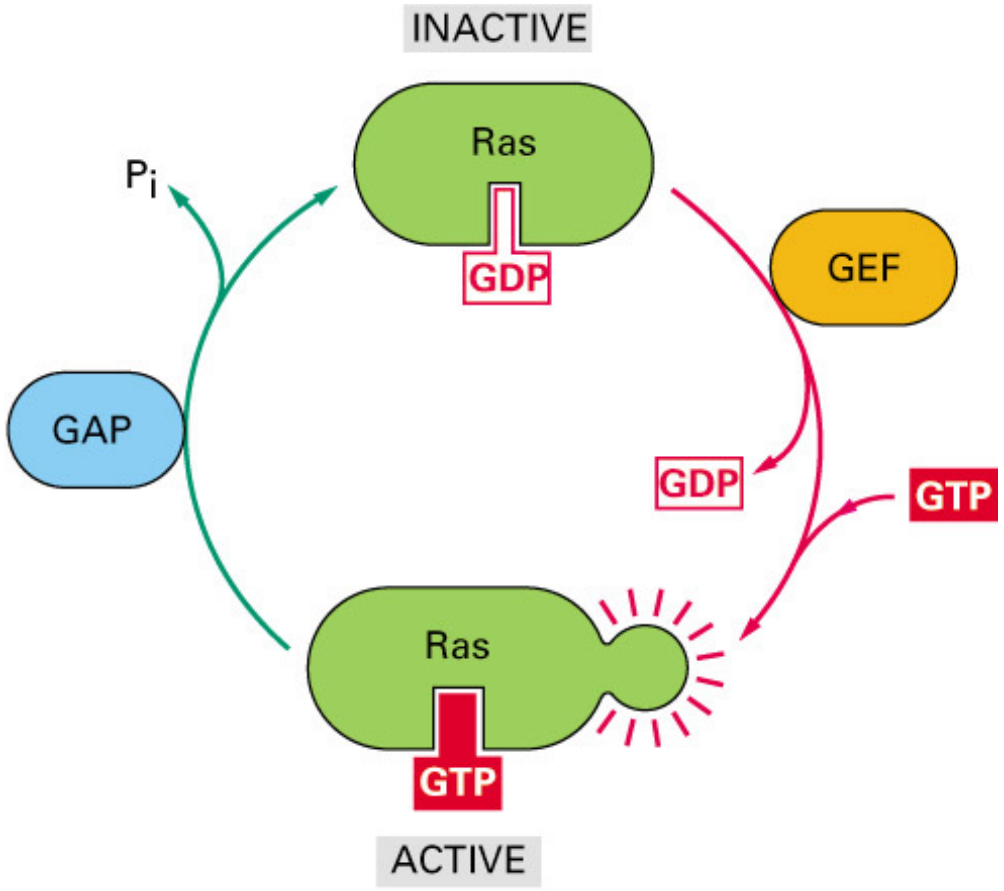
Ras (RAT-sarcoma) **is a monomeric GTPase**

- Ras is a G protein (guanosine-nucleotide-binding protein), a small GTPase
- The first two *ras* genes, *HRAS* and *KRAS*, were first identified from the Harvey sarcoma virus and Kirsten sarcoma virus, by Scolnick and colleagues at (NIH) in 1982. In 1982, activated and transforming human *ras* genes were discovered in human cancer cells. A third *ras* gene was subsequently discovered and named *NRAS*, for its initial identification in human neuroblastoma cells.
- Anchored at the plasma membrane owing to its prenylation and palmitoylation (HRAS and NRAS) or the combination of prenylation and a polybasic sequence adjacent to the prenylation site (KRAS).
- The C-terminal region of Ras first gets farnesylated at its Cys residue in the cytosol, allowing Ras to loosely insert into the membrane of the endoplasmatic reticulum and other cellular membranes.
- The three human *ras* genes encode extremely similar proteins made up of chains of 188 to 189 amino acids, designated H-Ras, N-Ras and K-Ras4A and K-Ras4B (from alternative splicing).

Ras is a monomeric GTPase

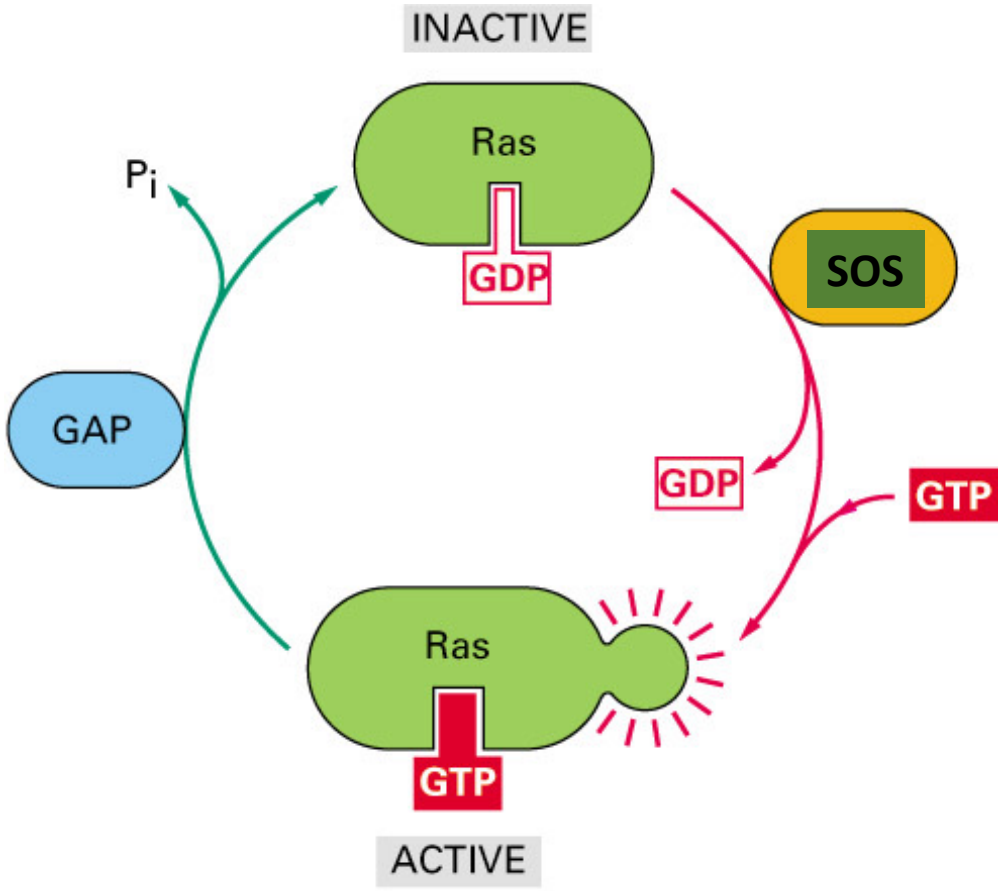


Ras has an intrinsic GTPase activity: the protein on its own will hydrolyze a bound GTP molecule into GDP. However this process is too slow for efficient function, and hence the GAP for Ras, *RasGAP*, may bind to and stabilize the catalytic machinery of Ras.

GEFs catalyze a "push and pull" reaction which releases GDP from Ras. Because intracellular GTP is abundant relative to GDP (approximately 10 fold more) GTP predominantly re-enters the nucleotide binding pocket of Ras and reloads the spring. Thus GEFs facilitate Ras activation.

The balance between GEF and GAP activity determines the guanine nucleotide status of Ras, thereby regulating Ras activity.

