



REPÚBLICA
PORTUGUESA

SAÚDE



15 Outubro 2019

Vias de sinalização na regulação da expressão génica

Mestrado Tecnologias Moleculares em Saúde, ESTeSL

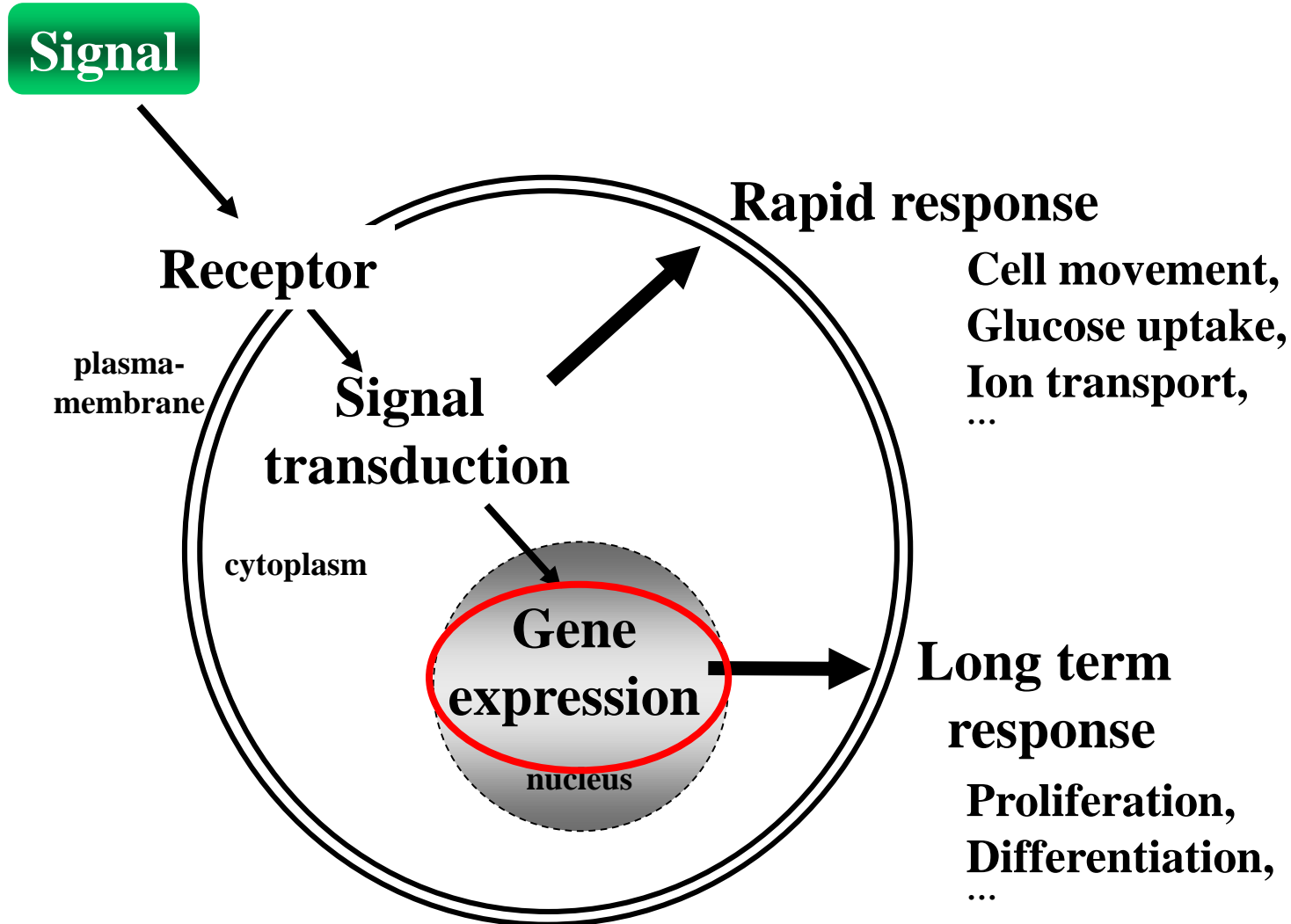
Peter Jordan,

[**\(peter.jordan@insa.min-saude.pt\)**](mailto:peter.jordan@insa.min-saude.pt)

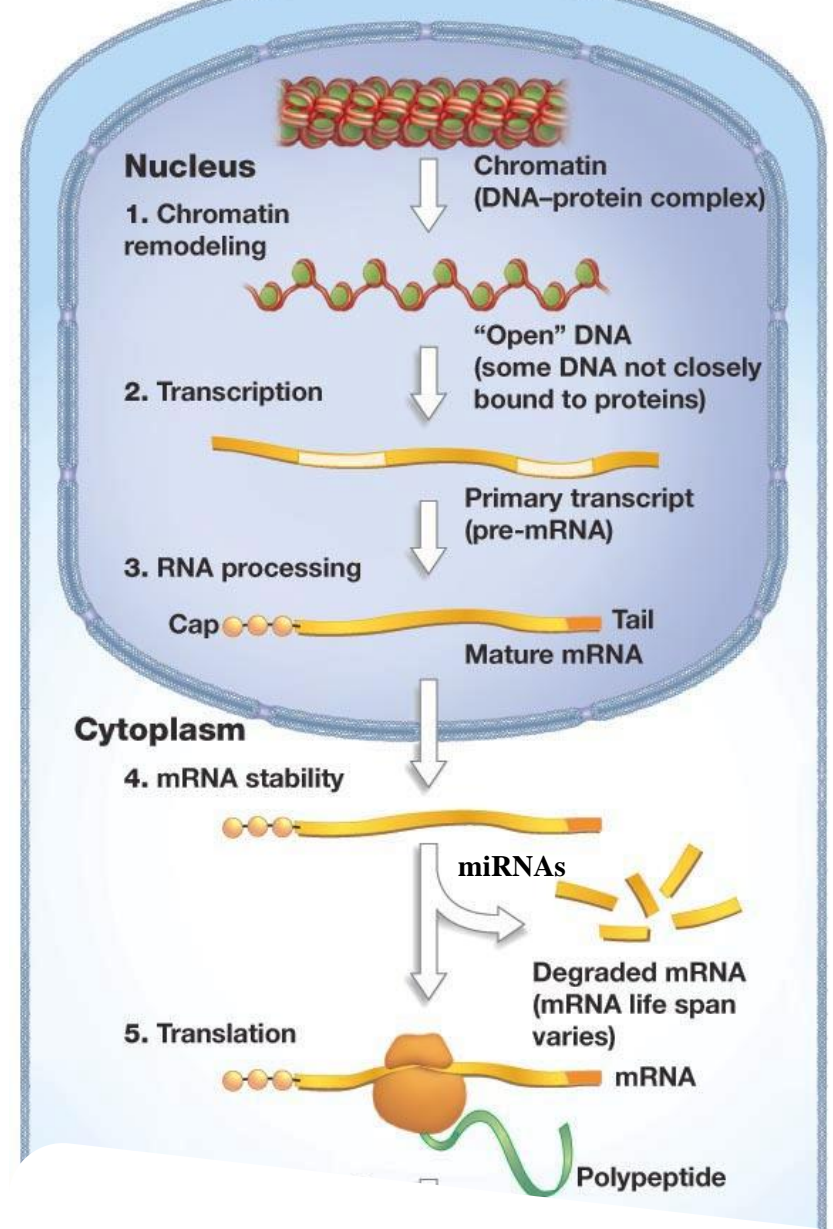
Departamento de Genética Humana,

Instituto Nacional de Saúde Doutor Ricardo Jorge, Lisboa

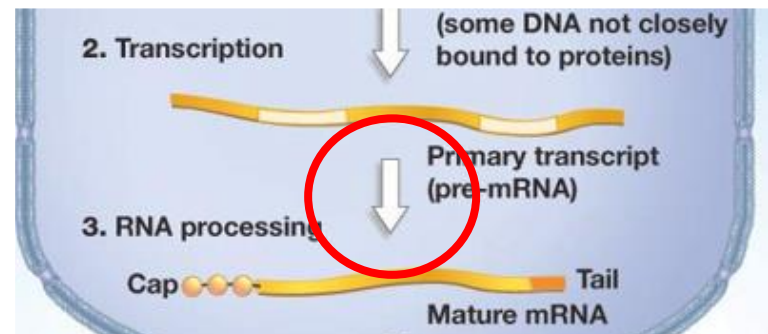
Extracellular signals and cellular response



Levels of gene expression regulation



Alternative splicing

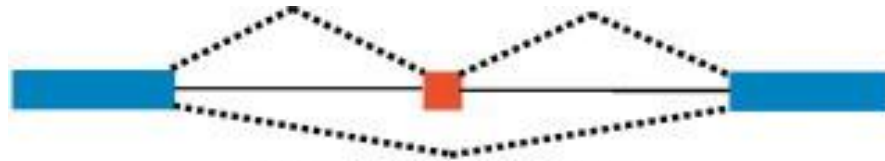


species	nb genes	% alt. splice
S. cerev.	6 000	0,002
C. elegans	19 000	15
D. melanog.	14 000	40
H. sapiens	25 000	>90

**...can occur in most human protein coding genes,
...tissues (or tumours) express specific sets of variants,**

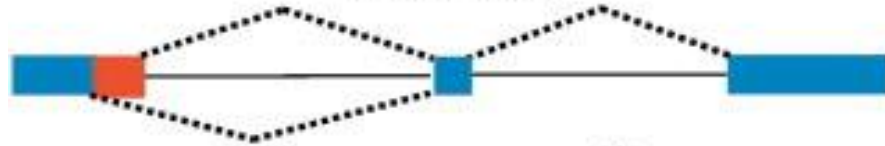
➔ lessons learned from the study of RAC1b

Alternative splicing



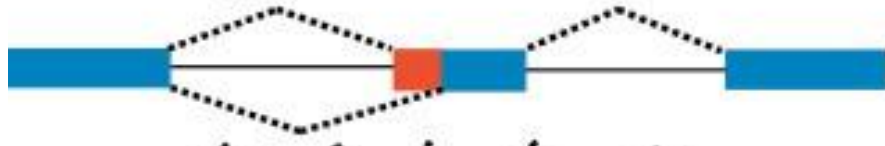
Simple cassette exon

~40%



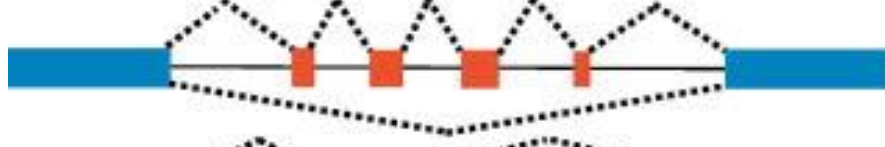
Alternative 5' splice sites

~8%

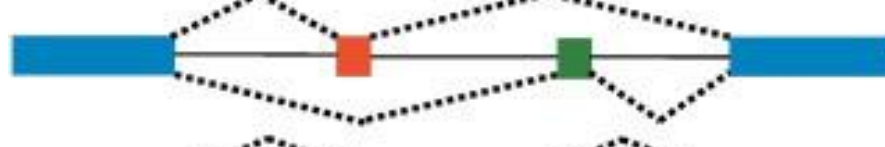


Alternative 3' splice sites

~18%



Tandem cassette exons

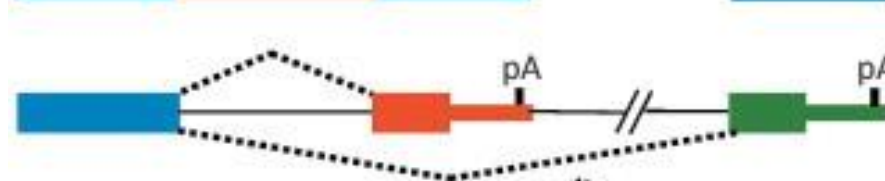


Mutually exclusive exons

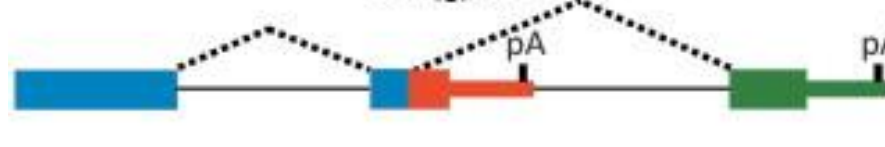


Intron retention

~3%



Alternative 3' terminal exon/
polyA site (APA3)



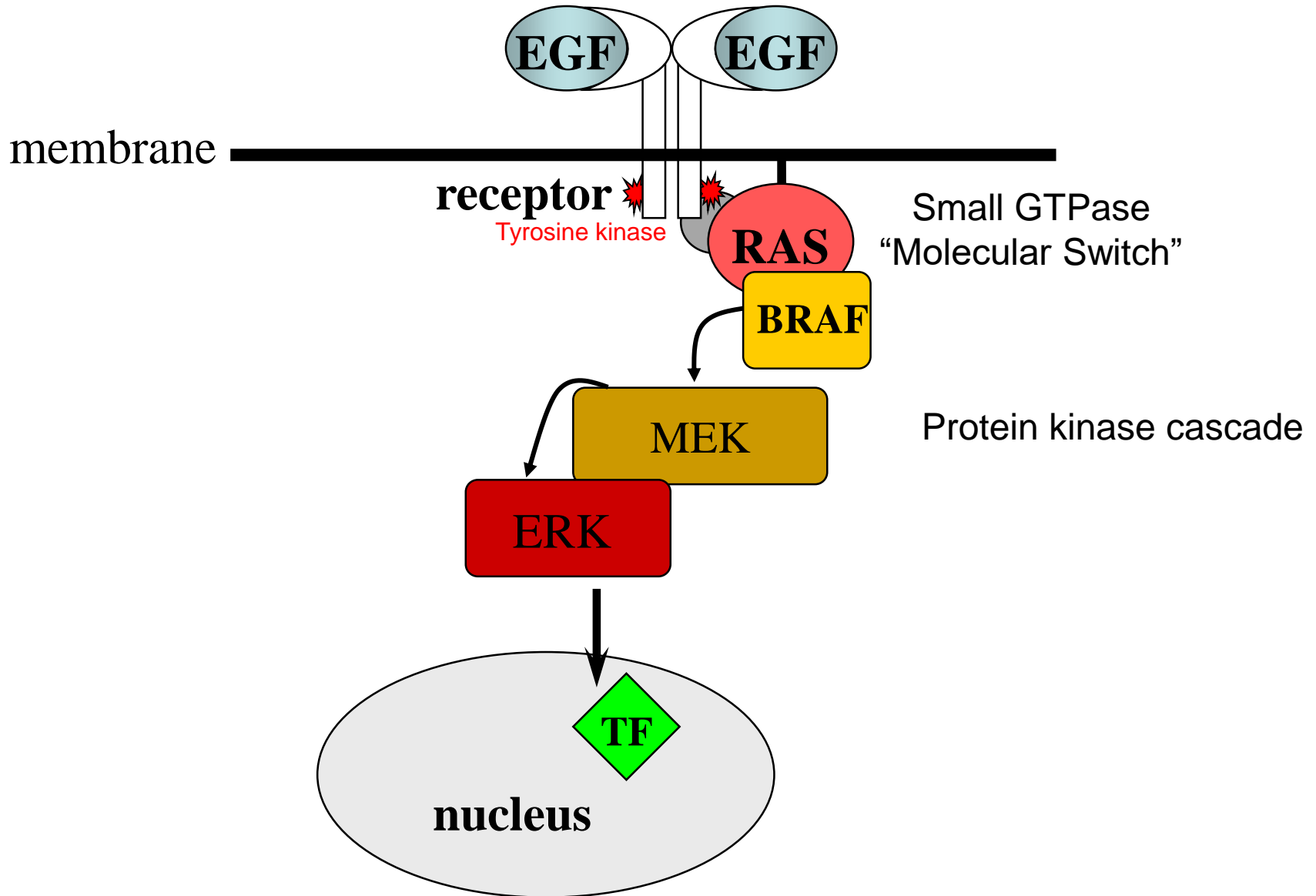
Alternative 3' terminal exon/
polyA site (APA5)



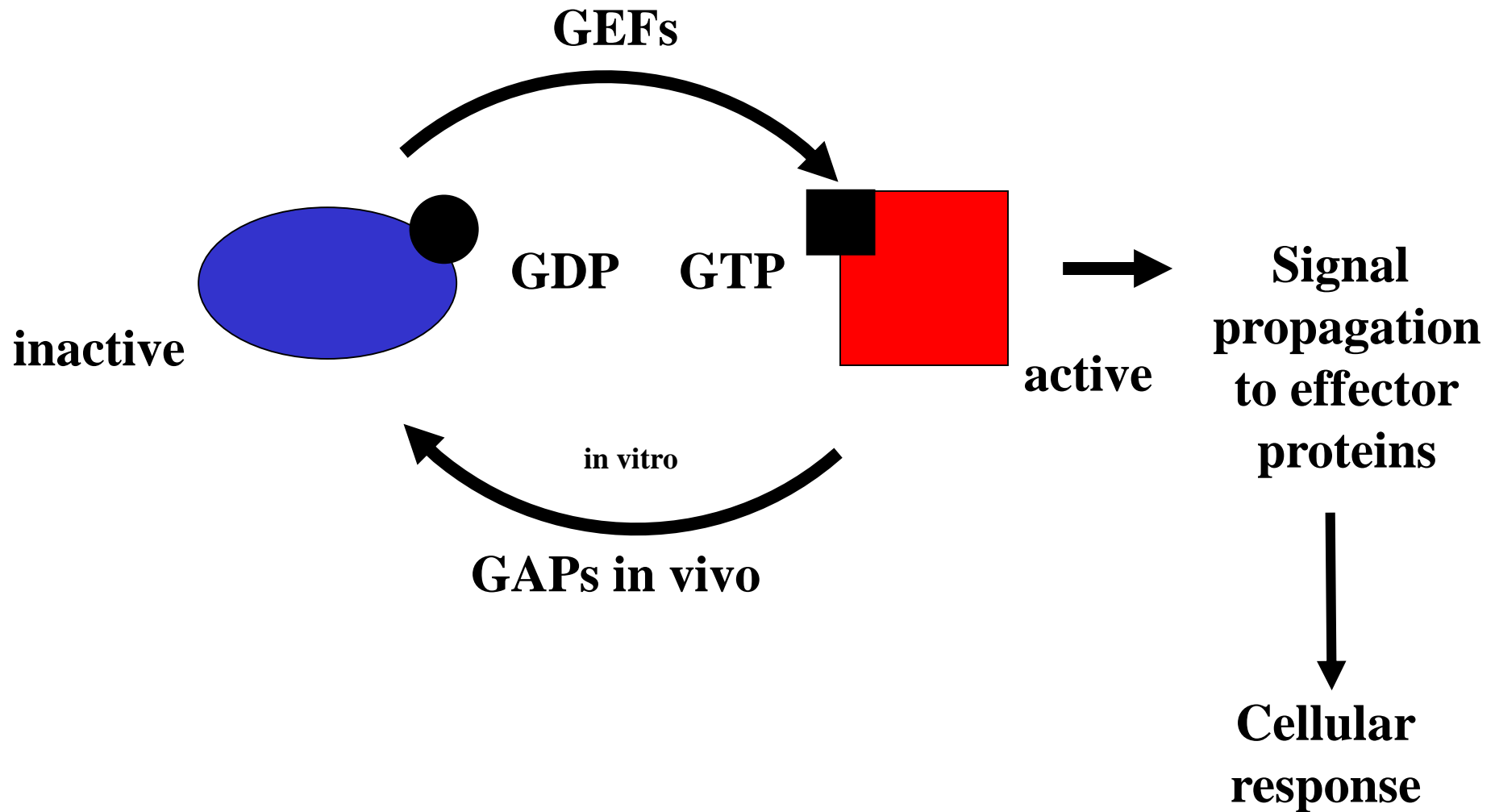
Alternative polyA (APA)

