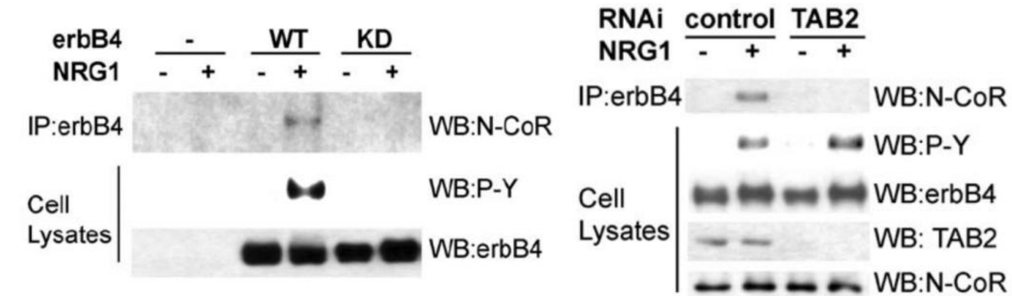
Advanced Cell Biology and Biotechnology

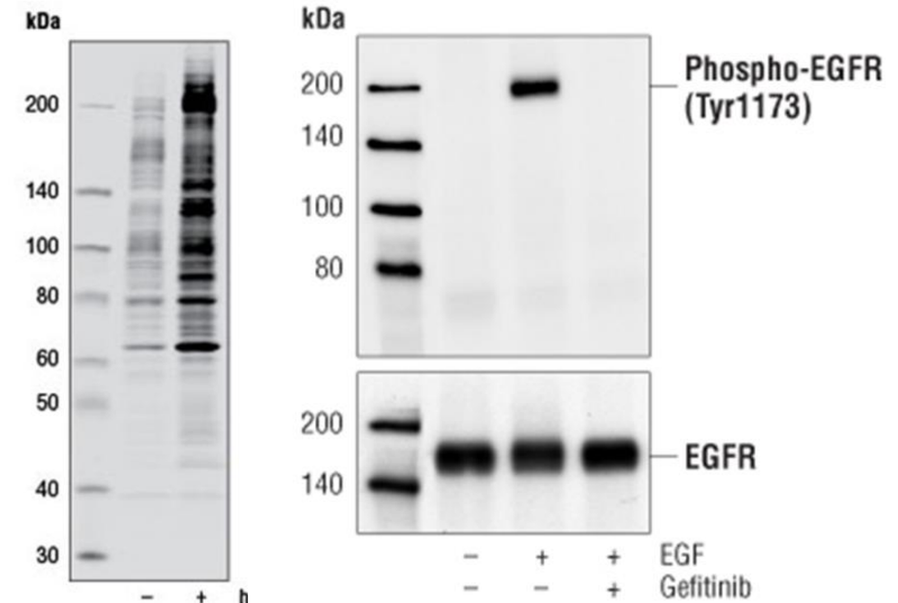
ACBB 2021/22

Summary of the previous lesson

- real examples and exercizes of co-IP to identify the proteins involved in a complex and the domain responsible of the interaction



- phosphorylation analysis using antibodies for generic phospho-tyrosines (p-Y) or for specific phosphotyrosines



How to study the activity of a kinase

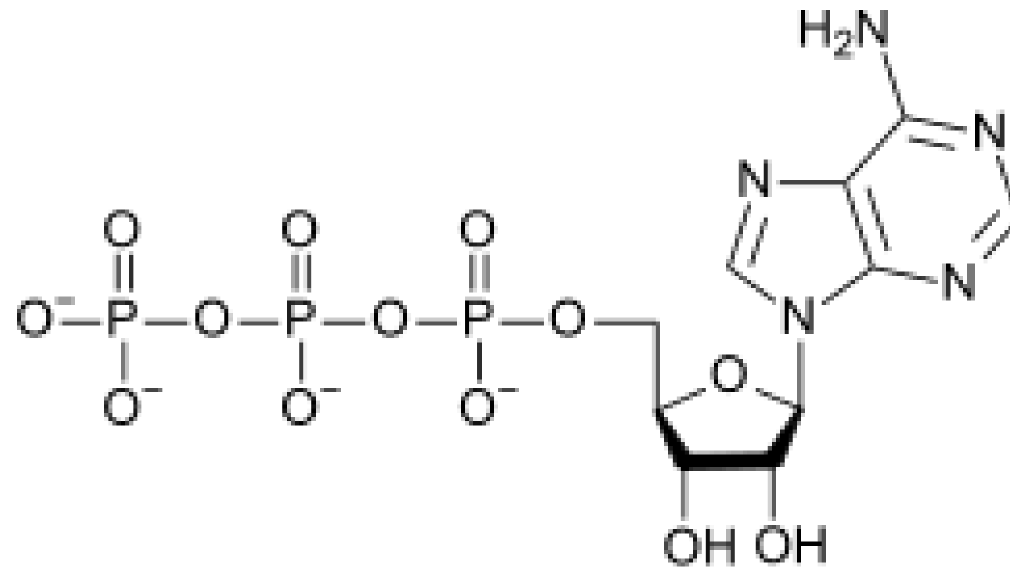
- phosphorylation of its substrates (using generic Ab α P-Y or Ab α specific phospho-tyrosines)
- kinase assay

How to study the role of a specific tyrosine (or serine or threonine)

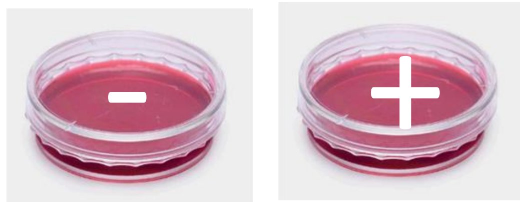
- use of phosphomimetics

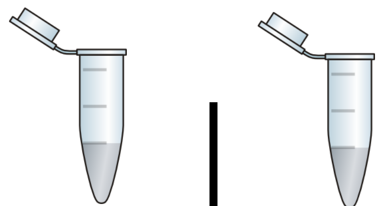
How to study the activity of a kinase

- phosphorylation of its substrate (using generic Ab α P-Y or Ab α specific phospho-tyrosines)
- **kinase assay**



Does my protein of interest have kinase activity?
Does a kinase interact with my protein of interest?





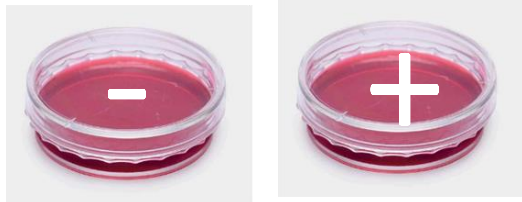
total protein extract

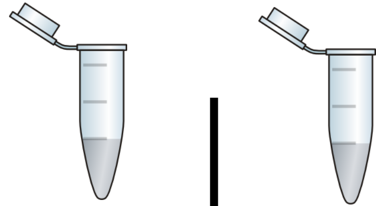
IP α protein of interest

Kinase assay

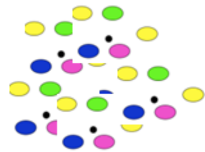
SDS-PAGE

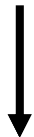
Does my protein of interest have Kinase activity?





total protein extract





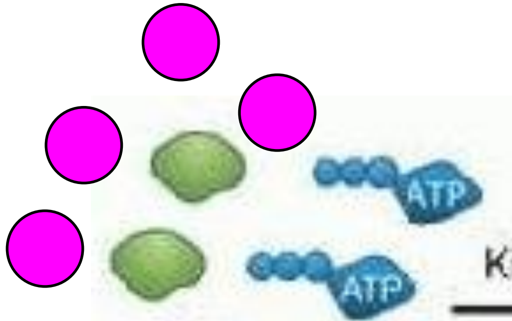
IP α protein of interest

Kinase assay

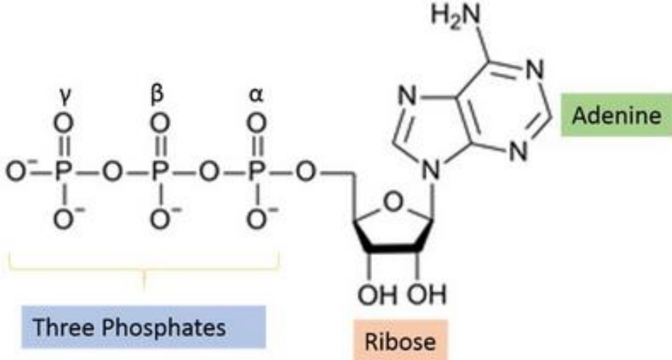
SDS-PAGE

KINASE ASSAY

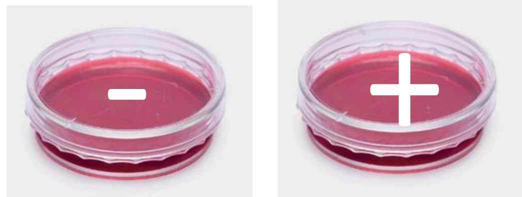
-to the co-IP add the substrate and ATP

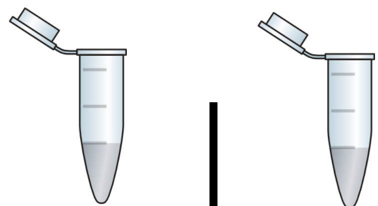


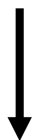
substrate



Does my protein of interest have Kinase activity?