Ras is a monomeric GTPase



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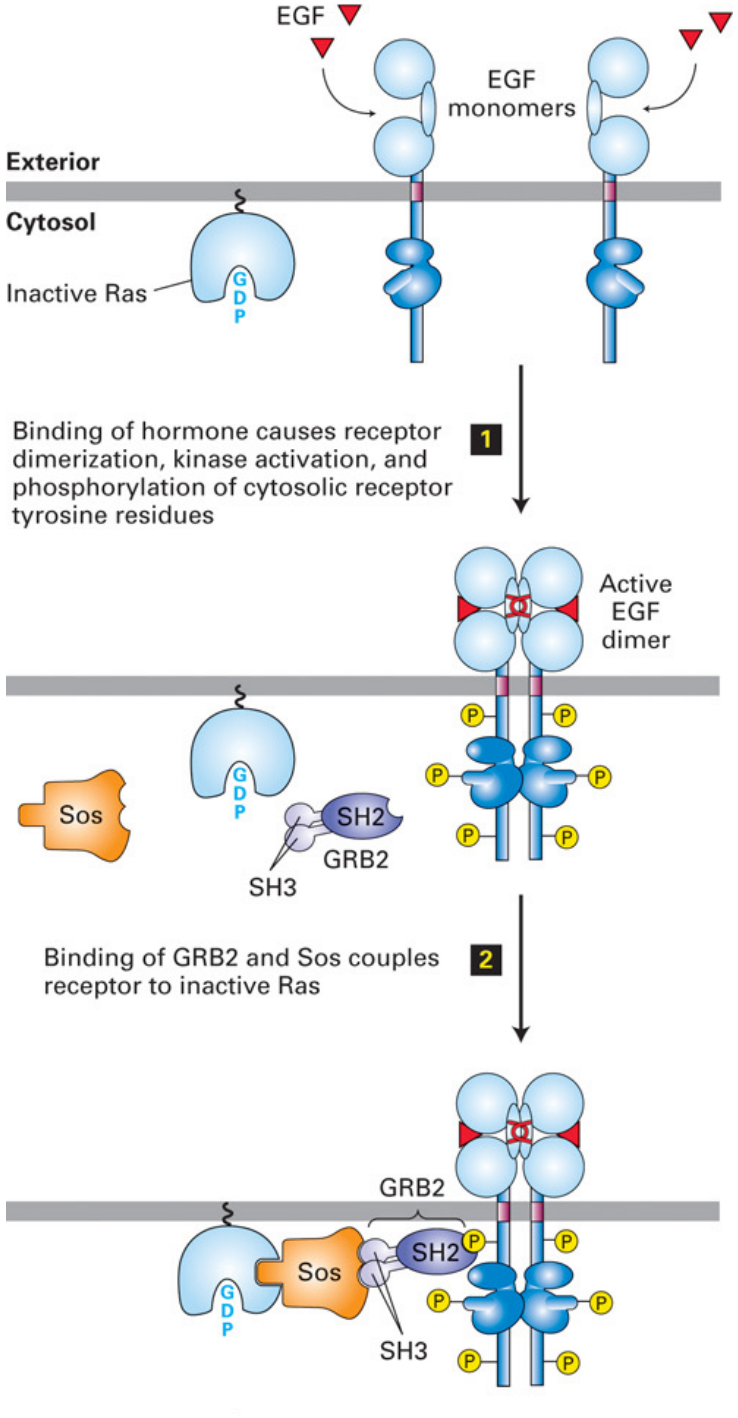
RTK Activation of Ras

EGF binding causes receptor clusterization and autophosphorylation on cytosolic tyrosines.

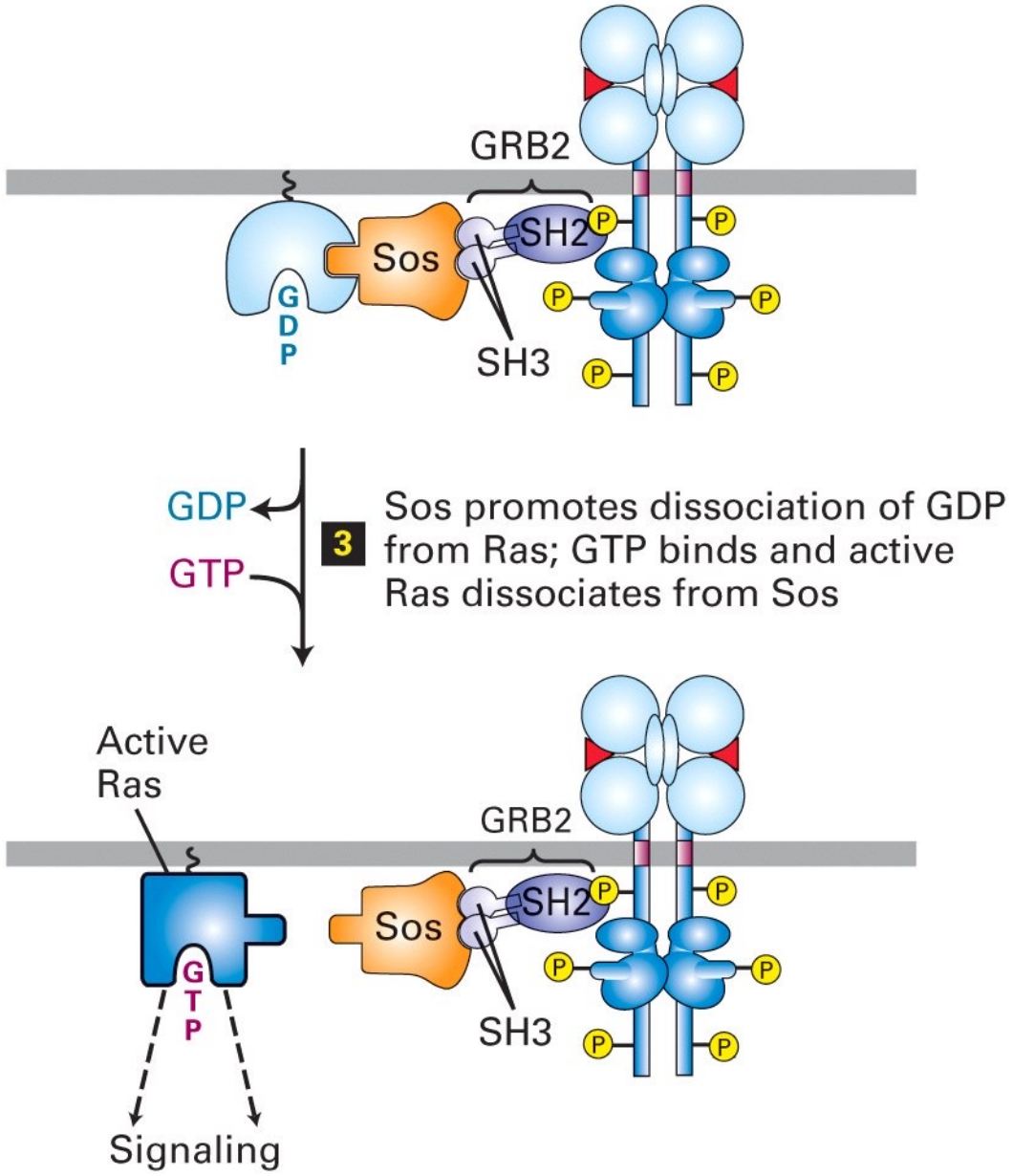
In Step 2, the adaptor protein GRB2 binds receptor phosphotyrosine residues via its SH2 domain. GRB2 contains SH3 domains that allow the GEF protein known as Sos to bind to the membrane complex.

