using **chemical genetics**.

- **Phosphoproteomics** - identifying and characterizing proteins that contain a phosphate as a post-translational modification

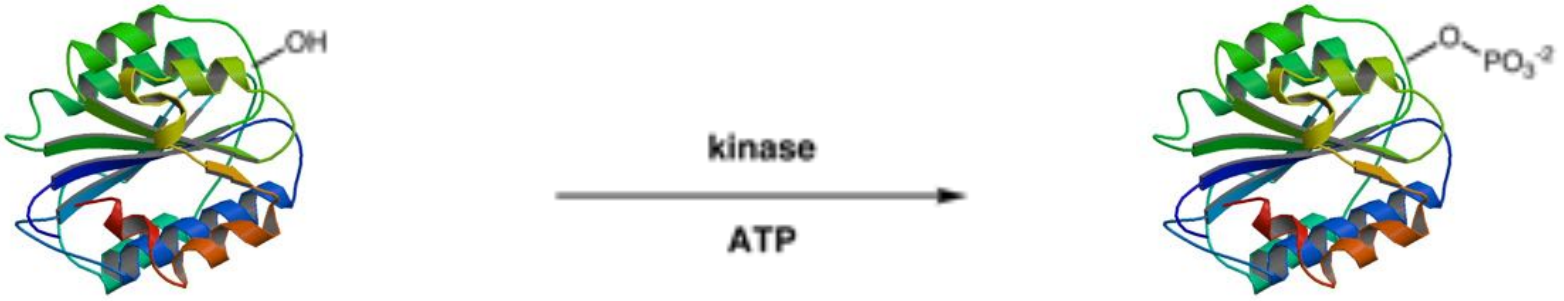
Signal transduction - a basic process in molecular cell biology where a signal from outside the cell is converted to a functional change within the cell



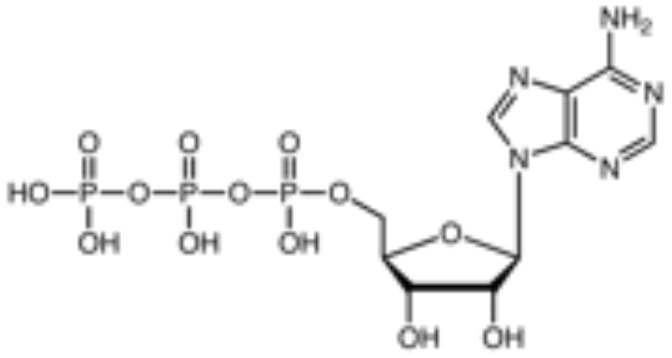
Cell communication that is often involved in cell proliferation, apoptosis, or changes in gene expression.

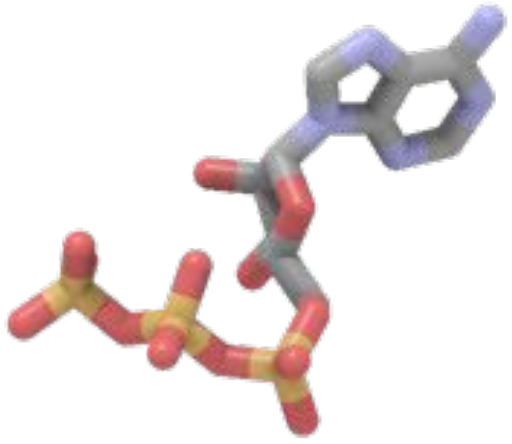
Kinases

Kinase - an enzyme that modifies other proteins by chemically appending a phosphate group



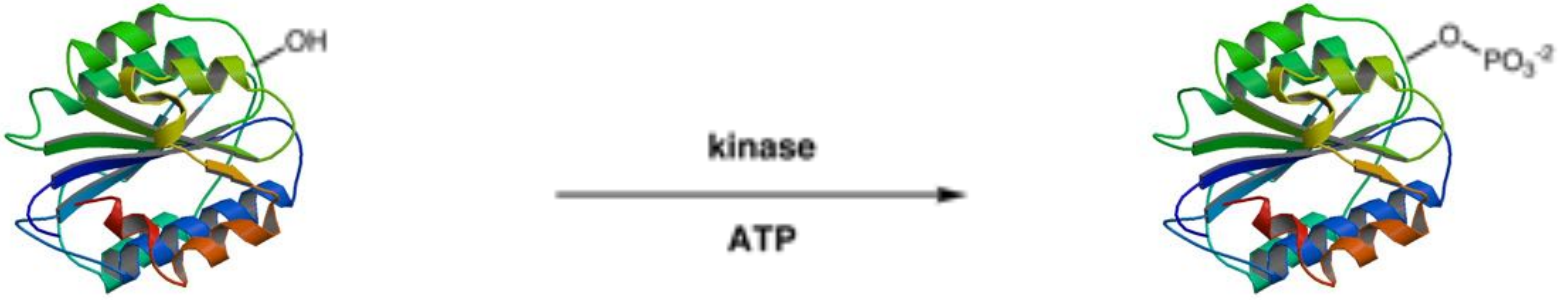
■ ATP - adenosine triphosphate - a coenzyme often called "the molecular unit of currency"





Kinases

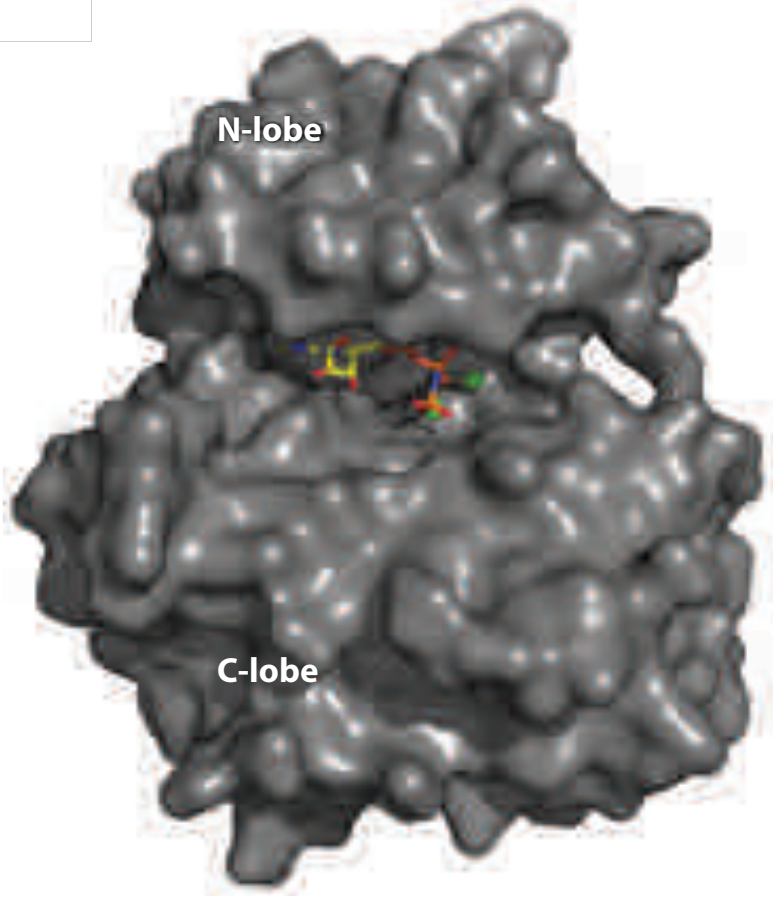
Kinase - an enzyme that modifies other proteins by chemically appending a phosphate group



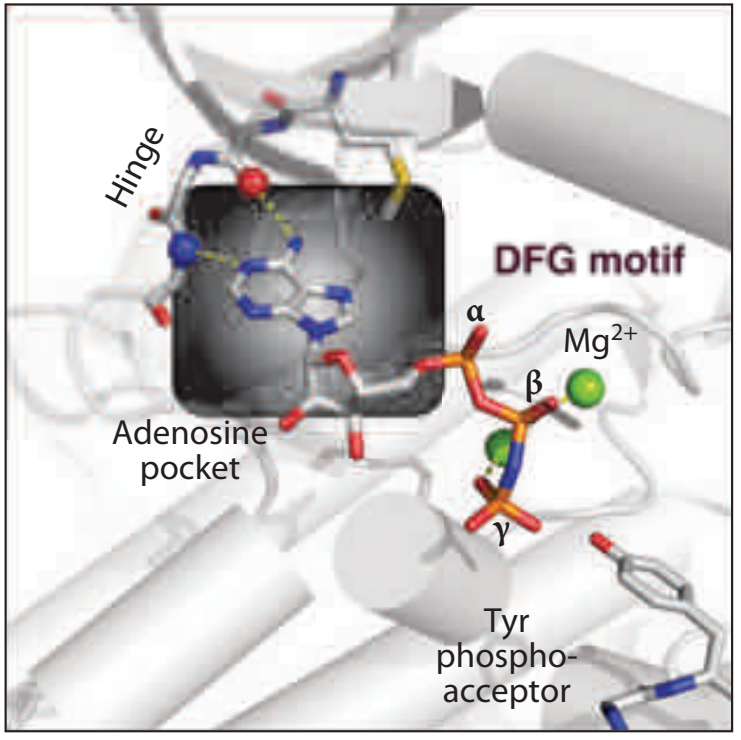
- Tyrosine, threonine, serine, and histidine residues can all be phosphorylated by a kinase.

Kinases

- insulin receptor kinase in complex with ATP and a small peptide substrate



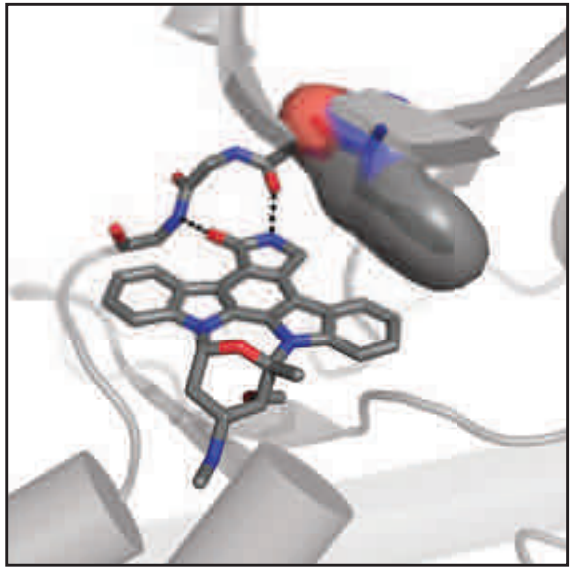
Protein kinase active site

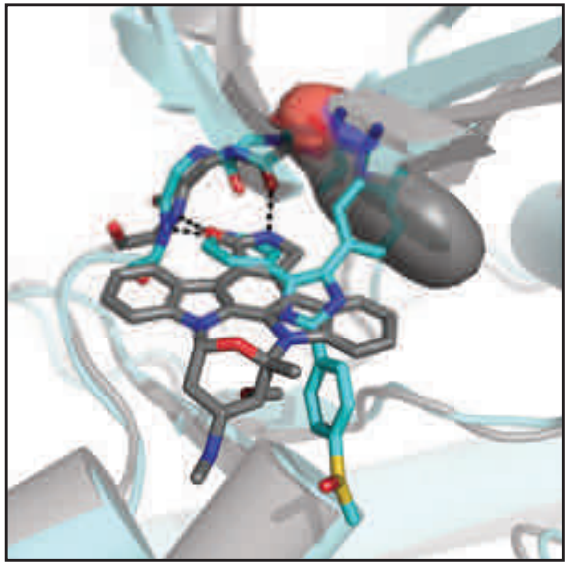


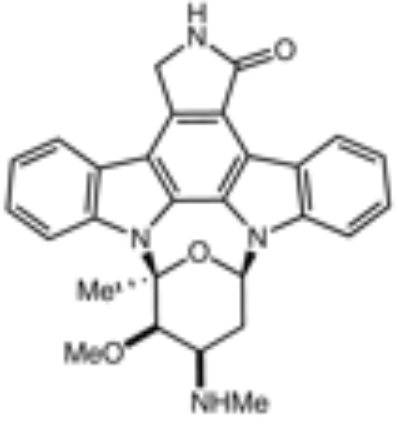
Dar, A. C.; Shokat, K. M. *Annu. Rev. Biochem.* **2011**, *80*, 769.

Gatekeeper Residue

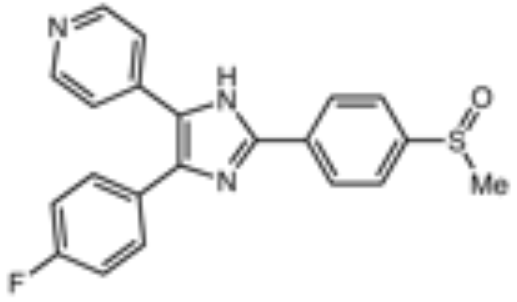
- staurosporine and SB203580, a p38 inhibitor, binding to CDK2







Staurosporine

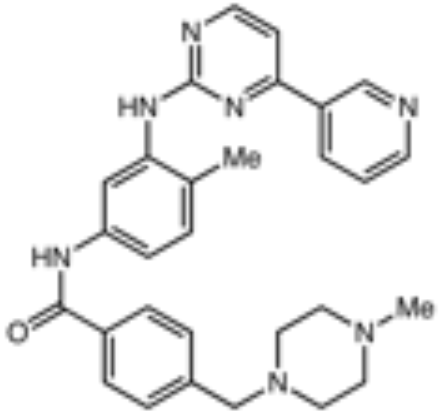


SB203580

Dar, A. C.; Shokat, K. M. *Annu. Rev. Biochem.* **2011**, *80*, 769.

Importance of Kinases

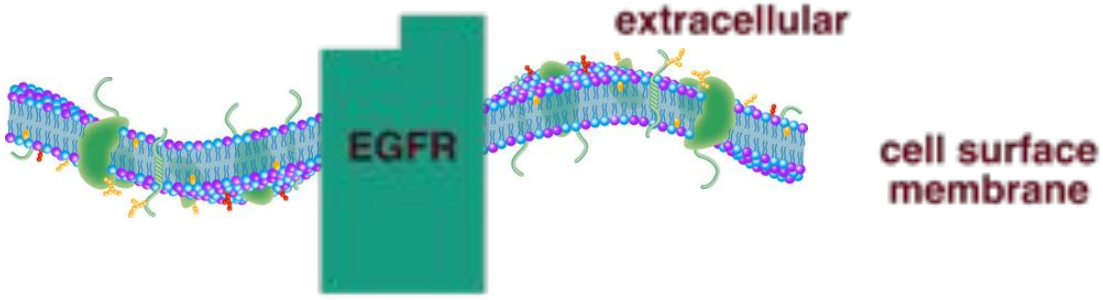
- There are roughly 500 protein kinase genes.
- Protein kinase genes constitute about 2% of the human genome.
- Up to 30% of all human proteins may be modified by kinase activity.
- Kinases are known to regulate the majority of cellular pathways.
- Protein kinases are often dysregulated in cancer and inflamatory diseases.
- Kinase inhibitors currently comprise up to 30% of drug-discovery programs in the pharmaceutical industry.
- Over 50 such compounds are now in clinical trials for a variety of diseases.



Gleevec is a competitive tyrosine-kinase inhibitor used in the treatment of multiple cancers, in particular chronic myelogenous leukemia (CML).