~50%

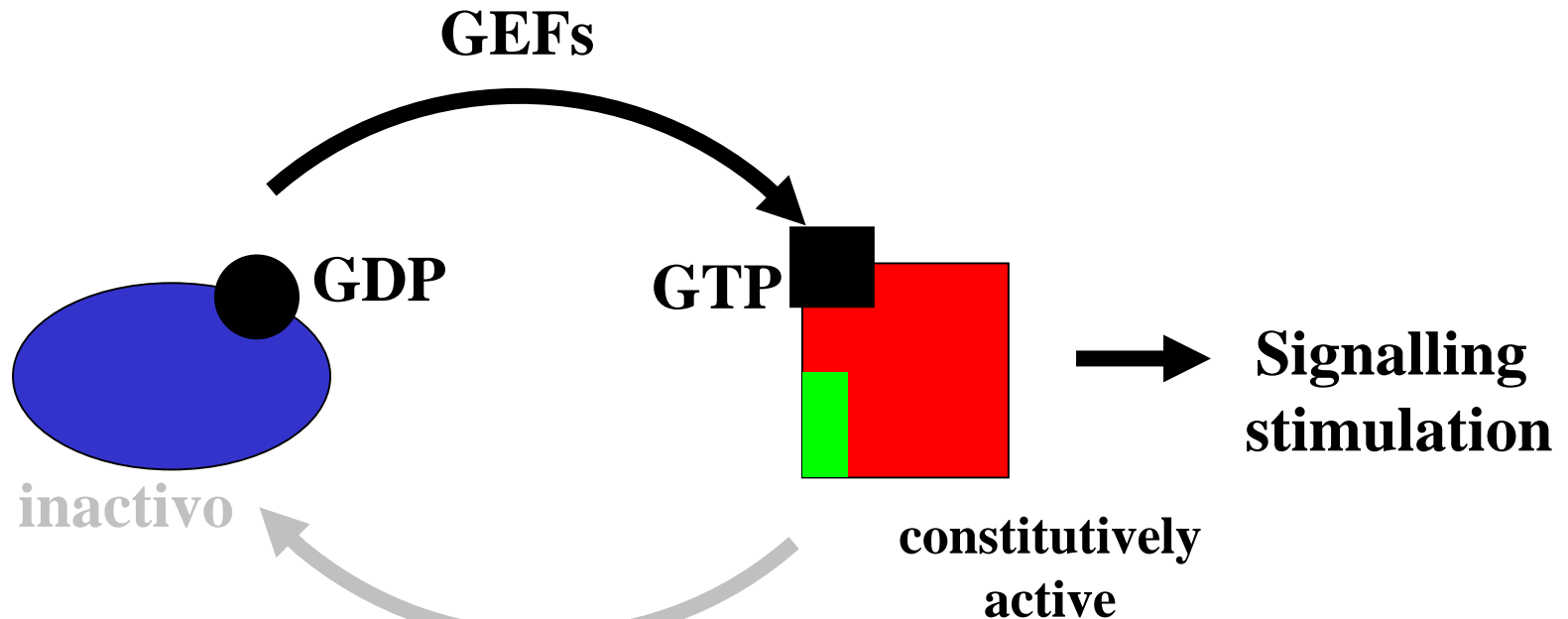
The MAP kinase pathway



The RAS superfamily and their GTPase cycle

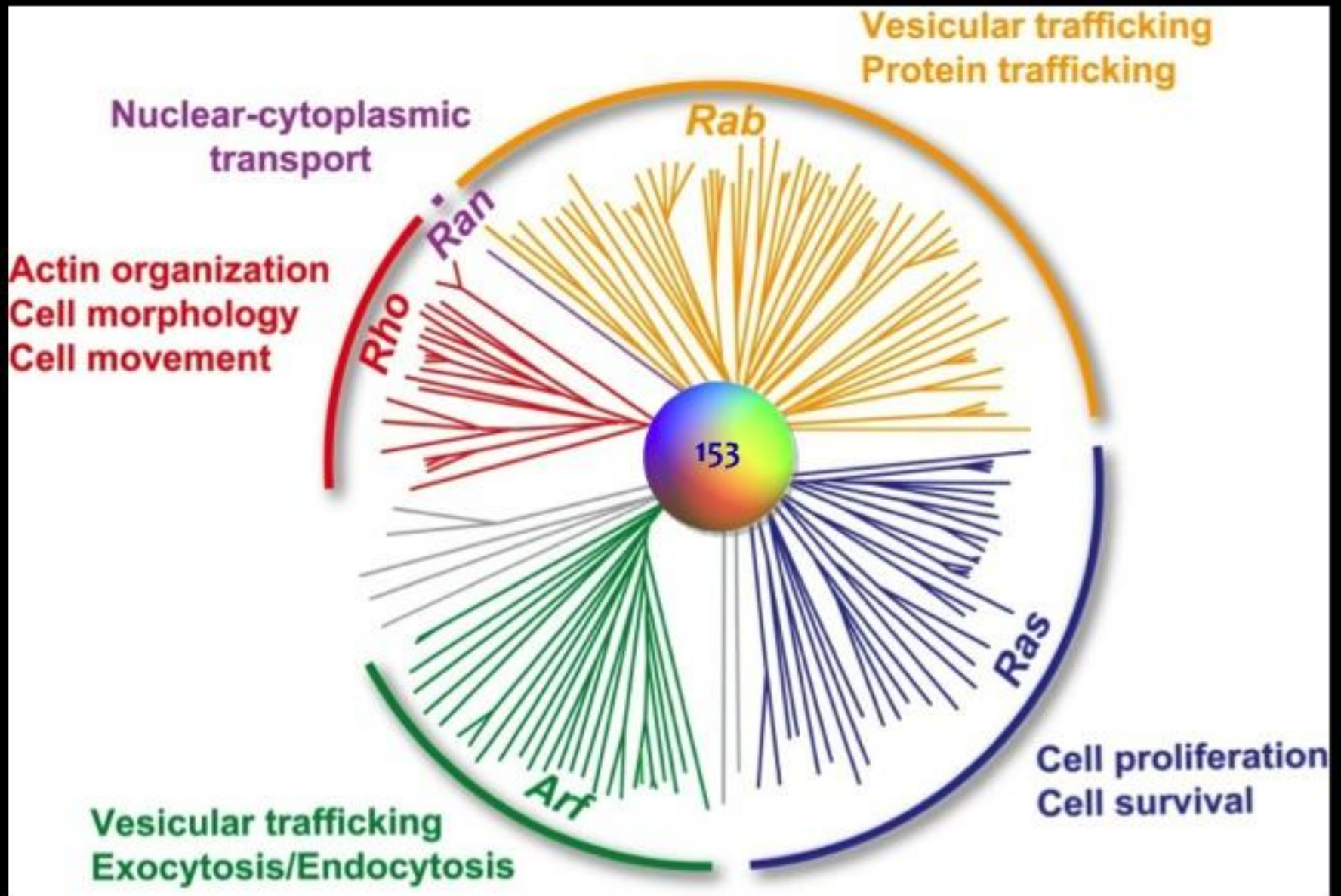


GTPase mutations found in tumors

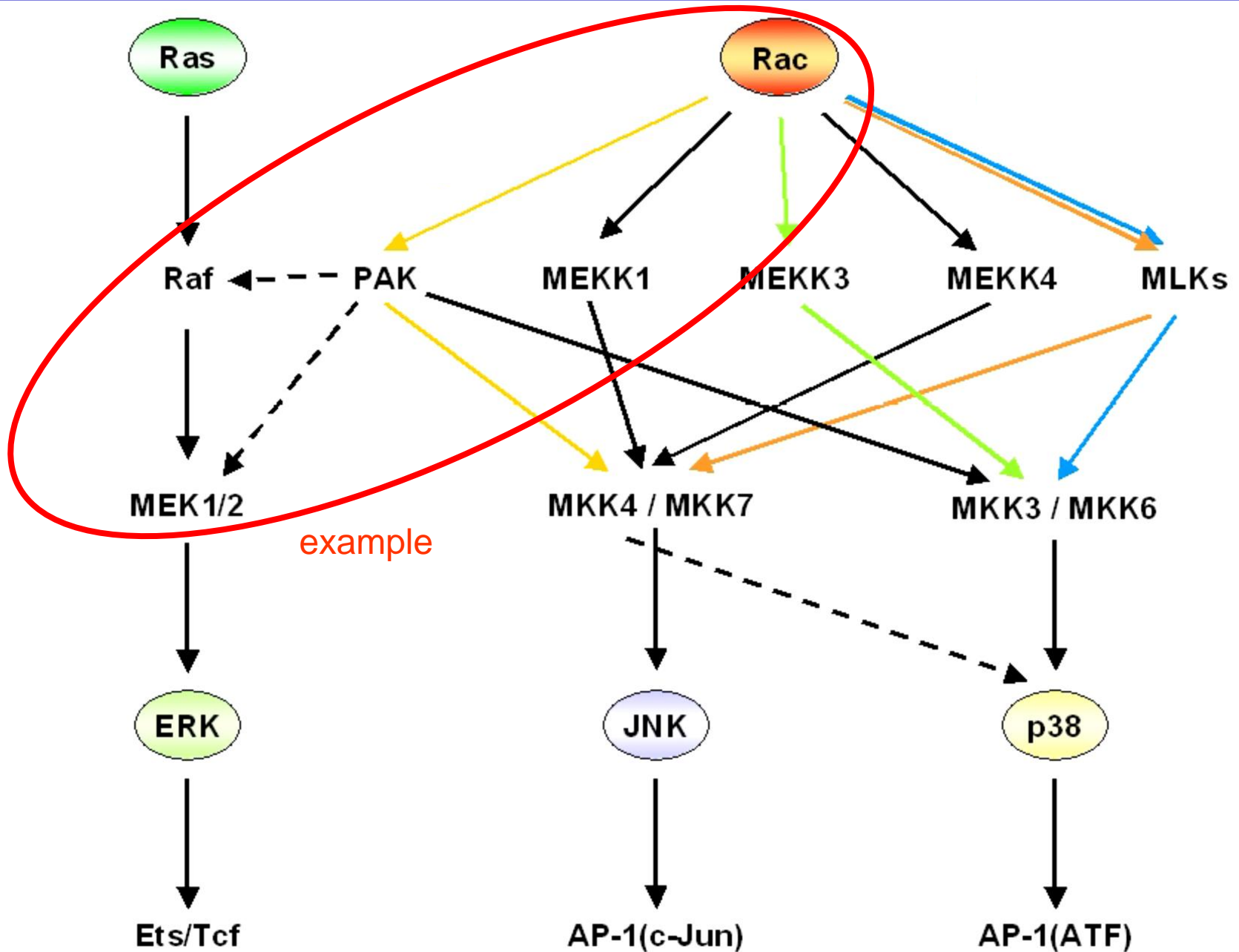


-GGT-GGC- = -G12-G13-
Single nucleotide point mutations:
-GTT=G12V; -GAC=G13D

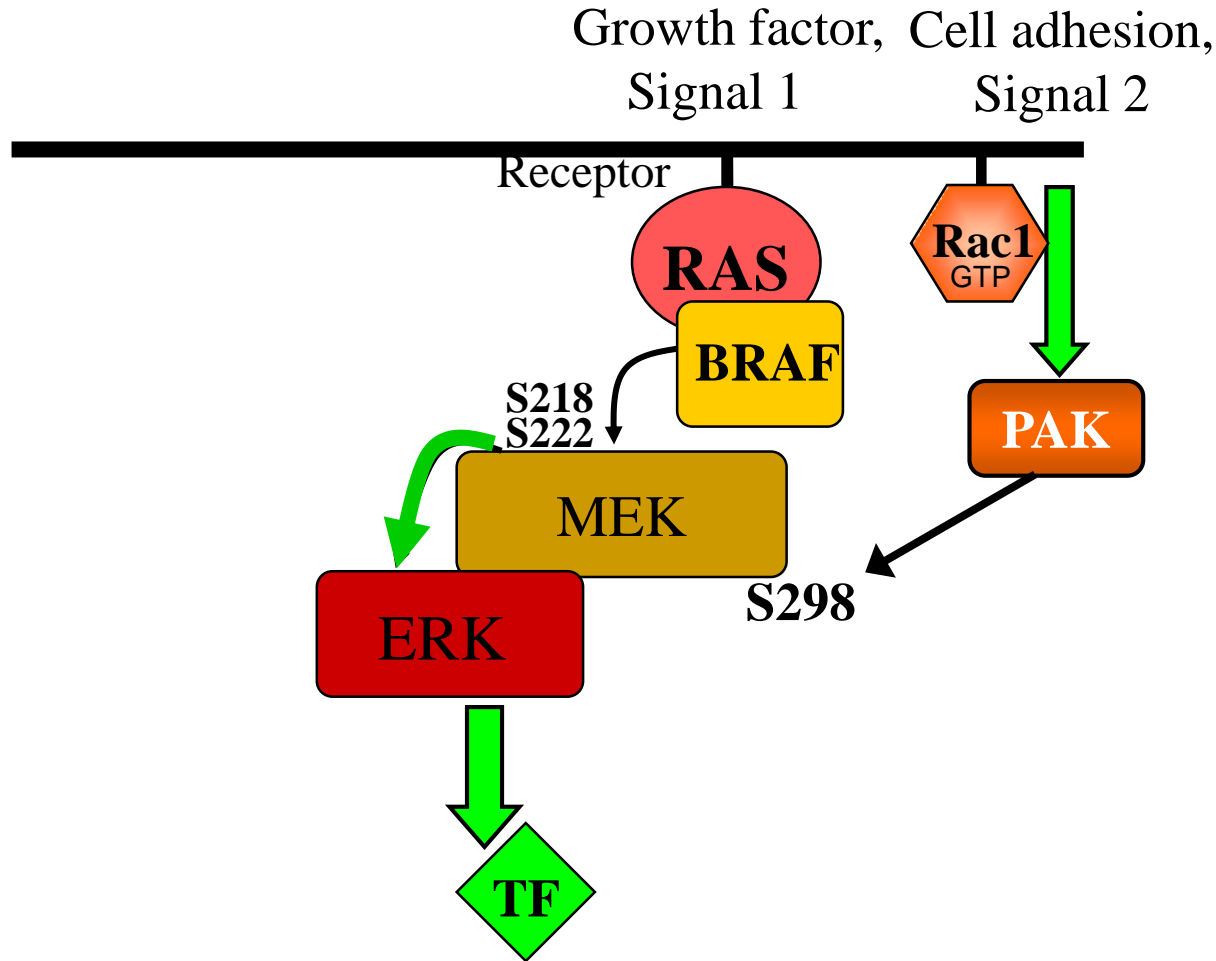
Human Ras superfamily of small GTPases

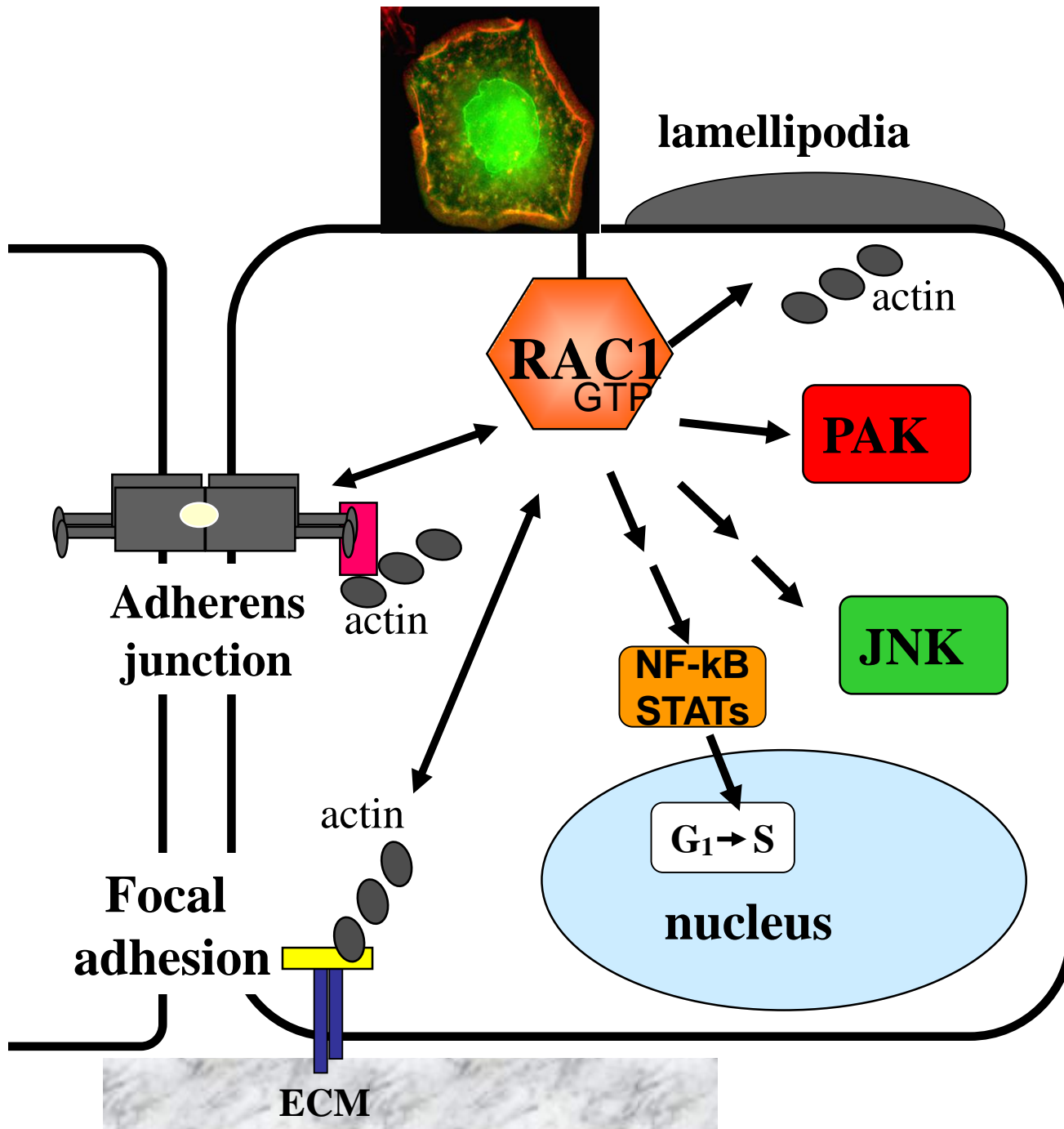


Signalling dynamics through **Cross-talk** – integrating complex signals



Modulation of ERK activation through *cross-talk*





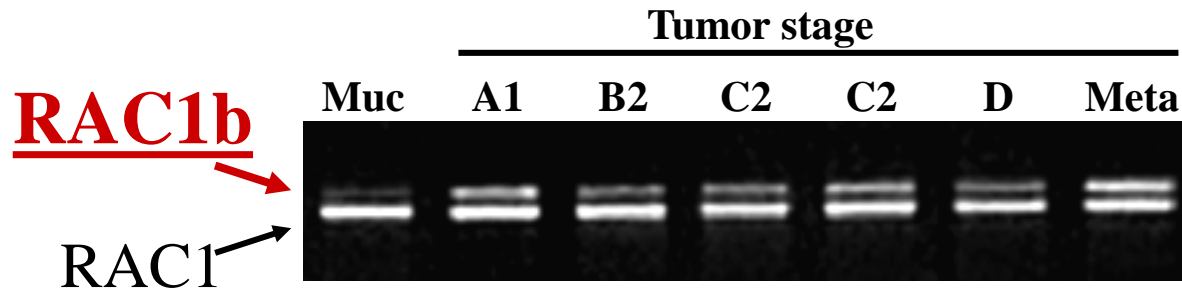
RAC1 signaling

- adhesion
- motility
- proliferation
- gene expression

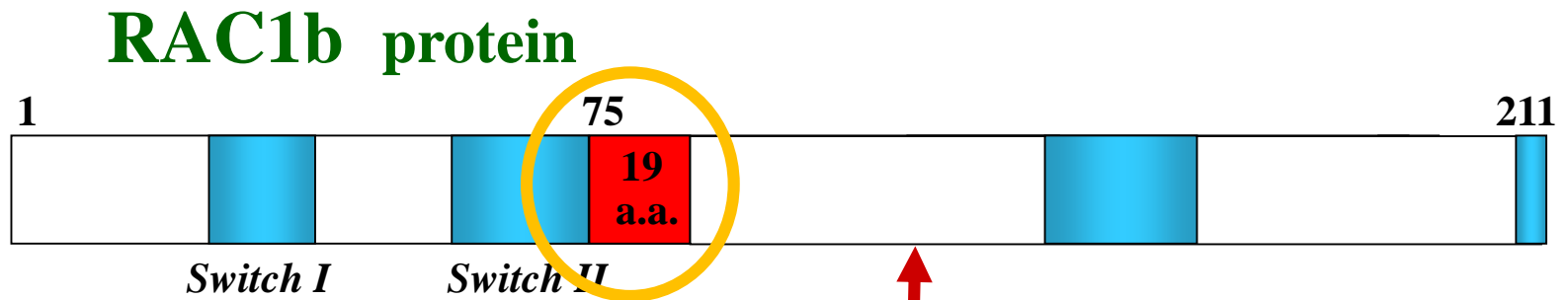


**Oncogenic
RAC1 mutations
in tumours ??**

RAC1 expression in colorectal cancer (CRC): discovery of RAC1b



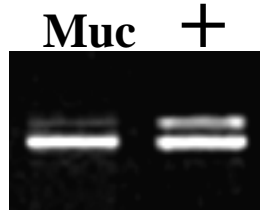
Jordan 1999, Oncogene 18:6835-39



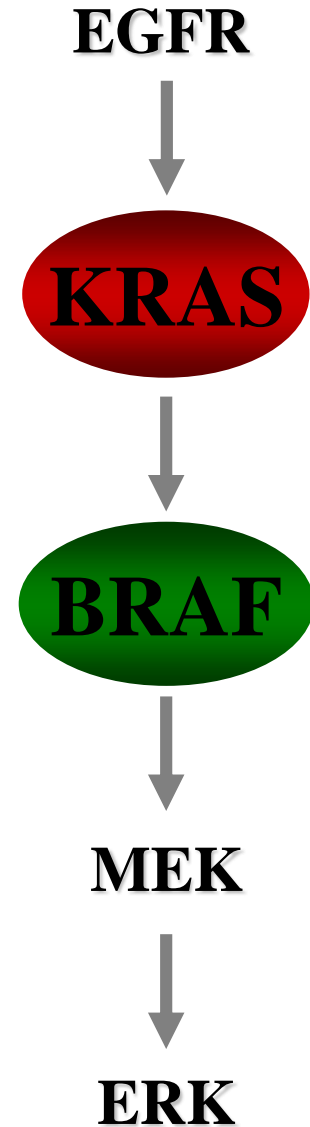
→ RAC1 alternative splicing

Matos 2000, BBRC 277:741-51

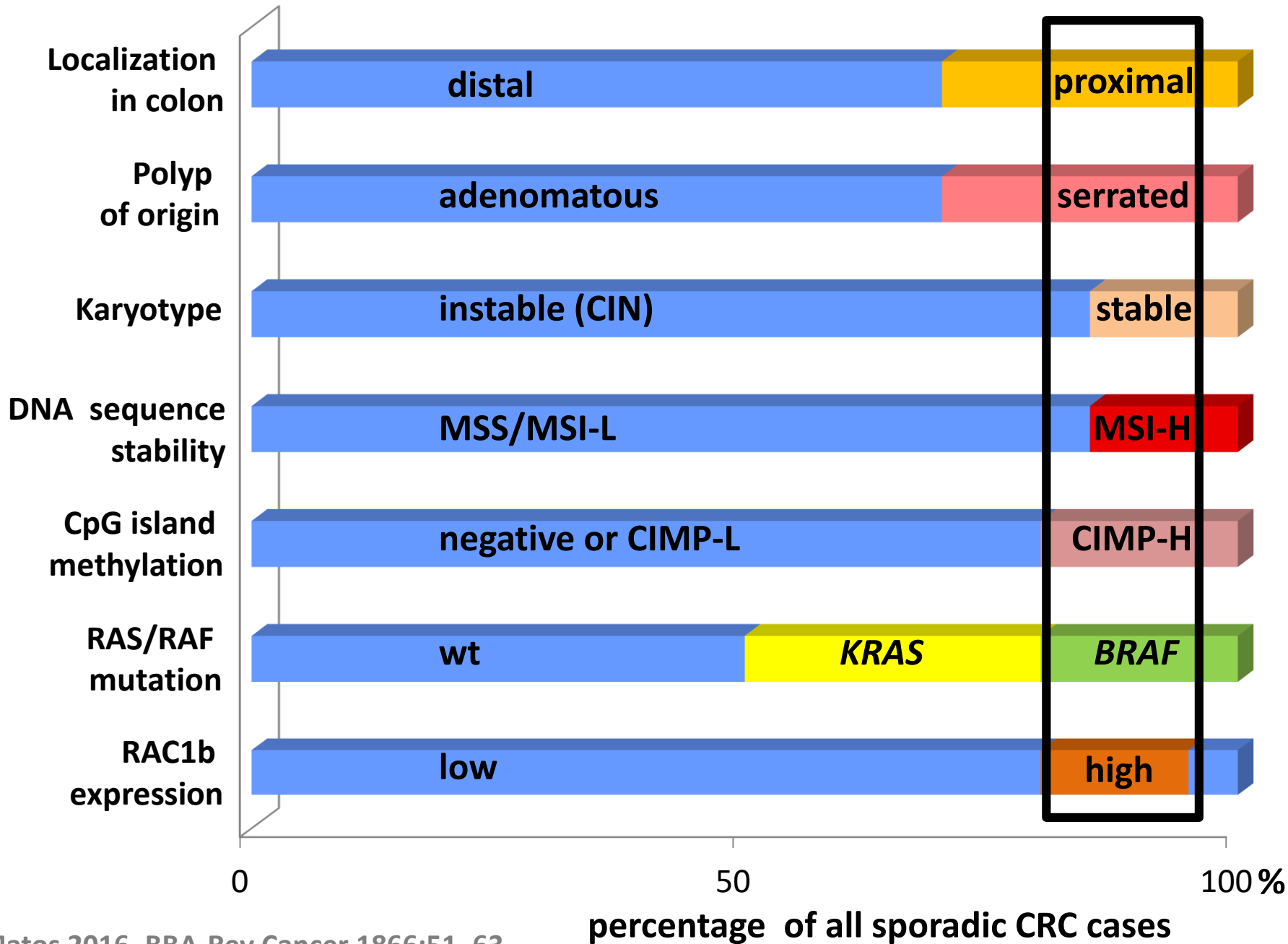
RAC1b expression and genotype of CRC cell lines