The C-terminus of Sos inhibits its nucleotide exchange activity; binding of GRB2 relieves this inhibition

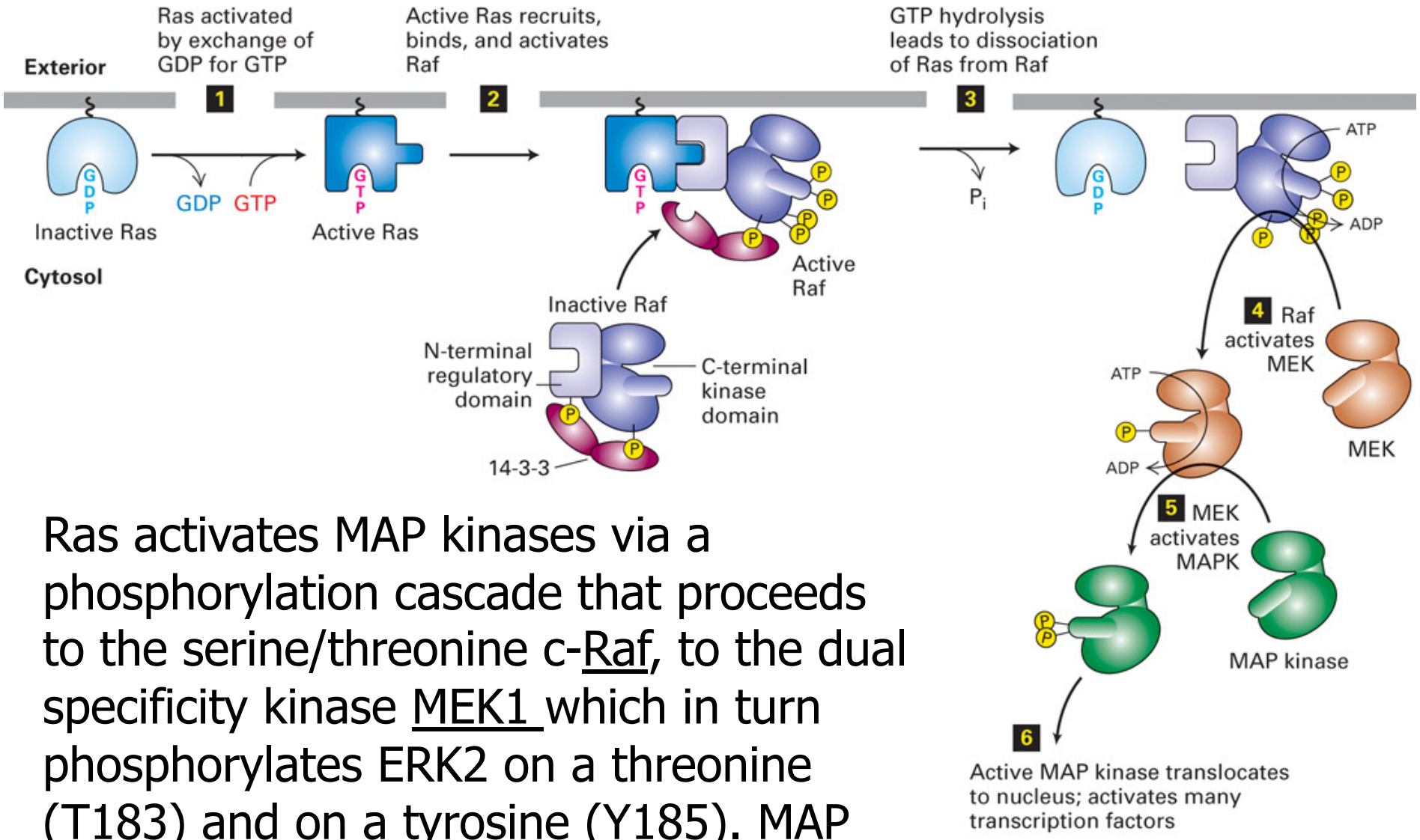
Sos converts inactive GDP-ras into active GTP-ras.



The activated Ras-GTP complex then dissociates from Sos, but remains tethered to the inner leaflet of the cytoplasmic membrane via a lipid anchor sequence. The active form of Ras then activates the MAP kinase portion of the signaling pathway.



Ras Activation of MAP Kinase

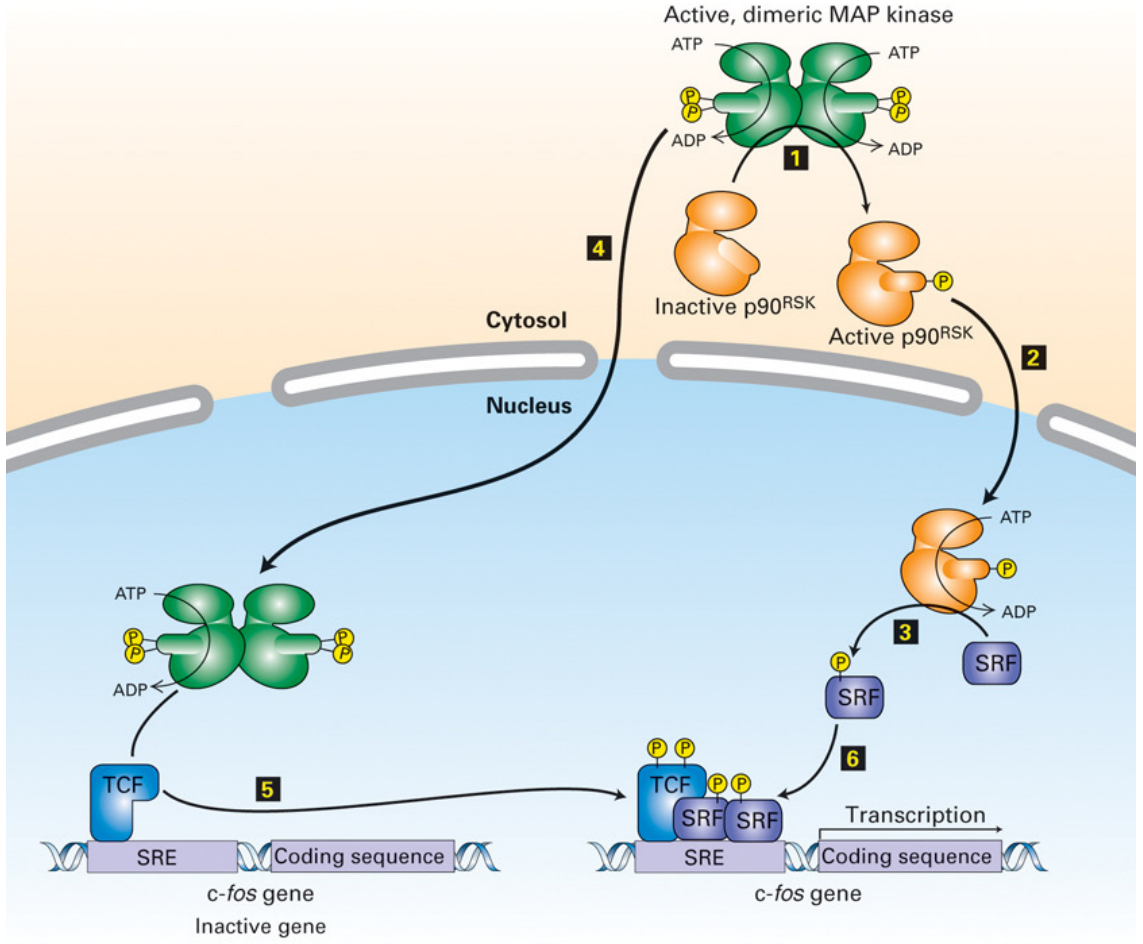


Ras activates MAP kinases via a phosphorylation cascade that proceeds to the serine/threonine c-Raf, to the dual specificity kinase MEK1 which in turn phosphorylates ERK2 on a threonine (T183) and on a tyrosine (Y185). MAP kinase then dimerizes and enters the nucleus.

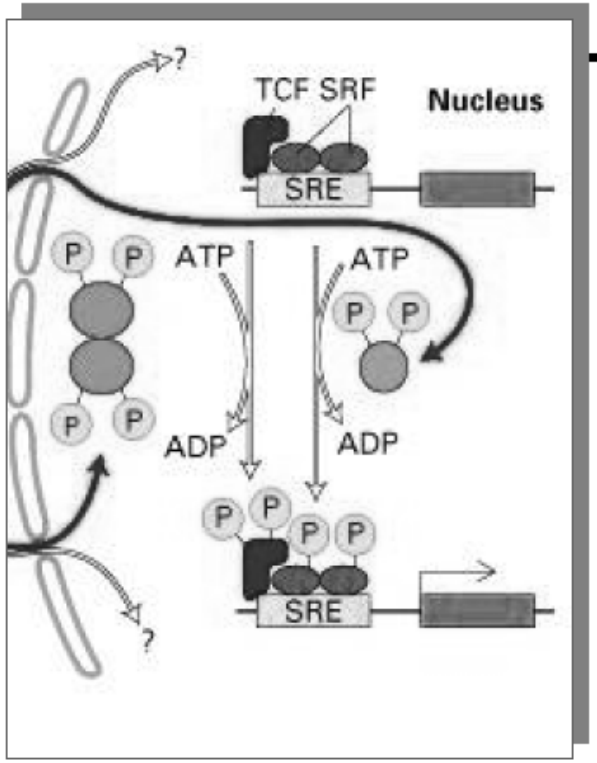
MAP Kinase Activation of Transcription

In the final steps of RTK-Ras/MAP kinase signaling, MAP kinase phosphorylates and activates the $p90^{RSK}$ kinase in the cytoplasm. Both kinases enter the nucleus where they phosphorylate ternary complex factor (TCF) and serum response factor (SRF), respectively.