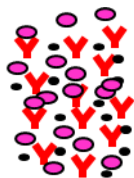






total protein extract

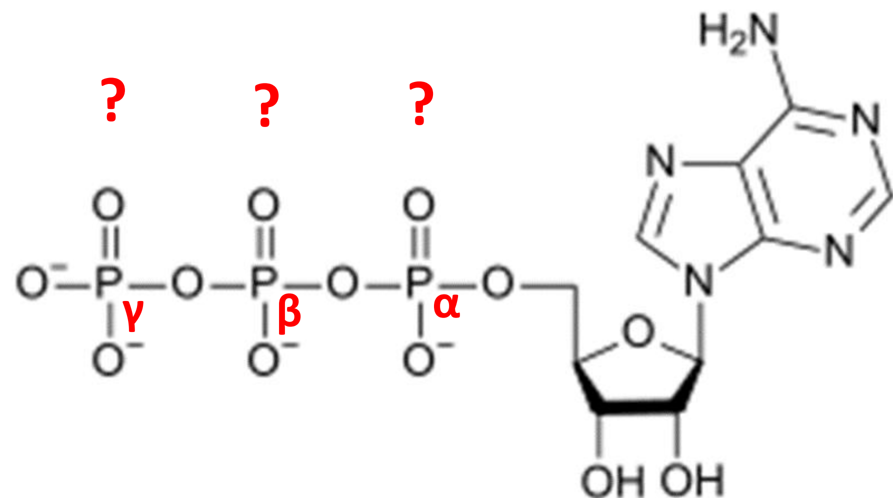
IP α protein of interest



Kinase assay

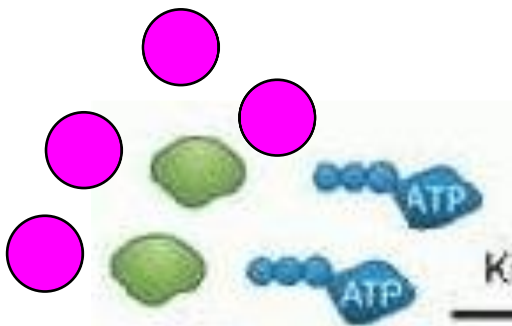
SDS-PAGE

wooclap

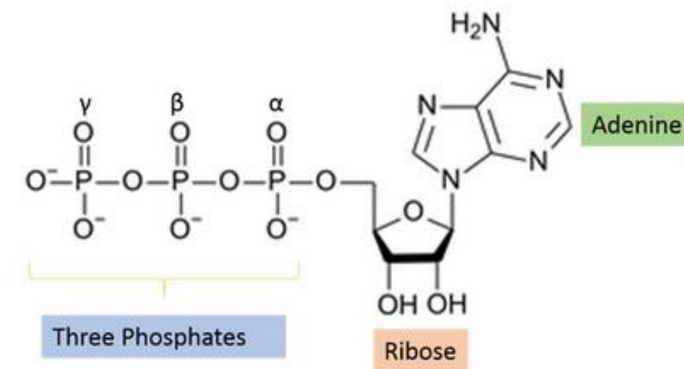


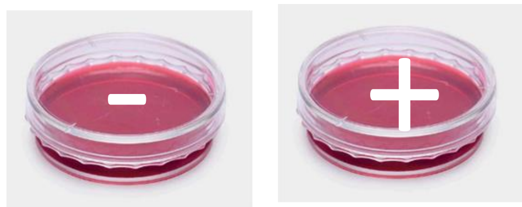
KINASE ASSAY

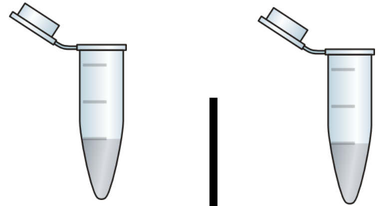
-to the co-IP add the substrate and ATP



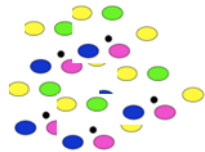
substrate







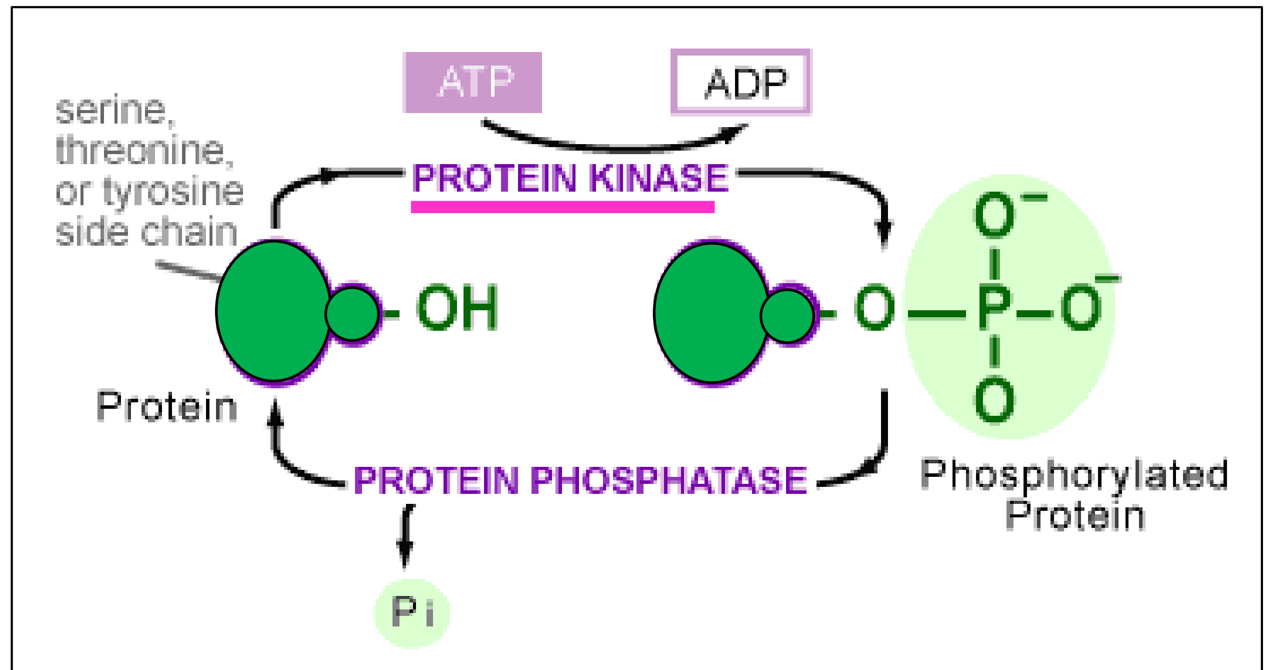
total protein extract



IP α protein of interest

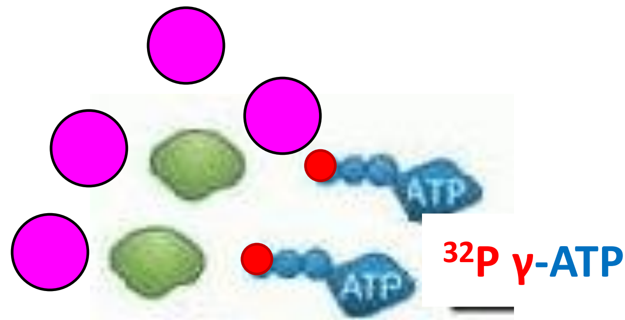
Kinase assay

SDS-PAGE

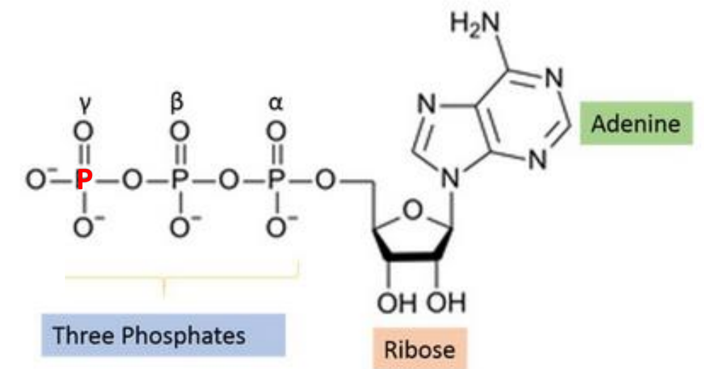


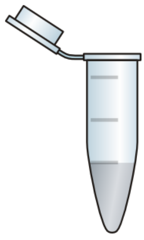
KINASE ASSAY

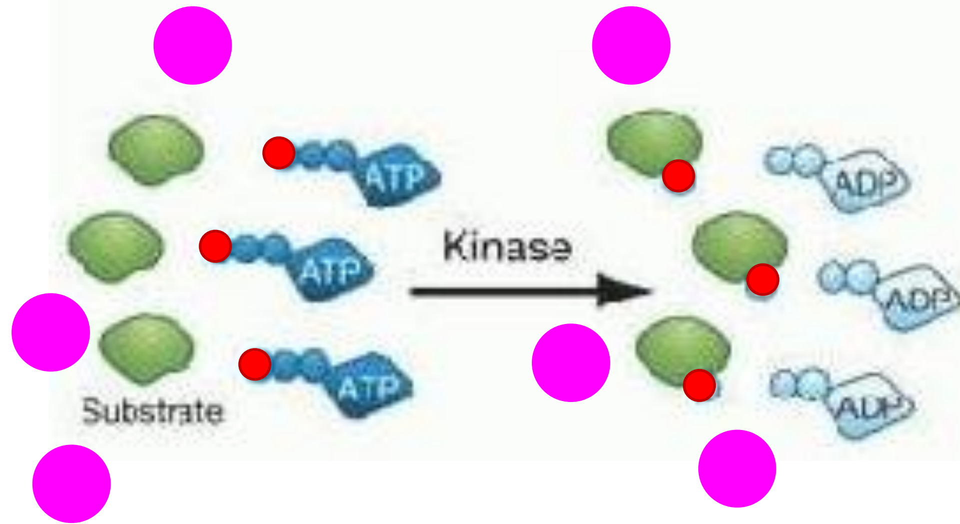
-to the co-IP add the substrate and ^{32}P γ-ATP



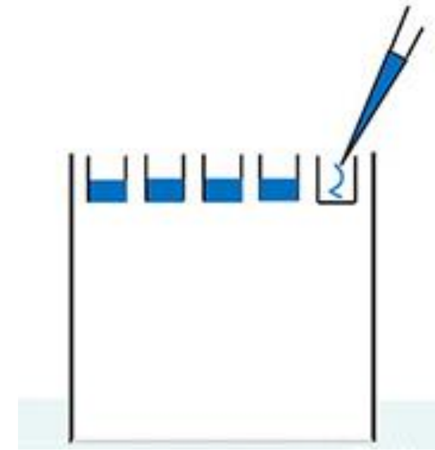
substrate

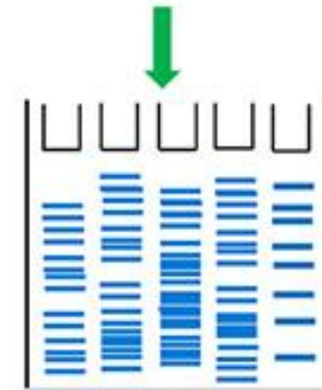


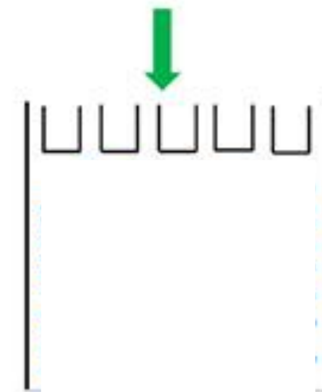


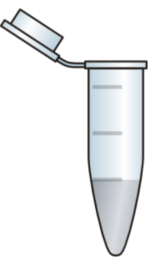
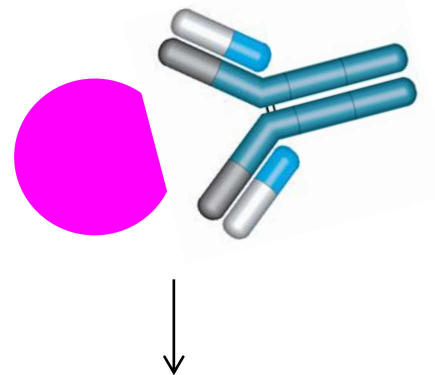