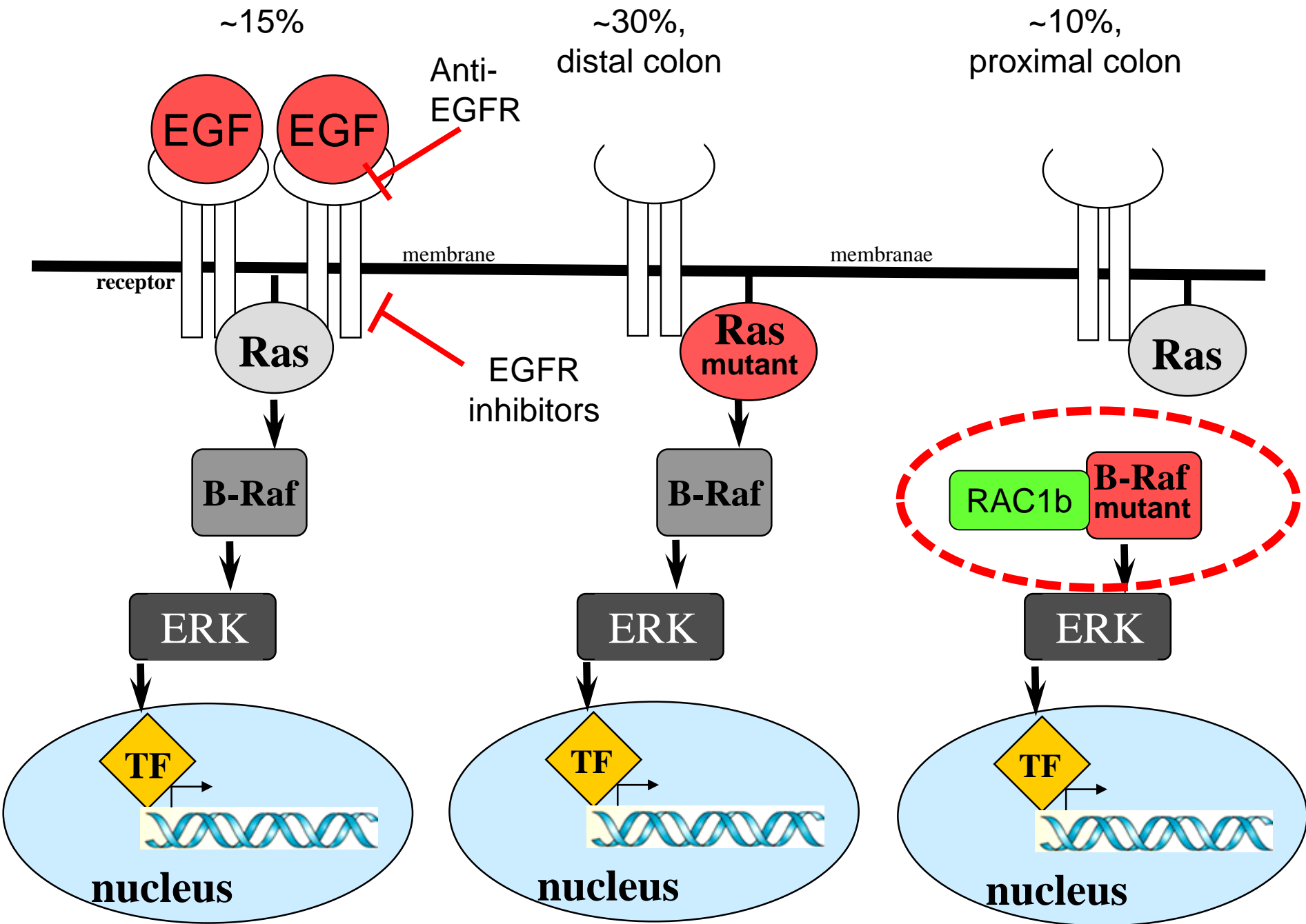


Cell line	Expression		Genotype	
	RAC1	RAC1b	<i>BRAF</i>	<i>KRAS</i>
V9P	+	-	wt	wt
SW48	+	-	wt	wt
LS174	+	-	wt	G12D
Lovo	+	-	wt	G13D
TC71	+	-	wt	G12D
HCT116	+	-	wt	G13D
HCT15	+	-	wt	G13D
IS513	+	-	wt	G12D
IS2	+	-	wt	G12D
Colo320	+	-	wt	G12D
SW480	+	-	wt	G12V
CO115	+	+	V600E	wt
HT29	+	+	V600E	wt
RKO	+	+	V600E	wt
Colo205	+	+	V600E	wt
Caco2	+	+	wt	wt

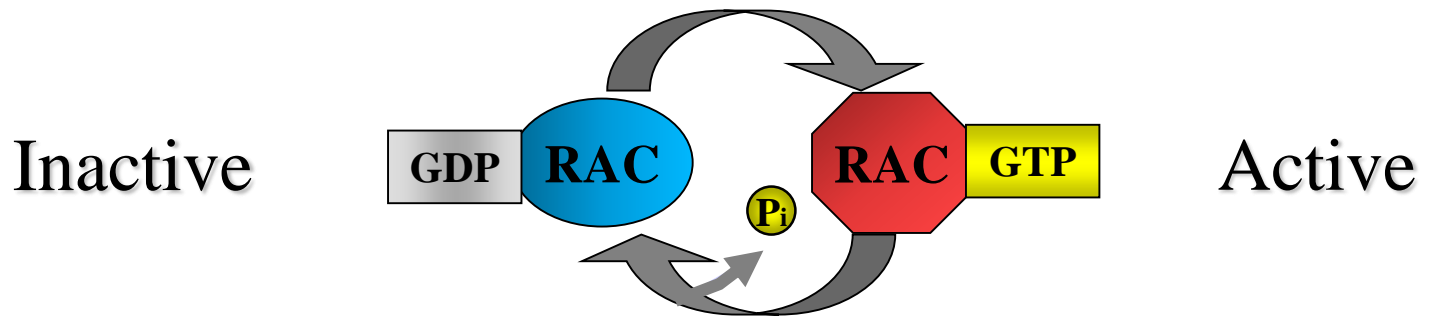
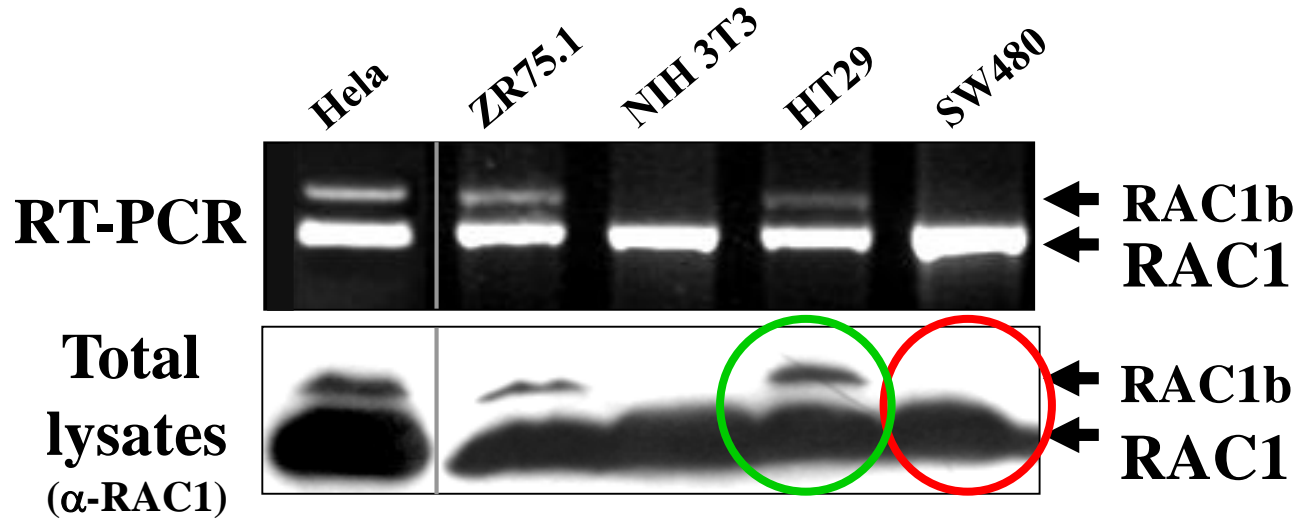


CRC classification: RAC1b overexpression characterizes a subtype

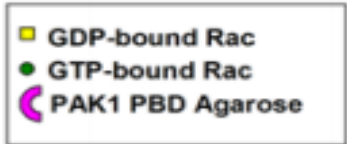
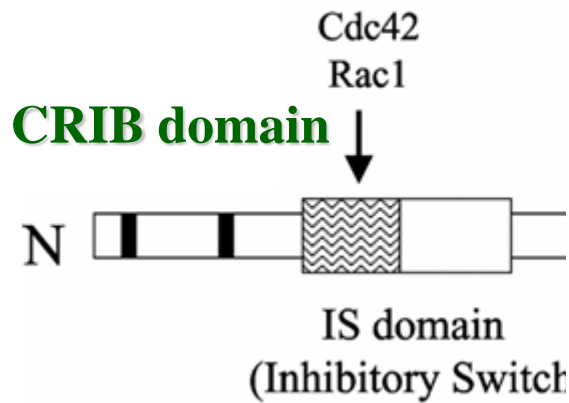




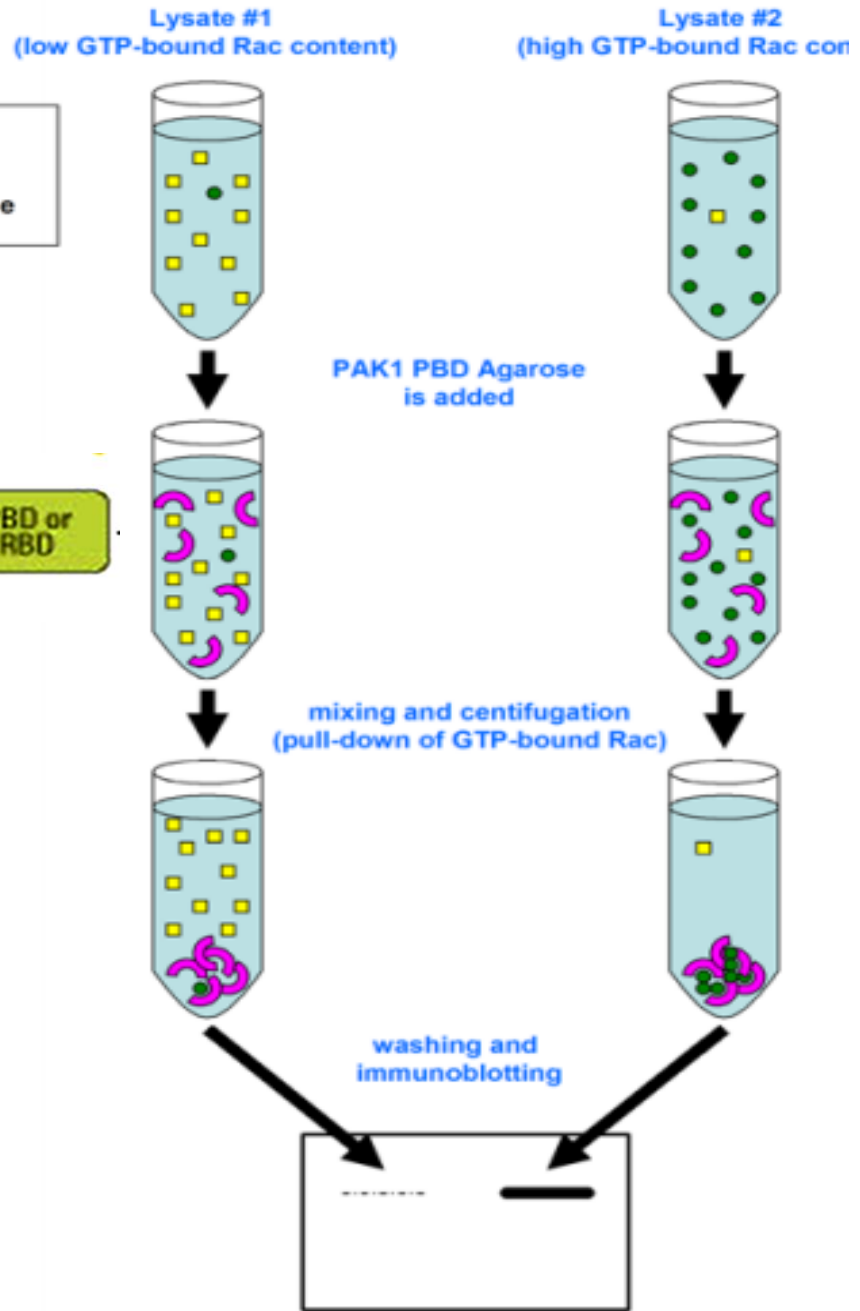
Study RAC1b in mammalian cell lines



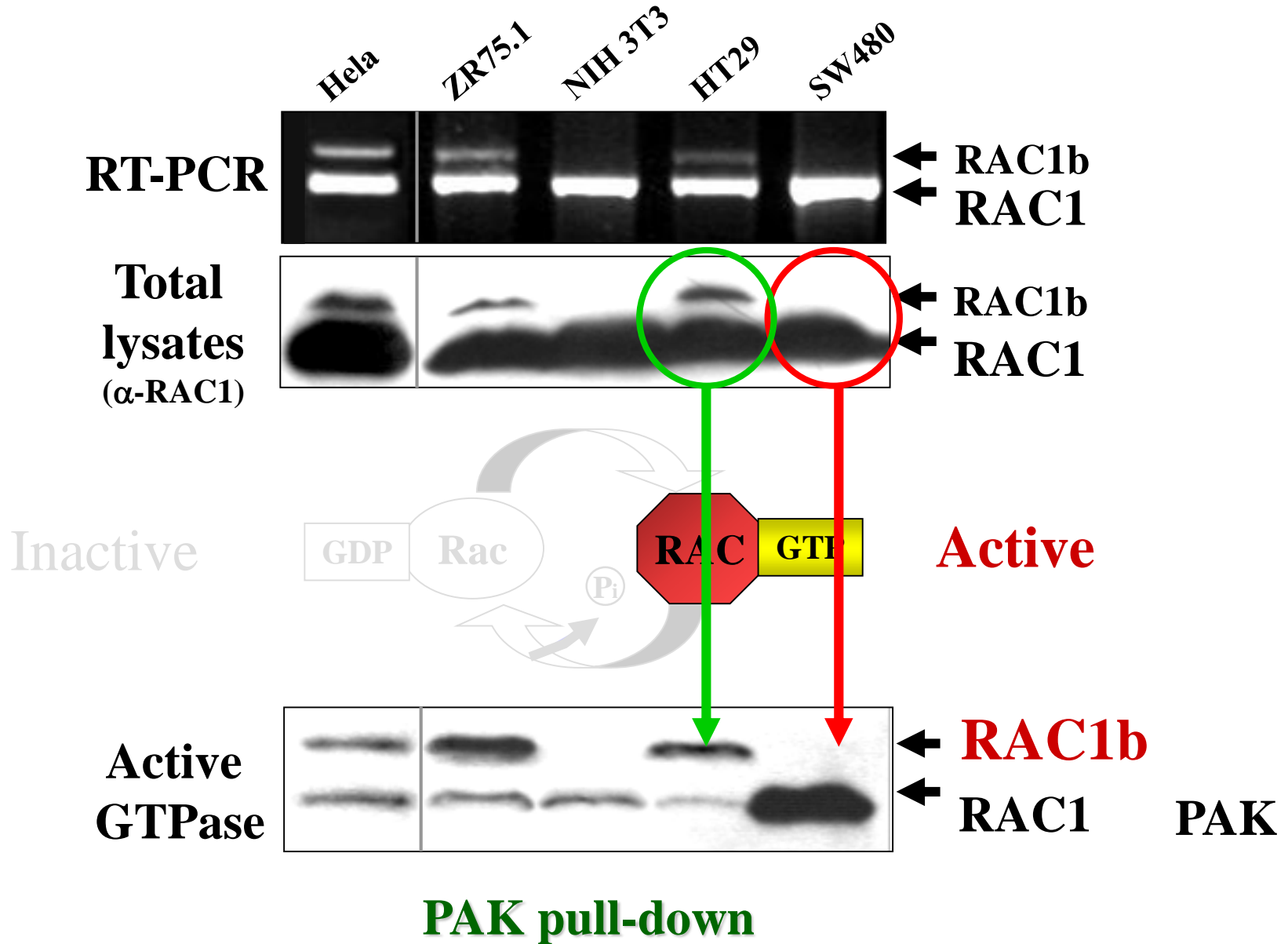
Assay Principle



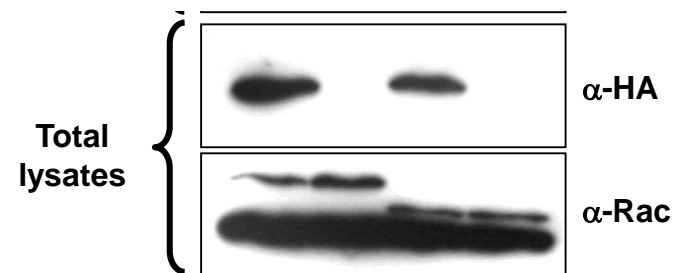
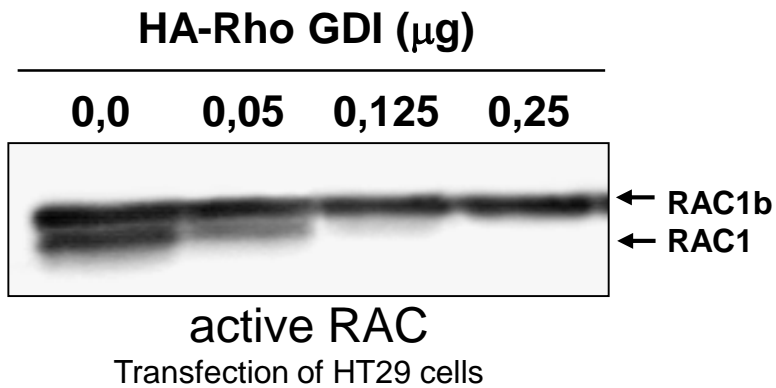
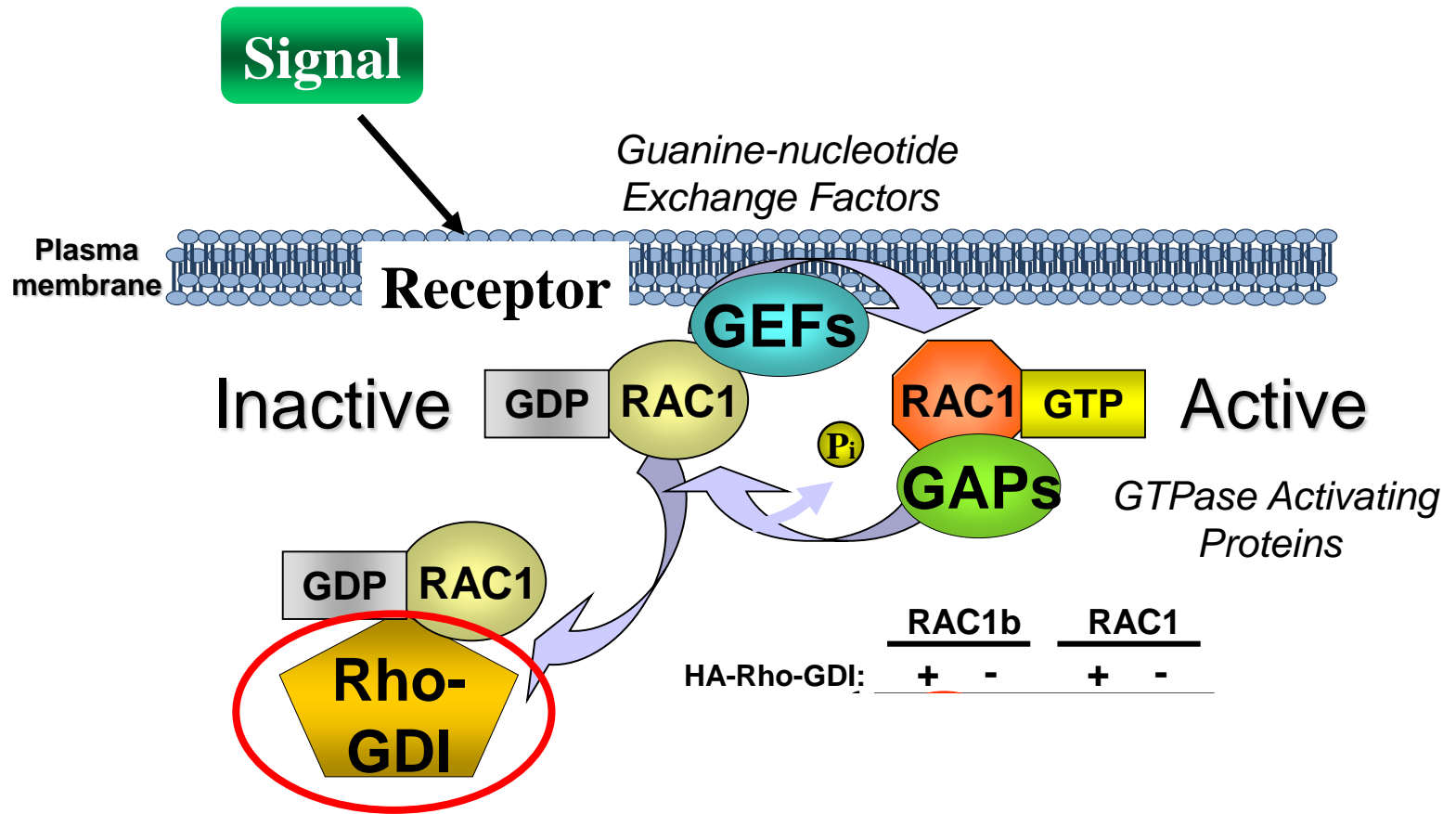
PAK pull-down assay