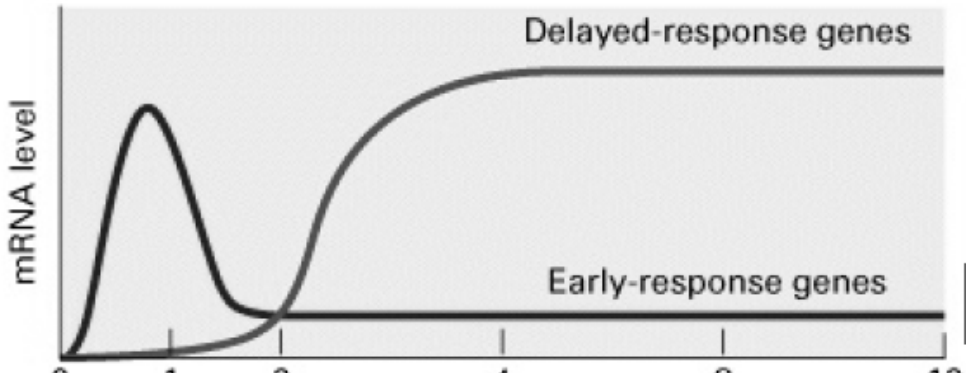
The phosphorylated forms of these TFs bind to serum response element (SRE) enhancer sequences that control genes regulated by growth factors present in serum (such as c-fos) and propel cells through the cell cycle.



Genes regulated by RTK/Ras pathway include early response genes.



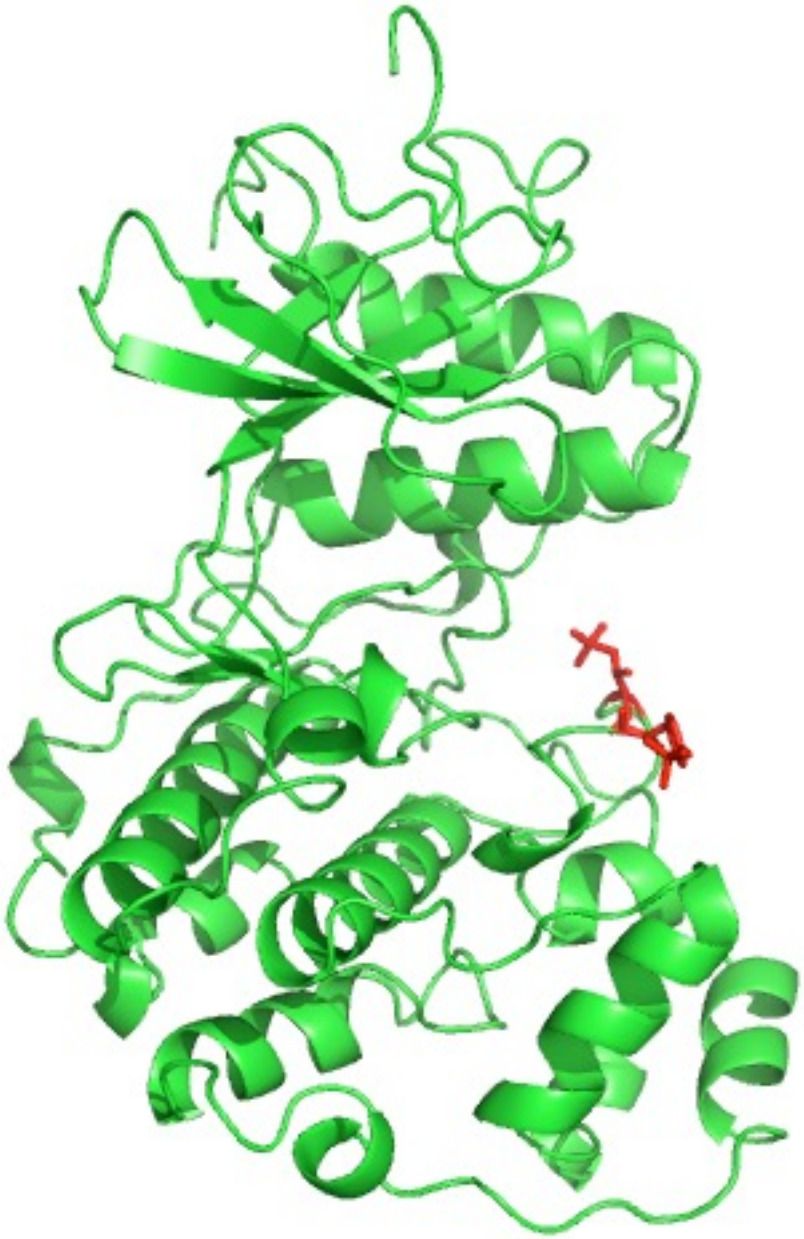
serum response elements;
-allows for activated transcription following growth factor (mitogen) stimulation
-found in genes involved in cellular proliferation.
Ex: c-fos gene
-c-fos is an early response gene
-required for the induction of delayed response genes including cyclin D.



D-type cyclins

C-fos transcription factor

MAP Kinases



- **Mitogen-activated protein kinases** are serine/threonine-protein kinases. They regulate proliferation, gene expression, differentiation, mitosis, cell survival, and apoptosis.

MAP kinases are found in eukaryotes only.

"Classical" MAPKs activation requires **two phosphorylation events**, both threonine and tyrosine residues, in order to lock the kinase domain in a catalytically competent conformation.

Inactivation of MAPKs is performed by a very conserved family of dedicated phosphatases is the so-called MAP kinase phosphatases (MKPs), dual-specificity phosphatases (DUSPs). They hydrolyze the phosphate from both phosphotyrosine and the phosphothreonine residues.

- Once activated, Ras propagates signaling further inside the cell via a kinase cascade that culminates in the activation of members of the MAP kinase family.
- MAP kinases phosphorylate TFs that regulate genes involved in the cell cycle, survival and in differentiation.

- As a result, mutations in *ras* genes can cause unintended and overactive signalling inside the cell and ultimately to cancer
- Ras is the most common oncogene in human cancer - mutations that permanently activate Ras are found in 20-25% of all human tumors and up to 90% in certain types of cancer (pancreatic cancer).