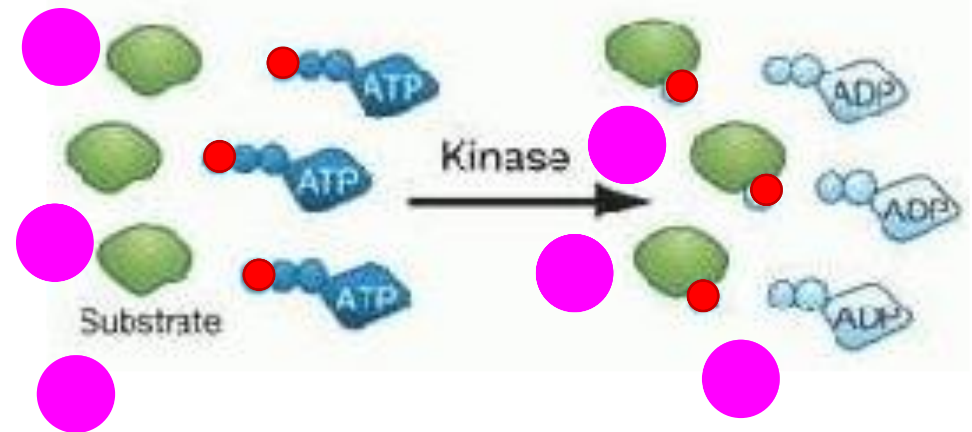
SDS-PAGE





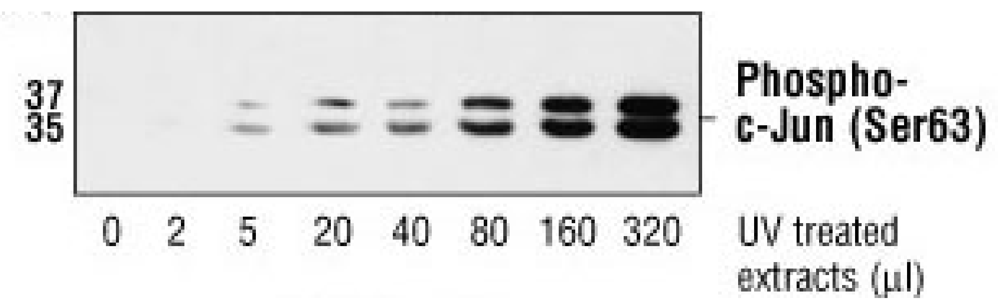


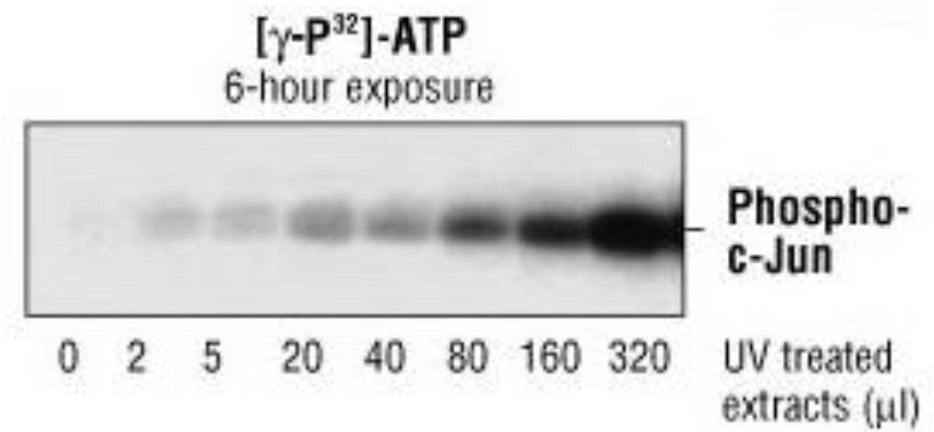


If the substrate is not a protein?

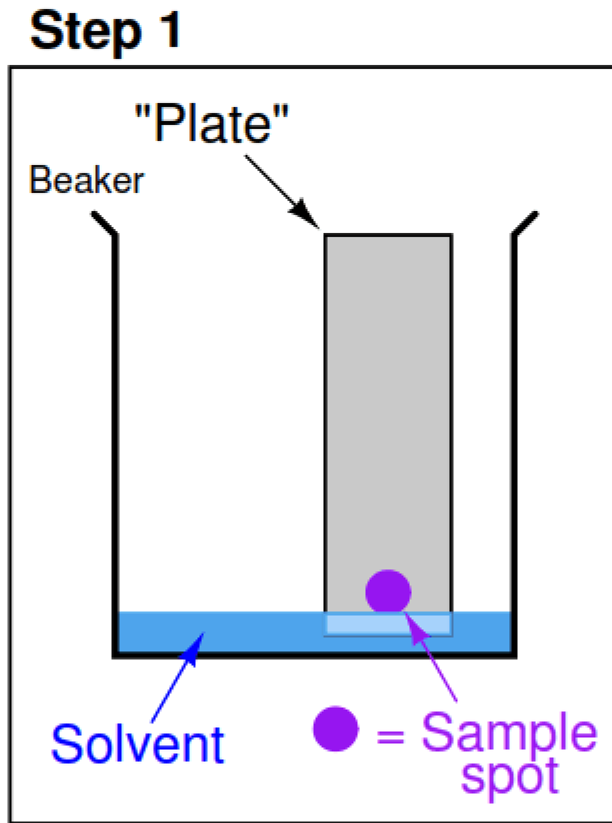
total cell extract -> WB α phospho-c-Jun (Ser63)

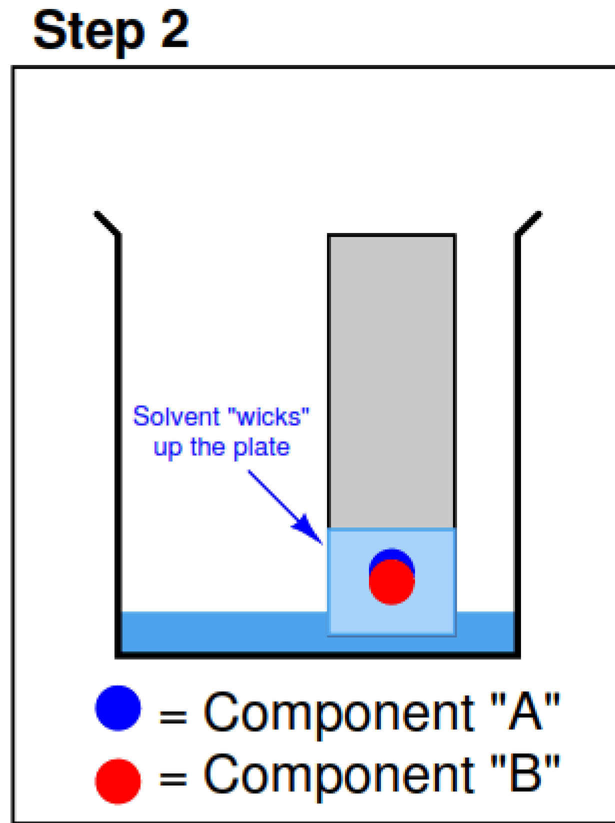


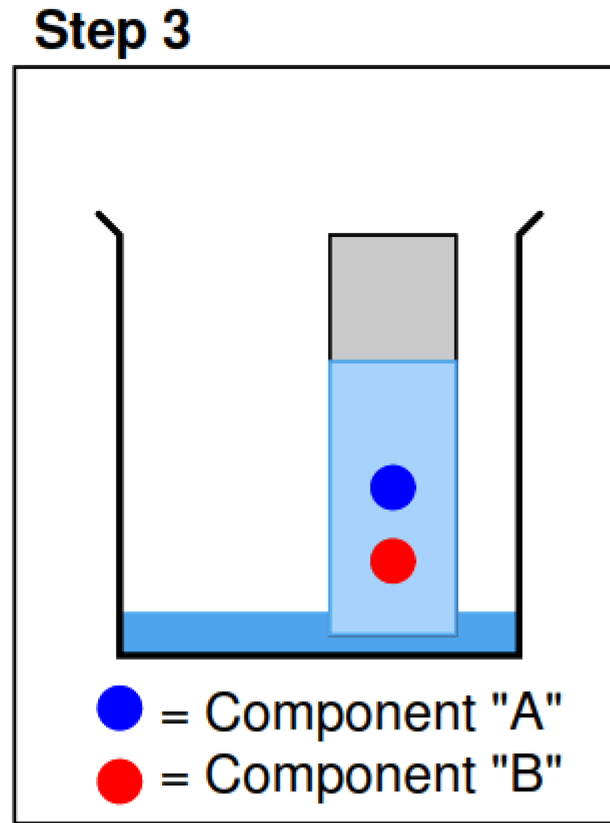
IP α JNK -> Kinase assay

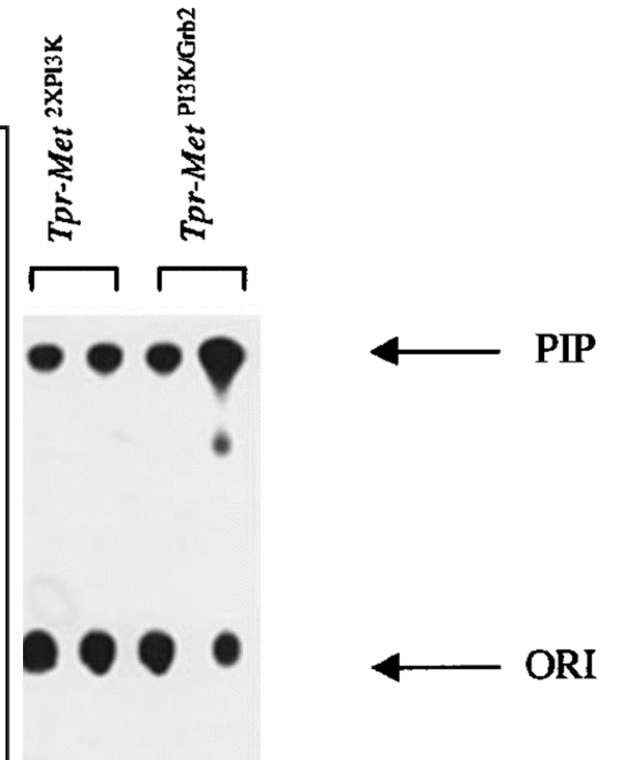


Thin layer cromatography







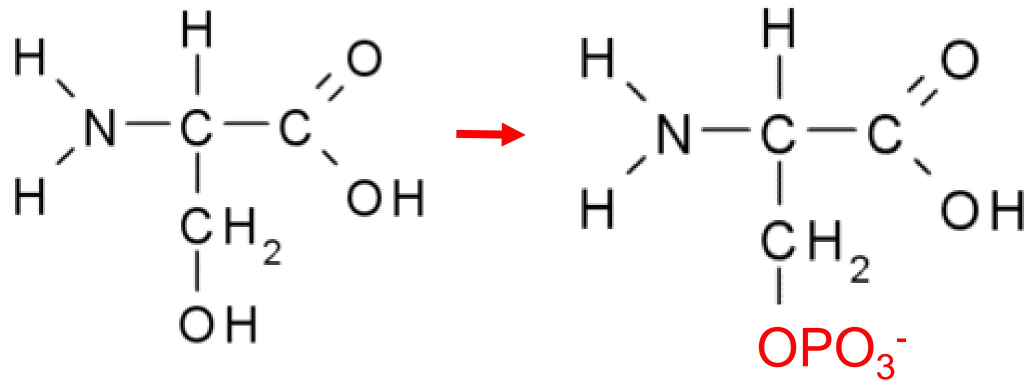


How to study the activity of a kinase

- analysis of the phosphorylation of its substrate
- kinase assay

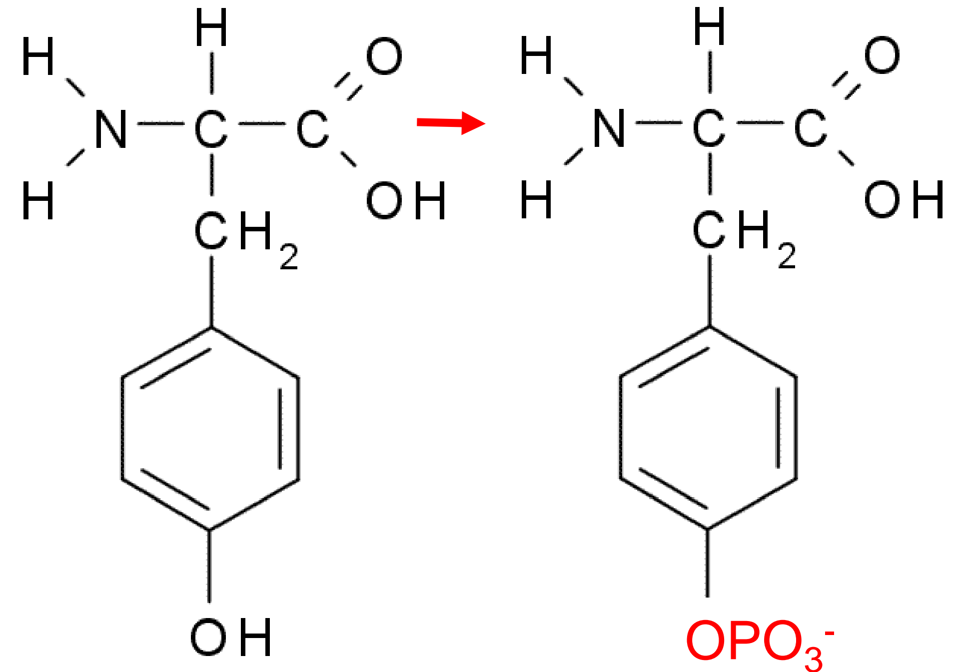
How to study the role of a specific tyrosine (or serine or threonine)