Imatinib, #35 selling drug in 2011

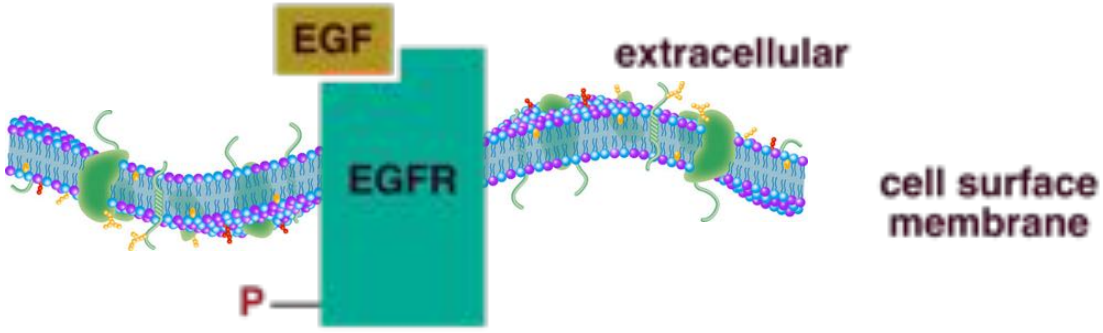
Signal Transduction Pathways are Quite Complicated



- MAPK/ERK pathway is a signal transduction pathway where a receptor outside the cell is able to communicate to DNA inside the nucleus.
- Proteins involved in this pathway are often mutated and is a necessary step in many cancers.
- Many drug therapy strategies focus on regulating this pathway.

Dhillon, A. S.; Hagan, S.; Rath, O.; Kolch, W. *Oncogene* **2007**, *22*, 3279.

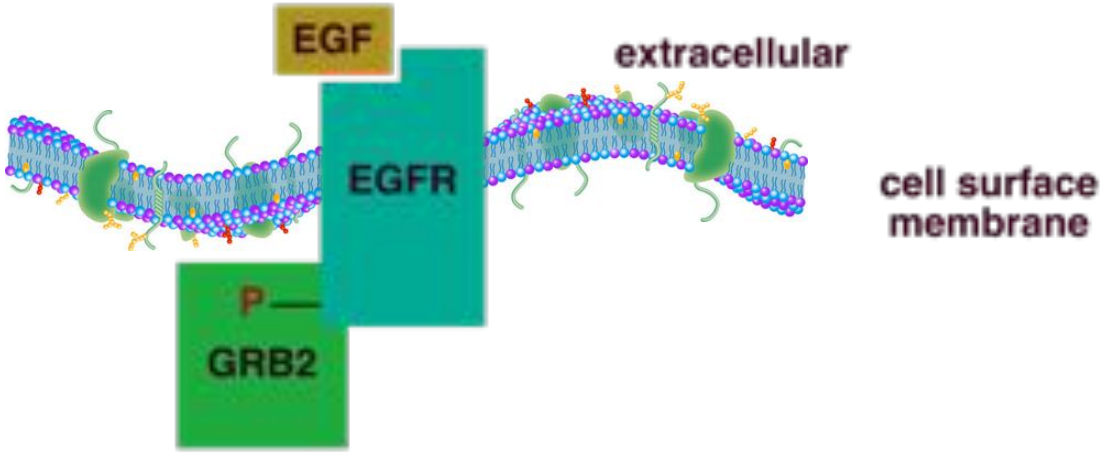
Signal Transduction Pathways are Quite Complicated



■ Binding of epidermal growth factor (EGF) to epidermal growth factor receptor (EGFR, receptor-linked tyrosine kinase), causing EGFR to become phosphorylated.

Dhillon, A. S.; Hagan, S.; Rath, O.; Kolch, W. *Oncogene* **2007**, *22*, 3279.

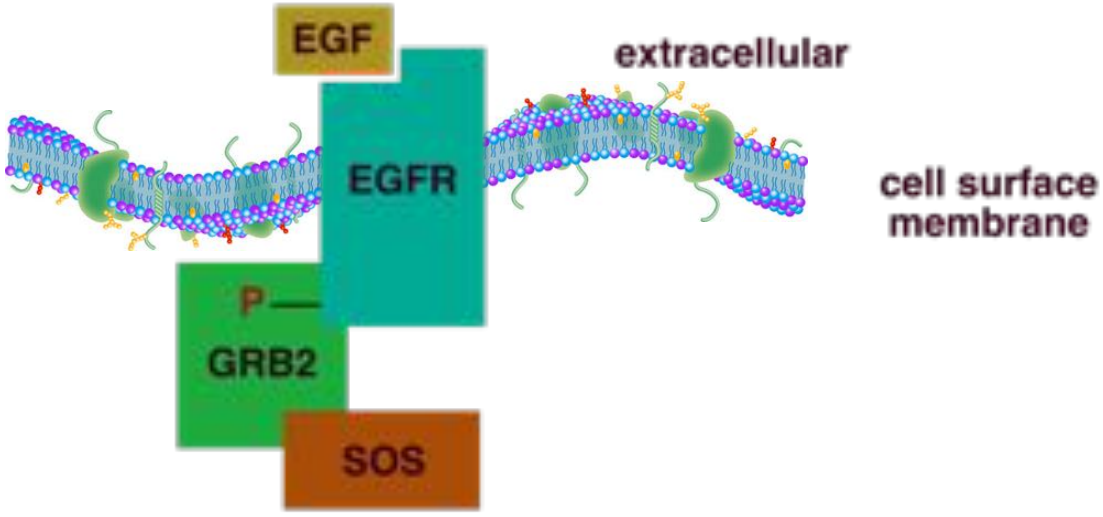
Signal Transduction Pathways are Quite Complicated



■ Once EGFR is phosphorylated, docking protein GRB2's SH2 domain can bind to the phosphotyrosine residue.

Dhillon, A. S.; Hagan, S.; Rath, O.; Kolch, W. *Oncogene* **2007**, *22*, 3279.

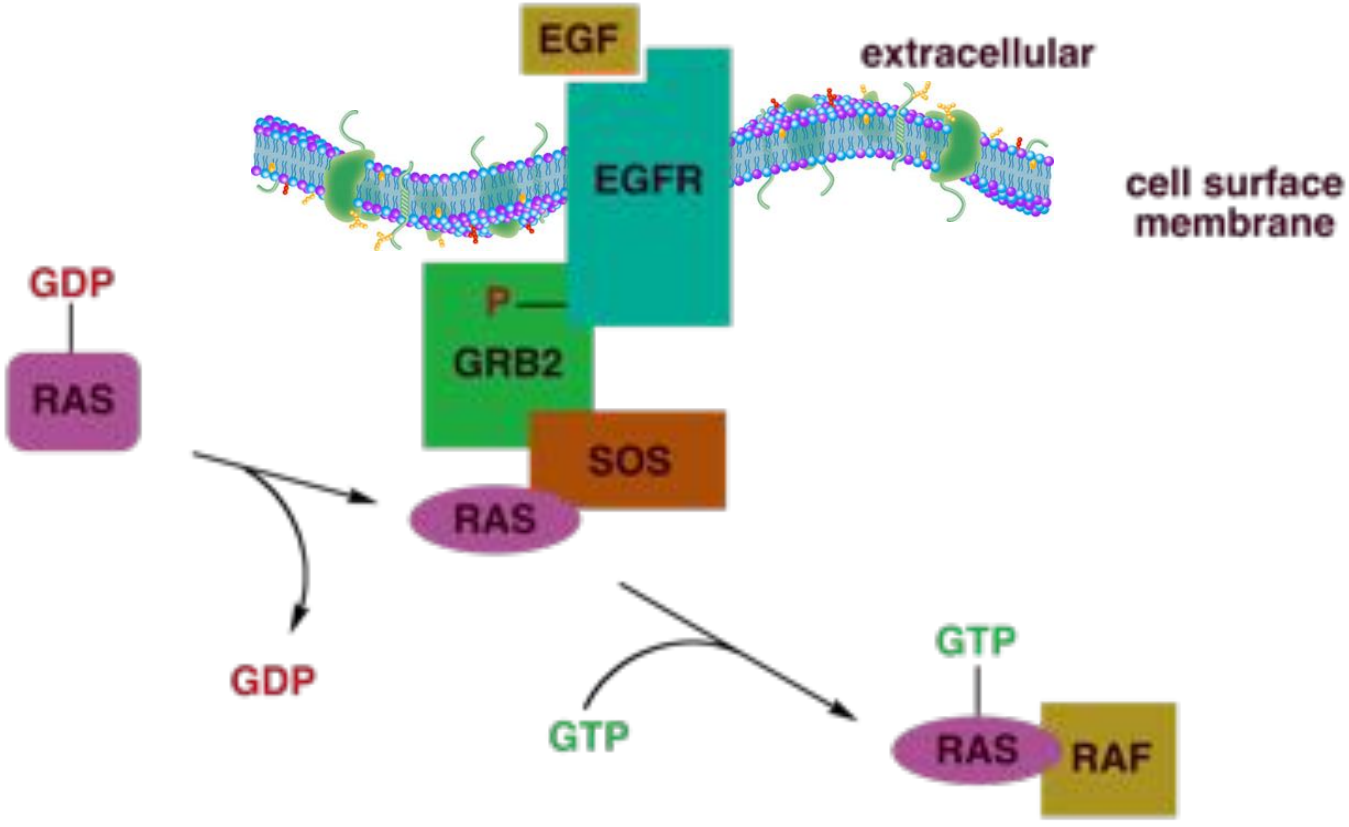
Signal Transduction Pathways are Quite Complicated



■ Growth factor receptor-bound protein 2, or GRB2, then binds to SOS, a guanine nucleotide exchange factor.

Dhillon, A. S.; Hagan, S.; Rath, O.; Kolch, W. *Oncogene* **2007**, *22*, 3279.

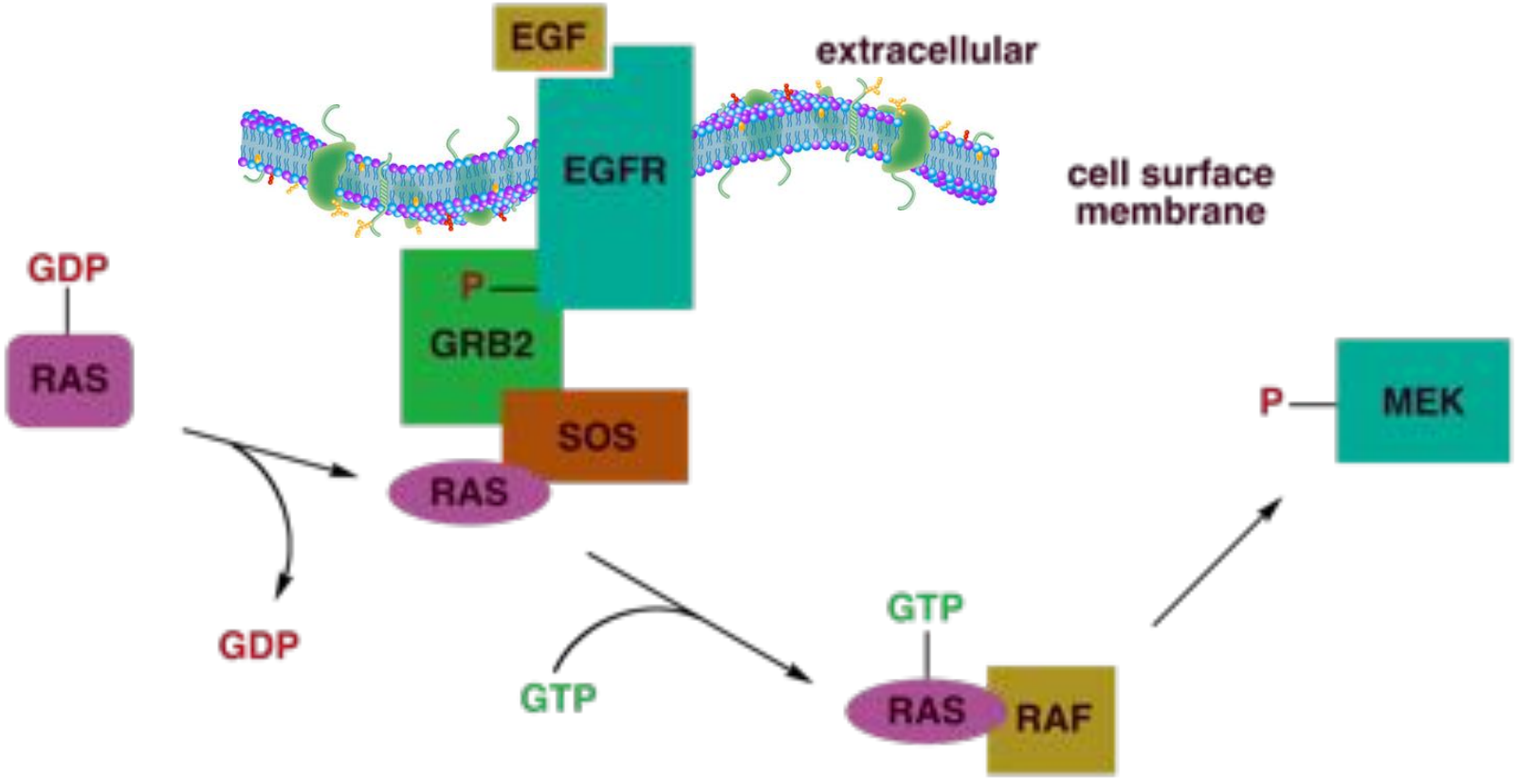
Signal Transduction Pathways are Quite Complicated



- Activated SOS promotes removal of GDP from a member of the Ras subfamily.
- When Ras binds to GTP, it is activated, causing it to bind to RAF.

Dhillon, A. S.; Hagan, S.; Rath, O.; Kolch, W. *Oncogene* **2007**, *22*, 3279.

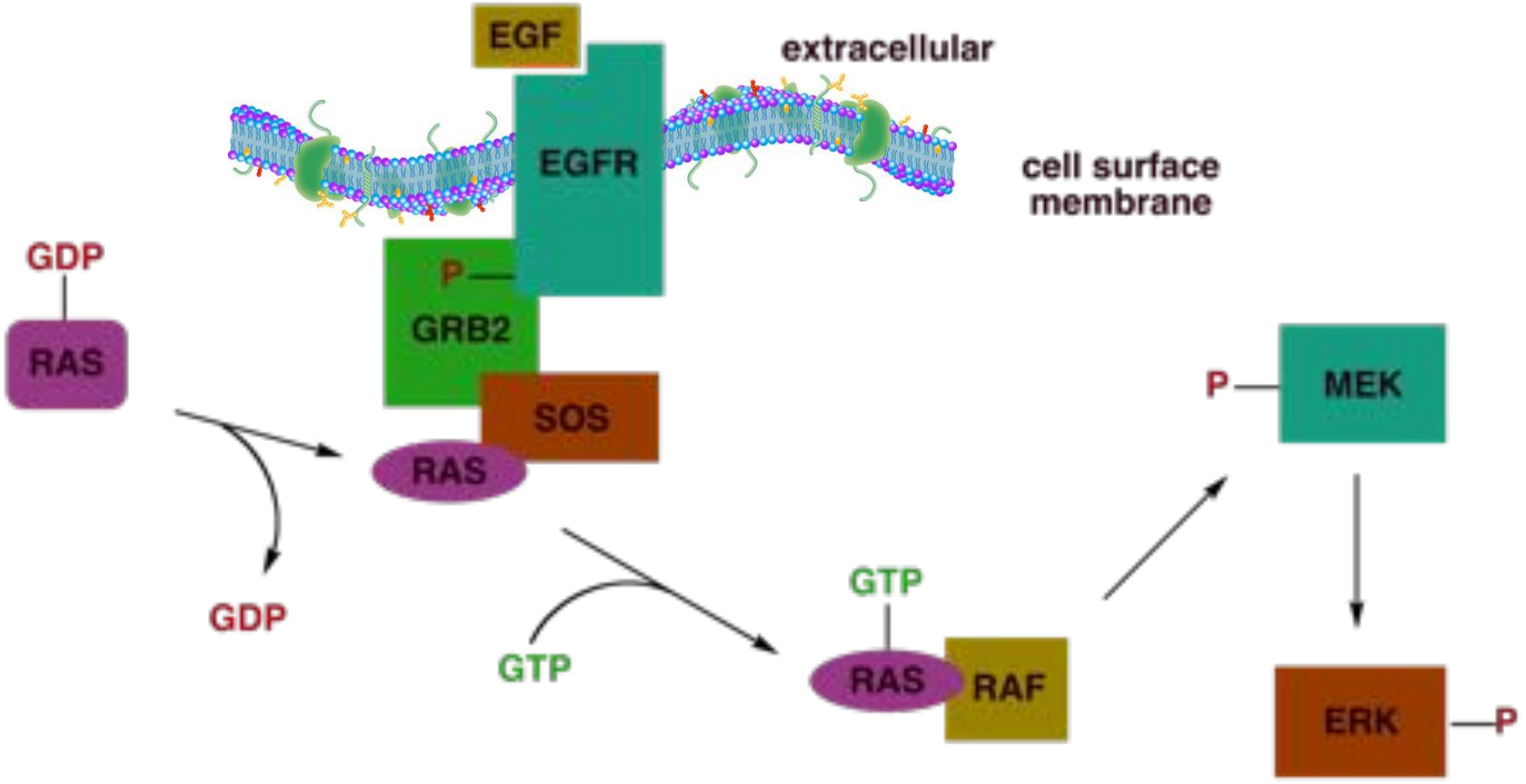
Signal Transduction Pathways are Quite Complicated



■ Ras activates RAF, a serine/threonine-specific kinase, to phosphorylate MEK.