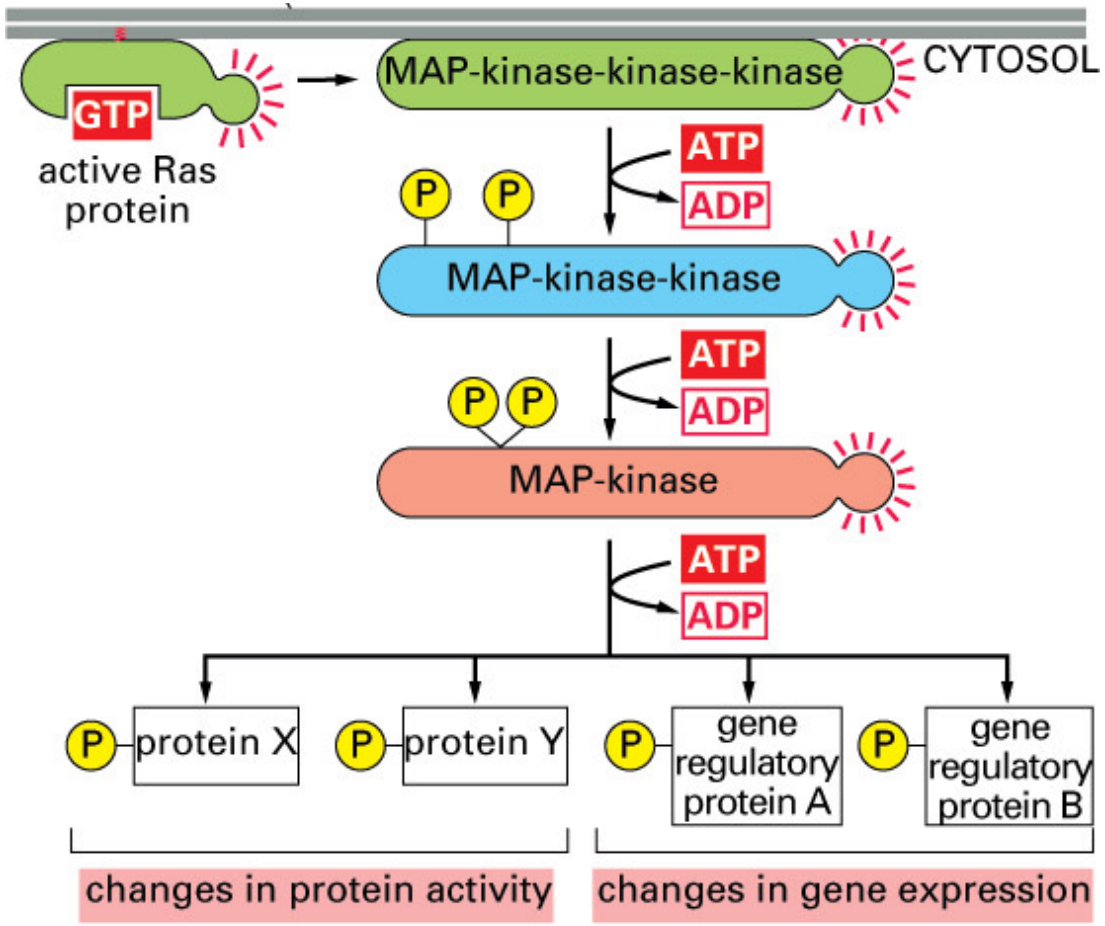


Table 2 | ***HRAS*, *KRAS*, *NRAS* and *BRAF* mutations in human cancer**

Cancer type	*HRAS*	*KRAS*	*NRAS*	*BRAF*
Biliary tract	0%	33%	1%	14%
Bladder	11%	4%	3%	0%
Breast	0%	4%	0%	2%
Cervix	9%	9%	1%	0%
Colon	0%	32%	3%	14%
Endometrial	1%	15%	0%	1%
Kidney	0%	1%	0%	0%
Liver	0%	8%	10%	3%
Lung	1%	19%	1%	2%
Melanoma	6%	2%	18%	43%
Myeloid leukaemia	0%	5%	14%	1%
Ovarian	0%	17%	4%	15%
Pancreas	0%	60%	2%	3%
Thyroid	5%	4%	7%	27%

The mutation data was obtained from the Sanger Institute Catalogue of Somatic Mutations in Cancer web site[148].

- Diversi tipi di cancro sembrano essere associati alla mutazione di una specifica isoforma RAS. Solitamente i carcinomi (in particolare quelli del colon e del pancreas) presentano mutazioni di KRAS, i tumori della vescica hanno mutazioni di HRAS e i tumori emopoietici presentano mutazioni di NRAS.

Oncogenes vs proto-oncogenes

- An **oncogene** is a gene that has the potential to cause cancer.
- In tumor cells, they are often mutated or expressed at high levels.
- The first confirmed oncogene was discovered in 1970 and was termed src. Src was in fact first discovered as an oncogene in a chicken retrovirus.
- In 1976 Dominique Stehelin, J. Michael Bishop and Harold E. Varmus demonstrated that oncogenes were activated proto-oncogenes, found in many organisms including humans (for this discovery Bishop and Varmus were awarded the Nobel Prize in Physiology or Medicine in 1989).

- A **proto-oncogene** is a normal gene that becomes an oncogene due to mutations or increased expression.
- Proto-oncogenes code for proteins that regulate cell growth and differentiation. Proto-oncogenes are often involved in signal transduction and execution of mitogenic signals.
- Upon *activation*, a proto-oncogene becomes a tumor-inducing agent, an oncogene.

CELLULAR ONCOGENES

- Present in cancer cells
- Contains introns characteristic of eukaryotic cells
- Encodes proteins triggering transformation of normal cells

VIRAL ONCOGENES

- Present in viruses
- Host cell origin
- Do not possesss introns
- Also called 'cancer genes'
- Encodes proteins triggering transformation of normal cells into cancer cells

VIRAL ONCOGENE	HUMAN ONCOGENE	ORIGIN	NATURE
V-src	C-src	Chicken	Sarcoma
V-ras	C-ras	Rat	Sarcoma
V-myc	C-myc	Chicken	Leukemia
V-fes	C-fes	Feline	Sarcoma
V-sis	C-sis	Simian	Sarcoma
V-mos	C-mos	Mouse	Sarcoma

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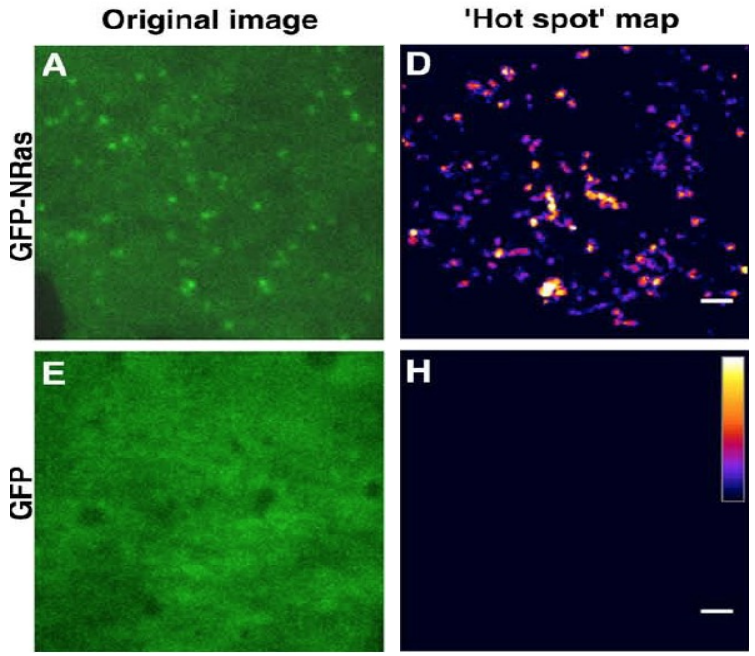
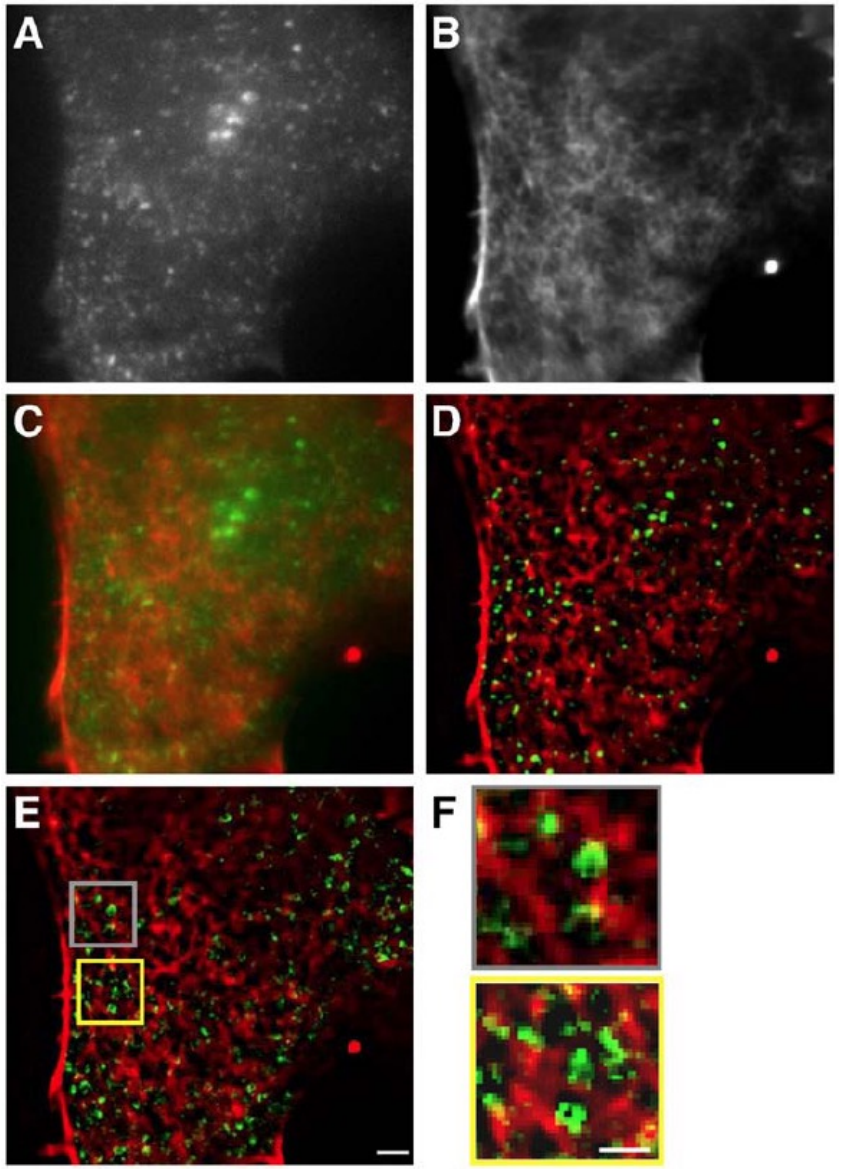
Rasosomes spread Ras signals from plasma membrane 'hotspots'