- **phosphomimetics**



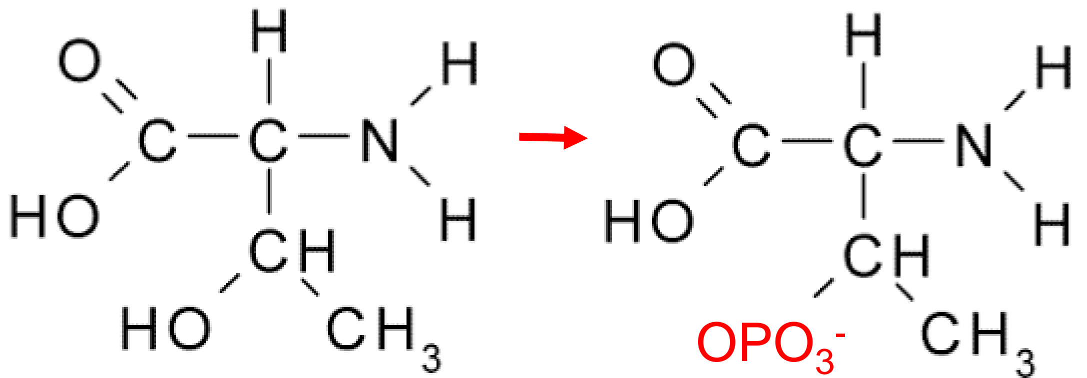
serine

phospho-serine



thyrosine

phospho-thyrosine

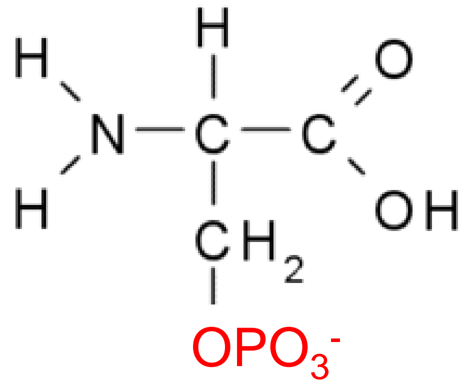


threonine

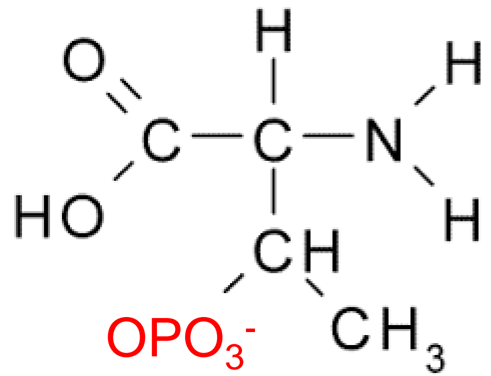
phospho-threonine

Phosphorylation is a common mode of activating or deactivating a protein as a form of regulation.

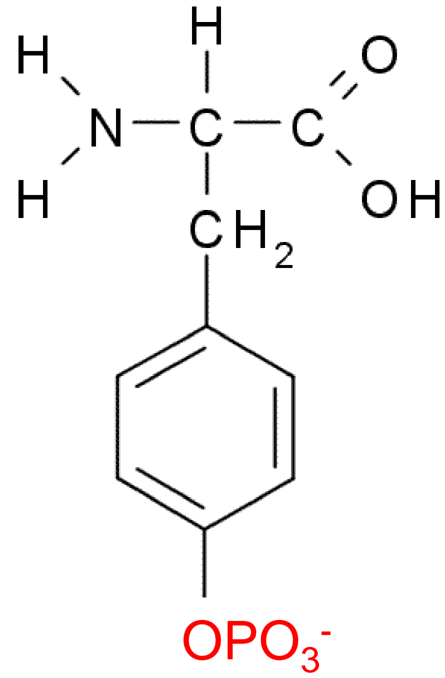
Within cells, proteins are commonly modified at **serine**, **tyrosine** and **threonine** amino acids by adding a phosphate group.



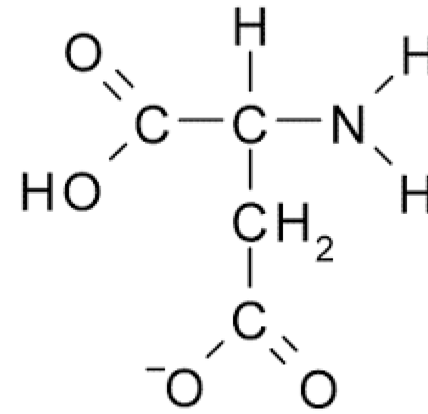
phospho-serine



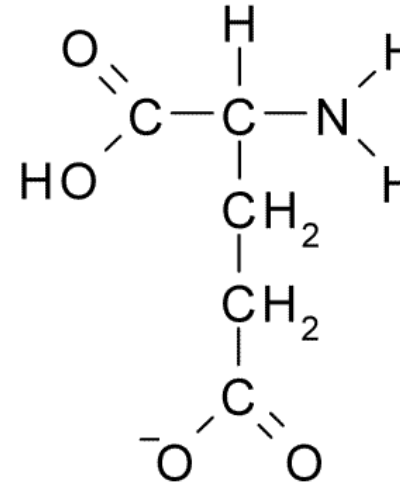
phospho-threonine



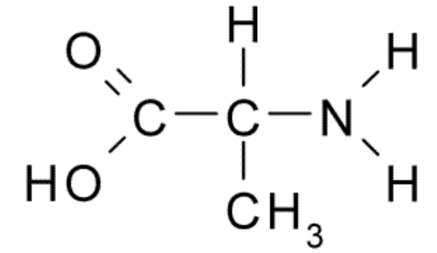
phospho-thyrosine



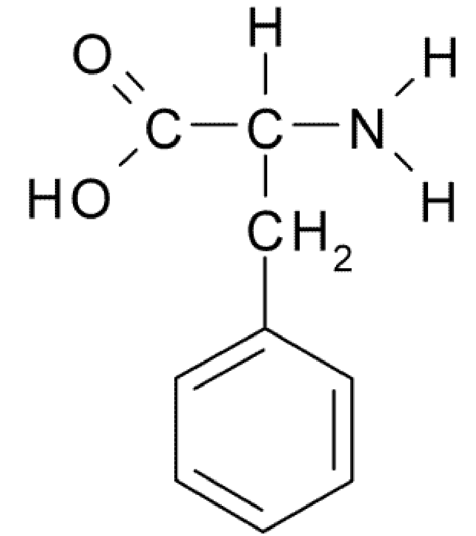
aspartate/
aspartic acid



glutamate/
glutamic acid



alanine

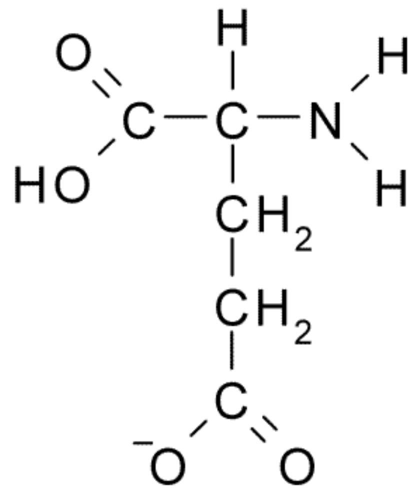


phenylalanine

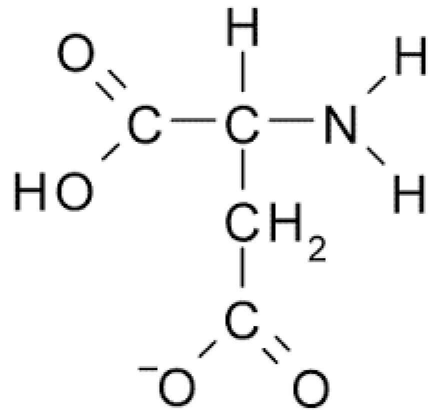
Phosphomimetics

Some amino acids appear chemically similar to phosphorylated amino acids. **Phosphomimetics** are amino acid substitutions that mimic a phosphorylated protein, thereby activating (or deactivating) the protein.

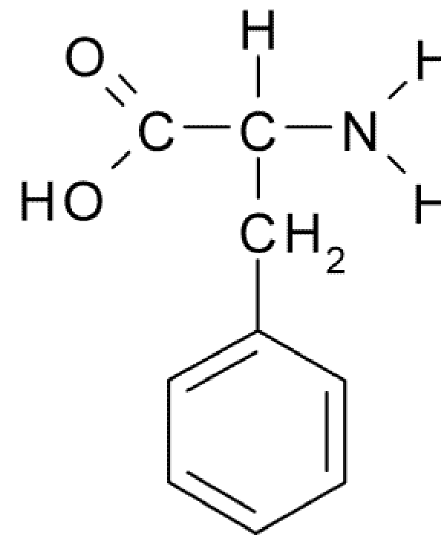
For example, **aspartic acid** and **glutamate** are used to obtain **pseudophosphorylation** while **alanine** and **phenylalanine** are used to obtain **not-phosphorylable aminoacids**.