Dhillon, A. S.; Hagan, S.; Rath, O.; Kolch, W. *Oncogene* **2007**, *22*, 3279.

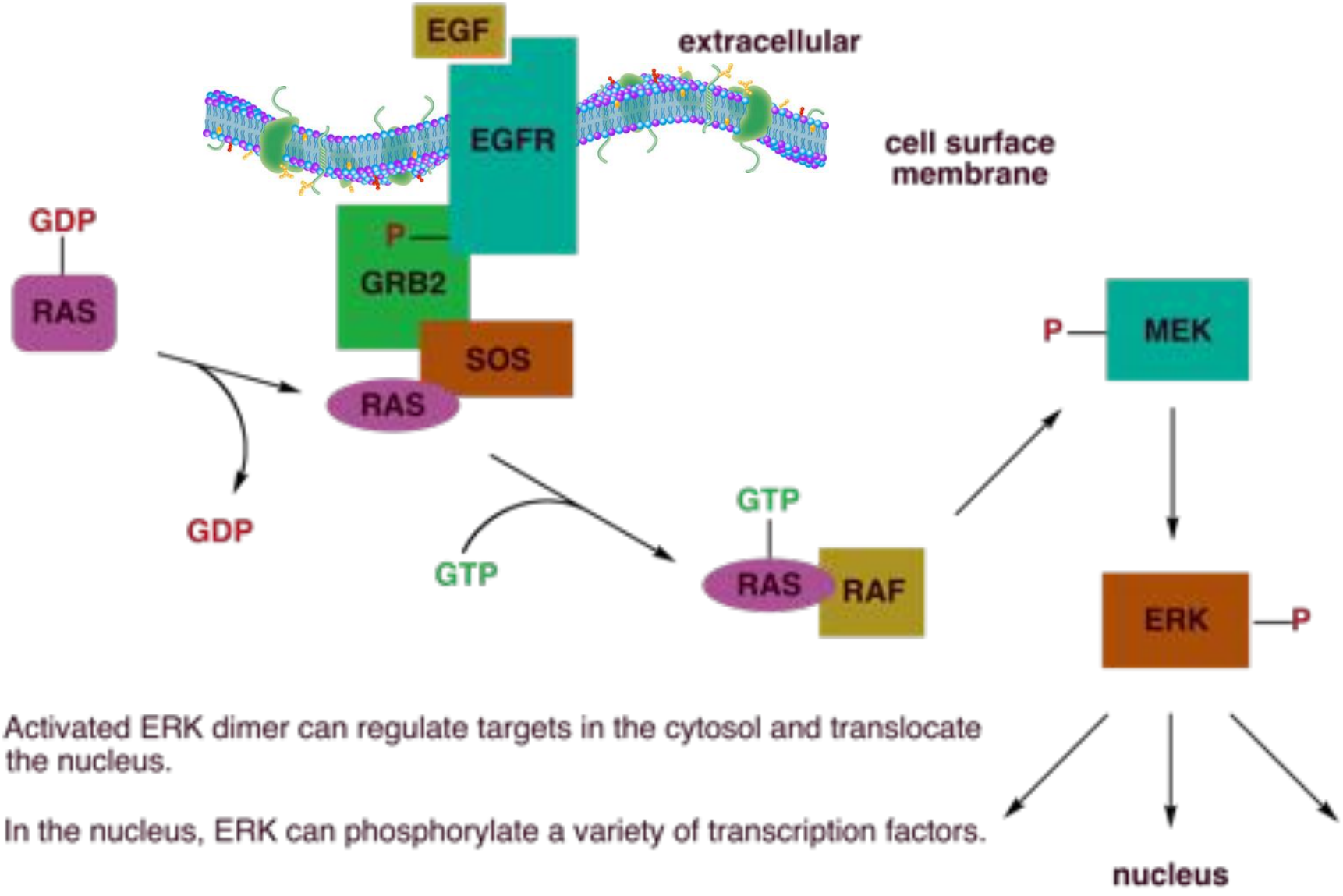
Signal Transduction Pathways are Quite Complicated



■ Activated MEK can phosphorylate ERK (or MAPK, mitogen-activated protein kinase).

Dhillon, A. S.; Hagan, S.; Rath, O.; Kolch, W. *Oncogene* **2007**, *22*, 3279.

Signal Transduction Pathways are Quite Complicated



- Activated ERK dimer can regulate targets in the cytosol and translocate to the nucleus.
- In the nucleus, ERK can phosphorylate a variety of transcription factors.

Dhillon, A. S.; Hagan, S.; Rath, O.; Kolch, W. *Oncogene* **2007**, *22*, 3279.

Classical Genetic Approaches

■ Forward Classical Genetics

Random Mutagenesis	→	**Phenotype of Interest**	→	**Identify Mutated Gene**
Irradiate fruit fly eggs.		Select flies born with specific wing defects.		Scan genome and find a certain mutation in these flies.

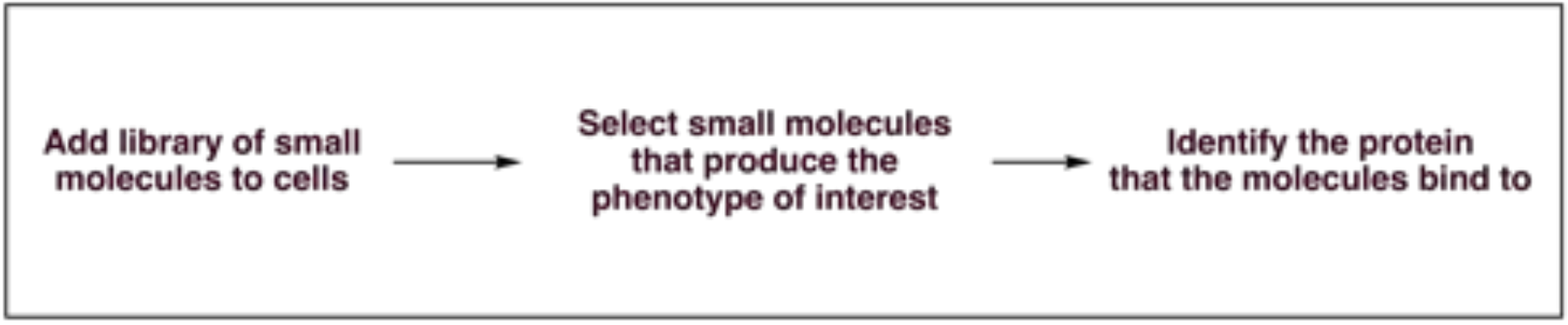
■ Reverse Classical Genetics

Mutate Single Gene of Interest	→	**Generate Cells or Animals with Mutation**	→	**Examine Phenotype**

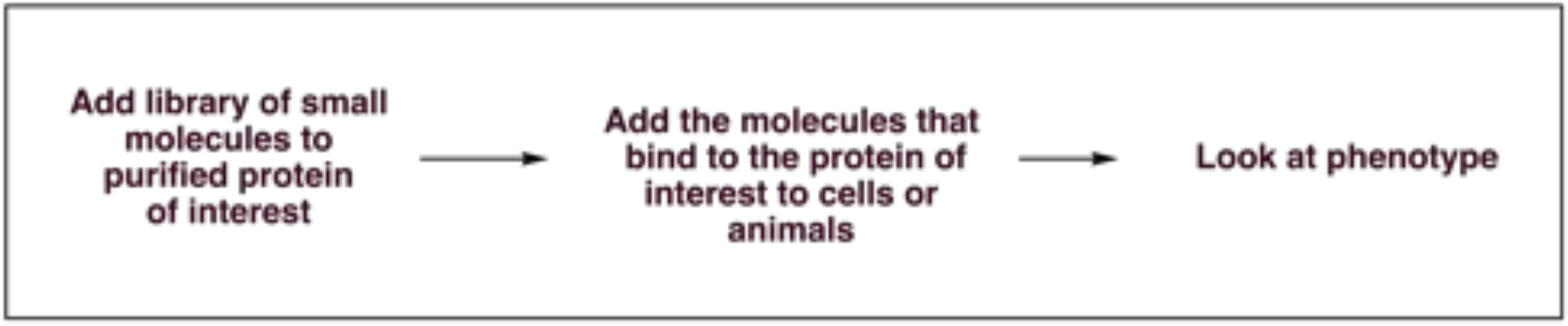
Alaimo, P. J.; Shogren-Knaak, M. A.; Shokat, K. M. *Curr. Opin. in Chem. Biol.* **2001**, *5*, 360.

The Chemical Genetic Approach

■ Forward Chemical Genetics



■ Reverse Chemical Genetics



Alaimo, P. J.; Shogren-Knaak, M. A.; Shokat, K. M. *Curr. Opin. in Chem. Biol.* **2001**, *5*, 360.

Disadvantages and Advantages

- Advantages to Classical Genetics

  This technique is highly specific (one single nucleotide change in 3 billion base pairs).

  Highly portable (can be applied to any animal or organism).

- Disadvantages to Classical Genetics

  Gene knock-out can be lethal.

  Genetic mutations are not conditional - gene is either or on off.

  Conditional mutations are possible through external stress, but this can produce unwanted side-effects.

  Knock-out phenotypes of non-essential genes can often be convoluted through compensation by related genes during the development of the organism.

Alaimo, P. J.; Shogren-Knaak, M. A.; Shokat, K. M. *Curr. Opin. in Chem. Biol.* **2001**, *5*, 360.

Disadvantages and Advantages

- Advantages to Chemical Genetics

  Chemical inhibition can be rapid, conditional, and dose-dependent.

  Functional compensation will be limited.

- Disadvantages to Chemical Genetics

  There are over 30,000 proteins.

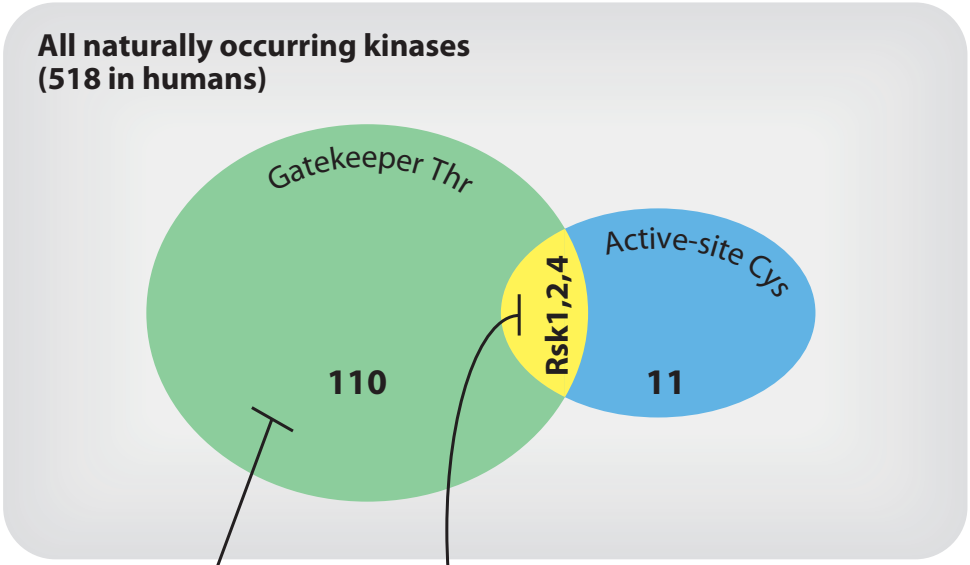
  Difficult to identify a small molecule that binds specifically to a single enzyme active site.

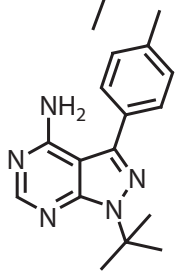
  In particular, the enzyme active site for kinases is highly conserved.

  The synthesis of small molecules is nontrivial.

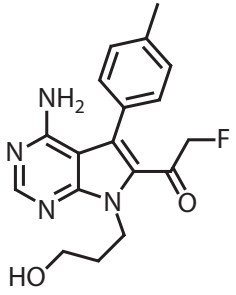
Alaimo, P. J.; Shogren-Knaak, M. A.; Shokat, K. M. *Curr. Opin. in Chem. Biol.* **2001**, *5*, 360.

Kinase Inhibition Strategies



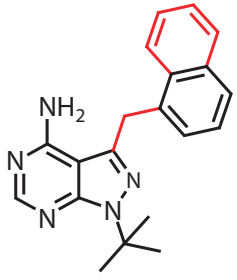


PP1

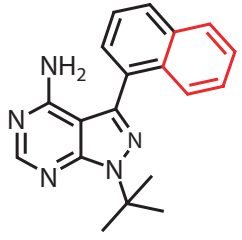


FMK

Gatekeeper
Gly, Ala



1NMPP1



1NAPP1

Dar, A. C.; Shokat, K. M. *Annu. Rev. Biochem.* **2011**, *80*, 769.

Cohen, M. S.; Zhang, C.; Shokat, K. M.; Taunton, J. *Science* **2005**, *308*, 1318.

Structural Bioinformatics-Based Design of Selective, Irreversible Kinase Inhibitors

- Use structural bioinformatics approach to selectively inhibit one kinase.

- Two selectivity filters used -

threonine as the gatekeeper (compact amino acid residue)

cysteine proximal to the active site

		nonconserved cysteine ↓		gatekeeper ↓
Src	[276]	LGQGCFGEVWMGTWNG	[333]	SEEP---IYIVTEYMSKGSL
RSK1	[424]	IGVGSYSVCKRCVHKA	[480]	DDGKH--VYLVTELMRGGEL
RSK2	[428]	IGVGSYSVCKRCIHKA	[484]	DDGKY--VYVVTELMKGGEL
RSK3	[421]	IGVGSYSVCKRCVHKA	[477]	DDGKF--VYLVMELMRGGEL
RSK4	[432]	IGVGSYSVCKRCIHAT	[488]	DDGRY--VYLVTDLMKGGEL
MSK1	[432]	LGEGSFSICRKCVHKK	[489]	HDQLH--TFLVMELLNGGEL
MSK2	[395]	LGQGSFSVCRRCRQRQ	[452]	HDQLH--TYLVLELLRGGEL
PLK1	[59]	LGKGGFAKCFEISDAD	[121]	EDNDF--VFVVLELCRRRSL
PLK2	[88]	LGKGGFAKCYEMTDLT	[150]	EDKEN--IYILLEYCSRR-S
PLK3	[29]	LGKGGFARCYEATDTE	[91]	EDADN--IYIFLELCSRK-S
NEK2	[14]	IGTGSYGRCQKIRRKS	[75]	IDRTNTTLYIVMEYCEGGDL
MEKK1	[1300]	IGLGAFSSCYQAQDVG	[1371]	CEKSN--YNLFIEWMAGGSV

- Applying these two filters, three closely related paralogs emerged -- RSK (p90 ribosomal protein S6 kinases)1, 2, and 4.