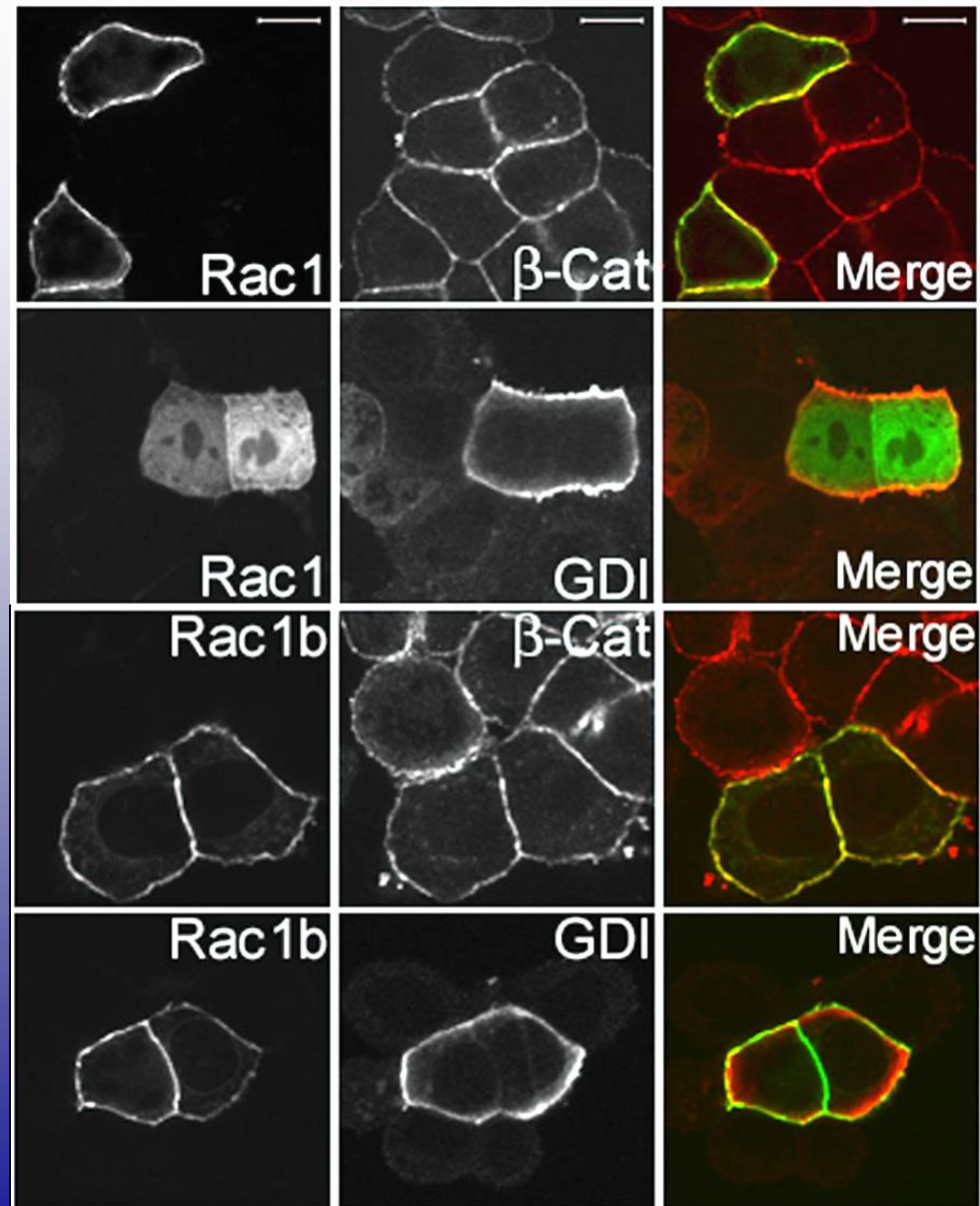
RAC1b in mammalian cell lines



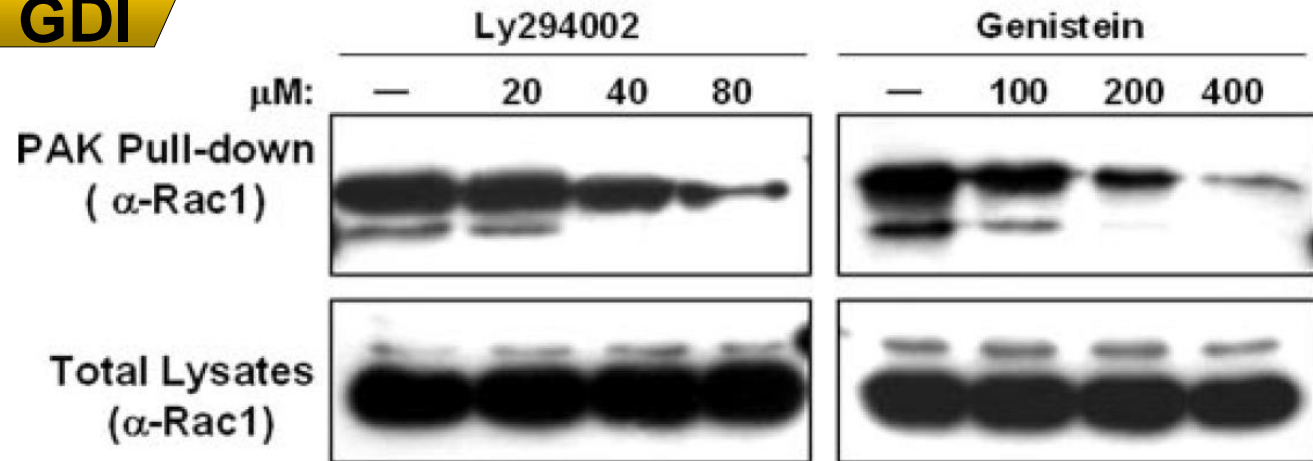
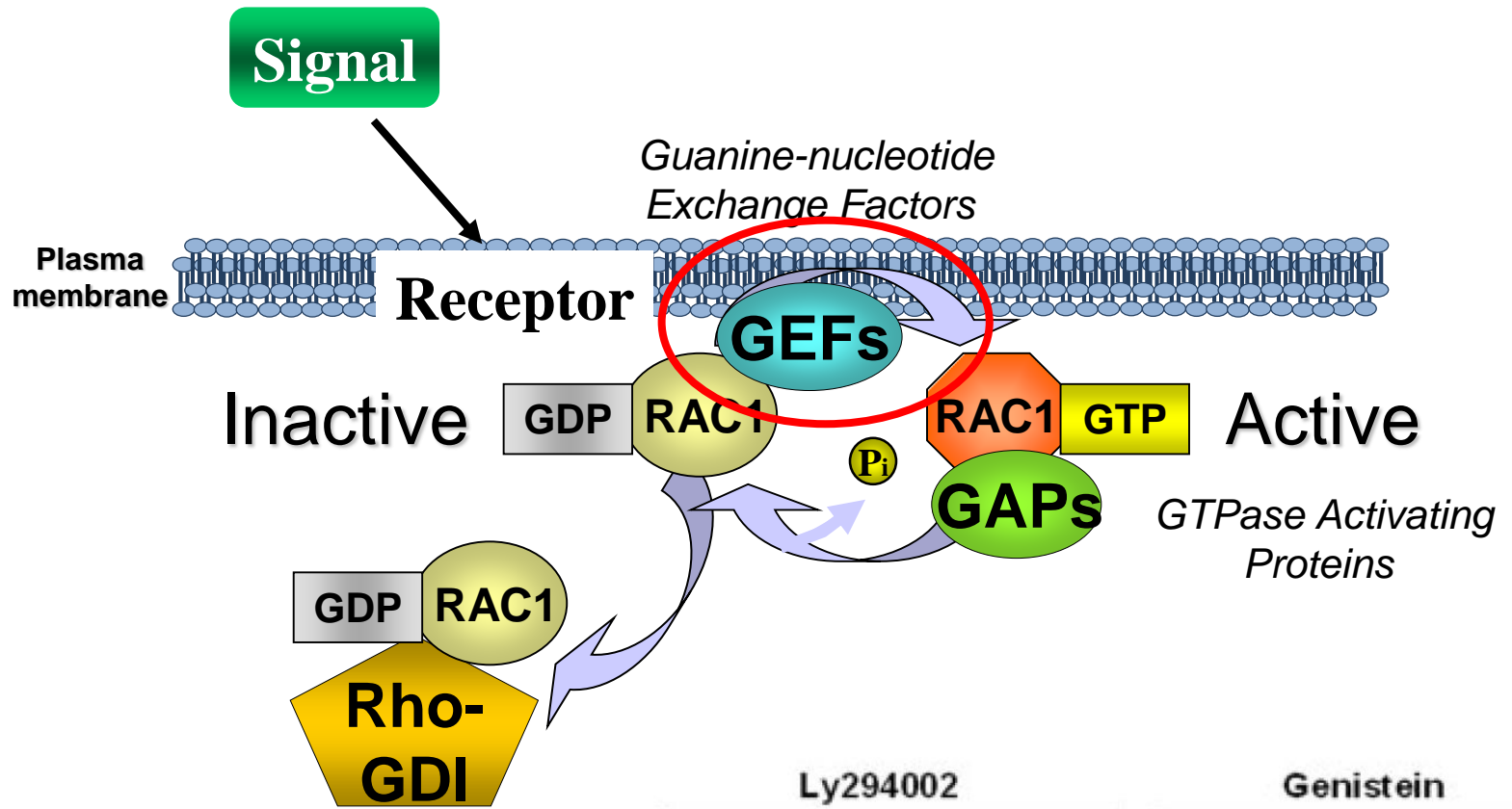
Regulation of RAC1 activation:



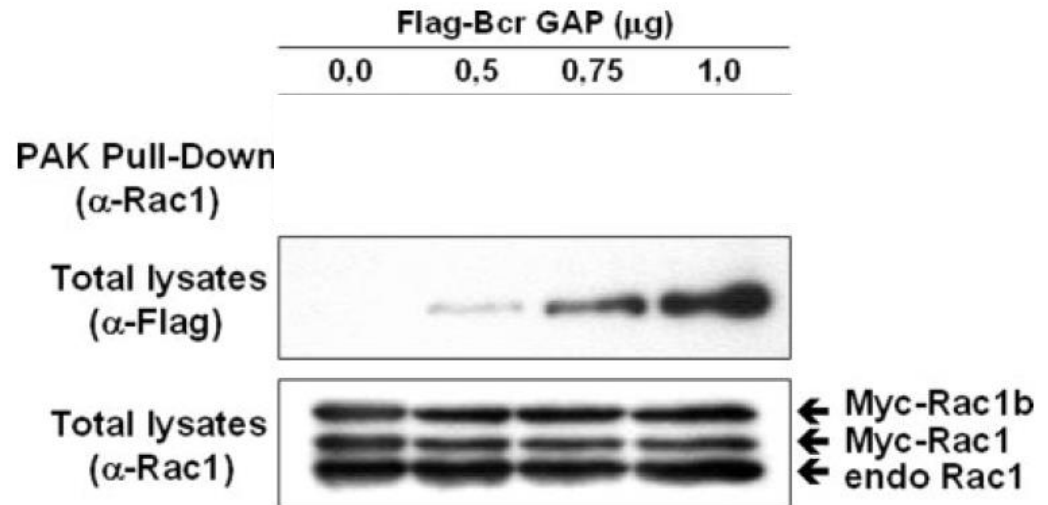
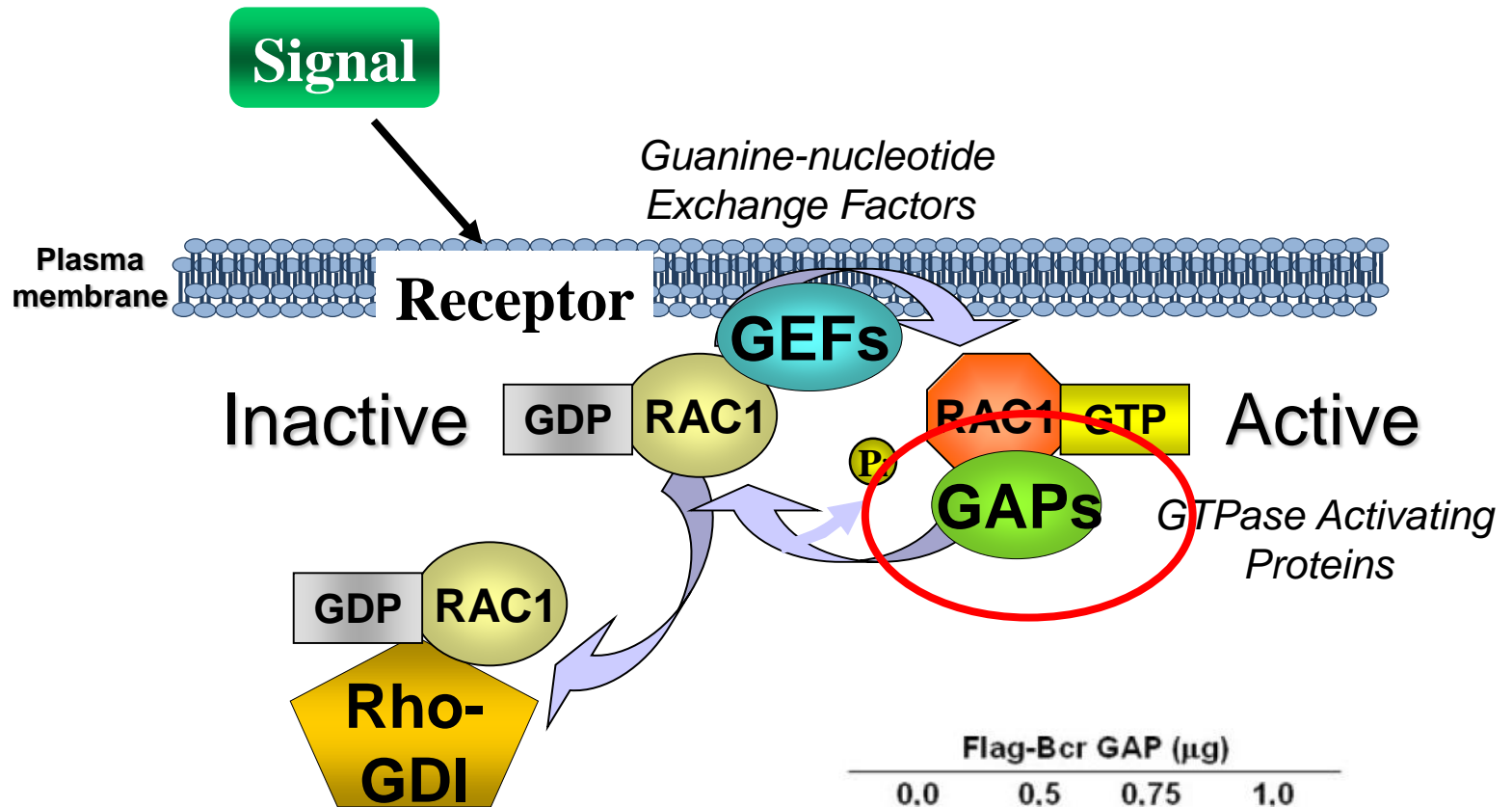
RAC1 but not RAC1b is sequestered by Rho-GDI



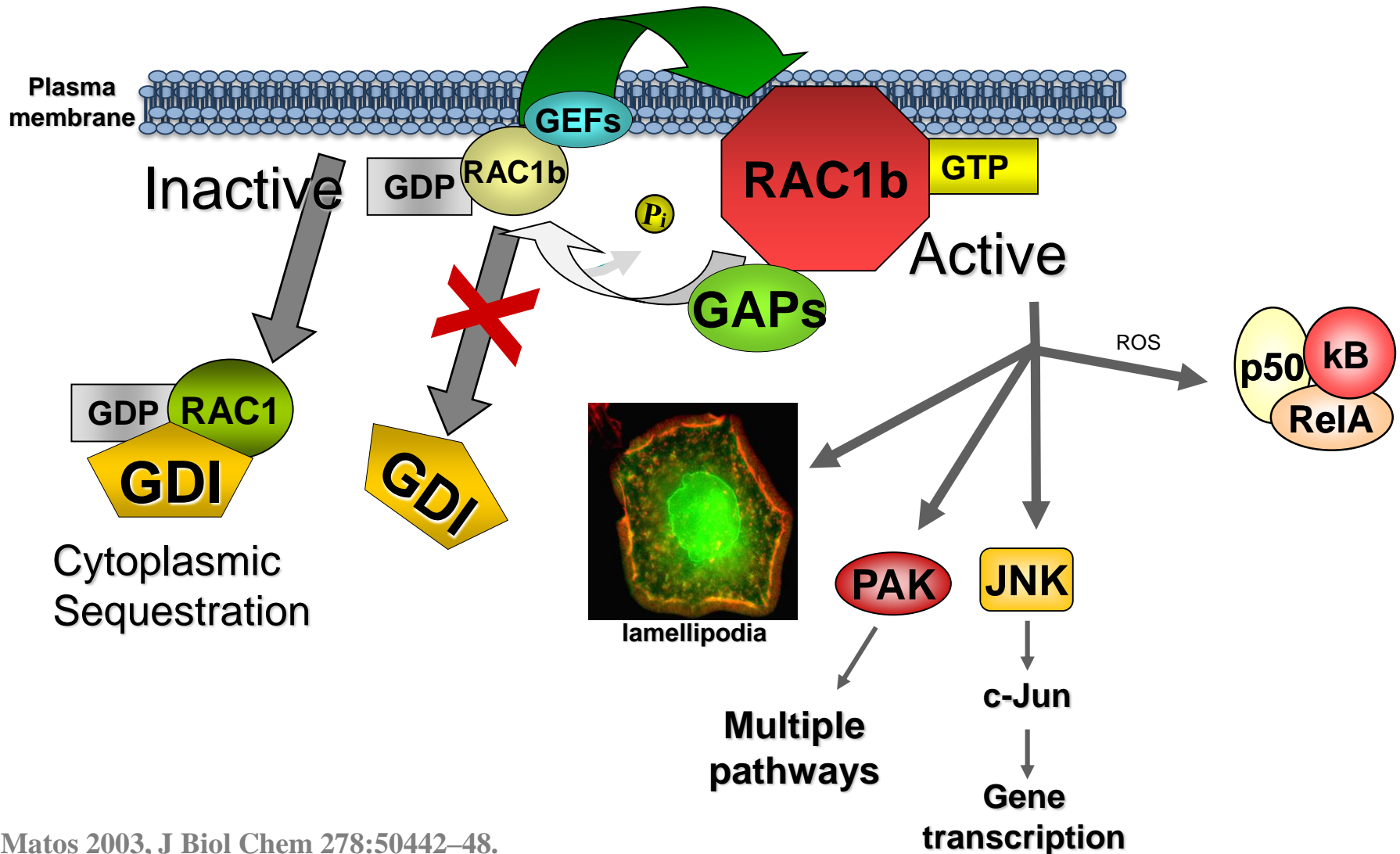
Regulation of RAC1 activation:



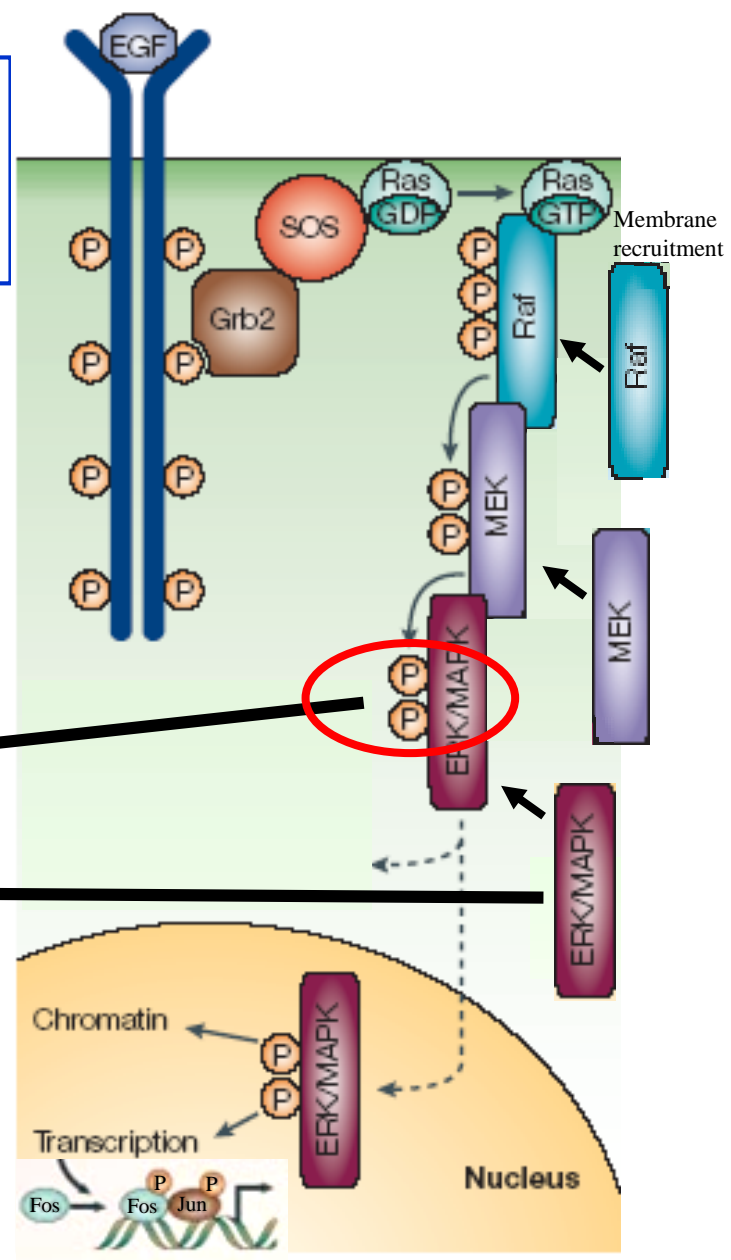
Regulation of RAC1 activation:



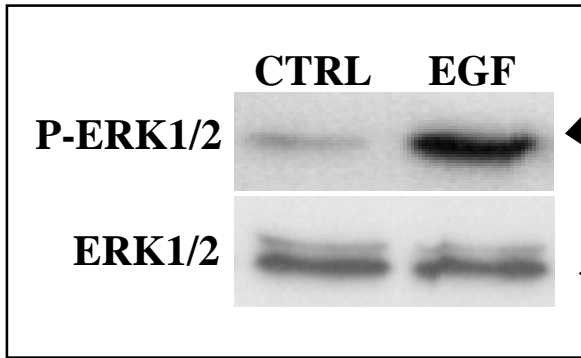
Summary of the unique properties of alternative spliced RAC1b



important research tools: 1. anti-phospho-antibodies

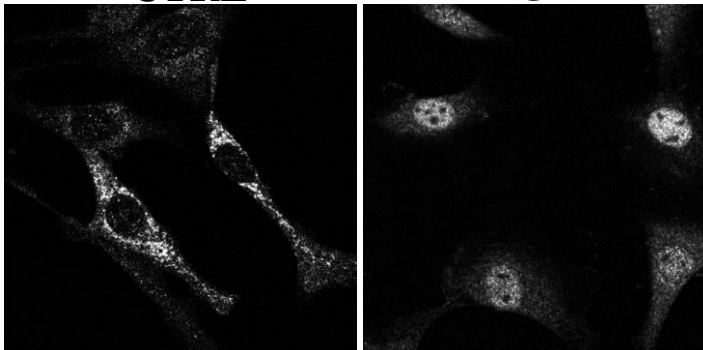


Anti-pThr183-Glu-pTyr185



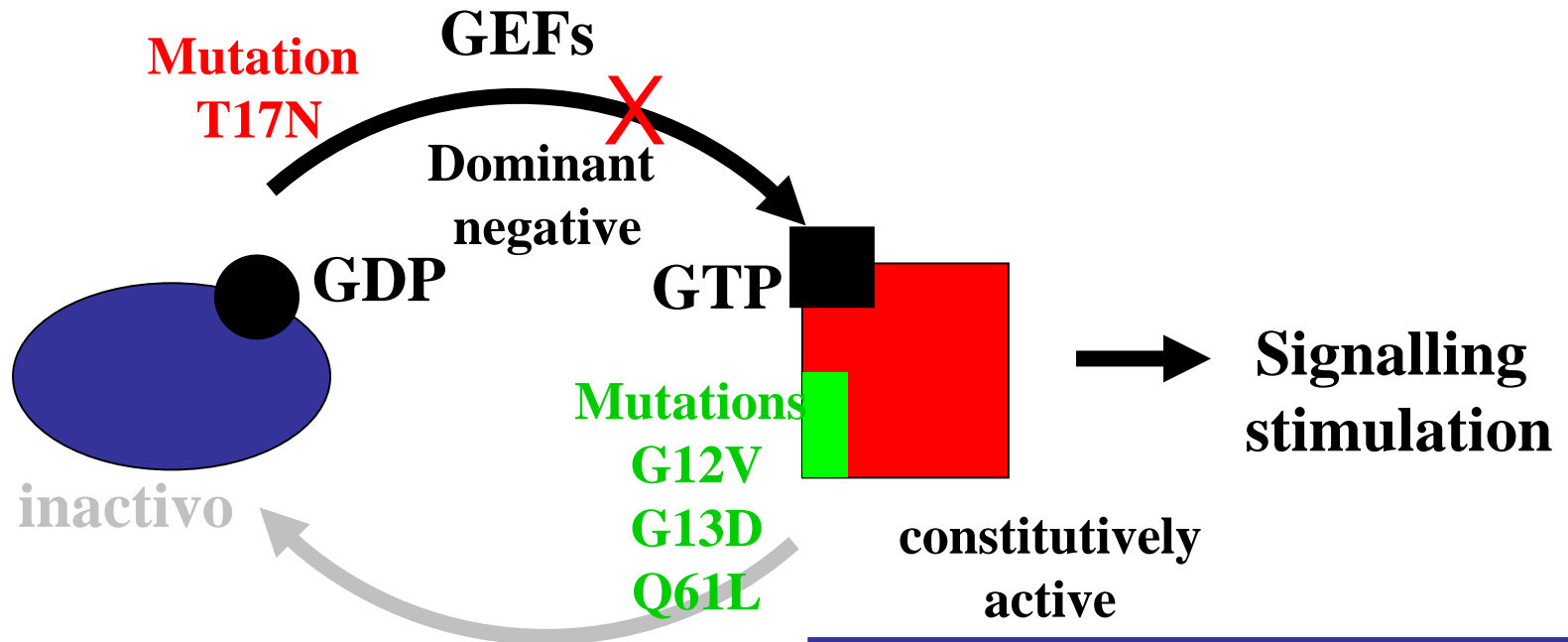
CTRL

EGF

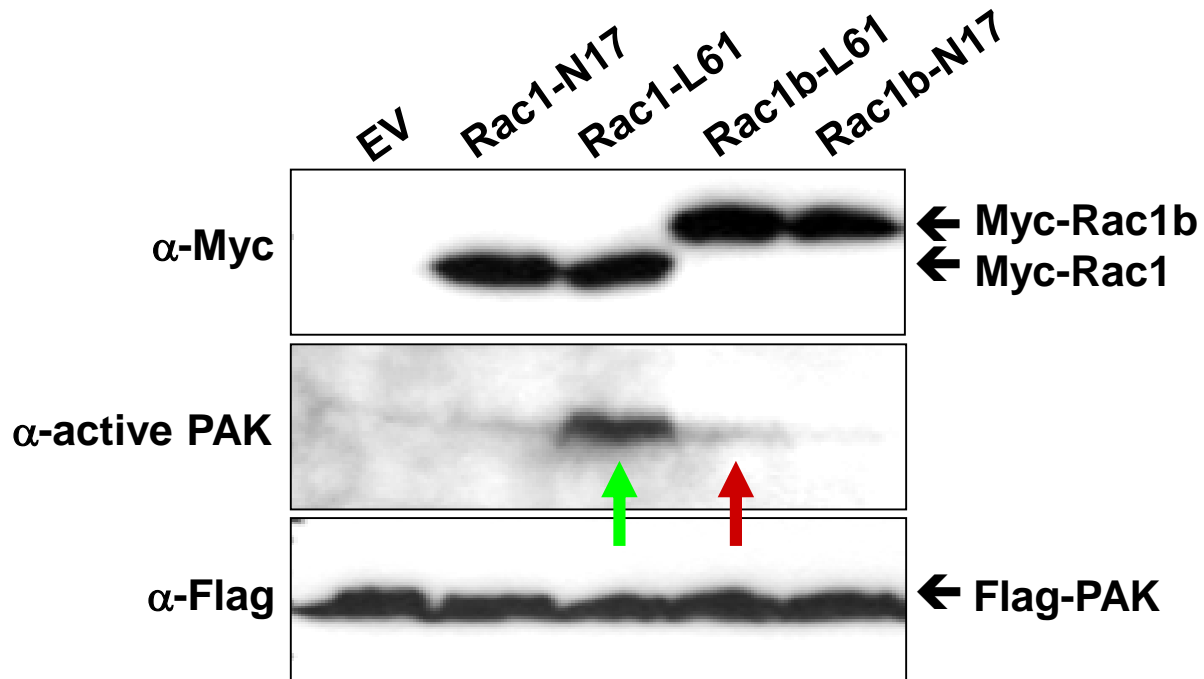
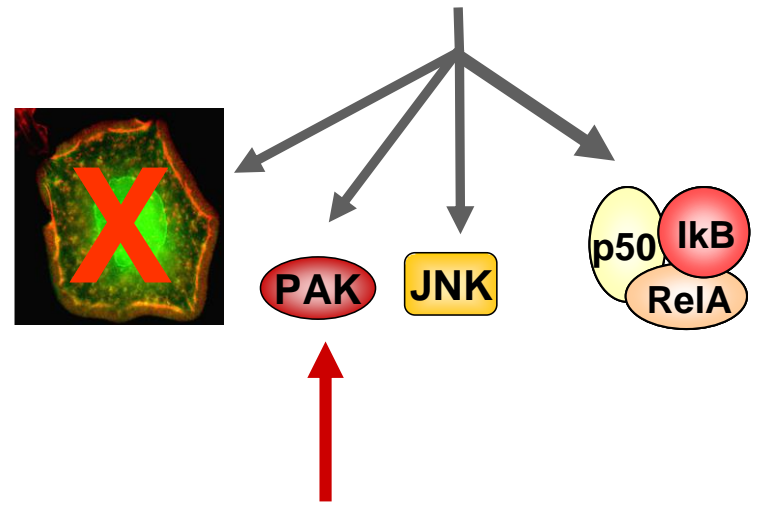


important research tools:

2. GTPase mutants

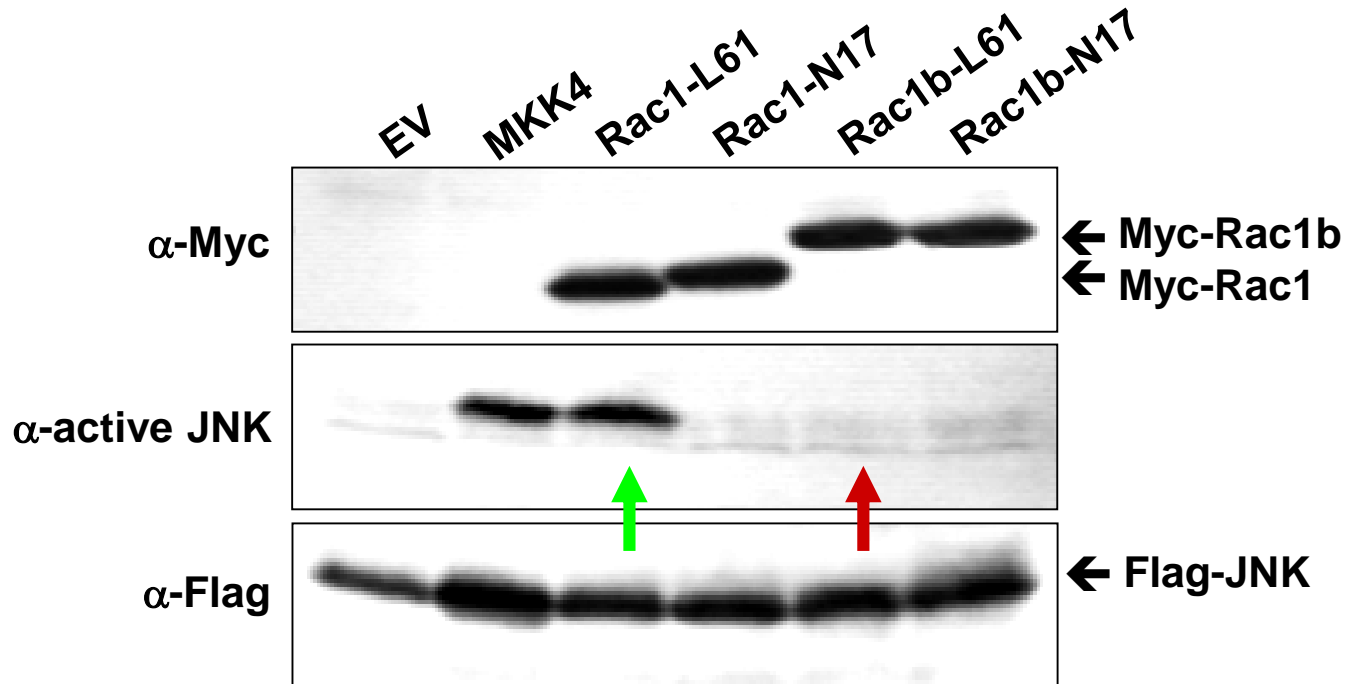
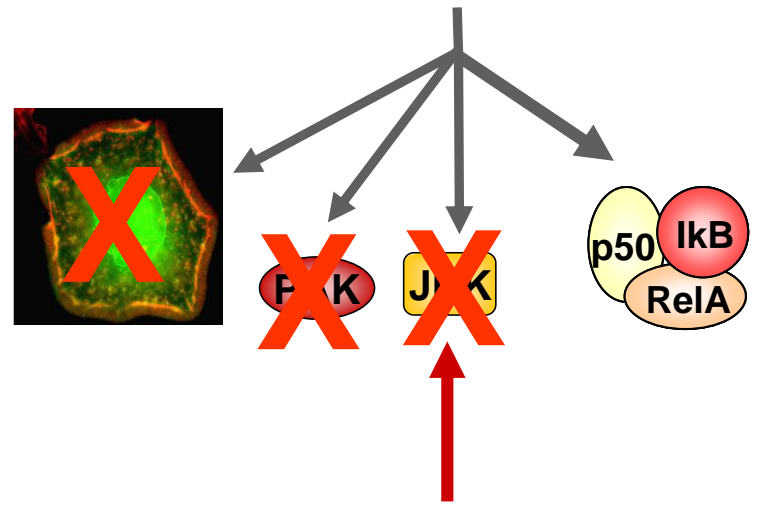


-GGT-GGC- = -G12-G13-
Single nucleotide point mutations:
-GTT=G12V; -GAC=G13D

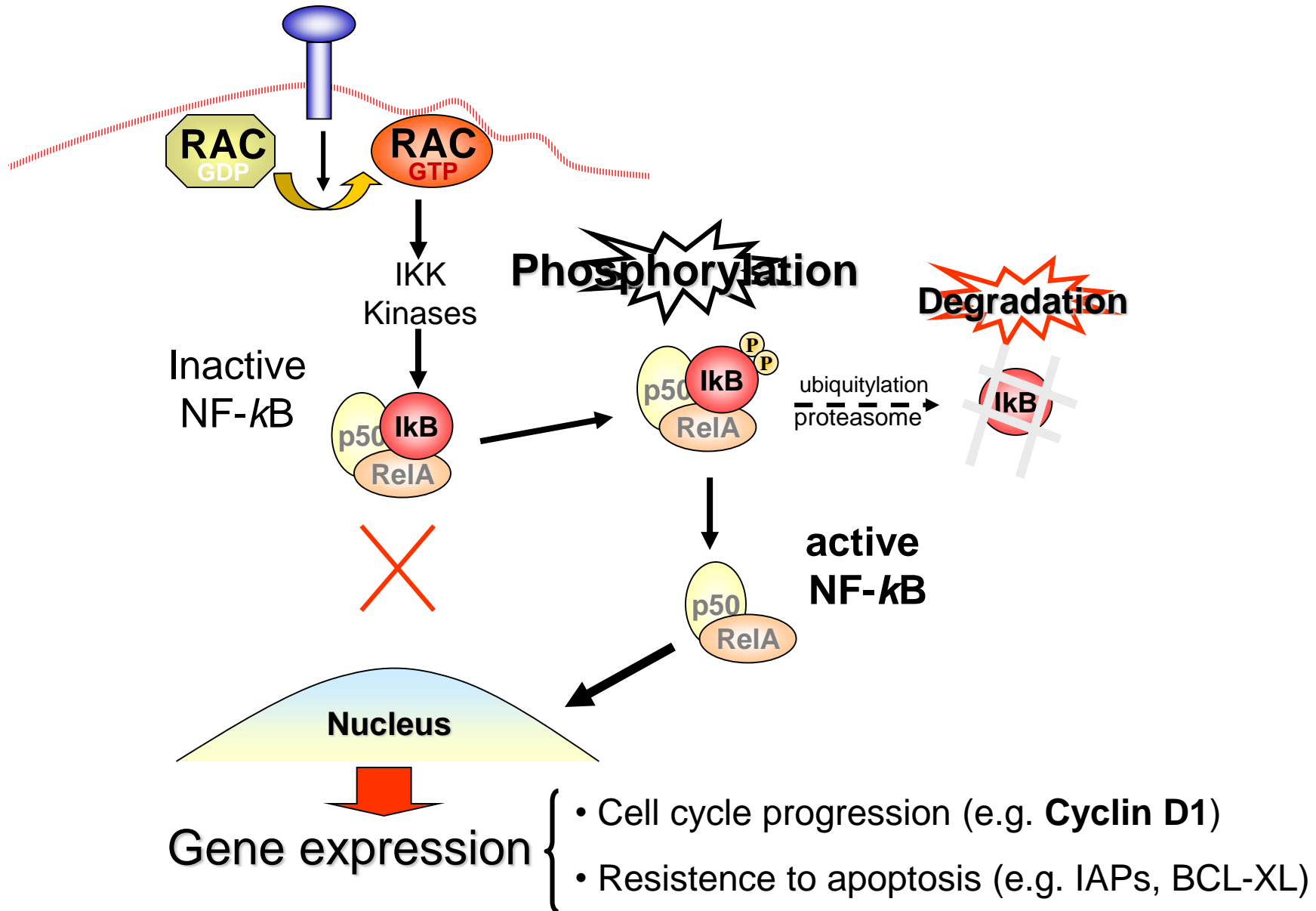


... but PAK-PD assays work

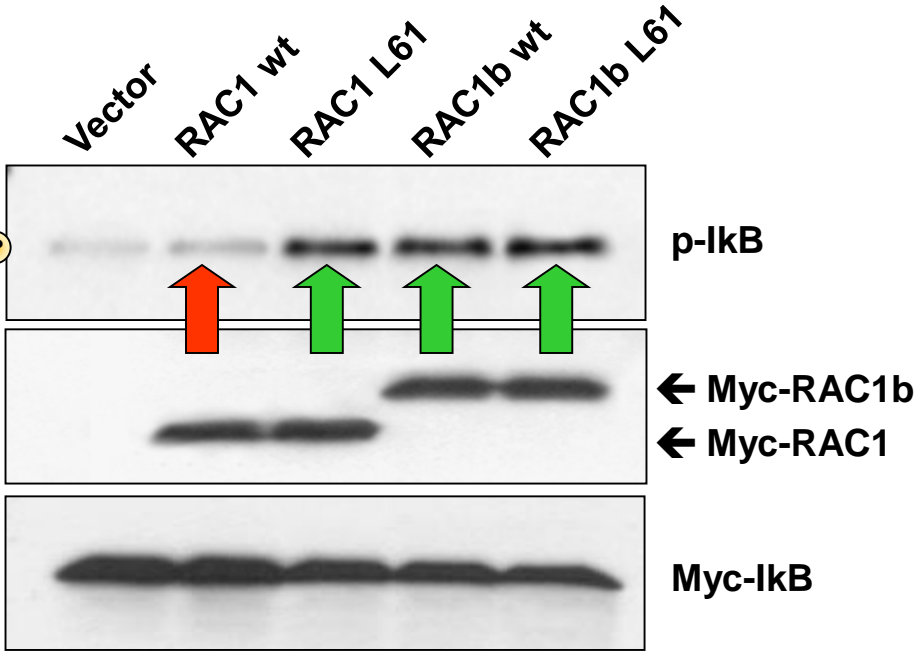
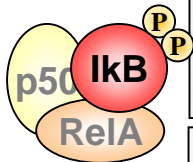
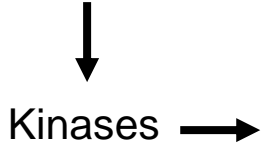
RAC1 but not RAC1b stimulates JNK activation



Activation of the transcription factor NF- κ B

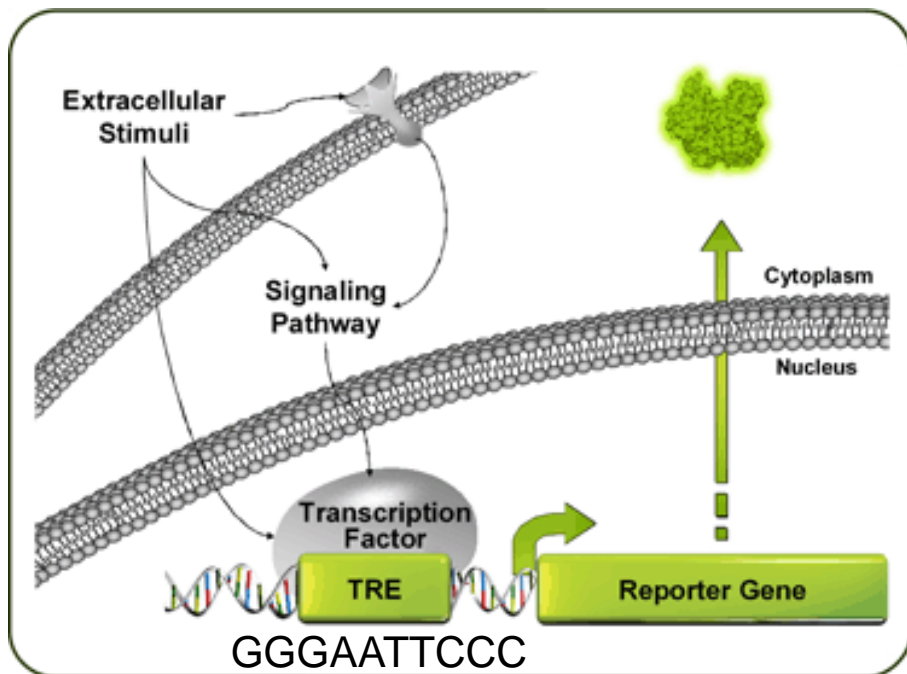
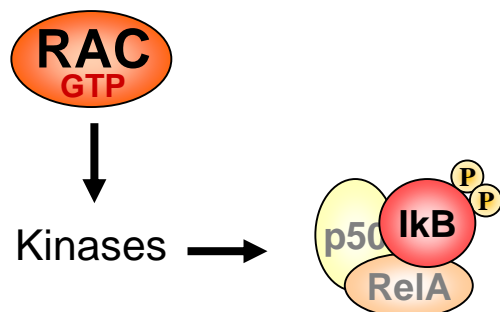


RAC1b-induced phosphorylation of IκB

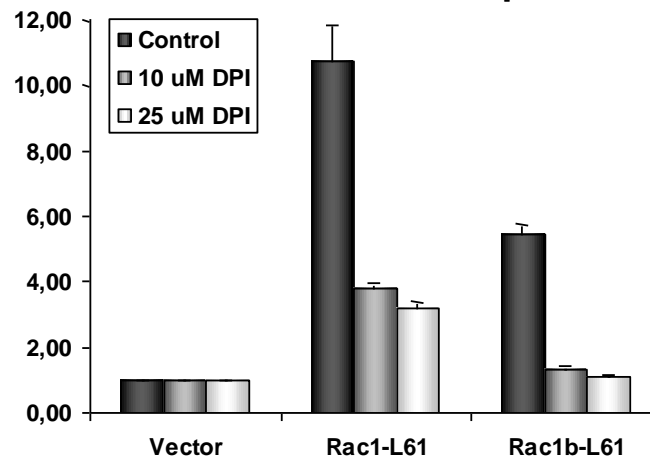


transfected NIH 3T3 cells

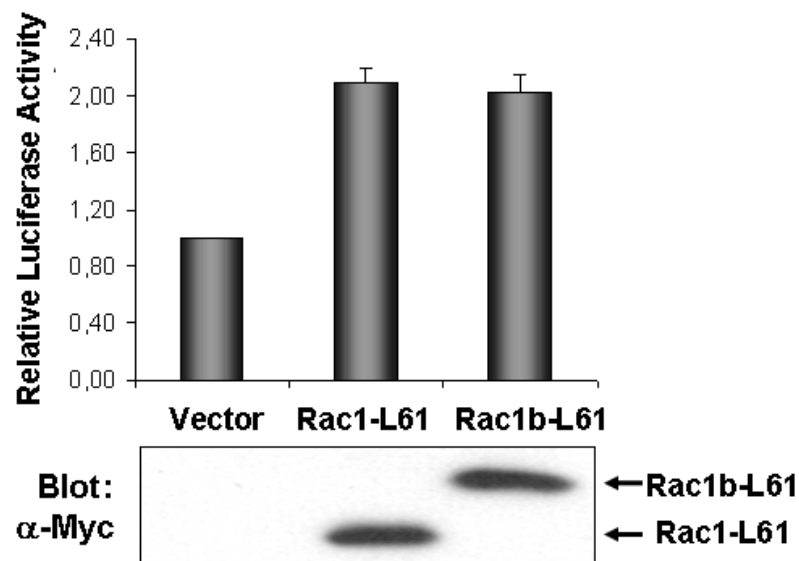
RAC1b-induced activation of NF- κ B transcriptional reporter gene activity