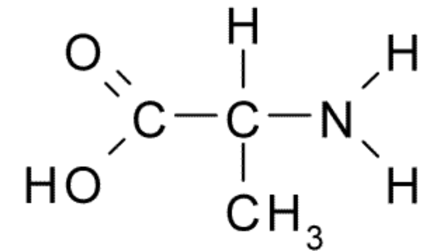
glutamate/
glutamic acid



aspartate/
aspartic acid

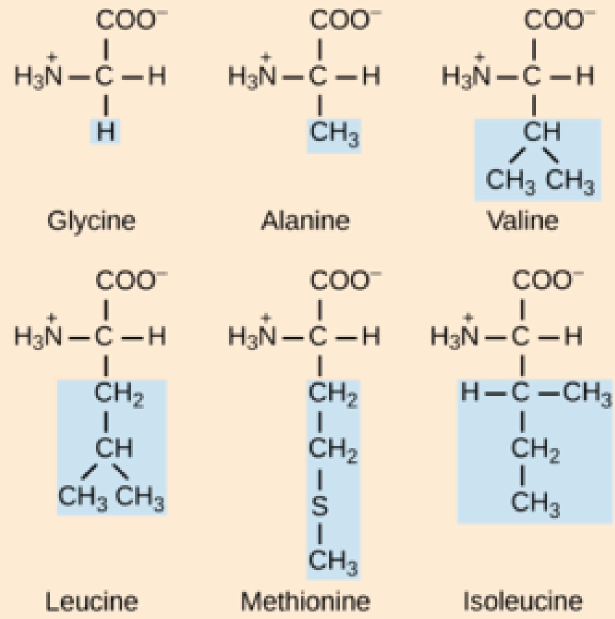


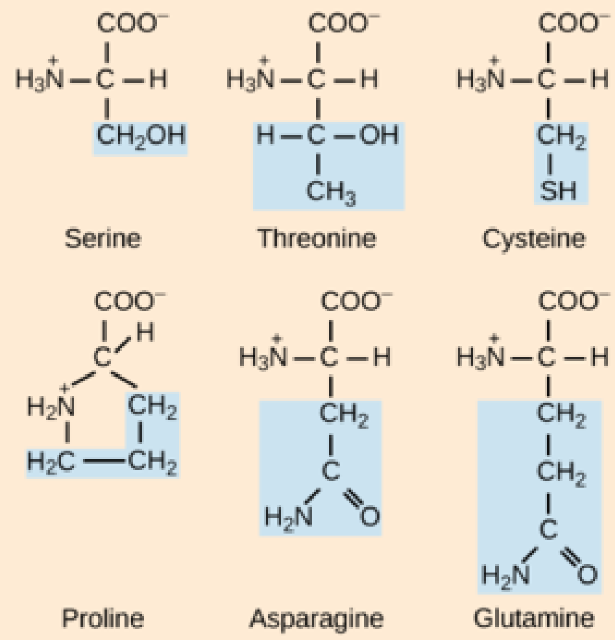
phenylalanine

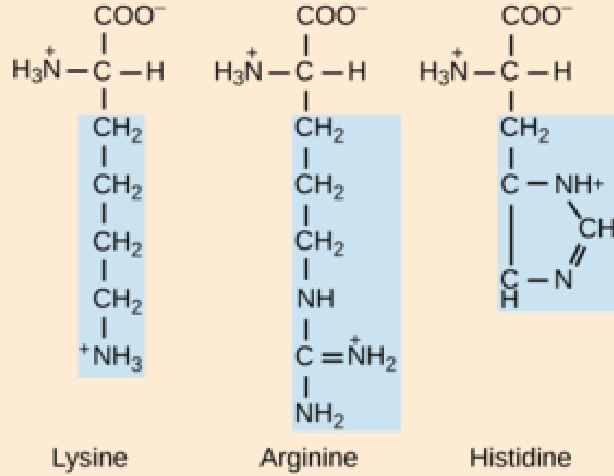


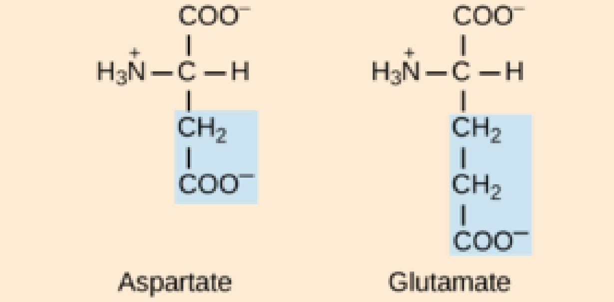
alanine

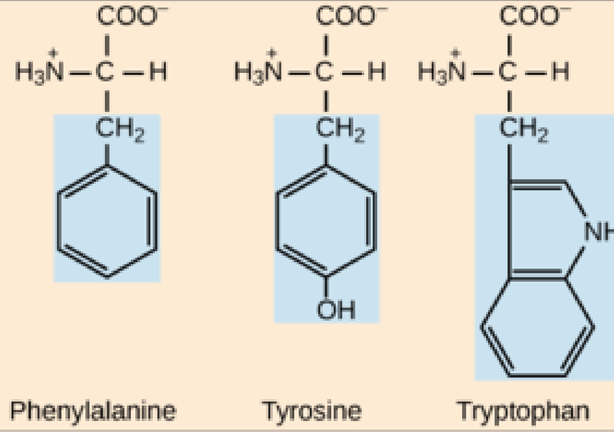
AMINO ACID	AMINO ACID











Second Letter

1st letter	U		C		A		G		3rd letter
U	UUU UUC UUA UUG	Phe Leu	UCU UCC UCA UCG	Ser	UAU UAC UAA UAG	Tyr Stop Stop	UGU UGC UGA UGG	Cys Stop Trp	U C A G
C	CUU CUC CUA CUG	Leu	CCU CCC CCA CCG	Pro	CAU CAC CAA CAG	His Gln	CGU CGC CGA CGG	Arg	U C A G
A	AUU AUC AUA AUG	Ile Met	ACU ACC ACA ACG	Thr	AAU AAC AAA AAG	Asn Lys	AGU AGC AGA AGG	Ser Arg	U C A G
G	GUU GUC GUA GUG	Val	GCU GCC GCA GCG	Ala	GAU GAC GAA GAG	Asp Glu	GGU GGC GGA GGG	Gly	U C A G

Ala = Alanine (A)
Arg = Arginine (R)
Asn = Asparagine (N)
Asp = Aspartate (D)
Cys = Cysteine (C)
Gln = Glutamine (Q)
Glu = Glutamate (E)
Gly = Glycine (G)
His = Histidine (H)
Ile = Isoleucine (I)
Leu = Leucine (L)
Lys = Lysine (K)
Met = Methionine (M)
Phe = Phenylalanine (F)
Pro = Proline (P)
Ser = Serine (S)
Thr = Threonine (T)
Trp = Tryptophan (W)
Tyr = Tyrosine (Y)
Val = Valine (V)

Standard genetic code

1st base	2nd base: U		C		A		G		3rd base
	U		C		A		G		
U	UUU	(Phe/F) Phenylalanine	UCU	(Ser/S) Serine	UAU	(Tyr/Y) Tyrosine	UGU	(Cys/C) Cysteine	U
	UUC		UCC		UAC		UGC		C
	UUA	(Leu/L) Leucine	UCA		UAA	Stop (*Ochre*)[B]	UGA	Stop (*Opal*)[B]	A
	UUG[A]		UCG		UAG	Stop (*Amber*)[B]	UGG	(Trp/W) Tryptophan	G
C	CUU		CCU	(Pro/P) Proline	CAU	(His/H) Histidine	CGU	(Arg/R) Arginine	U
	CUC		CCC		CAC		CGC		C
	CUA		CCA		CAA	(Gln/Q) Glutamine	CGA		A
	CUG[A]		CCG		CAG		CGG		G
A	AUU	(Ile/I) Isoleucine	ACU	(Thr/T) Threonine	AAU	(Asn/N) Asparagine	AGU	(Ser/S) Serine	U
	AUC		ACC		AAC		AGC		C
	AUA		ACA		AAA	(Lys/K) Lysine	AGA	(Arg/R) Arginine	A
	AUG[A]	(Met/M) Methionine	ACG		AAG		AGG		G
G	GUU	(Val/V) Valine	GCU	(Ala/A) Alanine	GAU	(Asp/D) Aspartic acid	GGU	(Gly/G) Glycine	U
	GUC		GCC		GAC		GGC		C
	GUA		GCA		GAA	(Glu/E) Glutamic acid	GGA		A
	GUG		GCG		GAG		GGA		G

UAU
UAC
Tyr/Y
→
UUU
UUC
Phe/F

UAU
UAC
Tyr/Y
→
GAU
GAC
Asp/D
→
GAA
GAG
Glu/E

ACU
ACC
ACA
ACG
Thr/T
→
GCU
GCC
GCA
GCG
Ala/A

ACU
ACC
ACA
ACG
Thr/T
→
GAU
GAC
Asp/D
→
GAA
GAG
Glu/E

UCU
UCC
UCA
UCG
Ser/S
→
GCU
GCC
GCA
GCG
Ala/A

UCU
UCC
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UCG
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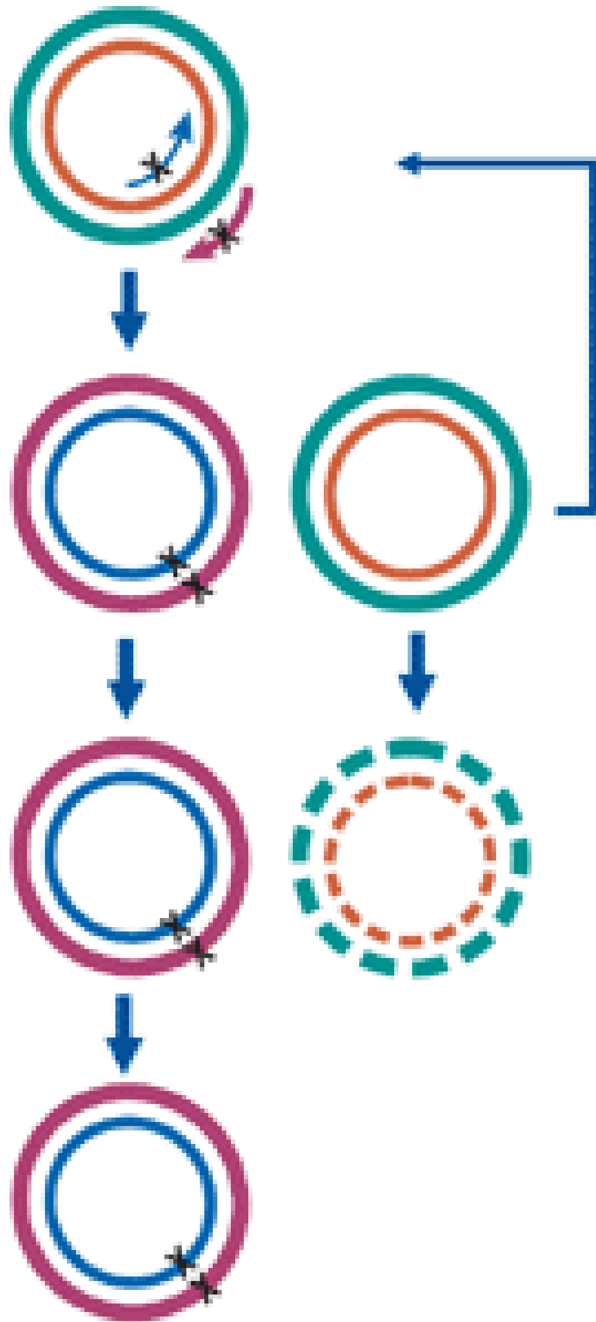
→
GAU
GAC
Asp/D
→
GAA
GAG
Glu/E



1. Mutant strand synthesis

Perform thermal cycling to:
- denature DNA template
- anneal mutagenic primers containing desired mutation
- extend and incorporate primers with *PfuUltra* DNA polymerase