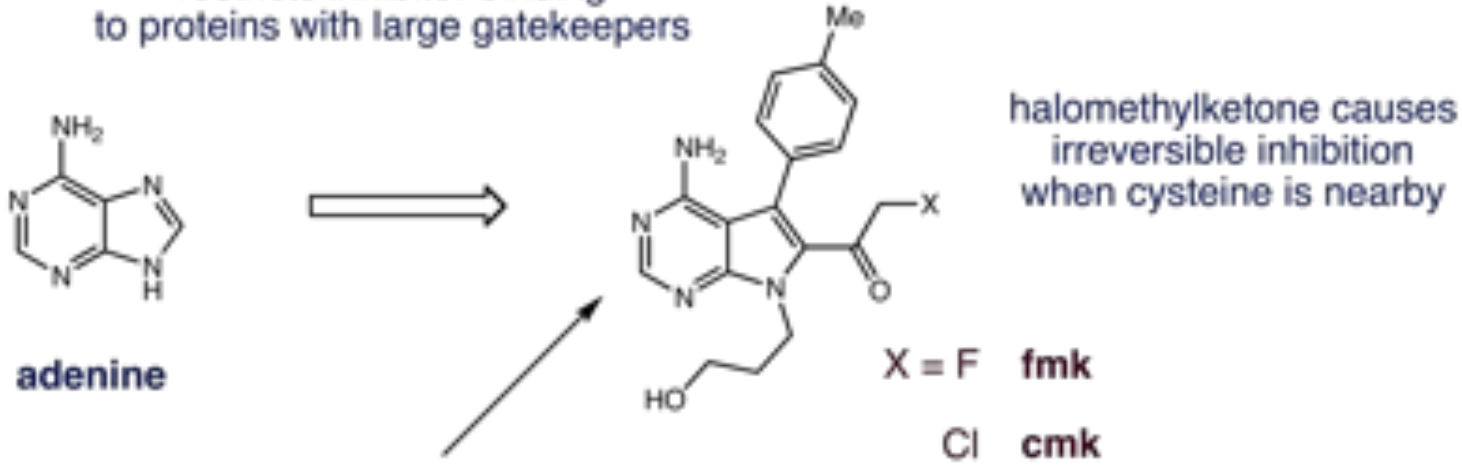
Dar, A. C.; Shokat, K. M. *Annu. Rev. Biochem.* **2011**, *80*, 769.

Cohen, M. S.; Zhang, C.; Shokat, K. M.; Taunton, J. *Science* **2005**, *308*, 1318.

Structural Bioinformatics-Based Design of Selective, Irreversible Kinase Inhibitors

- RSK1 and RSK2 are downstream effectors of the MAPK pathway.
- RSKs are directly activated by the MAPKs, ERK1, and ERK2.
- Mutations in the RSK2 gene cause Coffin-Lowry syndrome.

bulky aromatic group
restricts inhibitor binding
to proteins with large gatekeepers



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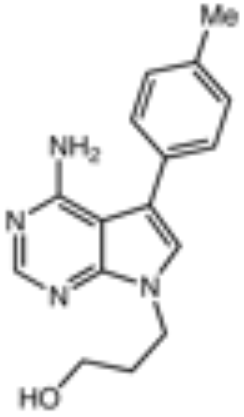
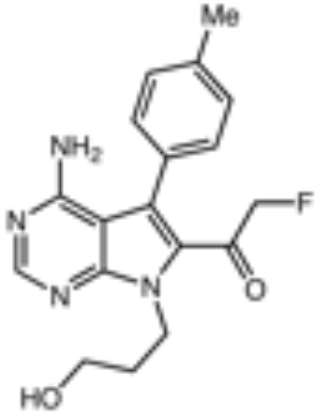
Structural Bioinformatics-Based Design of Selective, Irreversible Kinase Inhibitors

- Half-maximal inhibitory concentrations (IC_{50} in μM).



scaffold



fmk

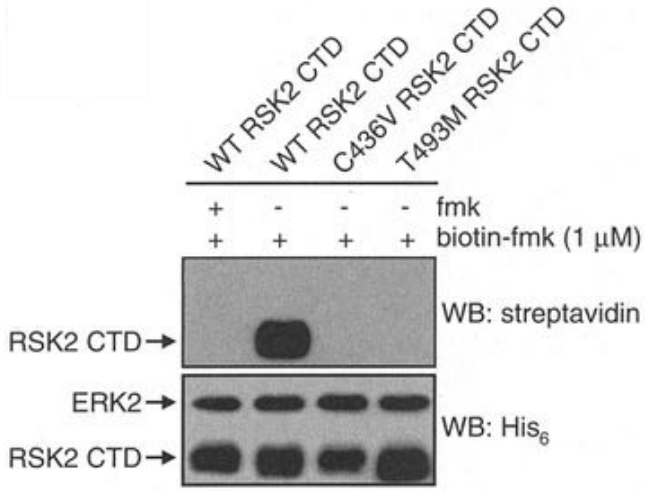
	WT	C436V	T493M
fmk	0.015	>10	3.4
scaffold	1.2	0.43	>30

Dar, A. C.; Shokat, K. M. *Annu. Rev. Biochem.* **2011**, *80*, 769.

Cohen, M. S.; Zhang, C.; Shokat, K. M.; Taunton, J. *Science* **2005**, *308*, 1318.

Structural Bioinformatics-Based Design of Selective, Irreversible Kinase Inhibitors

■ Does fmk bind irreversibly?



■ Denaturing gel electrophoresis and Western blot analysis with streptavidin-horseradish peroxidase used to show irreversible binding of fmk.

■ ERK2, required to activate RSK2 in vitro, was not labeled by biotin-fmk, despite the presence of a solvent-exposed cysteine in its ATP pocket.

■ In a human epithelial cell lysate and *in vivo*, biotin-fmk only binded with two proteins (RSK1 and RSK2).

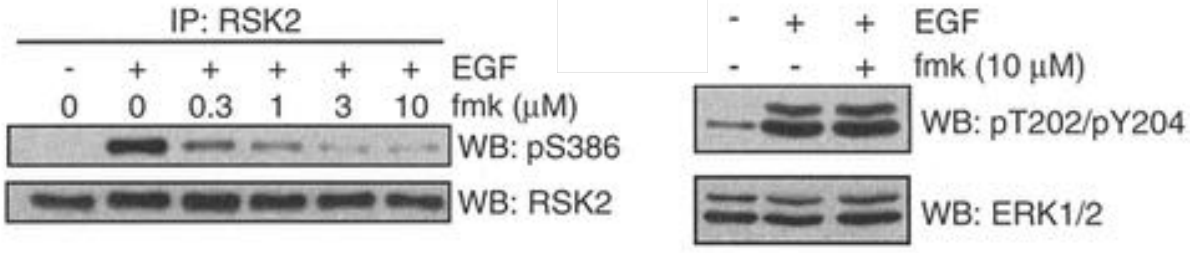
Dar, A. C.; Shokat, K. M. *Annu. Rev. Biochem.* **2011**, *80*, 769.

Cohen, M. S.; Zhang, C.; Shokat, K. M.; Taunton, J. *Science* **2005**, *308*, 1318.

Structural Bioinformatics-Based Design of Selective, Irreversible Kinase Inhibitors

■ RSK2 is known to phosphorylate itself at Ser^{386}, which creates a docking site for phosphoinositide-dependent kinase 1 (PDK1), which then phosphorylates N-terminal kinase domain (NTD) of RSK2.

■ Can fmk inhibit Ser^{386} phosphorylation?



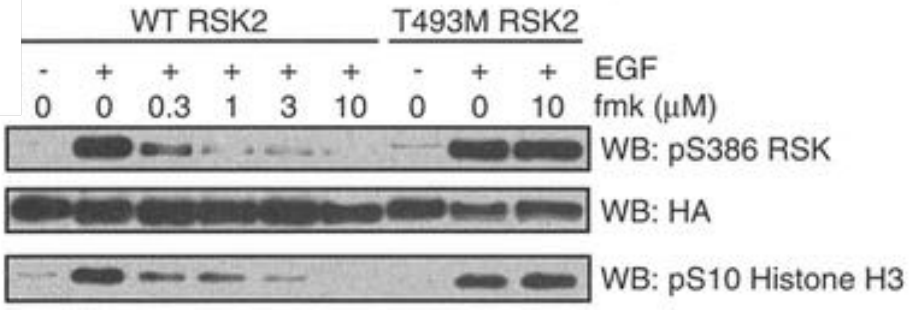
■ fmk had no effect on EGF-stimulated phosphorylation of ERK1 or ERK2.

Dar, A. C.; Shokat, K. M. *Annu. Rev. Biochem.* **2011**, *80*, 769.

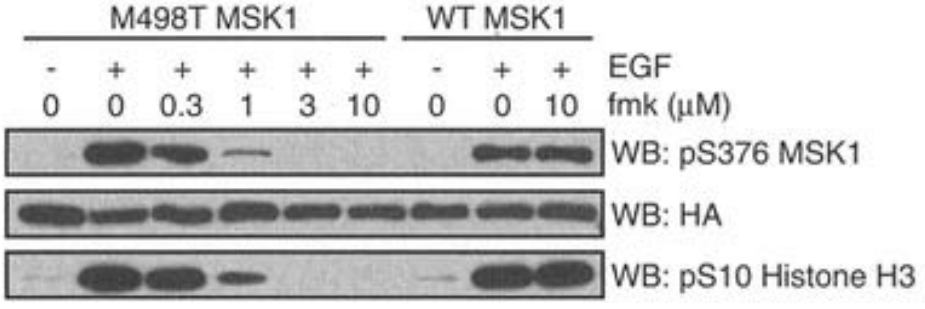
Cohen, M. S.; Zhang, C.; Shokat, K. M.; Taunton, J. *Science* **2005**, *308*, 1318.

Structural Bioinformatics-Based Design of Selective, Irreversible Kinase Inhibitors

■ Can fmk block signaling downstream of RSK2?



■ Can fmk also inhibit MSK1, mitogen- and stress-activated kinase, which has a methionine gatekeeper and proximal cysteine residue in the active site?



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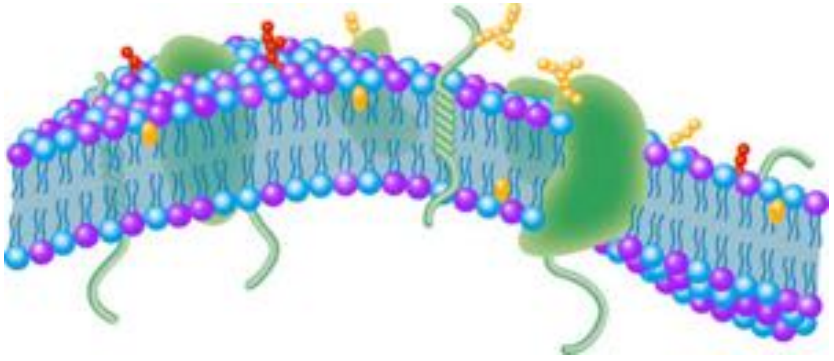
Inhibitor Hijacking of Akt Activation



cell membrane

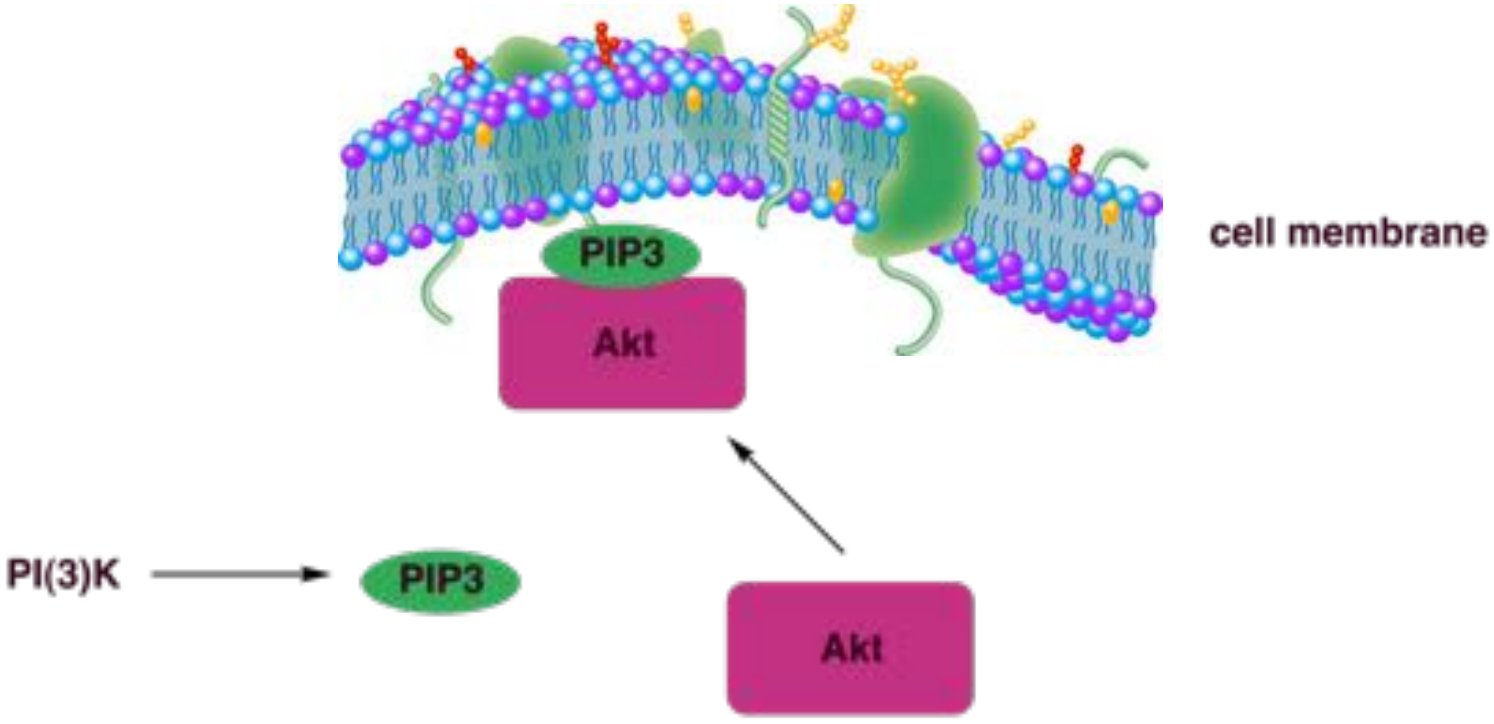
Akt

■ The kinase Akt is a part of the PI(3)K-Akt-mTORC1 pathway and regulates a number of growth factor signals that are related to proliferation and survival.

■ Without growth factors, Akt is in the cytoplasm and is inactive.

Dar, A. C.; Shokat, K. M. *Annu. Rev. Biochem.* **2011**, *80*, 769.
Okuzumi, T.; Fiedler, D.; Zhang, C.; Gray, D. C.; Aizenstein, B.; Hoffman, R.; Shokat, K. M. *Nat. Chem. Bio.* **2009**, *5*, 484.