Merav Kofer-Geles, Irit Gottfried, Roni Haklai, Galit Elad-Zefadia, Yoel Kloog *, Uri Ashery *

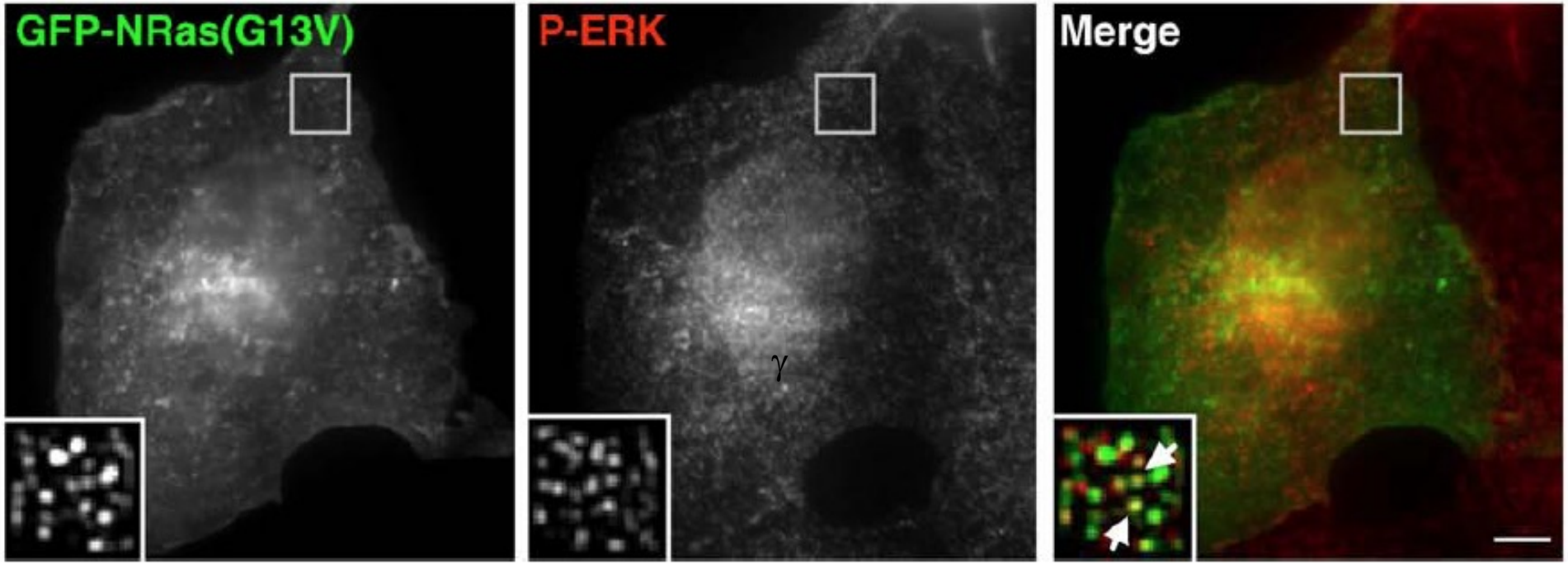
Department of Neurobiology, The George S. Wise Faculty of Life Sciences, Tel Aviv University, 69978 Tel Aviv, Israel



Ras-osomes move within distinct areas, rasosomal 'hotspots', near the PM.



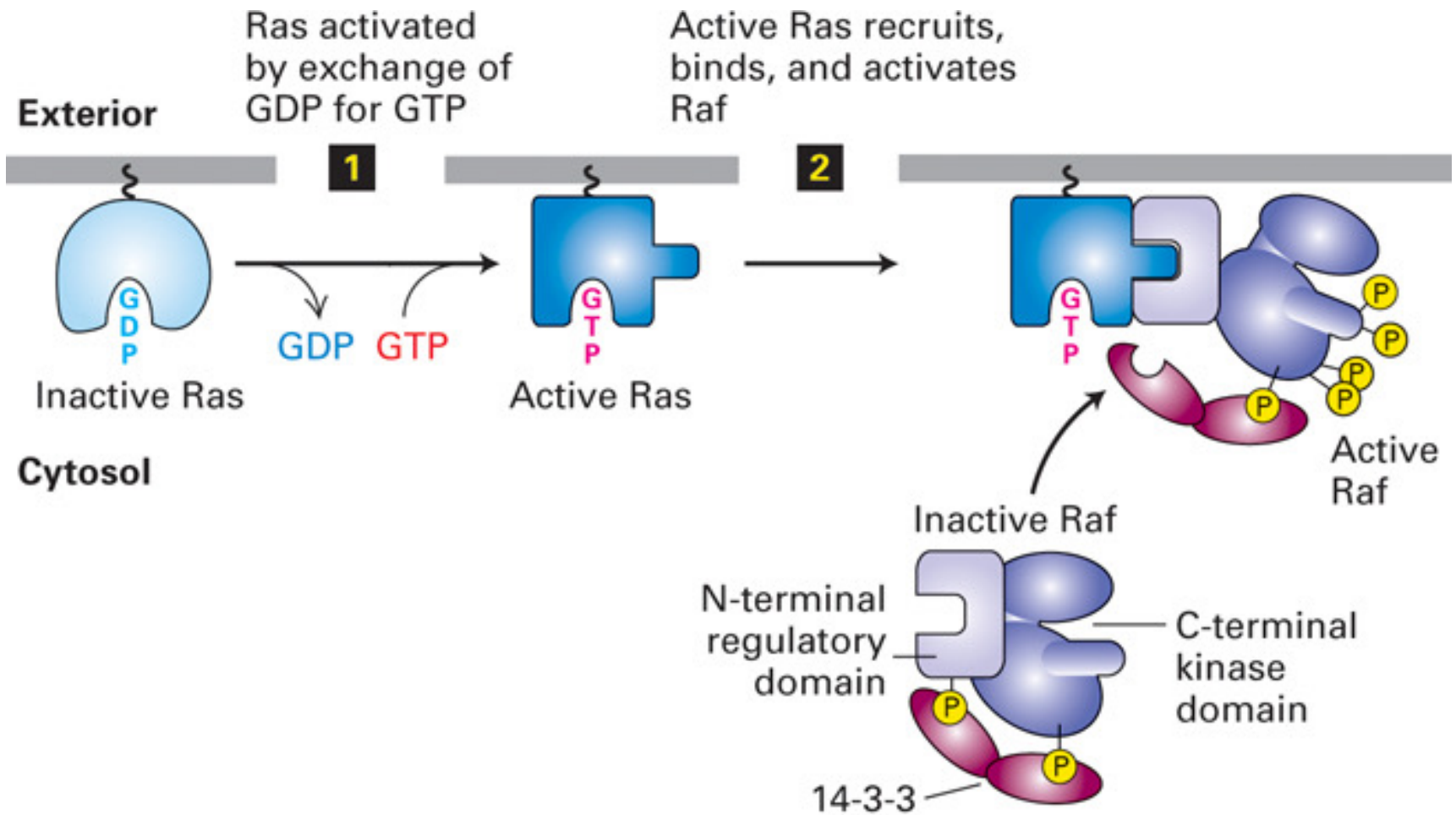
Rasosomes move within cortical actin cages.