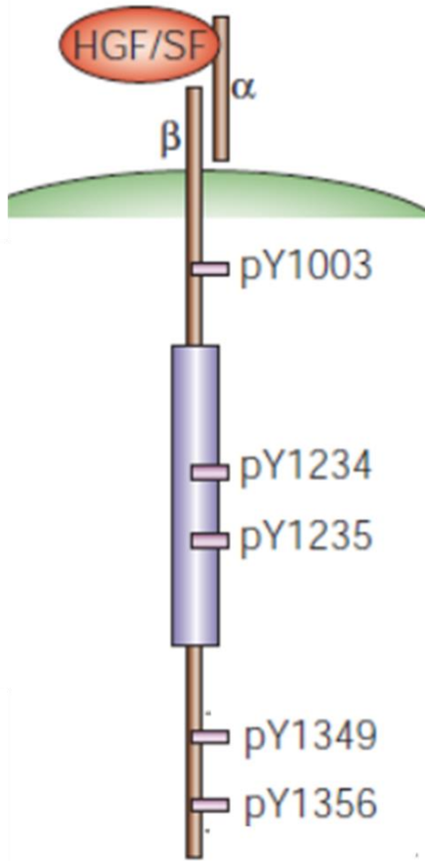
2. *DpnI* digestion of template

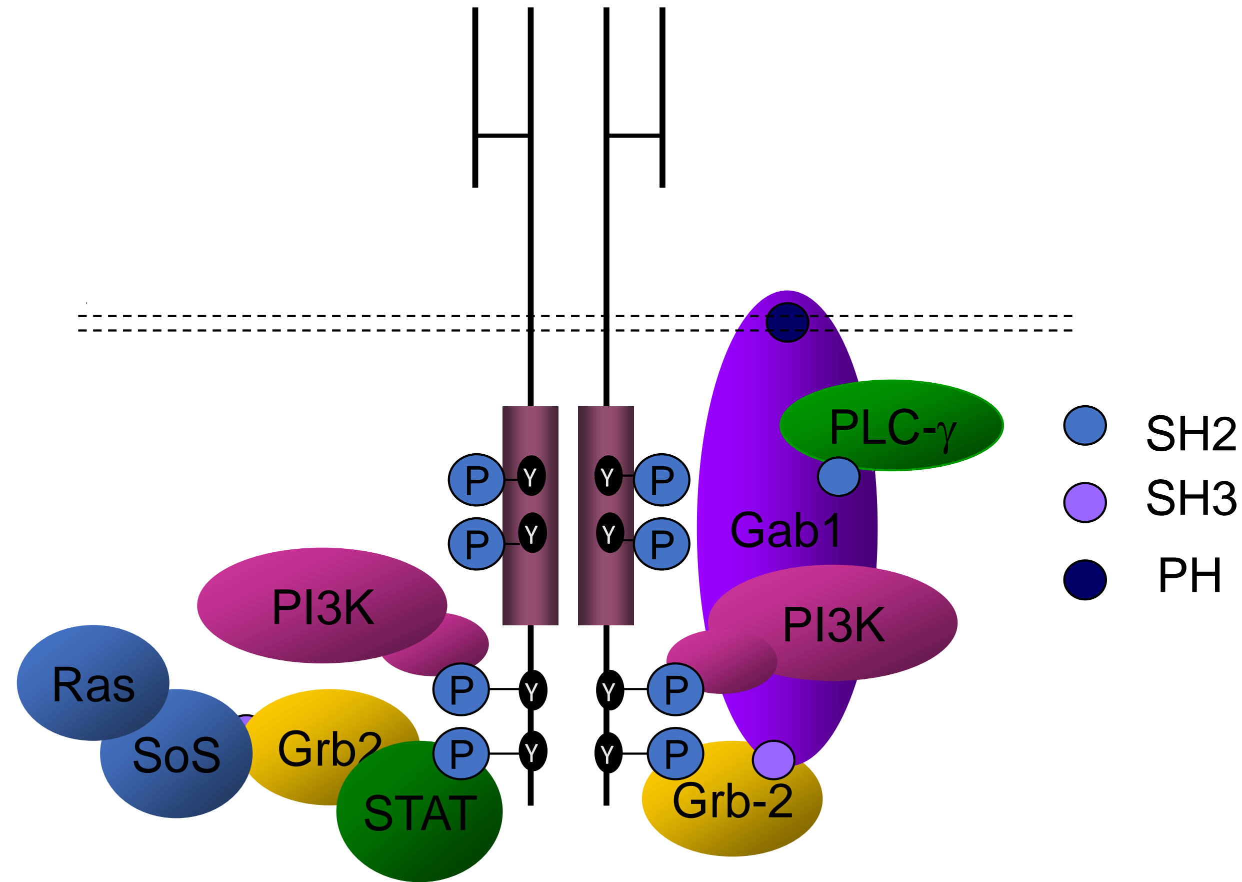
Digest parental methylated and hemimethylated DNA with *DpnI*

3. Transformation

Transform mutated molecule into competent cells for nick repair

DpnI cleaves only when its recognition site is methylated. DNA purified from a dam^+ strain will be a substrate for *DpnI*.





wooclap

Please describe in a very schematic manner how would you test if tyrosine 1349 of the Met oncogene is involved in the interaction between Met and PI3K following stimulation with HGF/SF (Ex: "transfect cells with..."; "extract proteins"; ...).

Please, indicate how many cell plates are necessary for the entire experiment and try to imagine the results you will obtain if the hypothesis is correct.

Please describe in a very schematic manner how would you test if tyrosine 1349 of the Met oncogene is involved in the interaction between Met and PI3K following stimulation with HGF/SF

A possible longer answer:

To answer this question we need 4 plates, two transfected with an expression vector for Met wild type (WT), two transfected with an expression vector for Met with tyrosine 1349 mutated to phenylalanine (Y1349F).
For each construct (WT and Y1349F) we have a mock sample and a sample stimulated with HGF.
After stimulation we extract the proteins, we save an aliquot of total cell lysate and we perform IP against Met.
Then a SDS-PAGE is carried out with total cell lysates and IP samples, followed by western blot for PI3K and Met.
In the total cell lysate I will verify the expression of Met and PI3K, in the IP I will verify if PI3K interact with Met and if this interaction is abolished when the tyrosine 1349 is mutated to phenylalanine. With WB anti Met I will verify if Met is correctly immunoprecipitated.

Please describe in a very schematic manner how would you test if tyrosine 1349 of the Met oncogene is involved in the interaction between Met and PI3K following stimulation with HGF/SF

A possible more schematic answer:

- 4 plates, two transfected with Met wild type (WT), two transfected with Met Y1349F,
- -/+ HGF treatment
- protein extraction
- IP against Met
- SDS-PAGE on total cell lysates and IP
- western blot for PI3K and Met
  - to verify if PI3K interacts with Met WT
  - to investigate if PI3K interacts or not with Met when tyrosine is mutated to phenylalanine.

If they do not interact when Y 1349 is mutated to F, it suggests that the tyrosine phosphorylation is necessary for PI3K-Met interaction.

MET-WT

transient trasfection

MET-Y1349F

mock + HGF

mock + HGF

total cell lysate (input)