Inhibitor Hijacking of Akt Activation

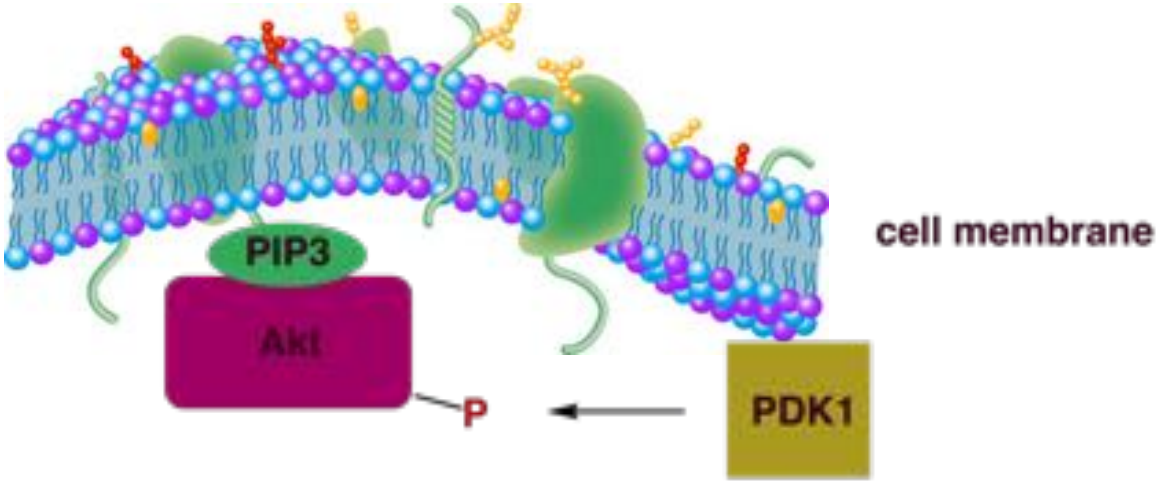


■ Growth factor stimulation of phosphatidylinositol-3-OH kinase (PI(3)K) causes phosphatidylinositol-1,4,5-triphosphate (PIP3) to be produced.

■ Akt binds to PIP3 through the pleckstrin homology (PH) domain, localizing Akt on the plasma membrane.

Dar, A. C.; Shokat, K. M. *Annu. Rev. Biochem.* **2011**, *80*, 769.
Okuzumi, T.; Fiedler, D.; Zhang, C.; Gray, D. C.; Aizenstein, B.; Hoffman, R.; Shokat, K. M. *Nat. Chem. Bio.* **2009**, *5*, 484.

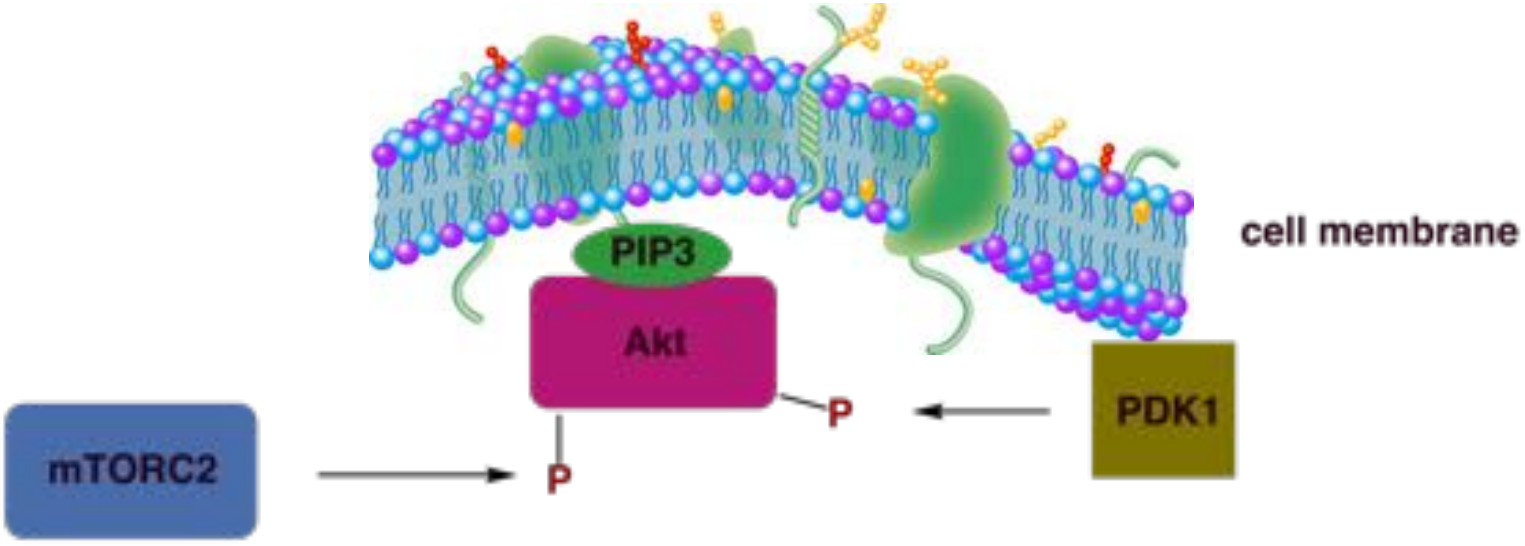
Inhibitor Hijacking of Akt Activation



- For Akt to become active, it must be phosphorylated at Thr^{308} and Ser^{473}.
- Thr^{308} is phosphorylated by membrane-localized phosphoinositide-dependent kinase 1 (PDK1).

Dar, A. C.; Shokat, K. M. *Annu. Rev. Biochem.* **2011**, *80*, 769.
Okuzumi, T.; Fiedler, D.; Zhang, C.; Gray, D. C.; Aizenstein, B.; Hoffman, R.; Shokat, K. M. *Nat. Chem. Bio.* **2009**, *5*, 484.

Inhibitor Hijacking of Akt Activation



■ Ser^{473} is phosphorylated by rapamycin-insensitive mTORC2.

■ Aberrant activation of Akt has been observed in a number of human cancers through a variety of pathways: PI(3)K-activating mutations, phosphatase and tensin homolog (PTEN) inactivation, Akt overexpression, Akt mutants that lead to membrane localization.

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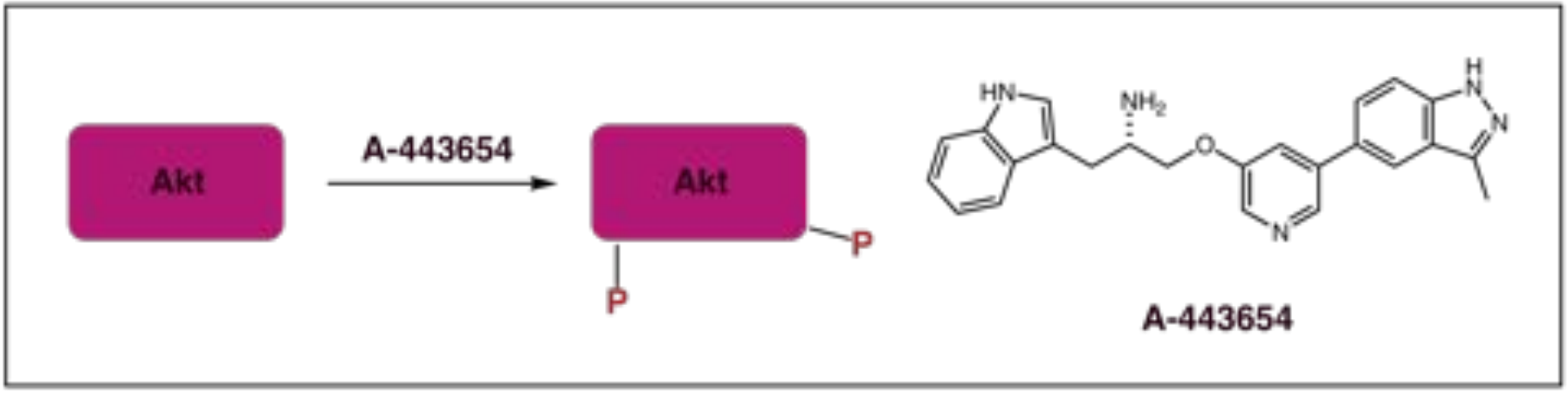
Okuzumi, T.; Fiedler, D.; Zhang, C.; Gray, D. C.; Aizenstein, B.; Hoffman, R.; Shokat, K. M. *Nat. Chem. Bio.* **2009**, *5*, 484.

Inhibitor Hijacking of Akt Activation

- Akt has become an attractive anticancer drug target.
- Not all of the inhibitors of the PI(3)K-Akt-mTORC1 pathway antagonize the pathway.
- For example, mTORC1 inhibitor rapamycin causes upstream activation, which leads to Akt activation.
- Similarly, A-443654, a potent Akt inhibitor developed from Abbott labs, causes hyper-phosphorylation of Akt while inhibiting downstream Akt signaling.



- Why does this happen?

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Okuzumi, T.; Fiedler, D.; Zhang, C.; Gray, D. C.; Aizenstein, B.; Hoffman, R.; Shokat, K. M. *Nat. Chem. Bio.* **2009**, *5*, 484.

Inhibitor Hijacking of Akt Activation

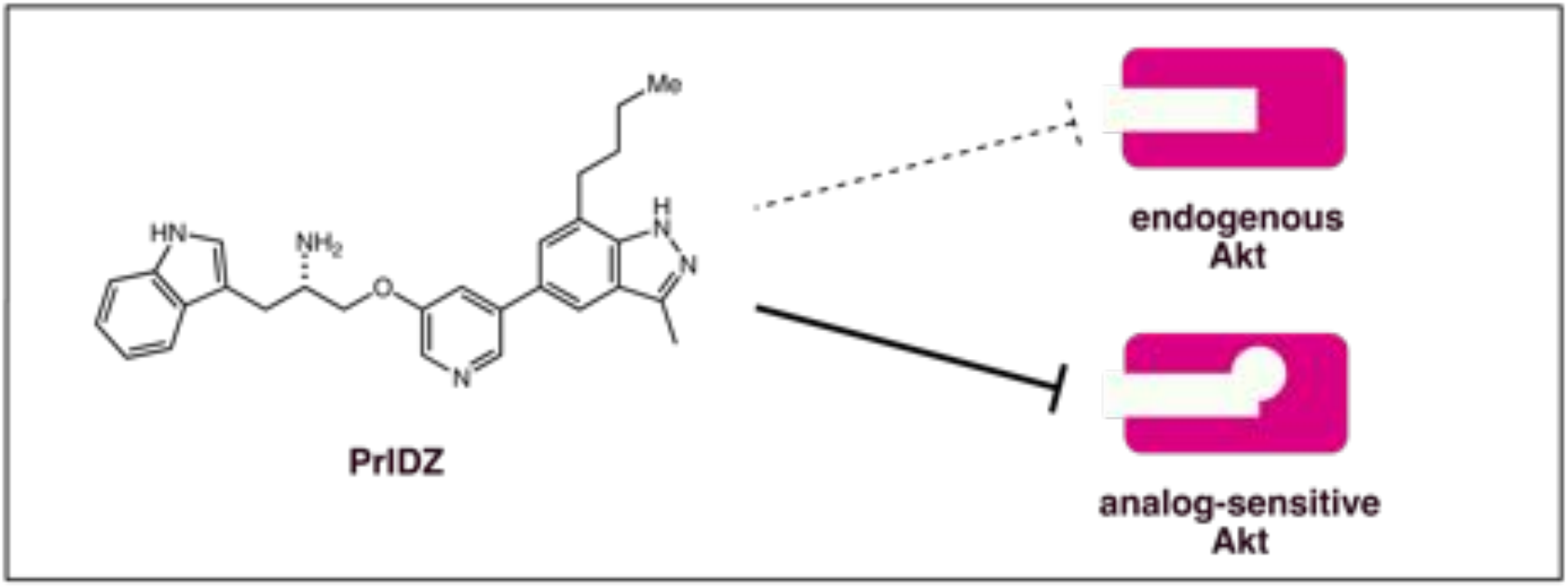
■ **Kinase-extrinsic mechanism**

A-443654 inhibits Akt, which reduces feedback inhibition of Akt phosphorylation.

■ **Kinase-intrinsic mechanism**

Hyperphosphorylation relies solely on A-443654 binding to Akt.

■ Problem - A-443654 is a promiscuous inhibitor (inhibits 47 of 220 kinases tested).



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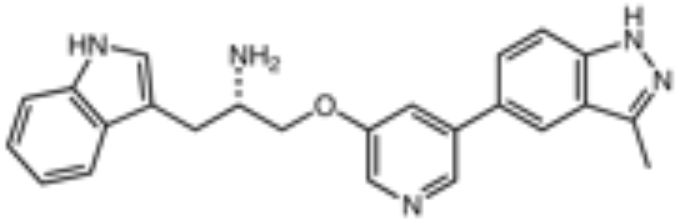
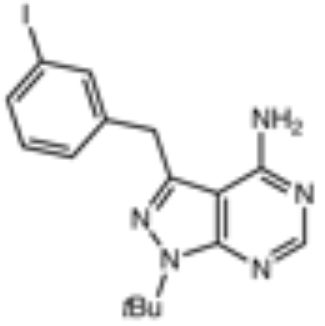
Inhibitor Hijacking of Akt Activation

■ Mutations were introduced for all three Akt isoforms to enlarge the ATP binding pocket by substituting the methionine gatekeeper for a glycine.

■ Pyrazolopyrimidine1 (PP1) was first screened but even the best inhibitor (3-IB-PP1) did not bind very well to *as*Akt2 and *as*Akt3.





IC_{50} (nM)	A-443654	3-IB-PP1
*wt*Akt1	2.5	>10,000
*as*Akt1	N.D.	28
*wt*Akt2	30	>10,000
*as*Akt2	N.D.	240
*wt*Akt3	51	>10,000
*as*Akt3	N.D.	120

Dar, A. C.; Shokat, K. M. *Annu. Rev. Biochem.* **2011**, *80*, 769.

Okuzumi, T.; Fiedler, D.; Zhang, C.; Gray, D. C.; Aizenstein, B.; Hoffman, R.; Shokat, K. M. *Nat. Chem. Bio.* **2009**, *5*, 484.

Inhibitor Hijacking of Akt Activation

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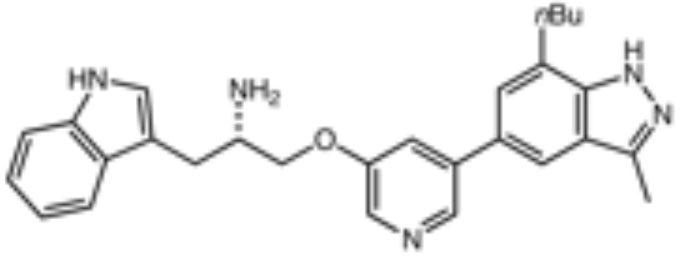

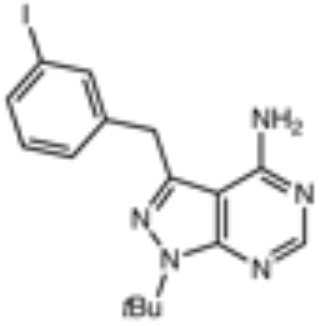

IC_{50} (nM)	PrIDZ	3-IB-PP1
*wt*Akt1	>1,000	>10,000
*as*Akt1	2.8	28
*wt*Akt2	>10,000	>10,000
*as*Akt2	31	240
*wt*Akt3	>10,000	>10,000
*as*Akt3	13	120

Dar, A. C.; Shokat, K. M. *Annu. Rev. Biochem.* **2011**, *80*, 769.