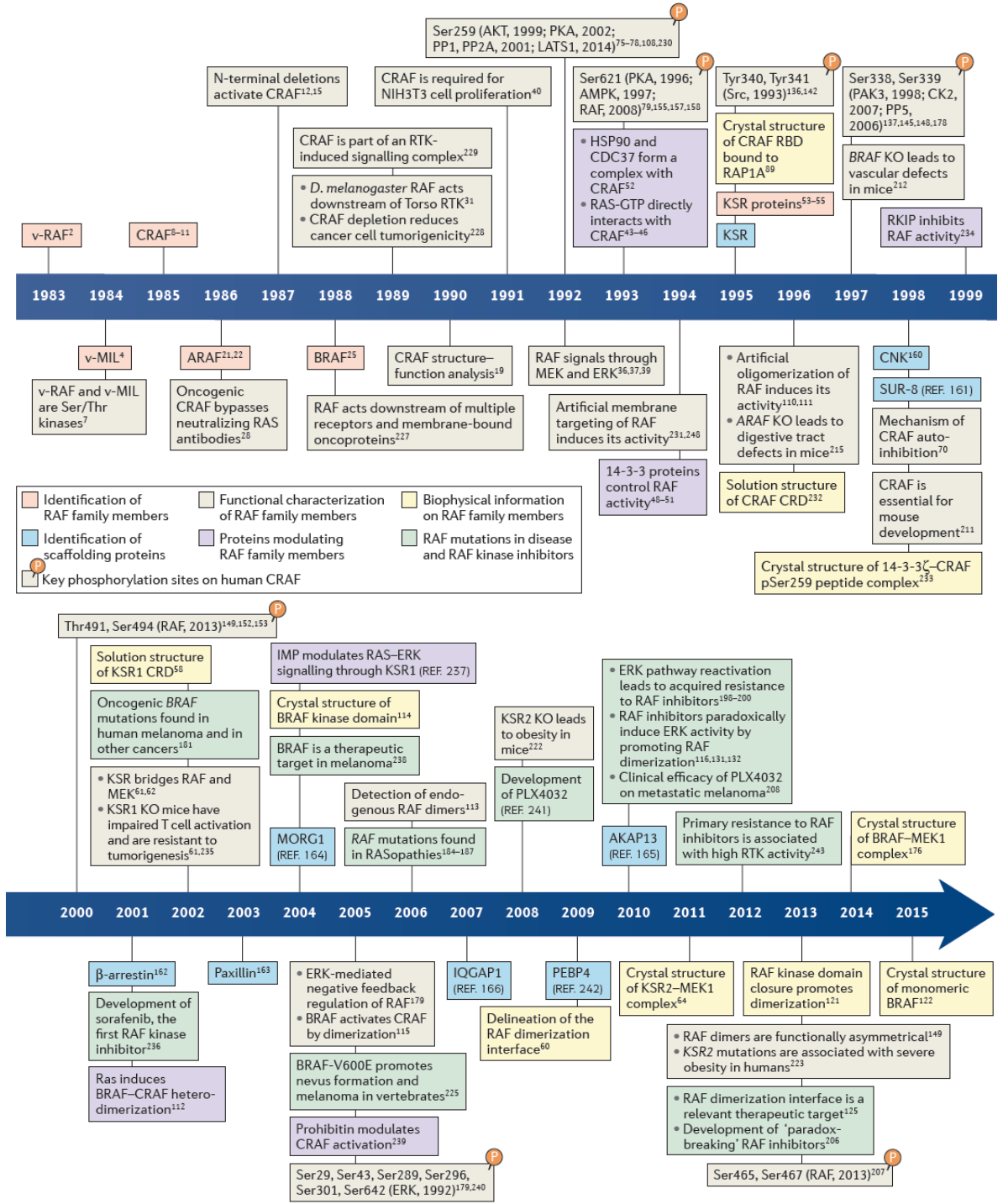
GFP-NRas expressing cells were labeled with anti-phosphorylated-ERK Abs. Insets show filtered images of the boxed regions with arrows that indicate phospho-ERK positive GFP-NRas rasosomes

Regulation of RAF protein kinases in ERK signalling

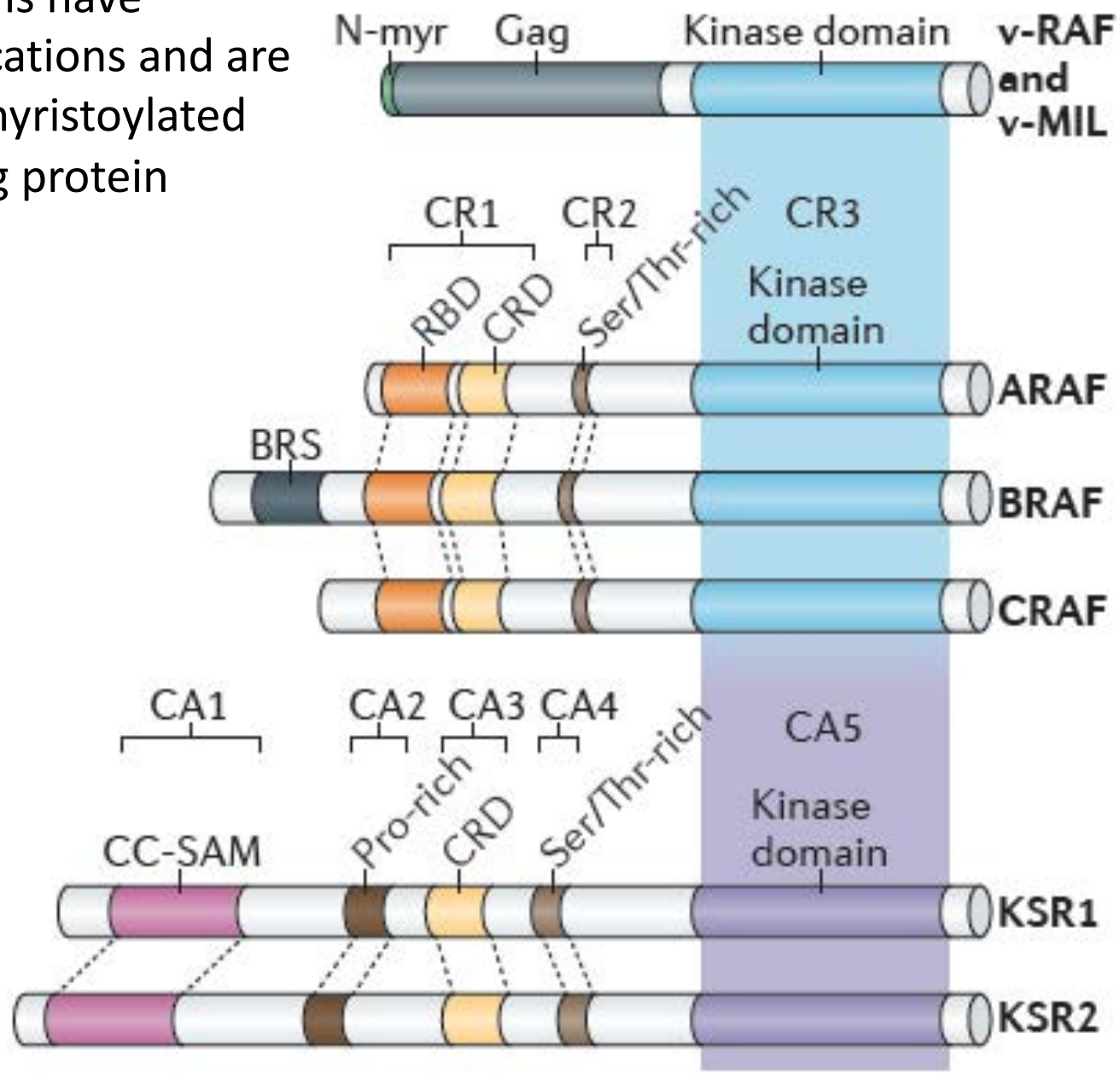
Hugo Lavoie[1] and Marc Therrien[1,2]