1-input 2-input 3-input 4-input

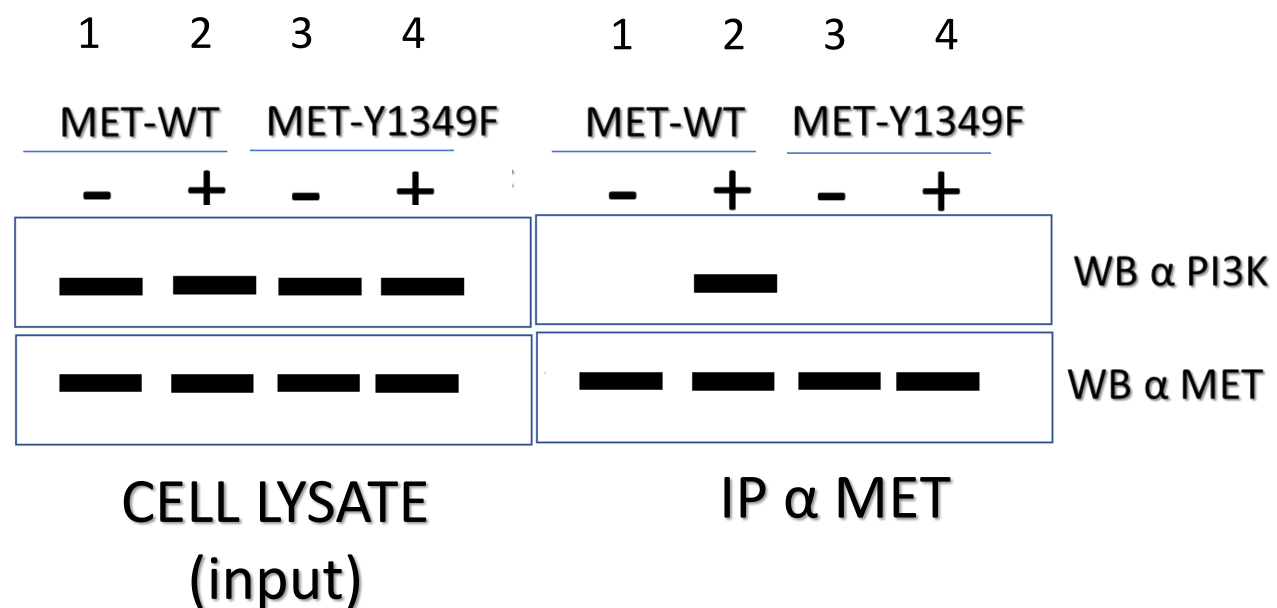
IP α Met

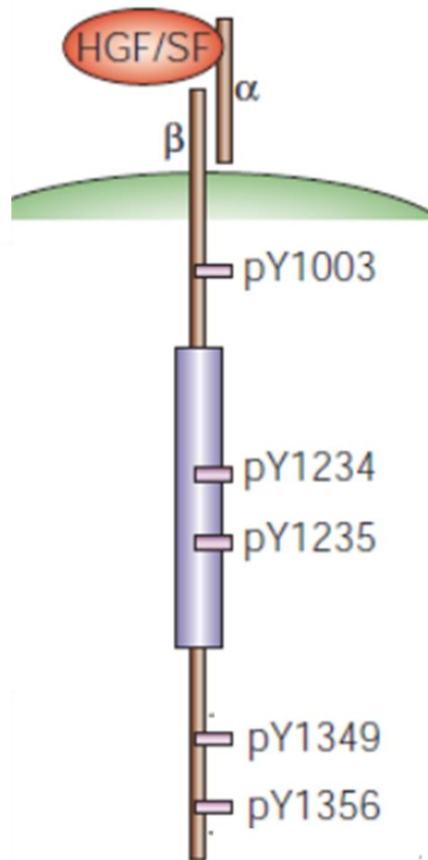
1-IP 2-IP 3-IP 4-IP

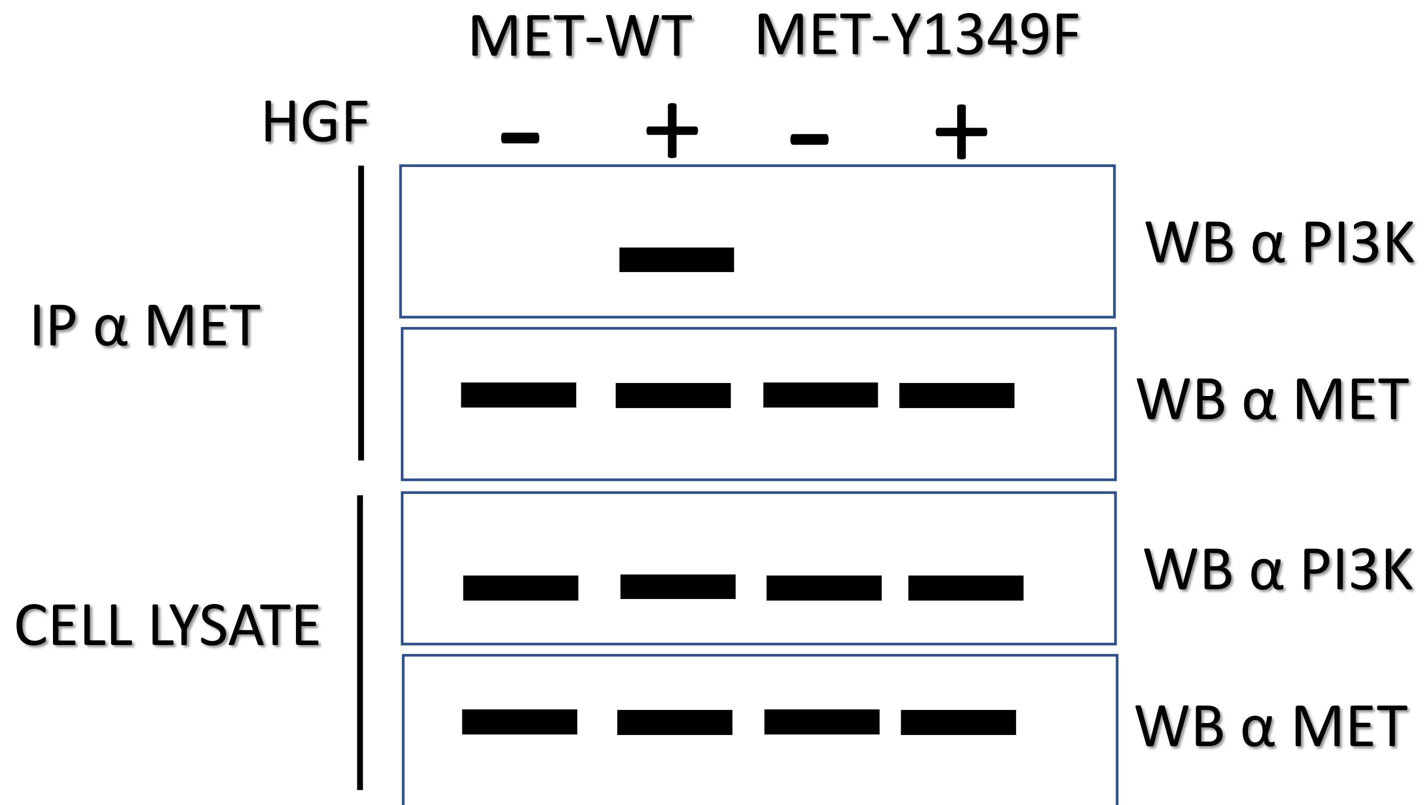
SDS-PAGE

WB α PI3K
WB α Met

In this panel Met and PI3K do not interact when Y 1349 is mutated to F,
thus suggesting that the phosphorylation of tyrosine 1349 is necessary for PI3K-Met interaction.

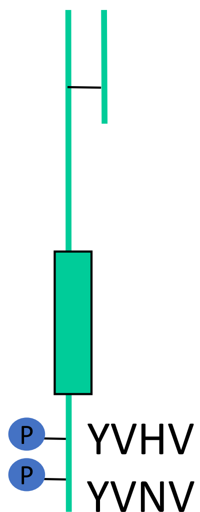



HGF/SF
α
β
pY1003
pY1234
pY1235
pY1349
pY1356


MET-WT
MET-Y1349F
HGF
–
+
–
+
IP α MET
WB α PI3K
WB α MET
CELL LYSATE
WB α PI3K
WB α MET

- identification of pathways involved in transformation and metastasis

Docking site mutants



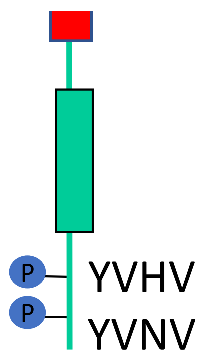
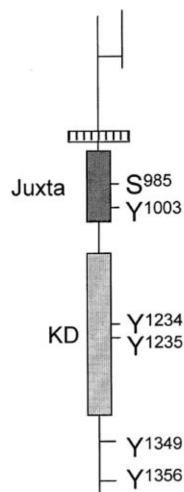
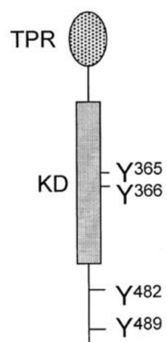


Table 1 *Tpr – Met* signaling mutants designed to preferentially bind Grb2 or PI 3-kinase

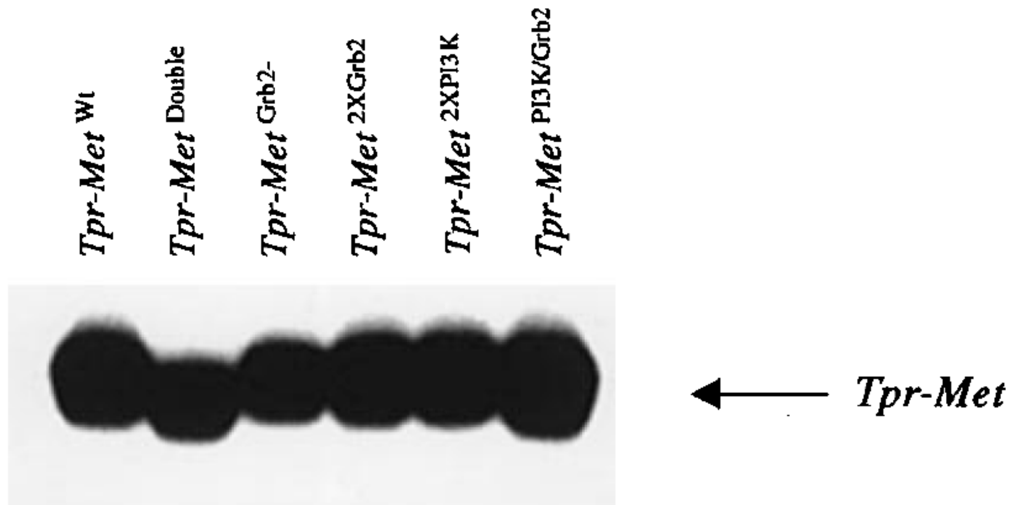
Tpr – Met^{Wt}	YVHVNATYVNV
Tpr – Met^{Double}	FVHVNATFVNV
Tpr – Met^{Grb2-}	YVHVNATYVHV
Tpr – Met$^{2 \times Grb2}$	YVNVNATYVNV
Tpr – Met$^{2 \times PI3K}$	YMPMNATYMDM
Tpr – Met$^{PI3K/Grb2}$	YMPMNATYVNV





Signalling mutants of the oncogenic form of the Met receptor (Tpr-Met) were generated by site-directed mutagenesis. The consensus sequences for the SH2 domains of Grb2 and p85 (the regulatory subunit of PI 3-kinase) were designed according to Songyang et al. (1993). Mutagenized residues are underlined.

Kinase activity of Tpr-Met mutants

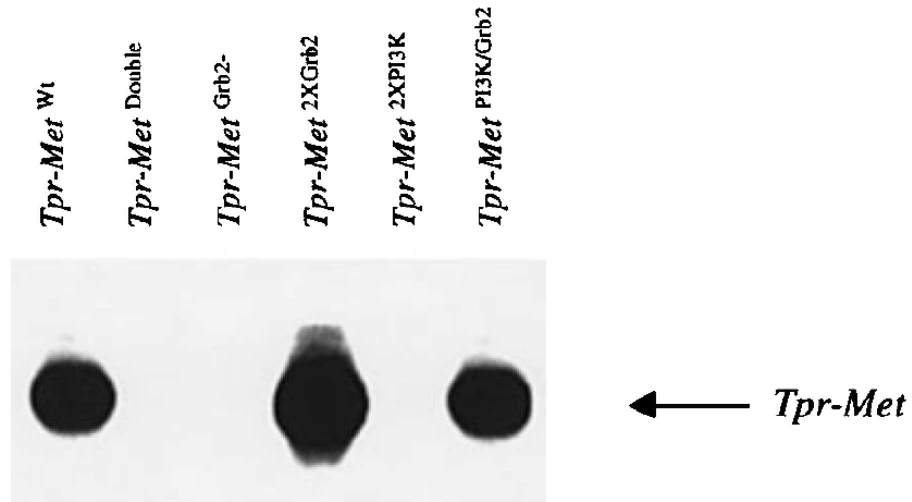


- transient trasfection of COS cells with different Tpr-Met constructs
- protein extraction
- IP anti Met
- kinase assay in vitro (with γ-^{32}P ATP)
- SDS-PAGE
- autoradiography

- the bands are radioactive, because they are phosphorylated with radioactively labeled γ-^{32}P ATP

- wild type and mutant Tpr - Met proteins were immunoprecipitated from COS-1 cells transfected with the corresponding constructs, using antibodies specific for human *Met*
- immunoprecipitated proteins were subjected to *in vitro* kinase assay with [γ-^{32}P]ATP
- labeled proteins were separated on 8% SDS-PAGE