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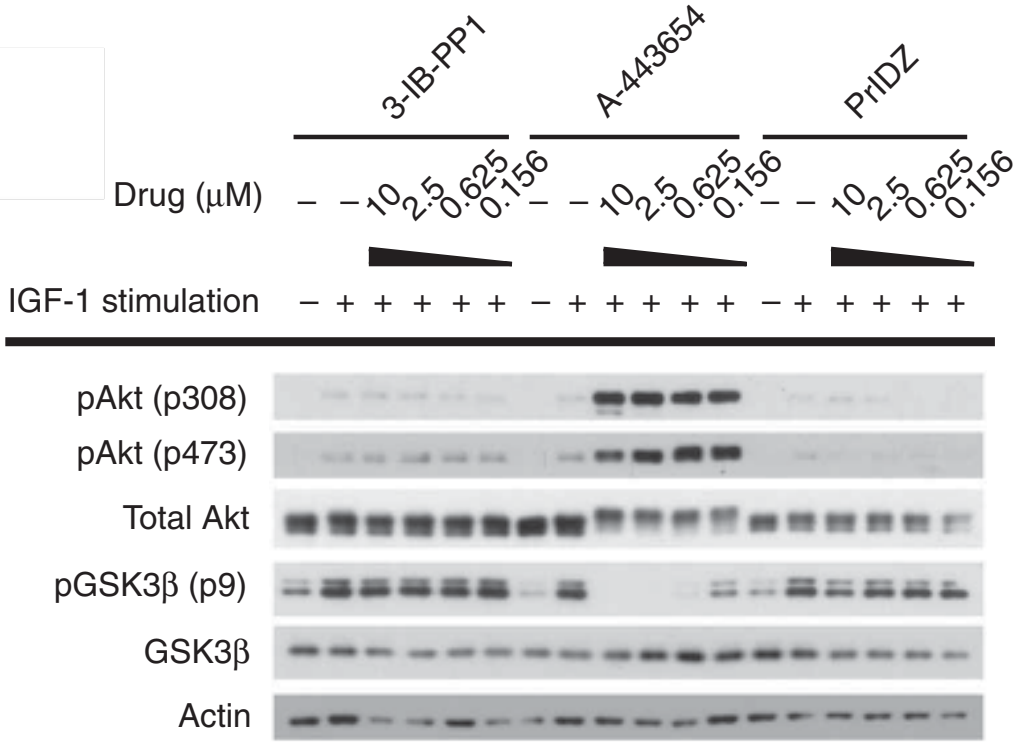
Inhibitor Hijacking of Akt Activation

■ A-443654 binds to *wt*Akt, inhibiting GSK3β phosphorylation and Akt hyperphosphorylation. 3-IB-PP1 and PrIDZ do not bind to *wt*Akt.



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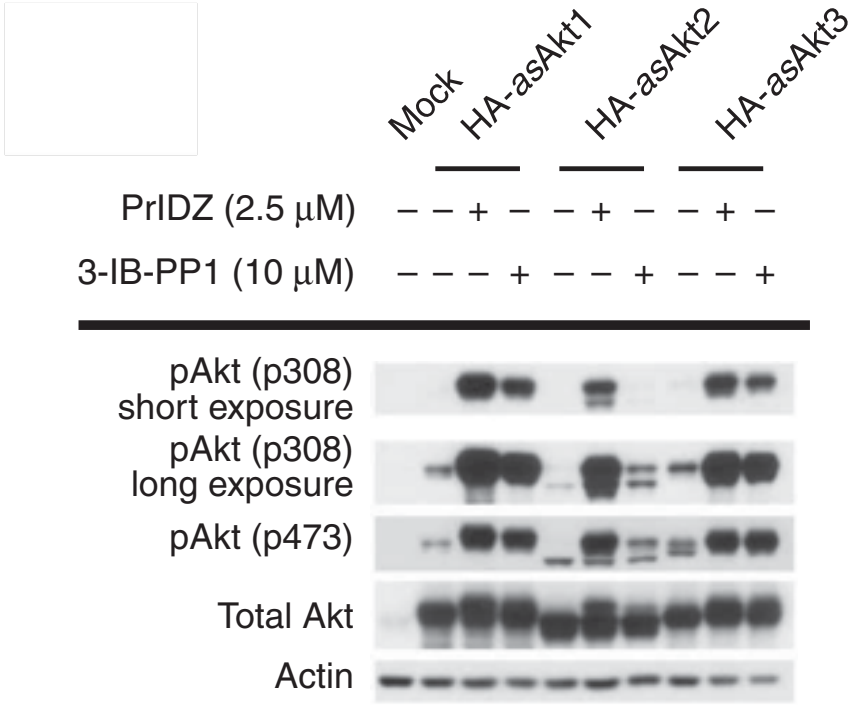
Inhibitor Hijacking of Akt Activation

- PrIDZ binds more strongly to all three *as*Akt than 3-IB-PP1.



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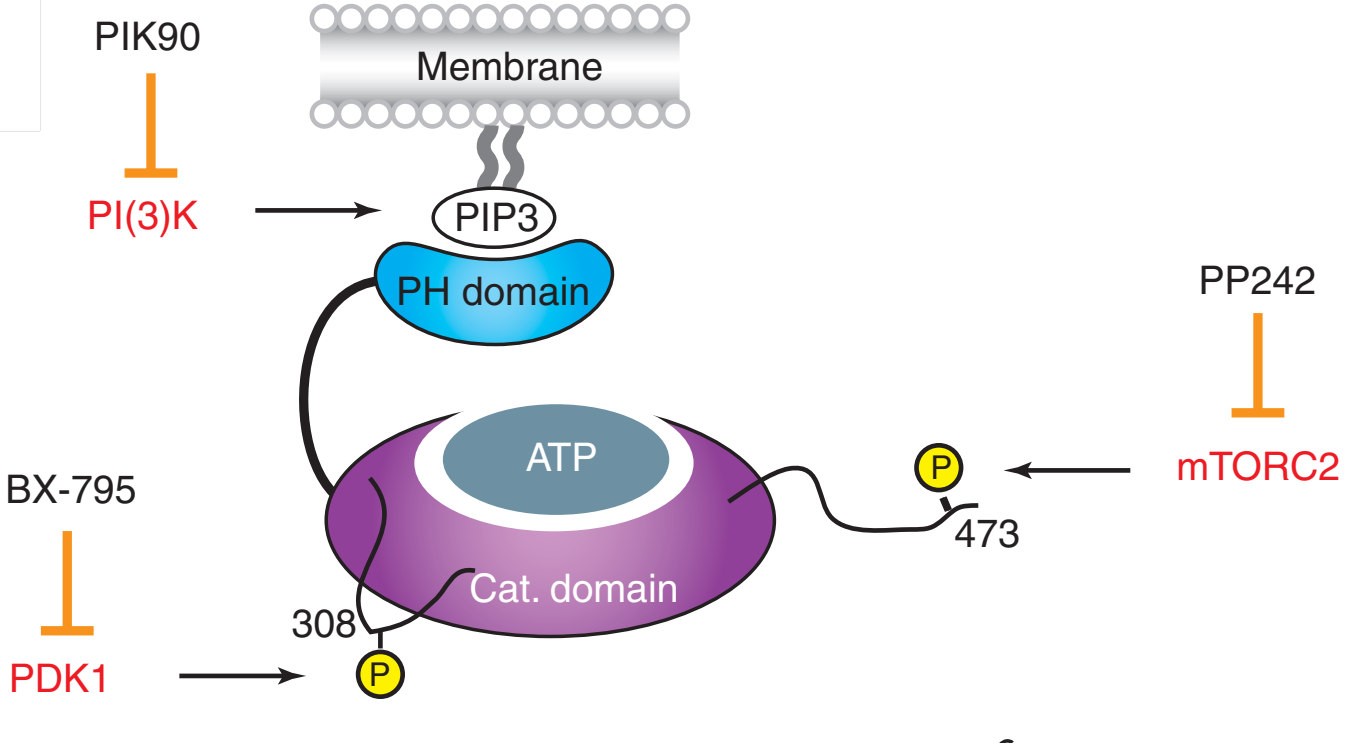
Inhibitor Hijacking of Akt Activation

■ Akt is regulated by three kinases.



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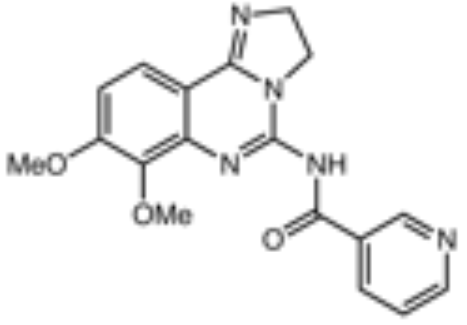
Okuzumi, T.; Fiedler, D.; Zhang, C.; Gray, D. C.; Aizenstein, B.; Hoffman, R.; Shokat, K. M. *Nat. Chem. Bio.* **2009**, *5*, 484.

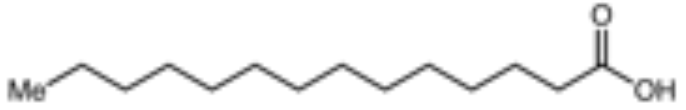
Inhibitor Hijacking of Akt Activation

■ PIK90, a selective pan-PI(3)K inhibitor, reduces hyperphosphorylation of *as*Akt in the presence of PrINZ, implying that PIP3 production is critical for Akt phosphorylation.

■ Similarly, mutant HA-*as*Akt1 R25C, a known mutation with decreased affinity for PIP3, greatly reduces PrINZ-induced phosphorylation.



PIK-90



myristic acid

■ Myristoylation of *as*Akt1 and treatment with PrINZ results in hyperphosphorylation.

■ myr-HA-*as*Akt is still hyperphosphorylated even if the cell is pretreated with PIK90 or R25C.

■ These results imply that membrane localization is necessary for hyperphosphorylation, that PH domain binding to PIP3 is not required.

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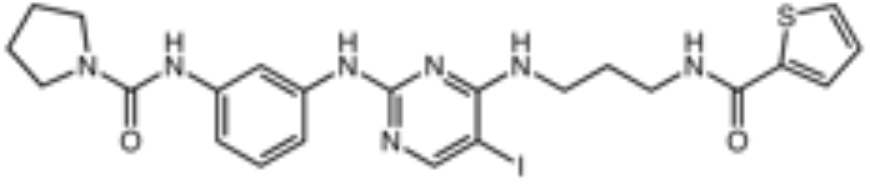
Inhibitor Hijacking of Akt Activation

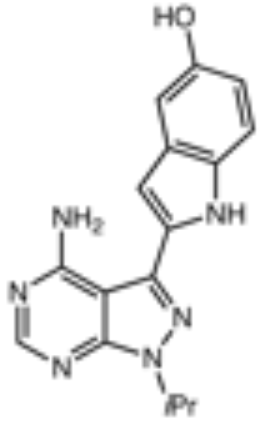
■ Pretreatment of cells with BX-795, a selective PDK1 inhibitor, resulted in a substantial reduction in PrINZ-induced Thr^{308} and Ser^{473} phosphorylation.

■ Addition of PP242, an ATP-competitive inhibitor of mTORC1 and mTORC2, inhibits phosphorylation of Ser^{473} while leaving Thr^{308} phosphorylation unaffected.

■ These results confirm that the same upstream kinases lead to both Akt activation in growth factor signaling and inhibitor-induced Akt hyperphosphorylation.



BX-795



PP242

Dar, A. C.; Shokat, K. M. *Annu. Rev. Biochem.* **2011**, *80*, 769.
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Inhibitor Hijacking of Akt Activation

- Does Akt hyperphosphorylation occur through a kinase-intrinsic or kinase-extrinsic mechanism?

- It is known that A-443654 can still cause hyperphosphorylation in *Tsc2*$^{-/-}$ cells. Tsc2 is a direct downstream target of Akt.

- If hyperphosphorylation occurs through a negative feedback loop, it could possibly occur through a S6K-mediated feedback, which has been reported for rapamycin.

- Addition of DG2, a new selective S6K inhibitor, does not inhibit Akt phosphorylation though.

- A catalytically inactive Akt is generated --

Two mutations --

gatekeeper mutation (M227G)

removal of aspartate from DFG motif (D292A)

Dar, A. C.; Shokat, K. M. *Annu. Rev. Biochem.* **2011**, *80*, 769.
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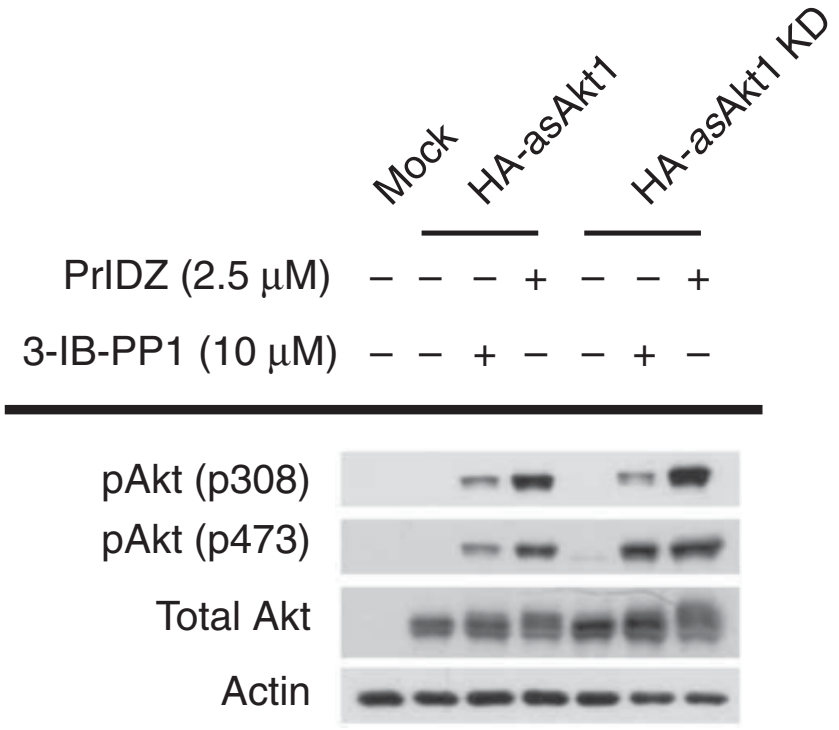
Inhibitor Hijacking of Akt Activation