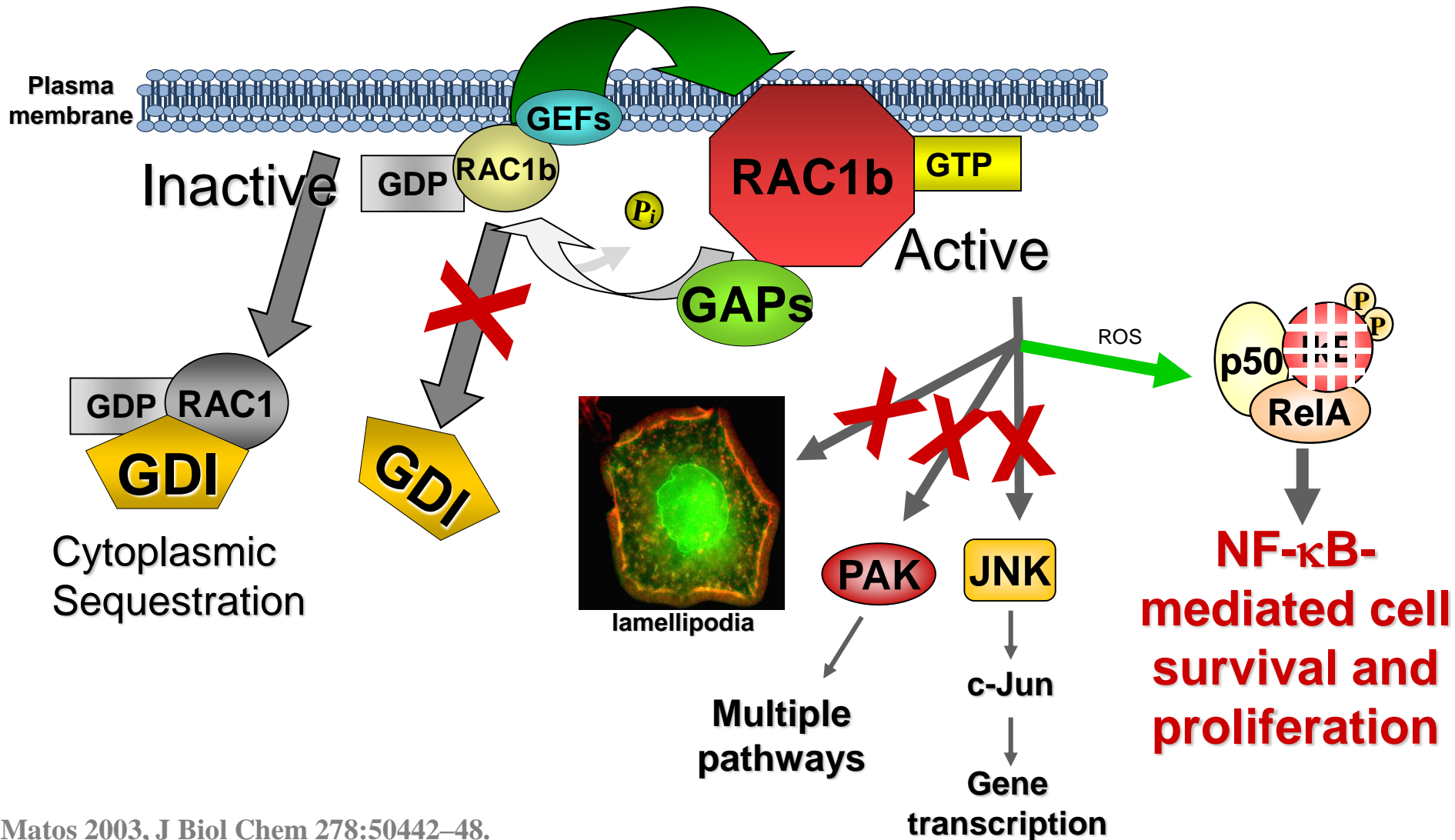
NF- κ B reporter



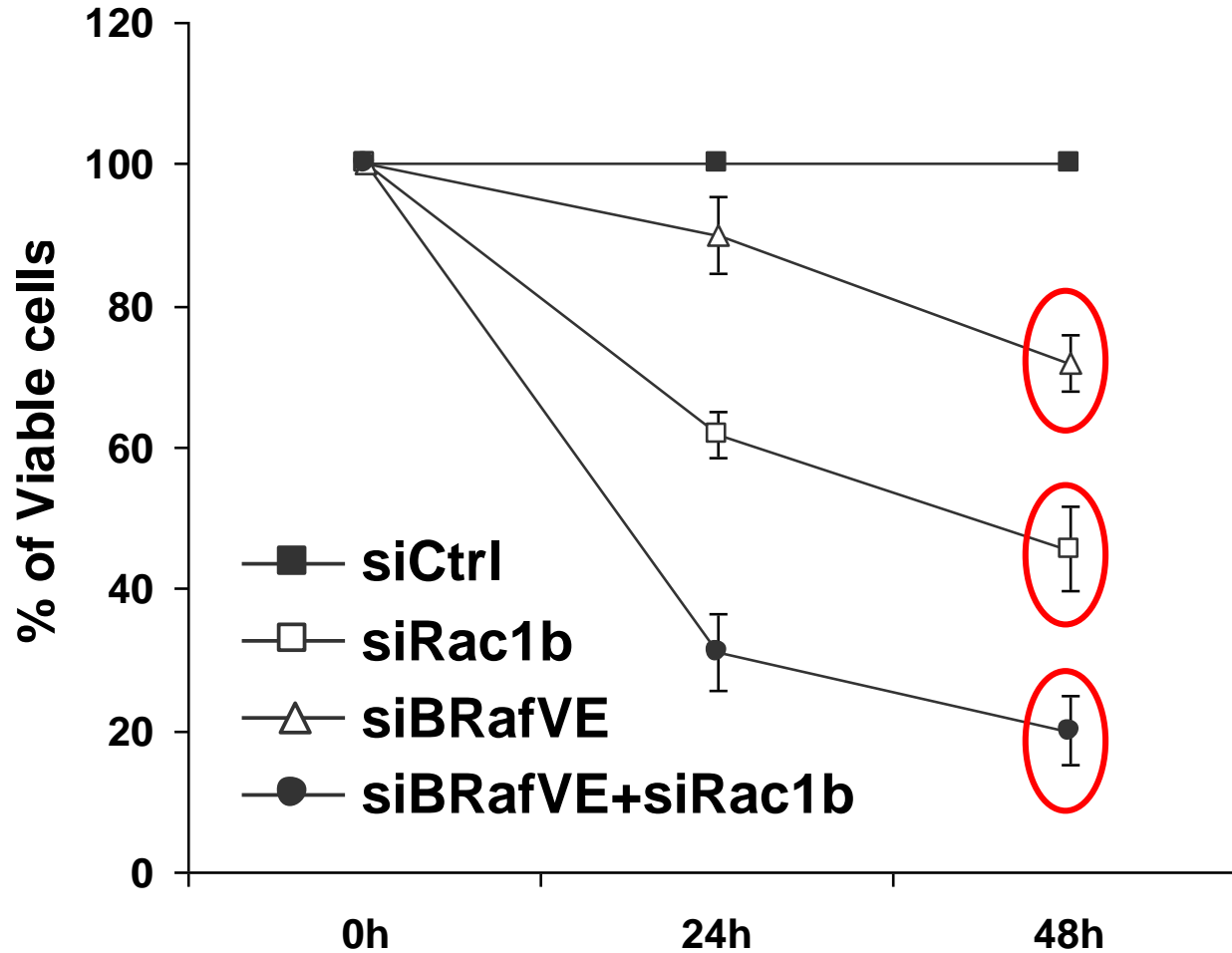
Cyclin D1 reporter



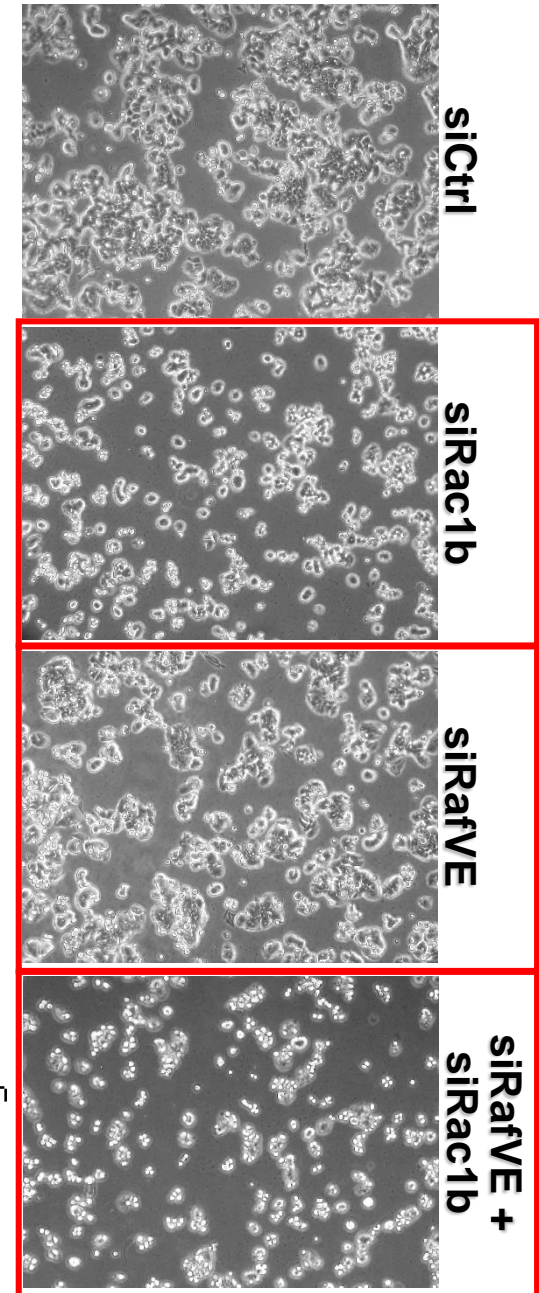
Summary of the unique properties of alternative spliced RAC1b



Effect of suppressing RAC1b and B-Raf on cell viability (HT29)



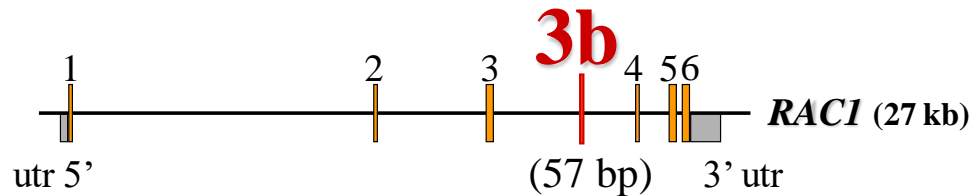
48h



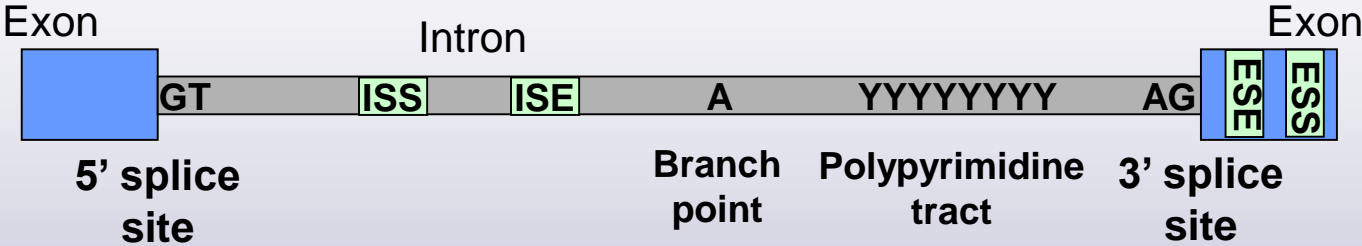
Summarizing so far...

- **Alternative splicing of *RAC1* pre-mRNA generates the highly activated variant RAC1b**
- **RAC1b protein differs in regulation and signaling**
- **Overexpression of RAC1b occurs in a subtype of CRC characterized by mutation in *BRAF* (10-15% of cases)**
- **Overexpression of RAC1b cooperates with mutant BRAF to sustain colorectal tumor cell survival**

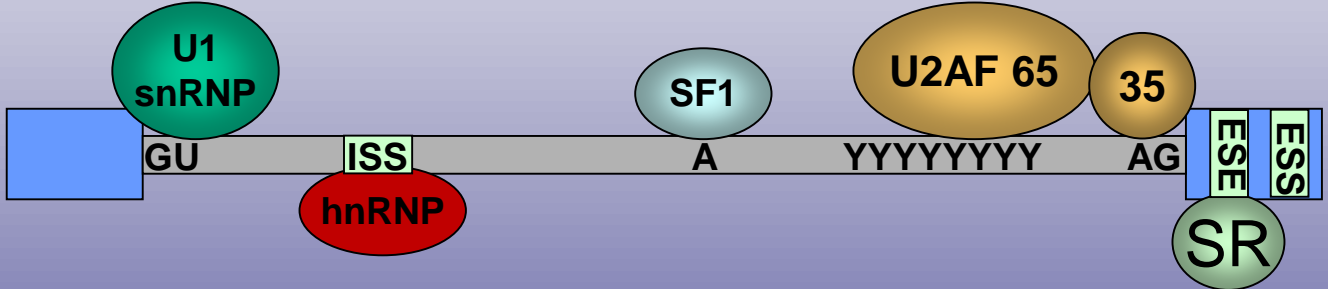
**How is
alternative splicing of RAC1b
regulated
in colorectal cells ?**



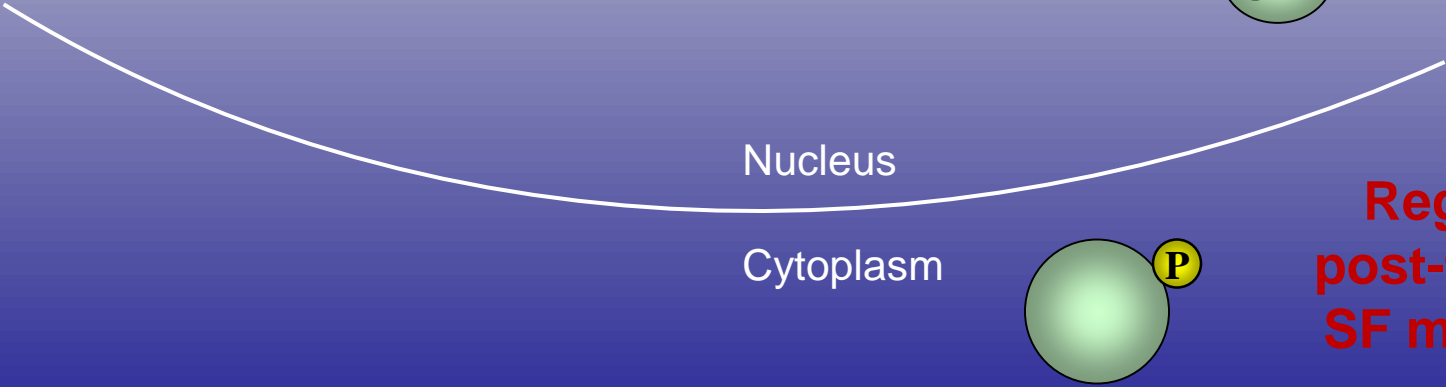
How can the expression of alternative splicing variants become altered in cells?



Mutation

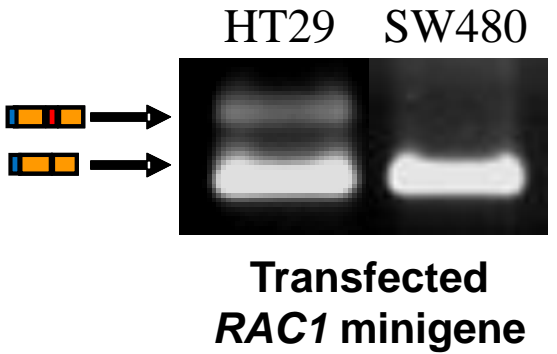
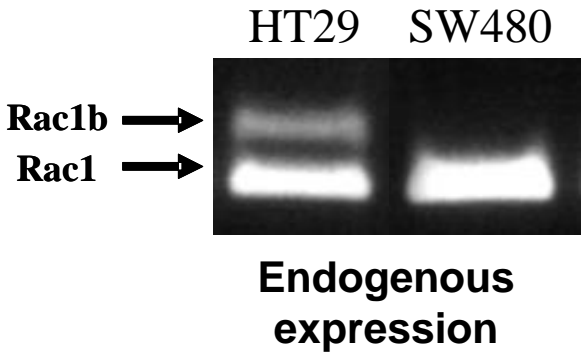
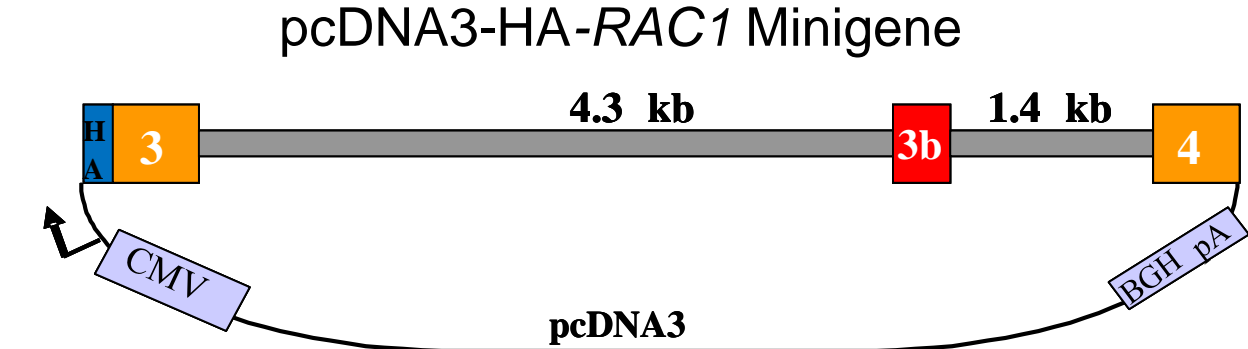
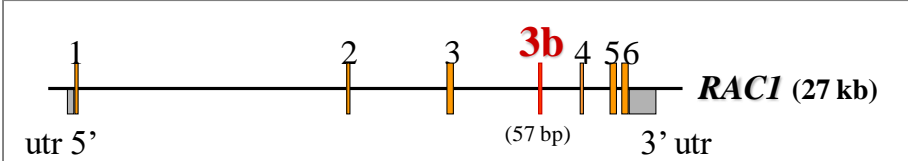


Regulated by relative SF concentration

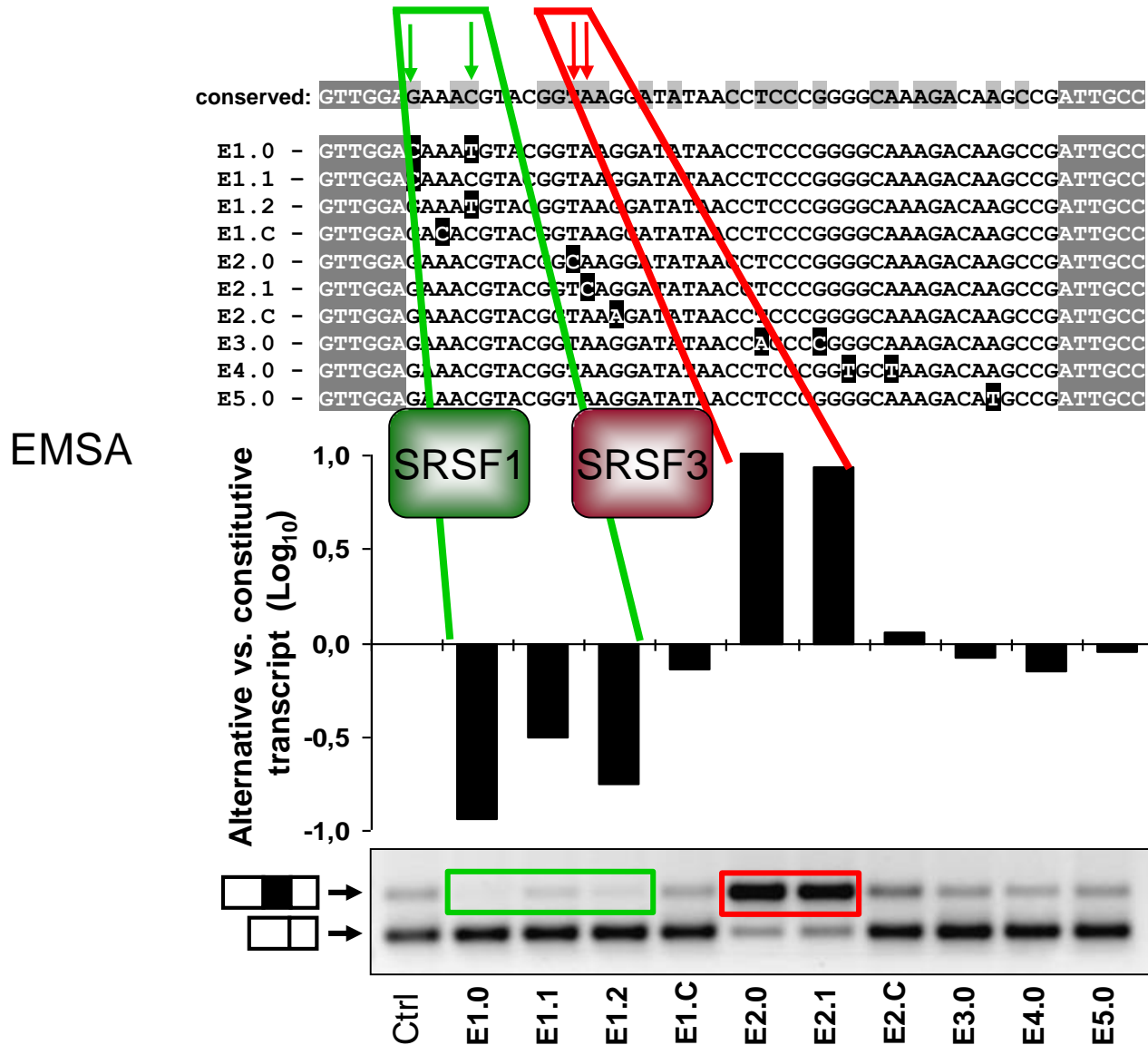


Regulated by post-translational SF modifications

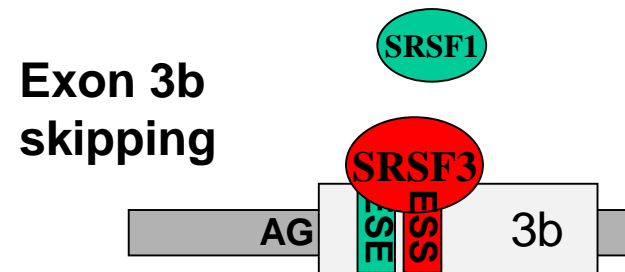
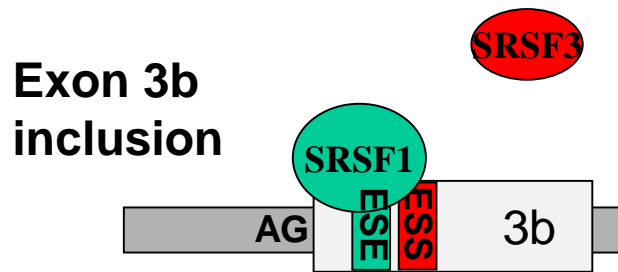
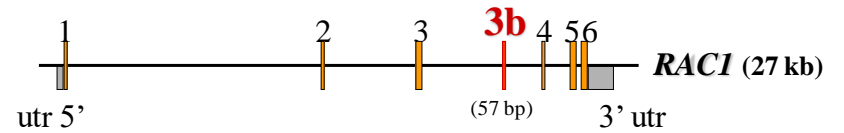
Dissection of splicing regulation with a *RAC1* minigene