Association of Tpr-Met mutants with Grb2



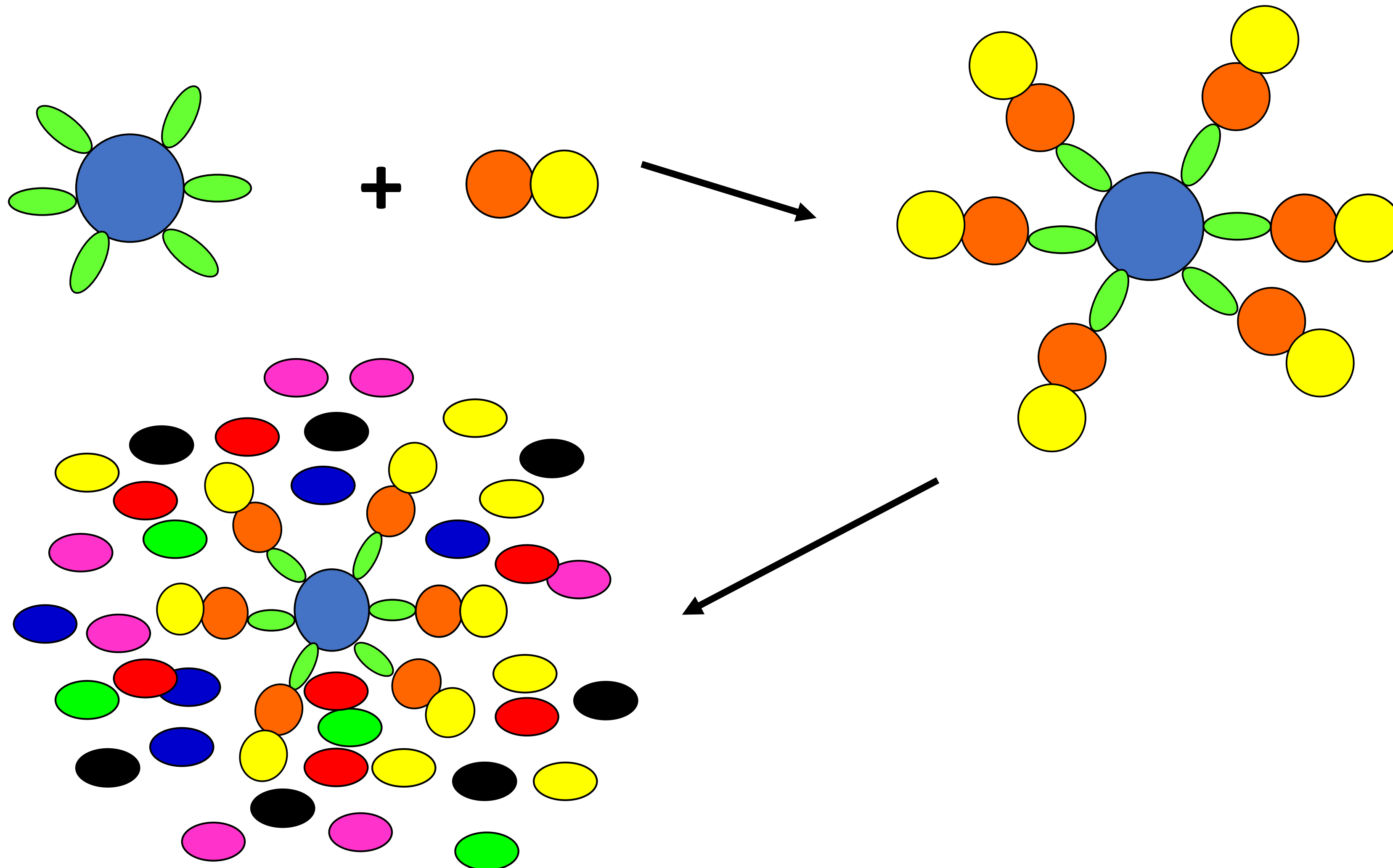
- fused protein: **Grb2-glutathione-transferase,** immobilized on **sepharose-glutathione beads**
- transient transfection of COS cells with various Tpr-Met constructs
- protein extraction
- "**pull down**": extracted proteins incubated together with the beads: some proteins bind to Grb2 and precipitate together with the beads
- kinase assay in vitro on precipitated proteins (with γ-^{32}P ATP)
- SDS-PAGE
- X-ray film exposure
- bands are proteins precipitated together with Grb2 and are radioactive because they are phosphorylated with radioactively labeled ATP

Grb2 fusion protein (approximately 500 ng/point) was immobilized on Glutathione-Sepharose beads and incubated with lysates of COS-1 cells containing comparable amounts of Tpr-Met mutants. Complexes were washed and the amount of Tpr-Met bound to Grb2 was visualized by in vitro kinase assay with [[γ-^{32}P]ATP. Labeled proteins were separated on 8% SDS± PAGE.

PULL-DOWN

Sepharose beads-glutatione

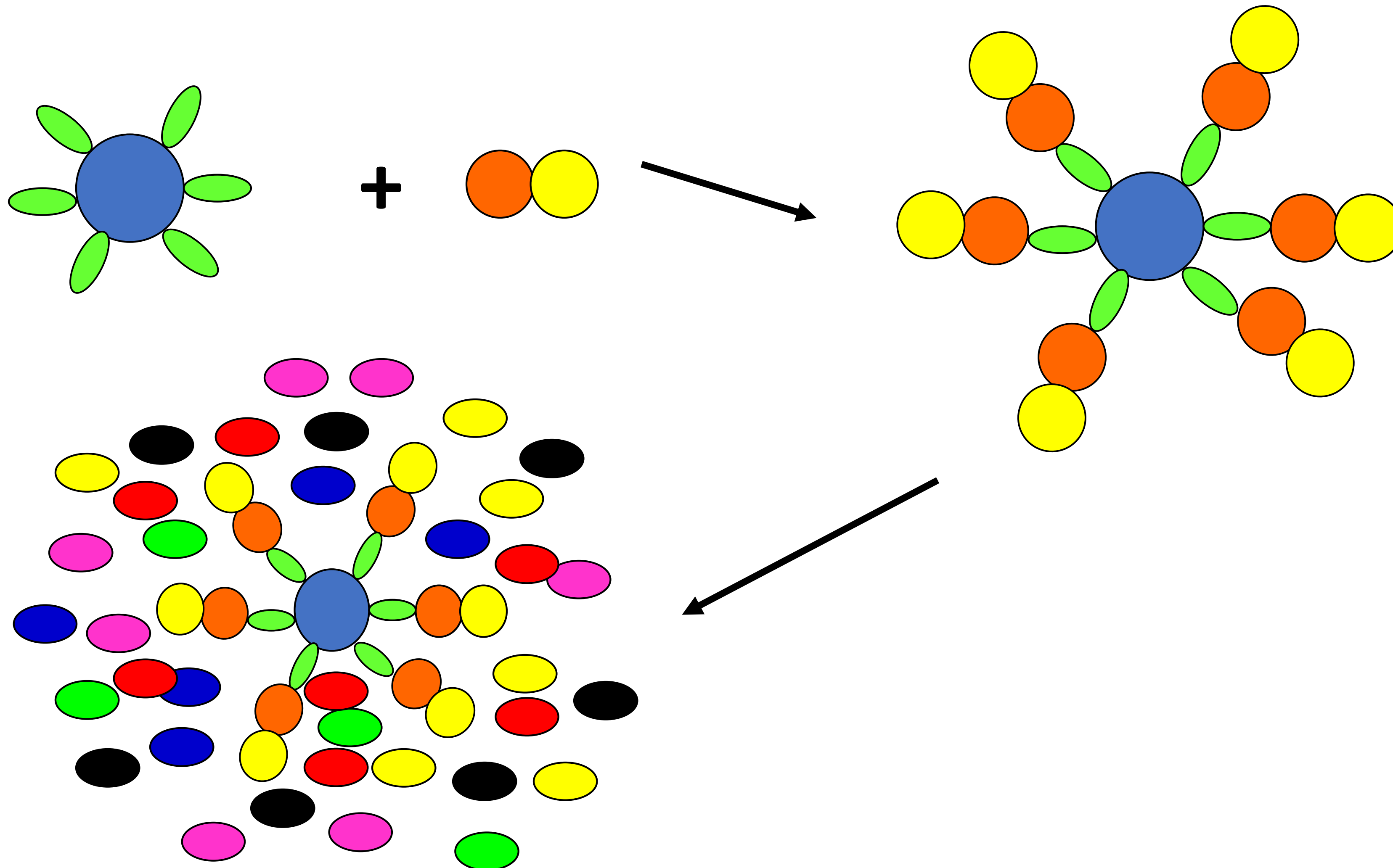
GLUTATIONE TRANSFERASE	**BAIT PROTEIN**



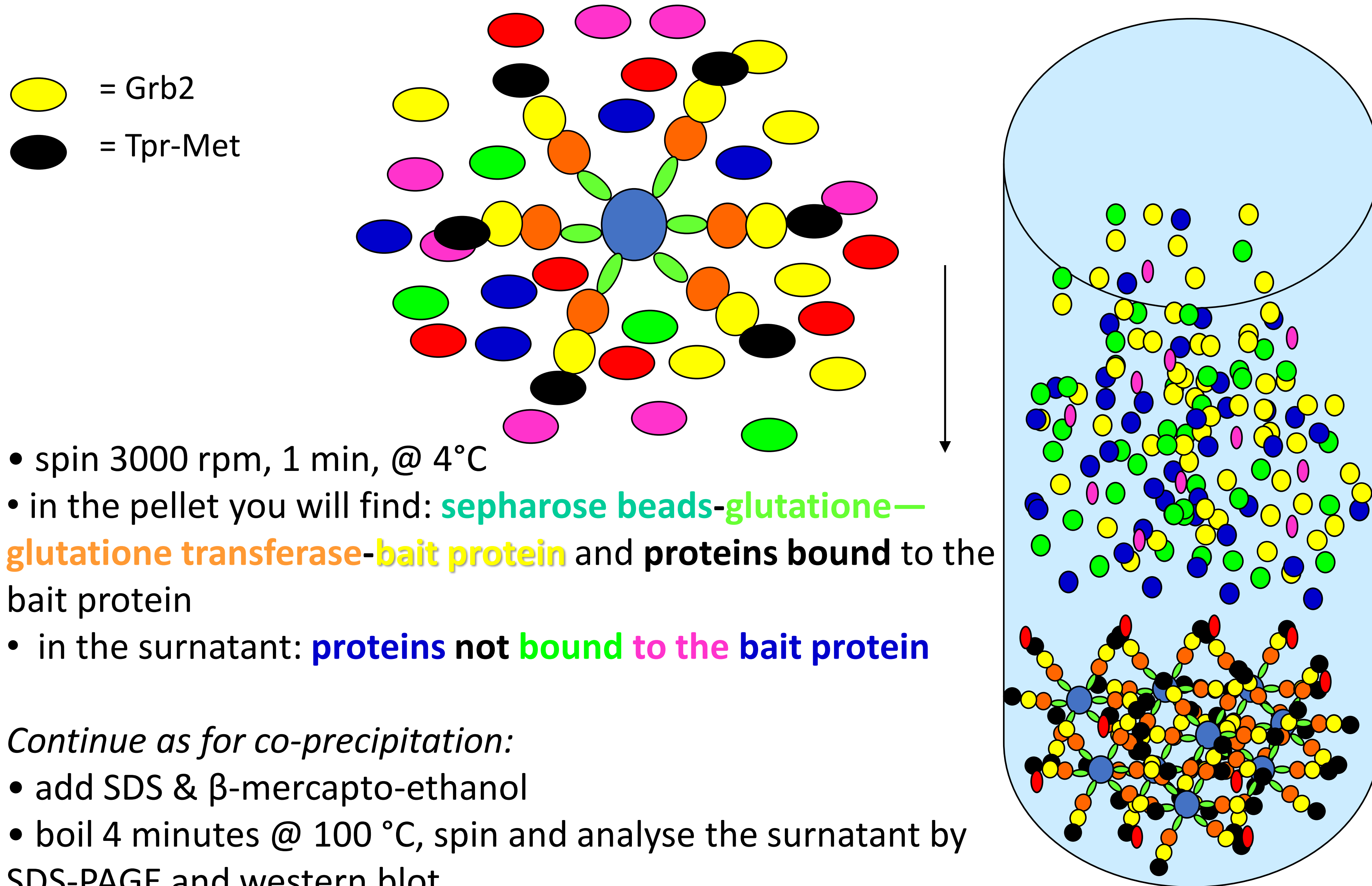
PULL-DOWN

Sepharose beads-glutatione

GLUTATIONE TRANSFERASE	Grb2



• slow rotation over-night @ 4°C in order that the “bait” protein (fused with glutatione transferase) meets all proteins you have in your protein extract



• spin 3000 rpm, 1 min, @ 4°C

• in the pellet you will find: **sepharose beads**-**glutatione—glutatione transferase**-**bait protein** and **proteins bound** to the bait protein

• in the surnatant: **proteins not bound to the bait protein**

Continue as for co-precipitation:

• add SDS & β-mercapto-ethanol

• boil 4 minutes @ 100 °C, spin and analyse the surnatant by SDS-PAGE and western blot