■ HA-*as*Akt1 displays similar behavior as HA-*as*Akt1 KD



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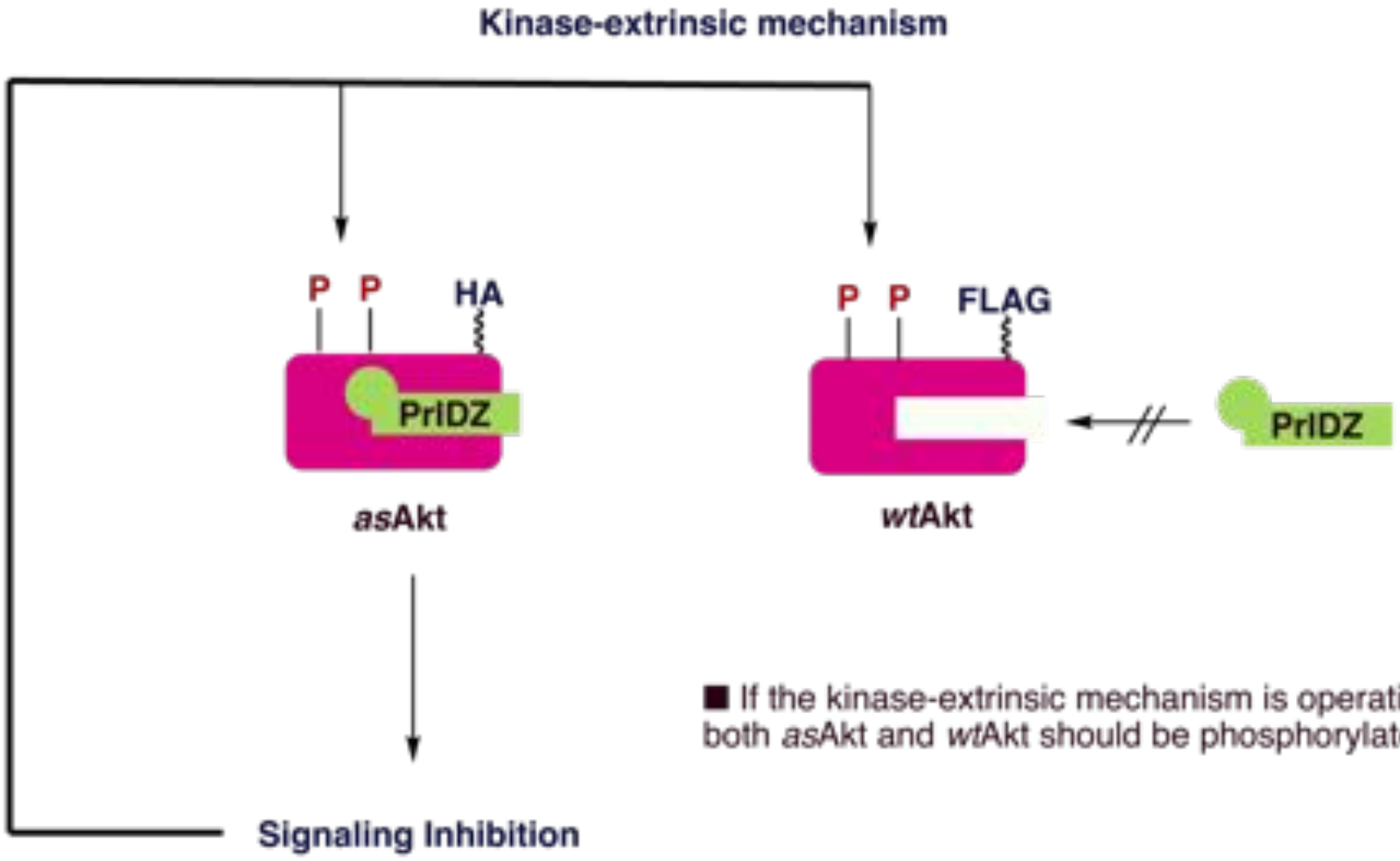
Inhibitor Hijacking of Akt Activation

■ Double Akt transfection experiment



■ If the kinase-extrinsic mechanism is operative, both *as*Akt and *wt*Akt should be phosphorylated.

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Inhibitor Hijacking of Akt Activation

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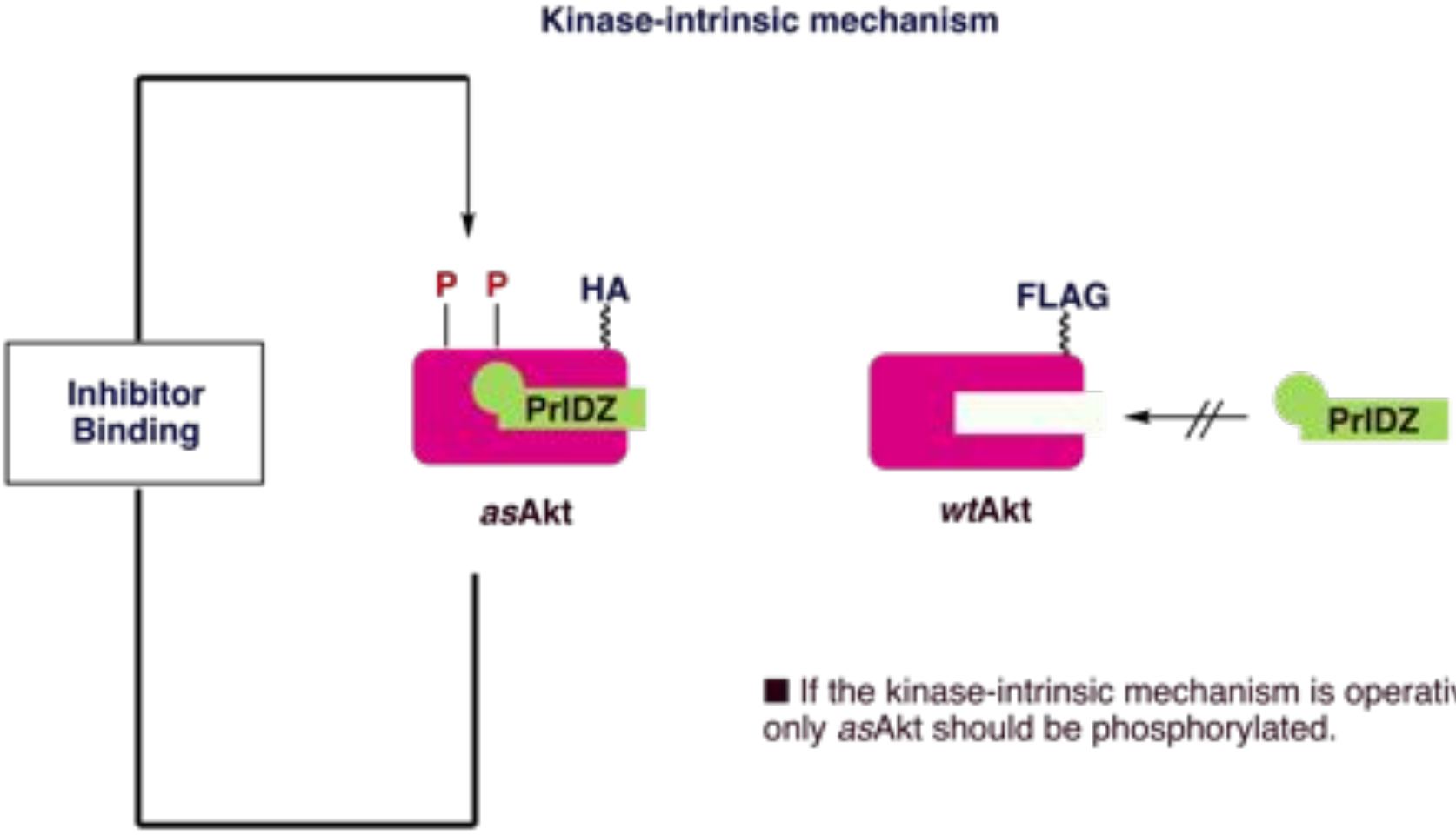

■ If the kinase-intrinsic mechanism is operative, only *as*Akt should be phosphorylated.

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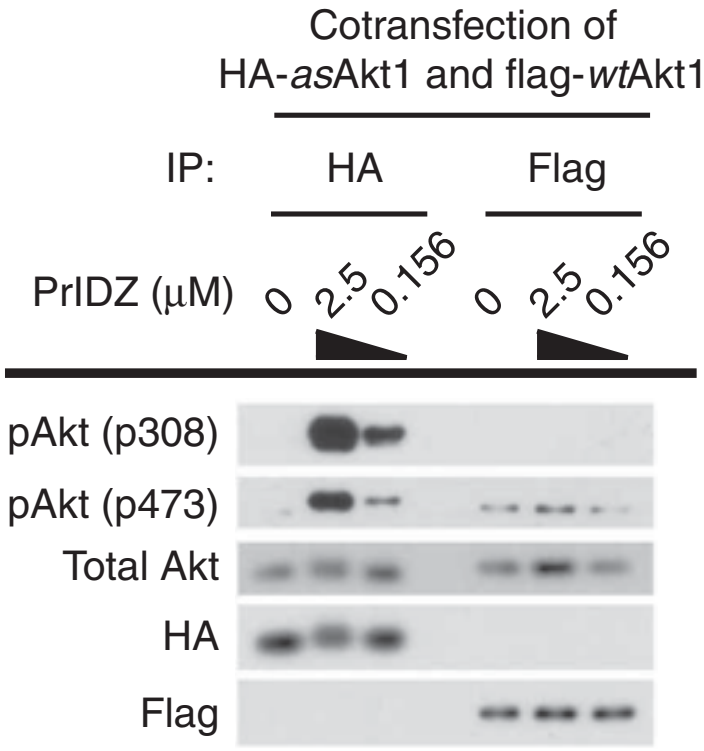
Okuzumi, T.; Fiedler, D.; Zhang, C.; Gray, D. C.; Aizenstein, B.; Hoffman, R.; Shokat, K. M. *Nat. Chem. Bio.* **2009**, *5*, 484.

Inhibitor Hijacking of Akt Activation

- Hyperphosphorylation is only observed on *as*Akt1



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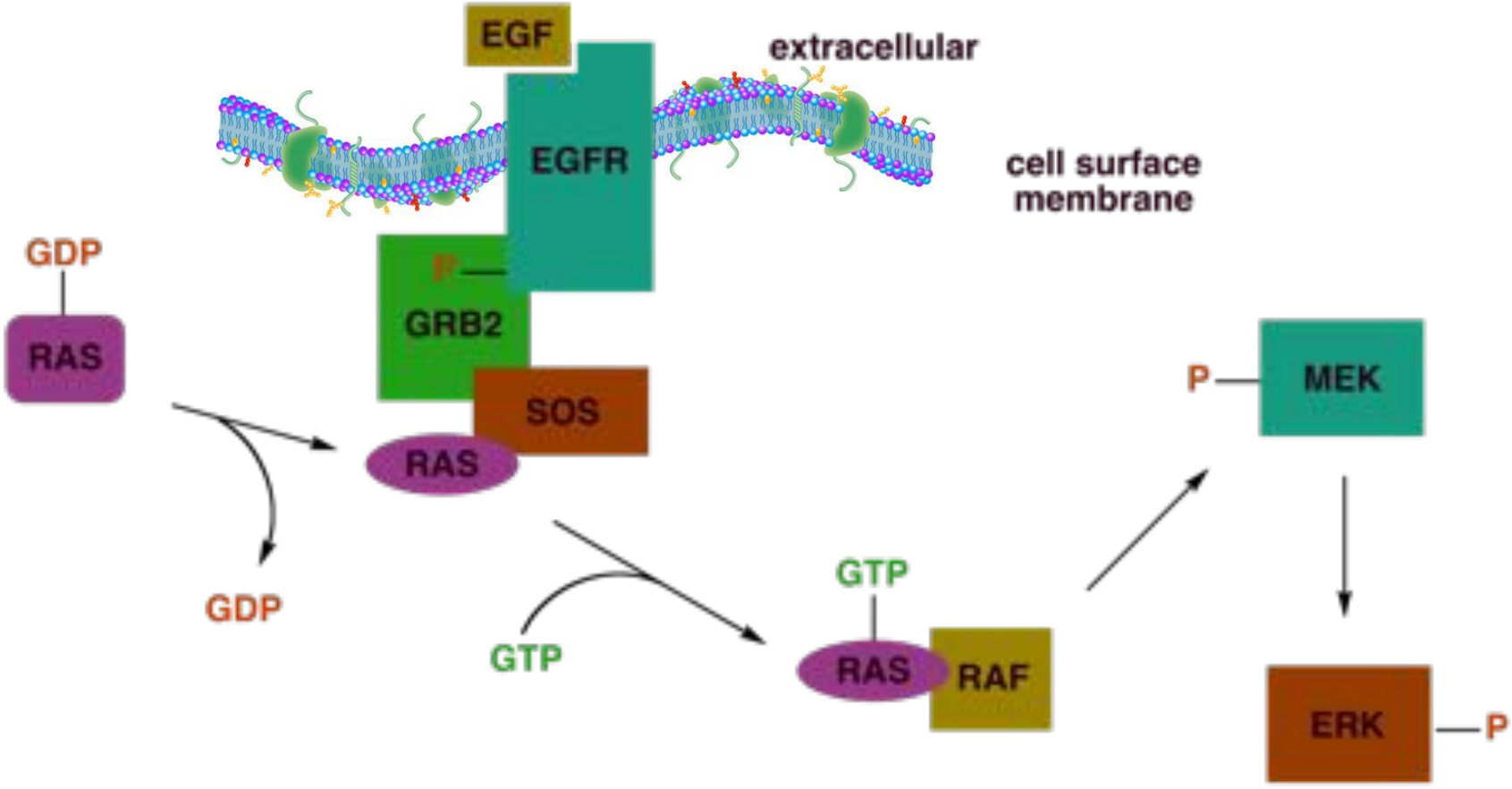
RAF Inhibitors Transactivate RAF Dimers and ERK Signalling in Cells with Wild-type BRAF



■ RAF is mutated in 6% of all cancers.

■ RAF is immediately downstream of Ras, which is one of the most frequently mutated oncogenes in cancer.

Dar, A. C.; Shokat, K. M. *Annu. Rev. Biochem.* **2011**, *80*, 769.

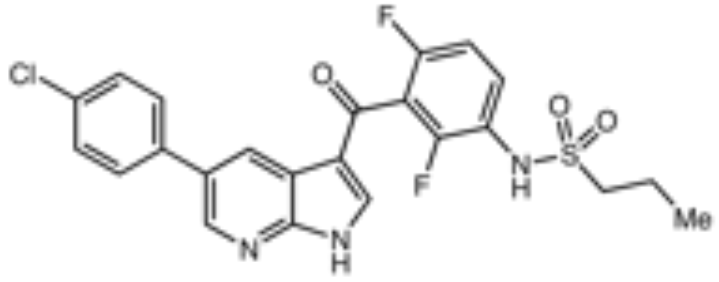
Poulikakos, P. I.; Zhang, C.; Bollag, G.; Shokat, K. M.; Rosen, N. *Nature* **2010**, *464*, 427.

RAF Inhibitors Transactivate RAF Dimers and ERK Signalling in Cells with Wild-type BRAF

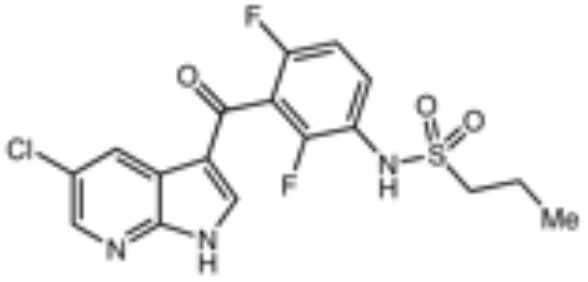
- B-RAF can have a variety of cancer mutations. V600E is the predominant form in melanoma.

- In V600E BRAF mutant cells, PLX-4720 is a potent inhibitor of MEK phosphorylation, and tumor regression was seen in 90% of patients.

- In studies of cells with other pathway-activating mutations, RAF inhibitors paradoxically activated MEK phosphorylation.



Vemurafenib



PLX-4720

- Vemurafenib is a commercial BRAF enzyme inhibitor developed by Roche used to treat late-stage melanoma.

- In clinical trials of vemurafenib, approximately one-third of melanoma patients treated at the maximum tolerated dose of PLX-4032 developed a different cancer, squamous cell carcinoma.