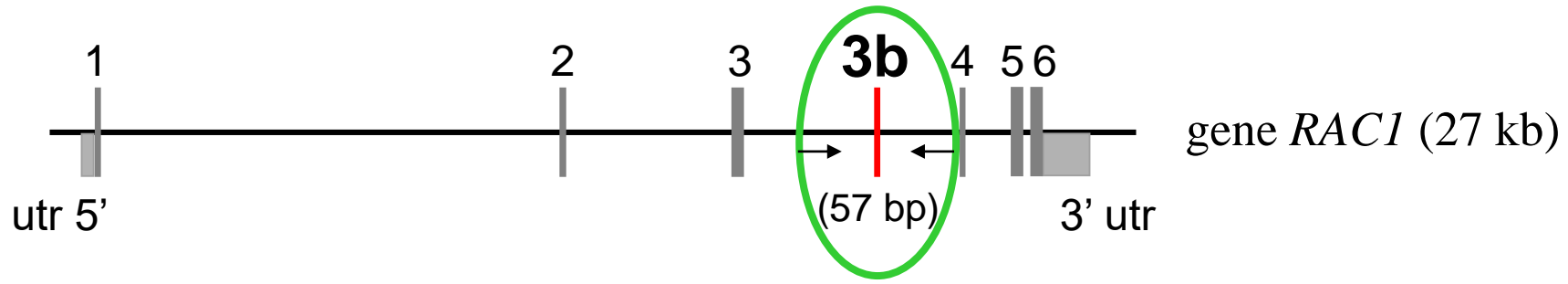
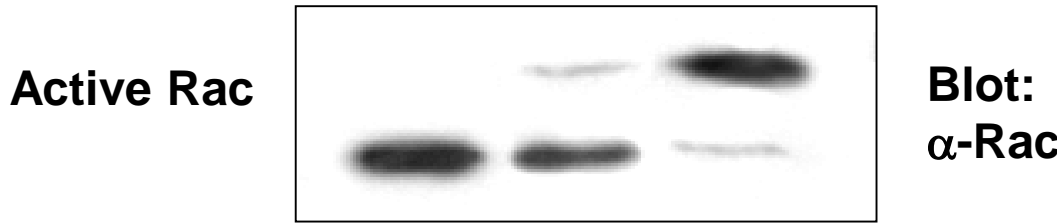
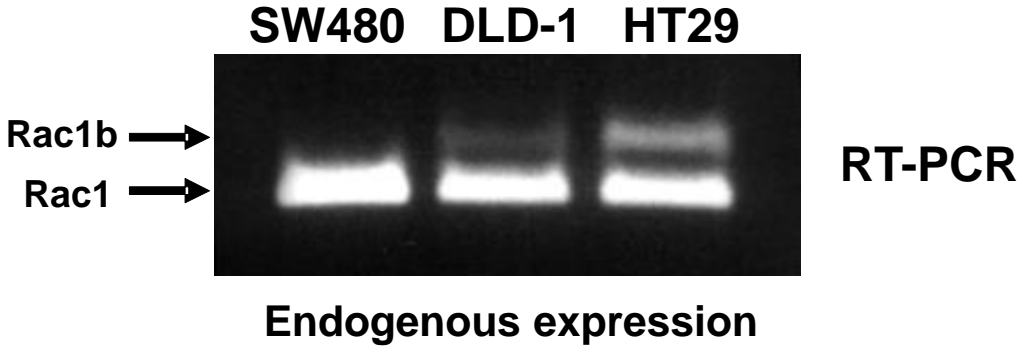
Two regulatory sequence elements determine exon 3b inclusion or skipping



Model for regulation of *RAC1* alternative splicing in CRC

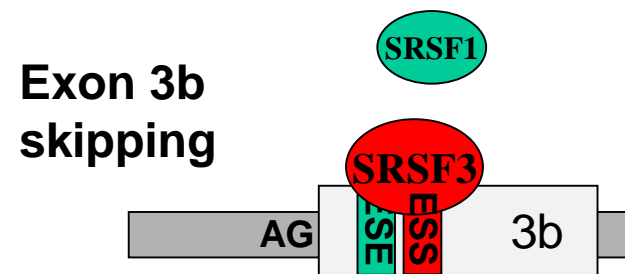
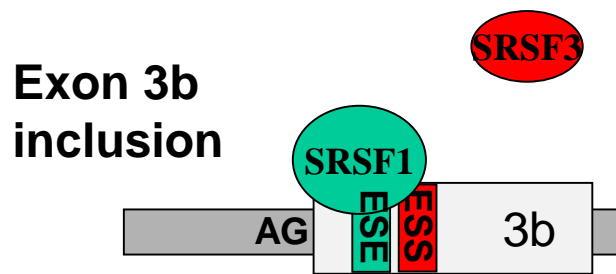
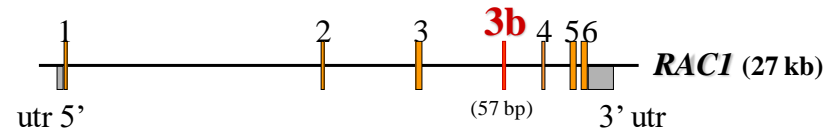


Do genomic mutations cause increased exon 3b inclusion?



➔ Cell lines have identical genomic *RAC1* sequence

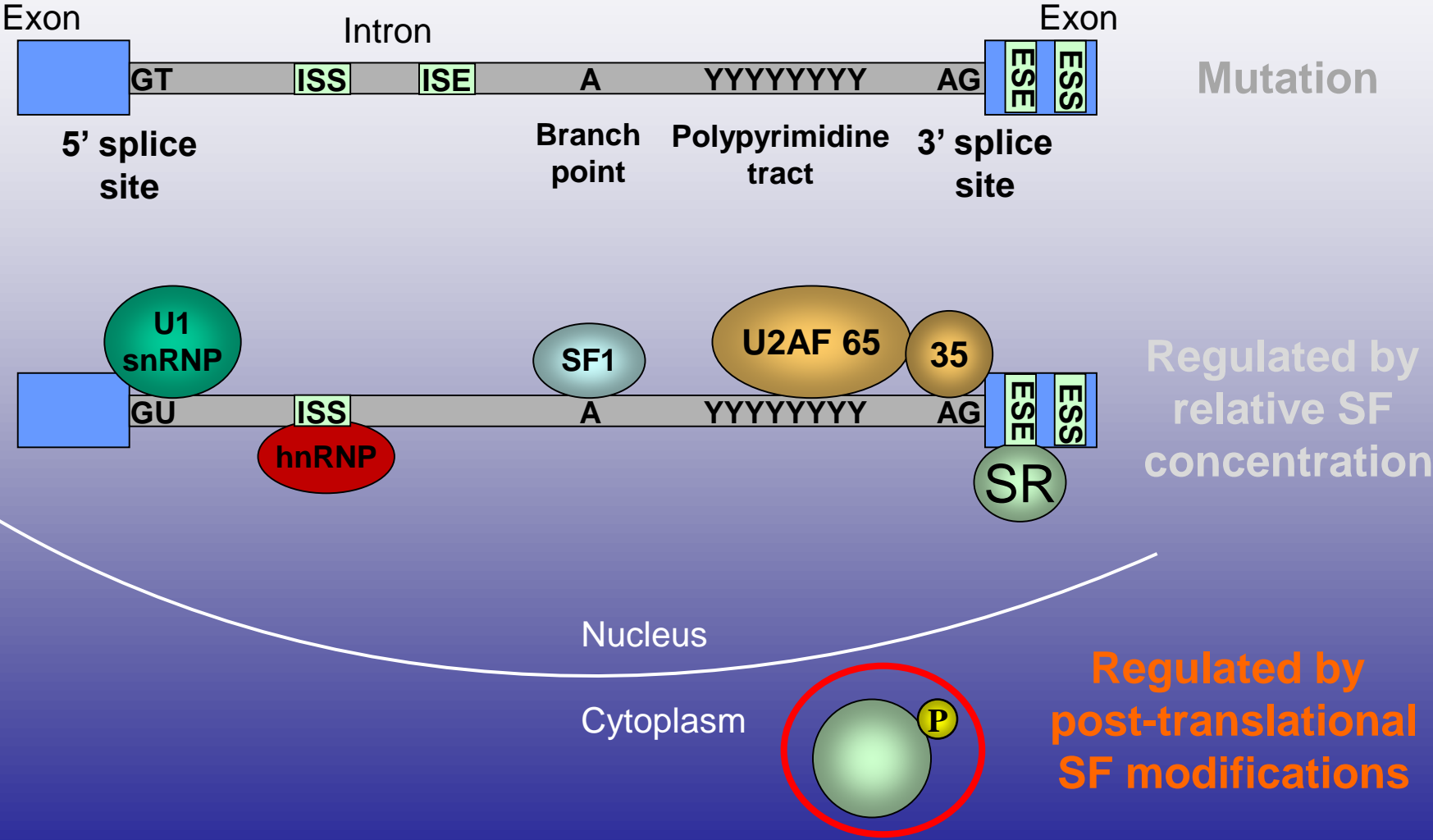
Model for regulation of *RAC1* alternative splicing in CRC



The changes in *RAC1* alternative splicing in colorectal cells are not caused by gene mutations....

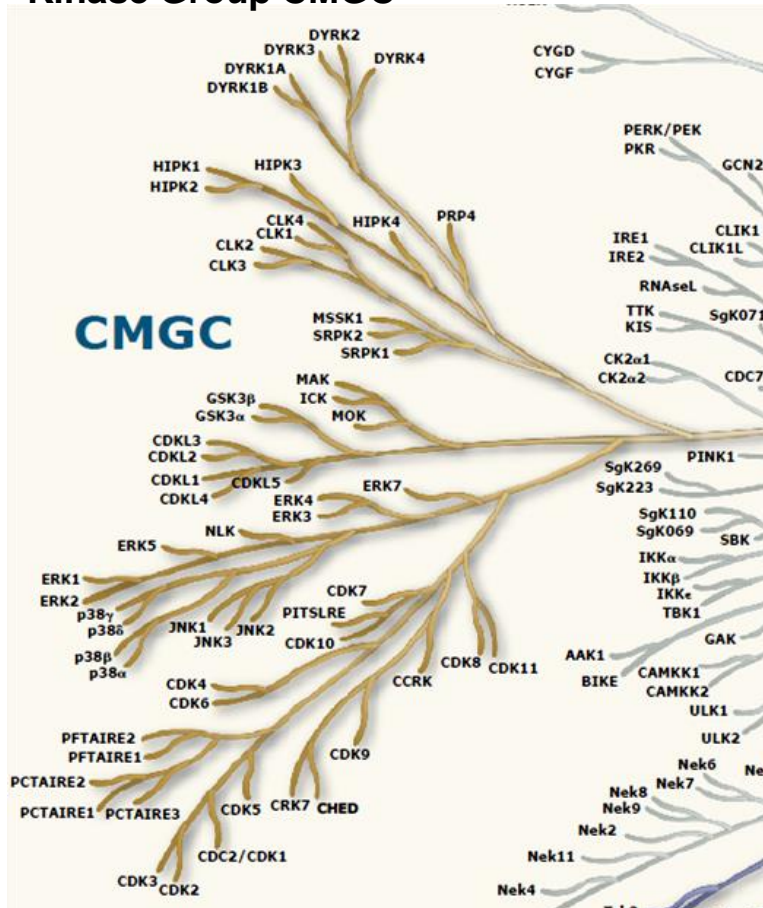
...so, how is the binding of SRSF1 and SRSF3 regulated?

How can the expression of alternative splicing variants become altered in cells?



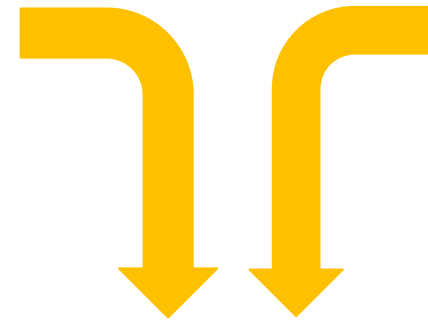
Protein kinases and phosphatases previously described to regulate alternative splicing events

Kinase Group CMGC



CLK1
CLK2
CLK3
CLK4
DYRK1a
DYRK1b
DYRK2
DYRK3
DYRK4
CDK11
CDK12
CDK13
GSK3alpha
GSK3beta
SRPK1
SRPK2
MSSK1
PRPF4B
PRP4

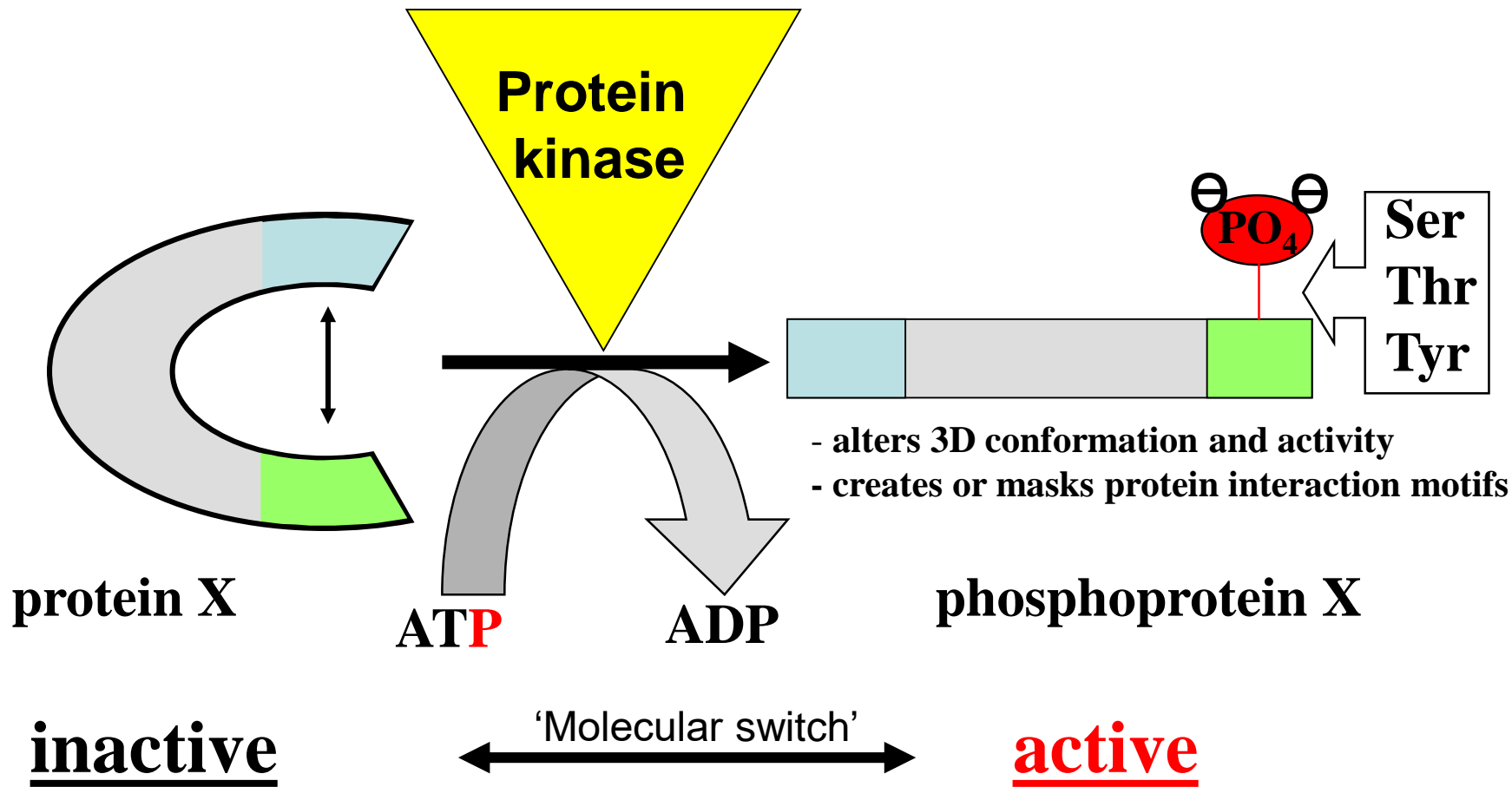
TOPOI
AKT1
AKT2
AKT3
PP1alpha
PP1beta
PP1gamma



Depletion by shRNAs

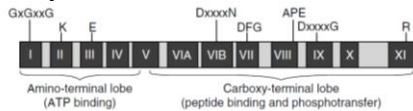


Differences in RAC1b protein levels in CRC cell lines ??



	-	EGF	+
pSer	89%		85%
pThr	10%		12%
pTyr	0.05%		2%

The human kinome



518
protein kinase genes

478 ePKs
conserved eucaryotic domain

40 aPKs
atypical catalytic domain

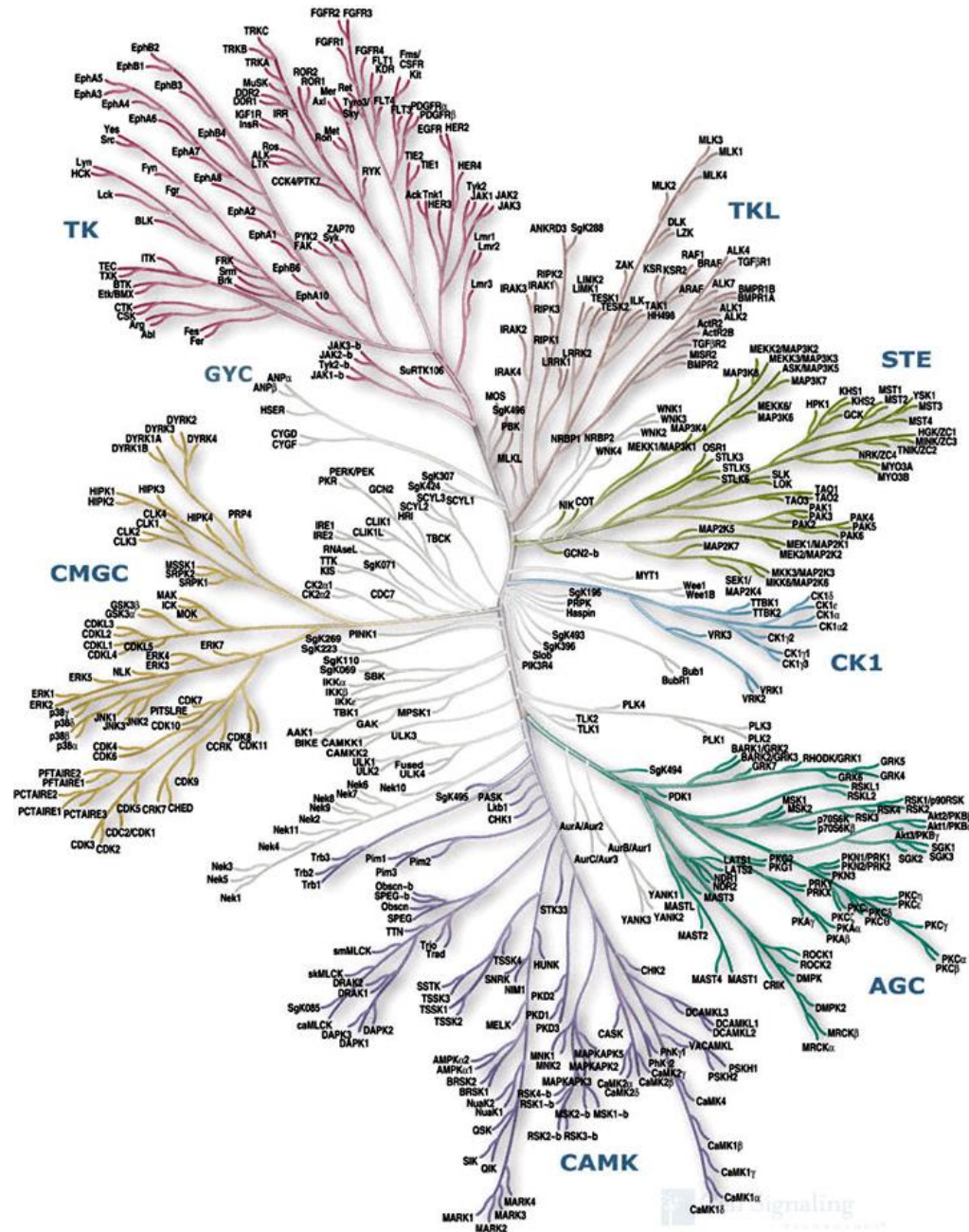
50 Pseudo-ePK kinases lacking conserved residues

428 ePKs with known or likely kinase activity

8 subgroups:

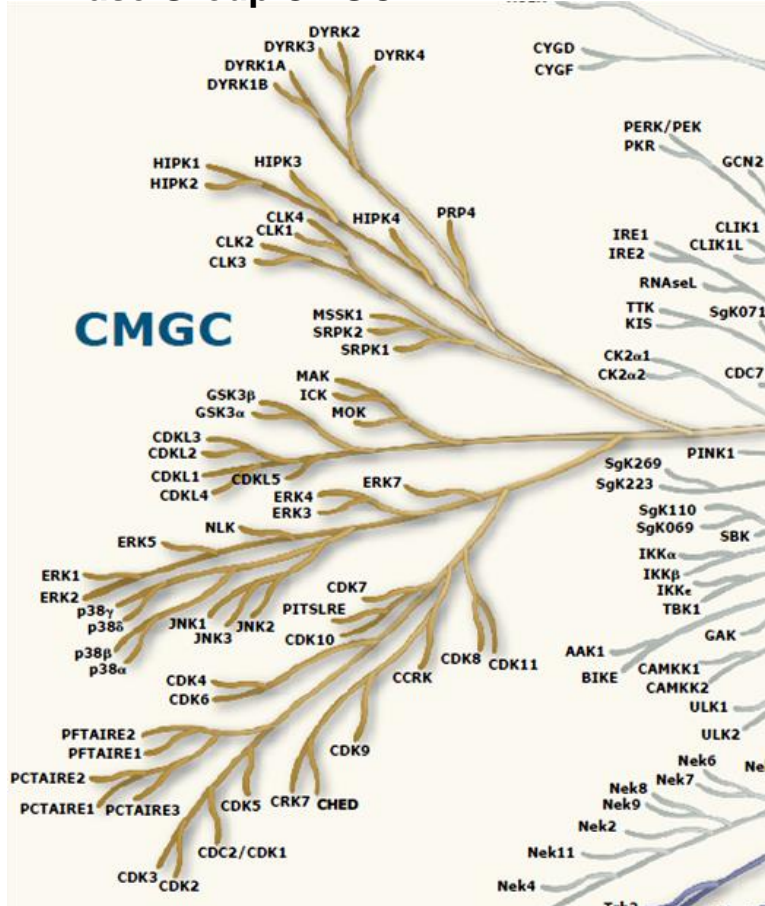
TK- 84; CAMK- 66; AGC- 61; CMGC- 61;
STE- 45; TKL- 37; CK1- 11; Other- 63

The human kinome tree:
 clustering by sequence
 similarity in the kinase
 domain led to identification
 of different subfamilies
 (incl. 60 receptor kinases)



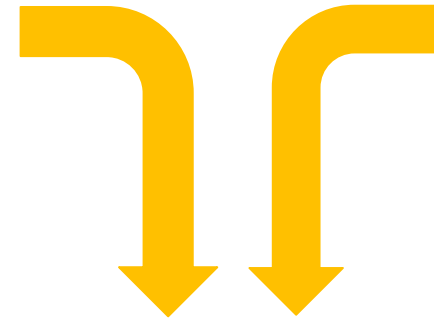
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CDK13
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PRP4

TOPOI
AKT1
AKT2
AKT3
PP1alpha
PP1beta
PP1gamma



Depletion by shRNAs

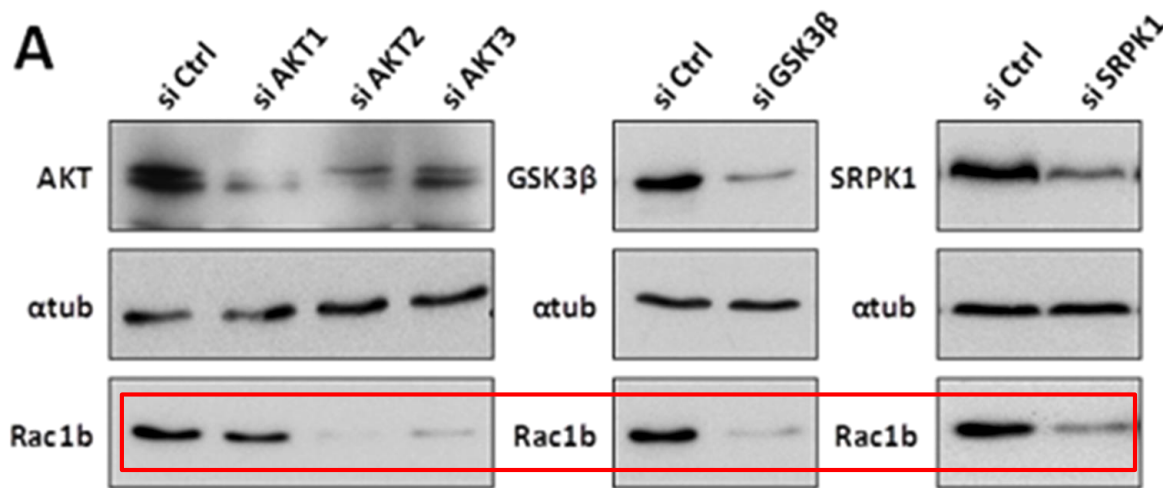


Differences in RAC1b protein levels in CRC cell lines ??