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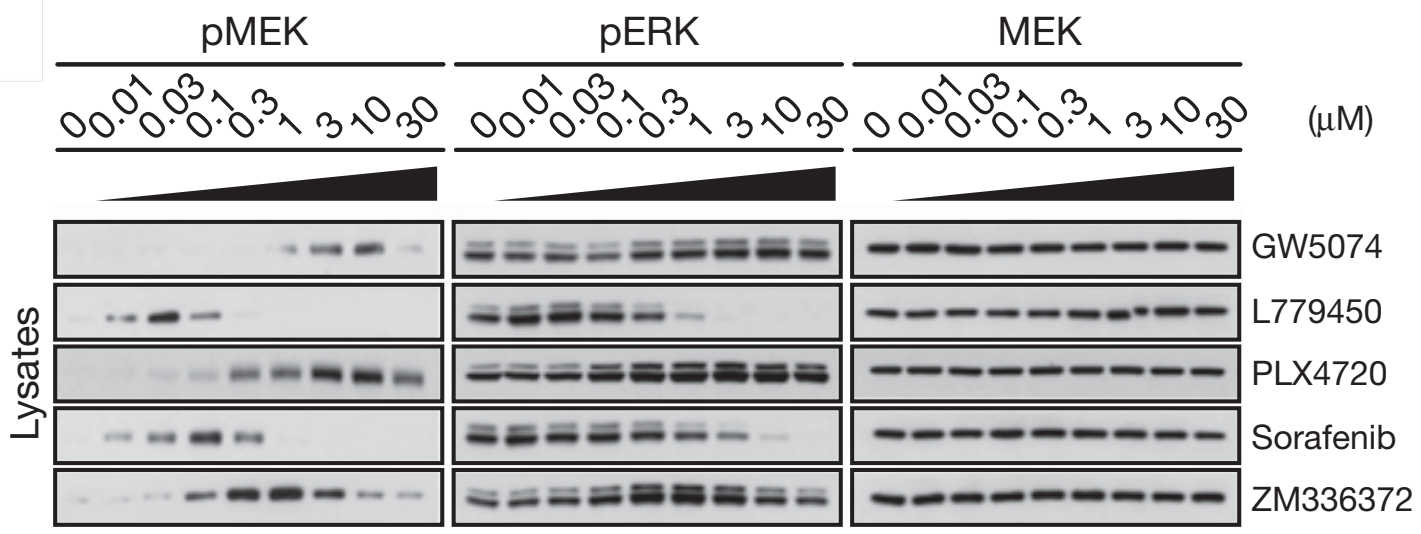
Poulikakos, P. I.; Zhang, C.; Bollag, G.; Shokat, K. M.; Rosen, N. *Nature* **2010**, *464*, 427.

RAF Inhibitors Transactivate RAF Dimers and ERK Signalling in Cells with Wild-type BRAF

■ Five distinct ATP-competitive RAF inhibitors induced MEK and ERK phosphorylation at intermediate concentrations in cells with *wt*BRAF.



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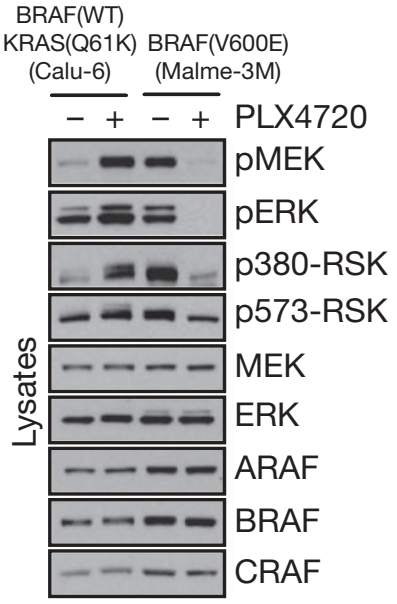
RAF Inhibitors Transactivate RAF Dimers and ERK Signalling in Cells with Wild-type BRAF

■ PLX4032 was assayed against 62 kinases in the kinome - only eight have an IC_{50} of 1-10 μM, while the rest are greater than 10 μM.

■ Vemurafenib (PLX4032) exhibits selective inhibition of the purified catalytic domains of BRAF (V600E), *wt*BRAF, and *wt*CRAF.

	IC_{50} (nM)
BRAF(V600E)	35
***wt*BRAF**	110
***wt*CRAF**	48



■ Yet PLX4720, an analog of PLX4032, induces ERK signalling in a rapid and reversible manner.

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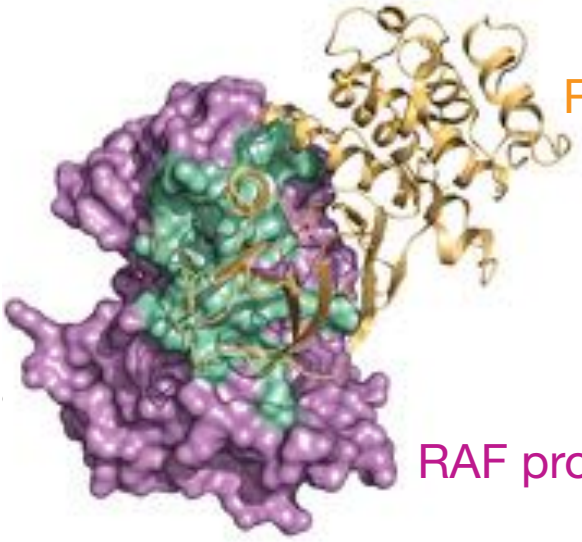
RAF Inhibitors Transactivate RAF Dimers and ERK Signalling in Cells with Wild-type BRAF

- MEK/ERK activation by RAF inhibitor is enhanced when RAS is overexpressed,
- It appears that RAS activity is required for MEK/ERK activation by RAF inhibitors.
- It is known that BRAF and CRAF kinases form homo- and heterodimers upon activation by RAS.



- Is BRAF or CRAF solely responsible for MEK/ERK activation by PLX4032/4720?

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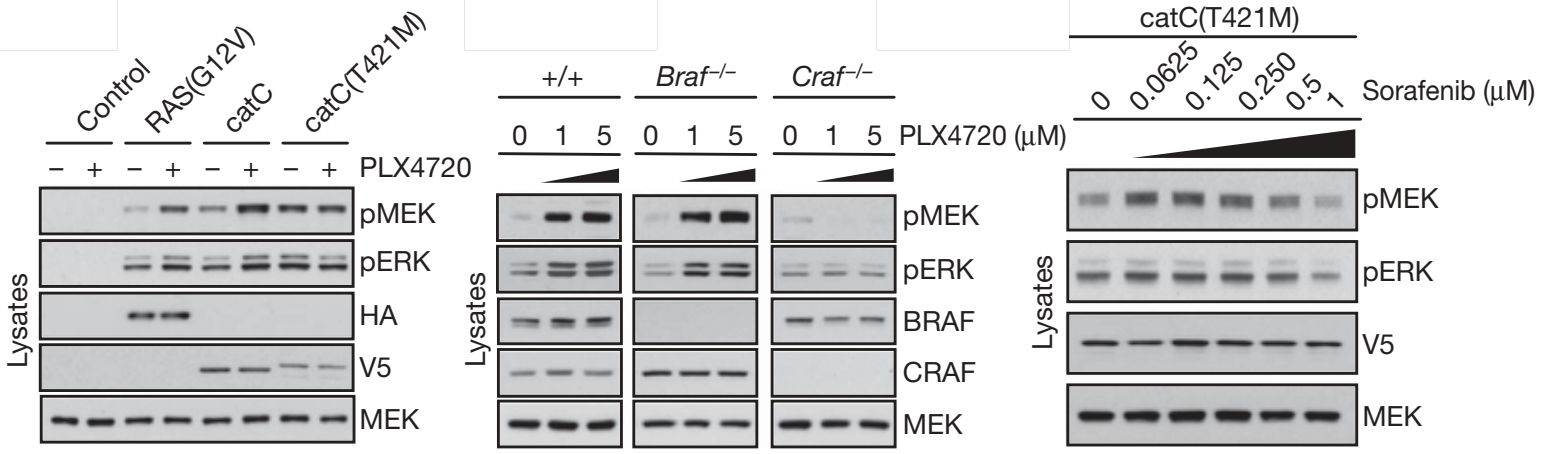
RAF Inhibitors Transactivate RAF Dimers and ERK Signalling in Cells with Wild-type BRAF

- CRAF expression is required for significant induction.
- Autoinhibition of RAF by its amino-terminal domain takes place unless RAS binds to RAF.
- Could overexpression of an N-truncated form (catC) of CRAF bypass required RAS activity?
- Is direct binding of catC to PLX4032/PLX4720 necessary?



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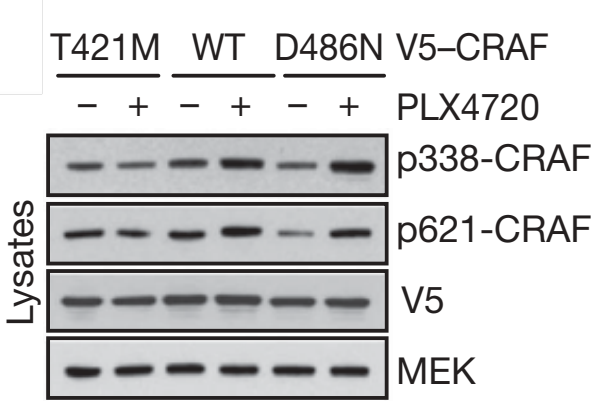
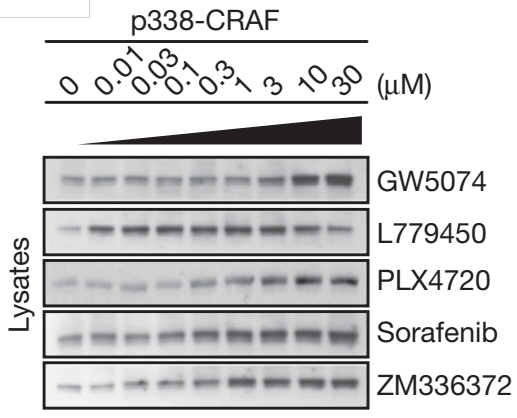
RAF Inhibitors Transactivate RAF Dimers and ERK Signalling in Cells with Wild-type BRAF

- Just like AKT, it is thought that binding of ATP-competitive inhibitors to RAF could induce phosphorylation and activation of the enzyme.
- BRAF, CRAF, and catC that is immunoprecipitated from PLX4032/PLX4720-treated cells and washed was more active than that isolated from untreated cells.
- Phosphorylation of CRAF at Ser^{338} and Ser^{621} is believed to be correlated to its activation.
- All RAF inhibitors tested induce phosphorylation of CRAF.





- Paradoxically, binding of ATP-competitive inhibitors to the catalytic domain of CRAF activates its function.

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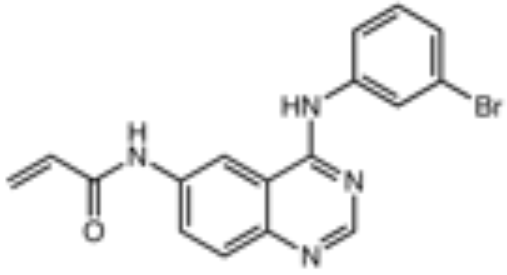
RAF Inhibitors Transactivate RAF Dimers and ERK Signalling in Cells with Wild-type BRAF

- Perhaps RAF inhibitors activate CRAF dimers through transactivation.

active RAF dimer

- A mutant catC (S428C) was generated to test this model with inhibitors JAB13 and JAB34.



JAB34

- catC(S428C) was inhibited by both JAB13 and JAB34 while catC was not.

- Similar to the other RAF inhibitors, low doses (40 nM to 1 μM) induced ERK signalling while higher doses (10 μM) caused inhibition.

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RAF Inhibitors Transactivate RAF Dimers and ERK Signalling in Cells with Wild-type BRAF

■ Two proteins are coexpressed -

V5-catC(S428C/D486N)

catalytically-inactive

able to bind to JAB34 (S428C)

unable to phosphorylate MEK (D486N)

Flag-catC

catalytically-active

unable to bind to JAB34

able to phosphorylate MEK

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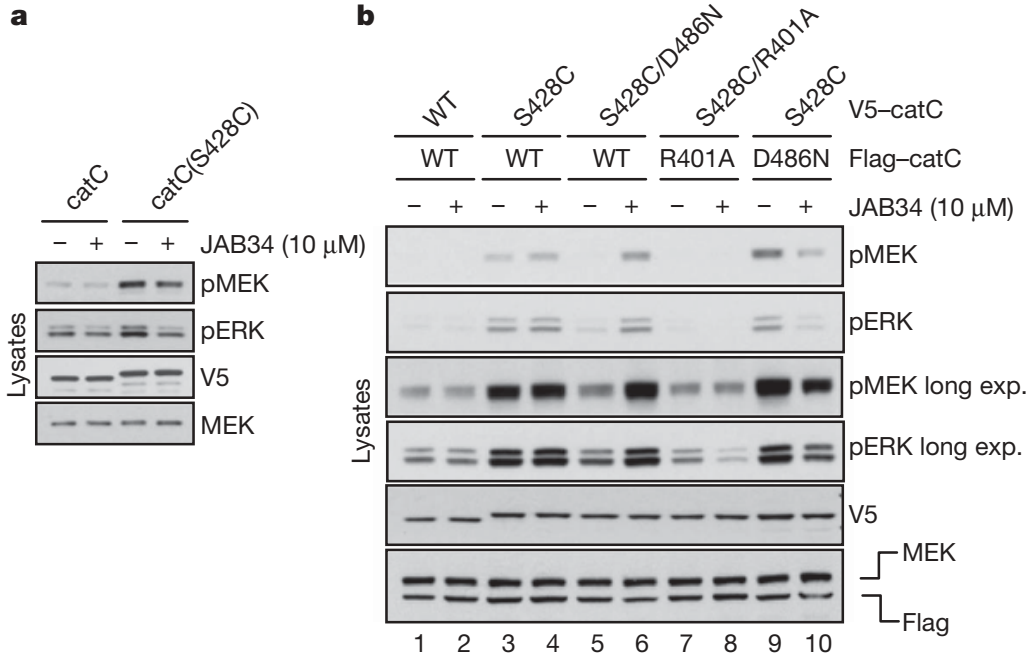
RAF Inhibitors Transactivate RAF Dimers and ERK Signalling in Cells with Wild-type BRAF

- High concentrations of JAB34 with catC(S428C) inhibit ERK signalling (a).
- There is a marked induction in ERK signalling when the two proteins are coexpressed (b).



- The same behavior is seen when Flag-BRAF or CRAF is coexpressed with V5-catC(S428C/D486N).
- A mutation to disrupt dimer formation (R401A) also results in a reduction of ERK signalling (b, lanes 7 and 8).

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RAF Inhibitors Transactivate RAF Dimers and ERK Signalling in Cells with Wild-type BRAF

■ A transactivation model seems to apply - binding of one RAF protomer activates the other RAF protomer.

■ RAS activation induces ERK signaling through BRAF and CRAF homo- and hetero-dimerization.

■ At low concentrations of RAF inhibitor, one protomer is inactivated from the RAF inhibitor while the other protomer is activated via transactivation.

■ At high concentrations of RAF inhibitor, all of RAF is inhibited and ERK signalling is not observed.

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RAF Inhibitors Transactivate RAF Dimers and ERK Signalling in Cells with Wild-type BRAF

- But why do RAF inhibitors work for cells expressing BRAF(V600E) and not for cells with *wt*BRAF?
- In cells overexpressing BRAF(V600E), ERK signalling was inhibited by PLX4032/PLX4720.
- If mutant RAS is coexpressed, ERK signalling becomes resistant to PLX4032/PLX4720.
- BRAF(V600E) tumors are believed to have insufficient levels of activated RAS.

- **Interesting clinical implications -**

  MEK inhibitors block ERK signaling in all tumor and normal cells, leading to toxicity.

  RAF inhibitors can have useful or deleterious effects, depending upon the circumstance.

  The absence of ERK inhibition in normal cells could allow higher doses of RAF inhibitor.

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Questions?