5 protein kinases affected RAC1b

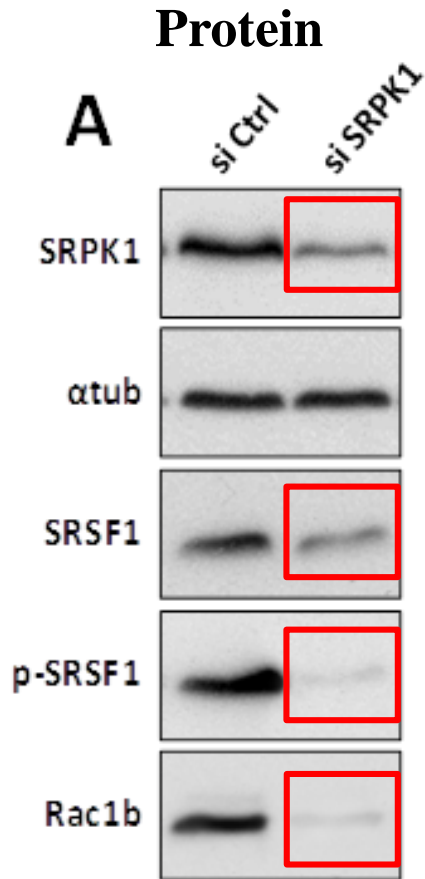
Validation of 5 protein kinases affecting RAC1b splicing



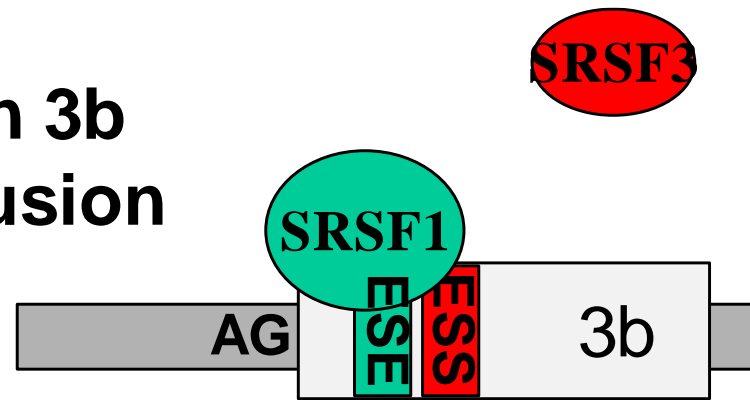
At RAC1b protein level:
Secondary screen with commercial siRNAs clearly reproduced the effects of 4 out of 5 shRNAs, (with exception of siAKT1)

At RAC1b transcript level:
only the depletion of **GSK3 β** and **SRPK1** decreased endogenous RAC1b expression

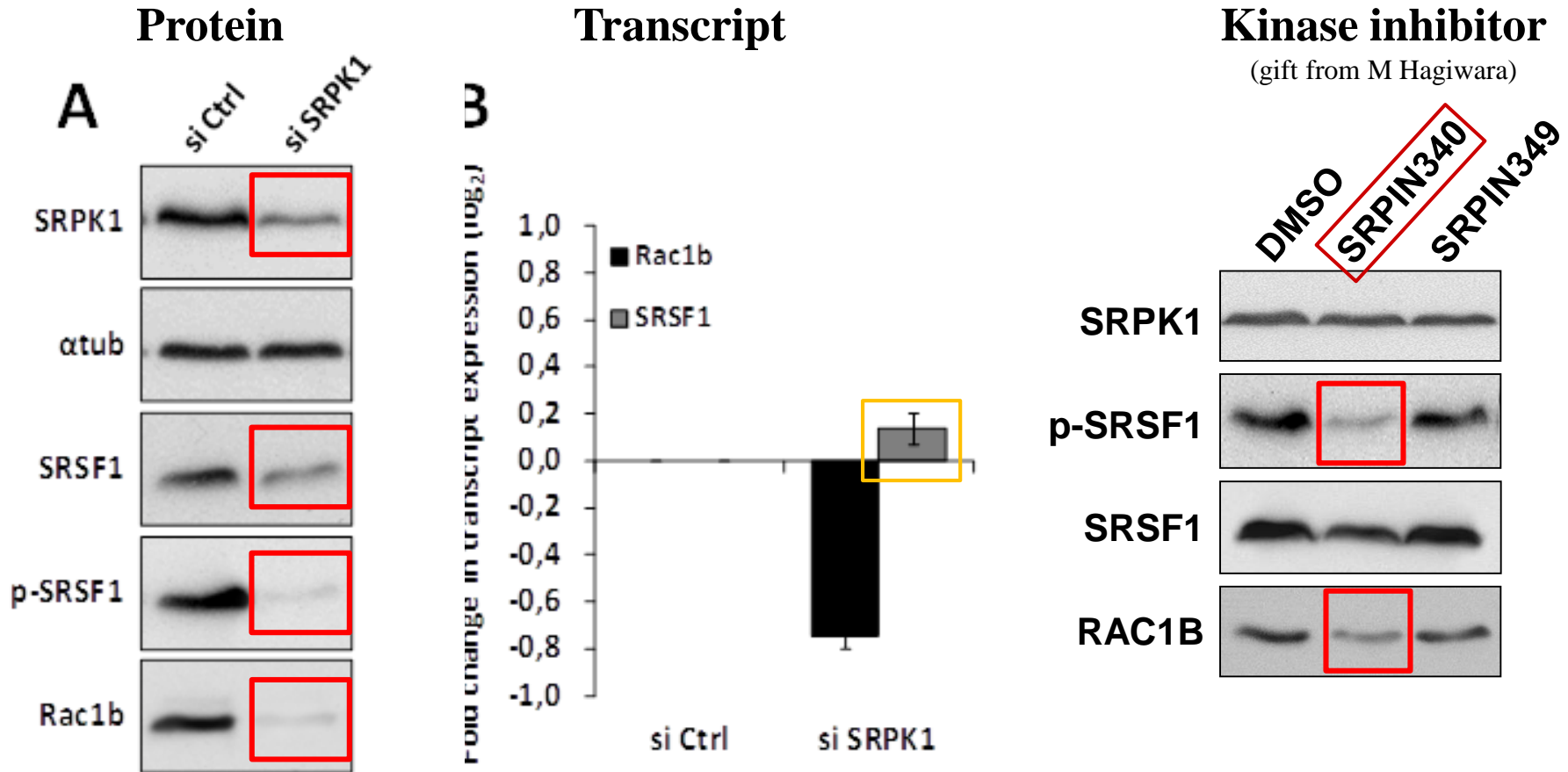
Candidate 1: role of protein kinase **SRPK1** in RAC1b splicing



**Exon 3b
inclusion**

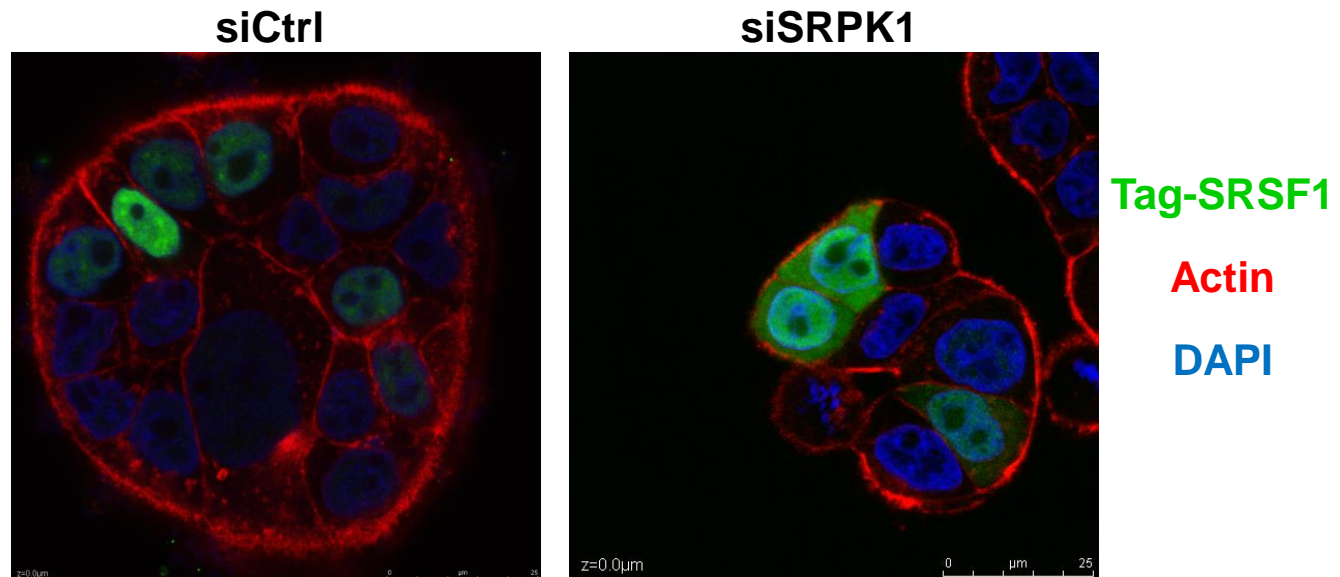
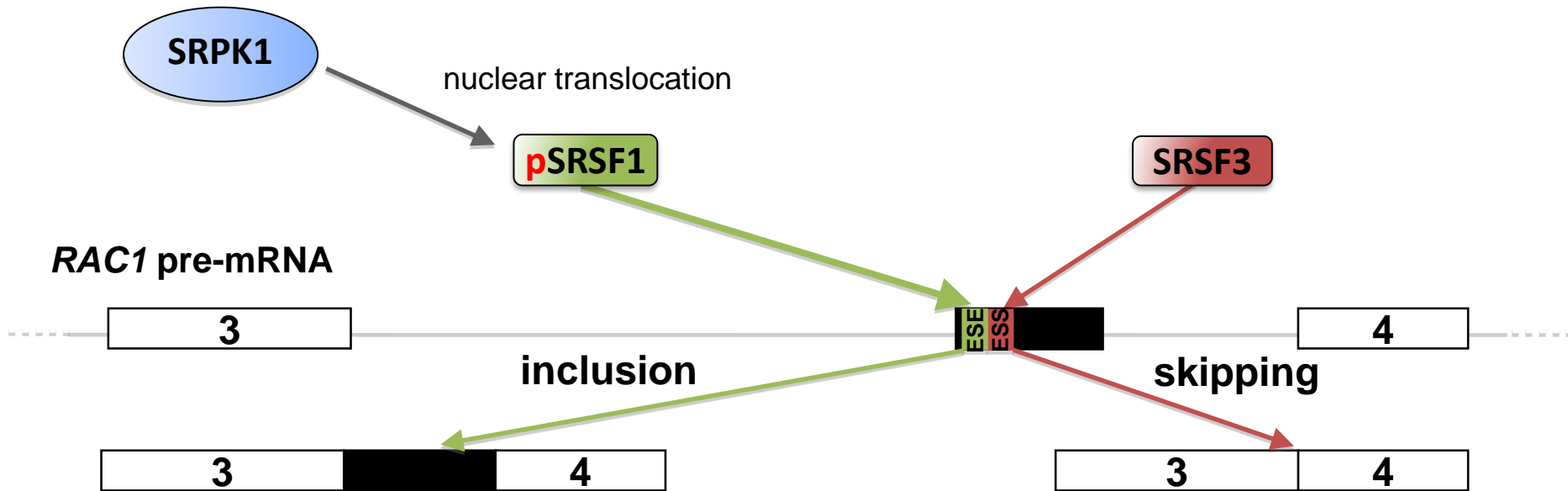


Candidate 1: role of protein kinase **SRPK1** in RAC1b splicing

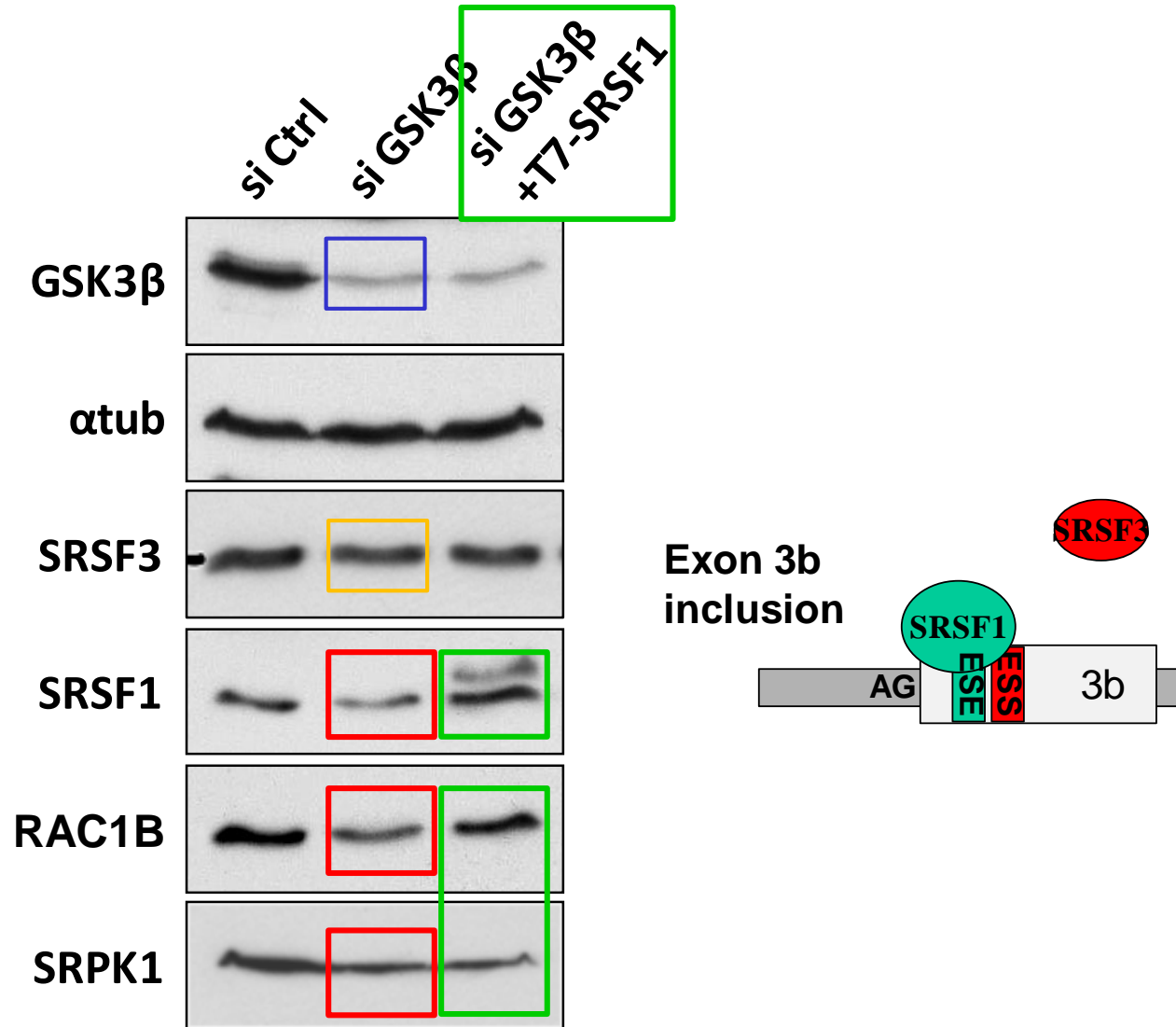


Inhibition of SRPK1 decreases phosphorylation of SRSF1 (but not its transcription), leading to decreased SRSF1-dependent alternative splicing of RAC1b

Role of protein kinase SRPK1 in RAC1b splicing

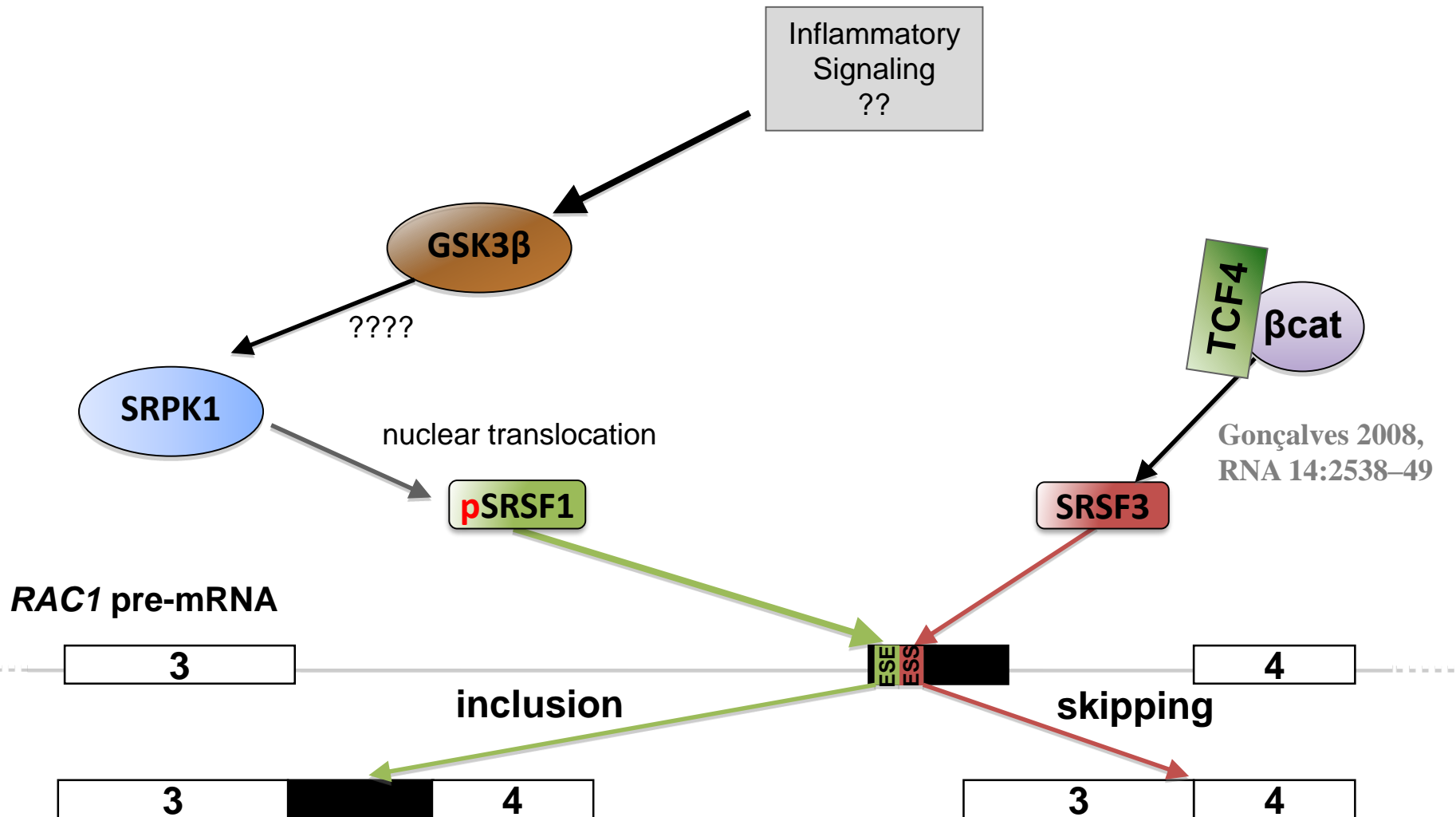


Candidate 2: role of protein kinase GSK3 β in RAC1b splicing



Inhibition of GSK3 β decreases RAC1b alternative splicing through SRSF1

Signaling pathways regulating RAC1b splicing

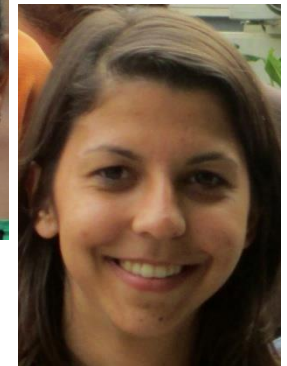


Summary-2

- **SRSF1 is a key splicing factor required for inclusion of alternative exon 3b to generate RAC1b**
- **Protein kinases GSK3 β and SRPK1 are upstream regulators of SRSF1-mediated RAC1b overexpression in CRC**
- **Small molecule protein kinase inhibitors can correct *RAC1* aberrant splicing events in cancer cells.**

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