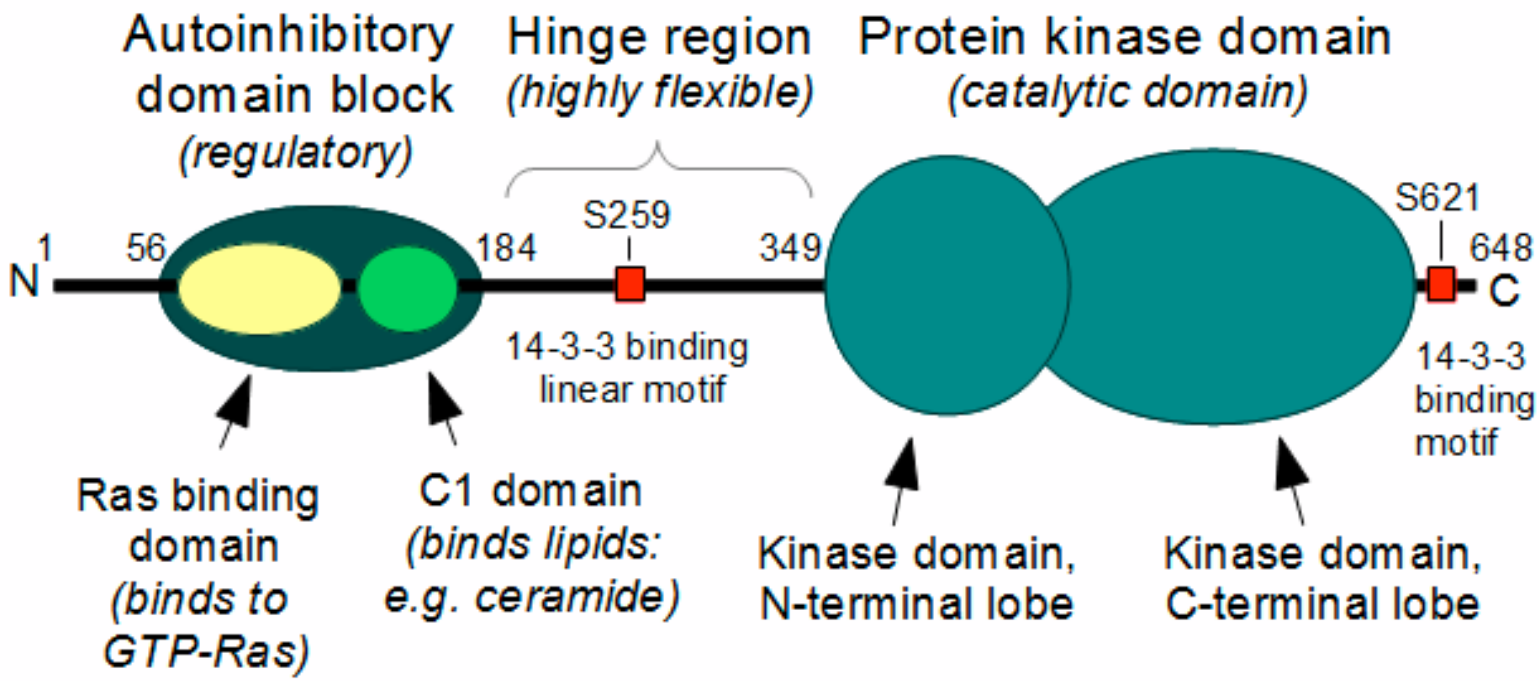
c-Raf


Ser259 (AKT, 1999; PKA, 2002; PP1, PP2A, 2001; LATS1, 2014)[75–76,108,230]
N-terminal deletions activate CRAF[12,15]
CRAF is required for NIH3T3 cell proliferation[40]
Ser621 (PKA, 1996; AMPK, 1997; RAF, 2008)[79,155,157,158]
Tyr340, Tyr341 (Src, 1993)[136,142]
Ser338, Ser339 (PAK3, 1998; CK2, 2007; PP5, 2006)[137,145,148,178]
CRAF is part of an RTK-induced signalling complex[229]
• HSP90 and CDC37 form a complex with CRAF[52]
• RAS-GTP directly interacts with CRAF[43–46]
Crystal structure of CRAF RBD bound to RAP1A[89]
BRAF KO leads to vascular defects in mice[212]
• D. melanogaster RAF acts downstream of Torso RTK[31]
• CRAF depletion reduces cancer cell tumorigenicity[228]
KSR proteins[53–55]
KSR
v-RAF[2]
CRAF[8–11]
RKIP inhibits RAF activity[234]
1983 1984 1985 1986 1987 1988 1989 1990 1991 1992 1993 1994 1995 1996 1997 1998 1999
v-MIL[4]
ARAF[21,22]
BRAF[25]
CRAF structure–function analysis[19]
RAF signals through MEK and ERK[36,37,39]
• Artificial oligomerization of RAF induces its activity[110,111]
• ARAF KO leads to digestive tract defects in mice[215]
CNK[160]
SUR-8 (REF. 161)
v-RAF and v-MIL are Ser/Thr kinases[7]
Oncogenic CRAF bypasses neutralizing RAS antibodies[28]
RAF acts downstream of multiple receptors and membrane-bound oncoproteins[227]
Artificial membrane targeting of RAF induces its activity[231,248]
14-3-3 proteins control RAF activity[48–51]
Solution structure of CRAF CRD[232]
Mechanism of CRAF auto-inhibition[70]
CRAF is essential for mouse development[211]
Crystal structure of 14-3-3ζ–CRAF pSer259 peptide complex[233]
Identification of RAF family members
Functional characterization of RAF family members
Biophysical information on RAF family members
Identification of scaffolding proteins
Proteins modulating RAF family members
RAF mutations in disease and RAF kinase inhibitors
Key phosphorylation sites on human CRAF
Thr491, Ser494 (RAF, 2013)[149,152,153]
Solution structure of KSR1 CRD[58]
IMP modulates RAS–ERK signalling through KSR1 (REF. 237)
Oncogenic BRAF mutations found in human melanoma and in other cancers[181]
Crystal structure of BRAF kinase domain[114]
KSR2 KO leads to obesity in mice[222]
• ERK pathway reactivation leads to acquired resistance to RAF inhibitors[198–200]
• RAF inhibitors paradoxically induce ERK activity by promoting RAF dimerization[116,131,132]
• Clinical efficacy of PLX4032 on metastatic melanoma[208]
BRAF is a therapeutic target in melanoma[238]
Development of PLX4032 (REF. 241)
• KSR bridges RAF and MEK[61,62]
• KSR1 KO mice have impaired T cell activation and are resistant to tumorigenesis[61,235]
Detection of endogenous RAF dimers[113]
MORG1 (REF. 164)
RAF mutations found in RASopathies[184–187]
AKAP13 (REF. 165)
Primary resistance to RAF inhibitors is associated with high RTK activity[243]
Crystal structure of BRAF–MEK1 complex[176]
2000 2001 2002 2003 2004 2005 2006 2007 2008 2009 2010 2011 2012 2013 2014 2015
β-arrestin[162]
Paxillin[163]
• ERK-mediated negative feedback regulation of RAF[179]
• BRAF activates CRAF by dimerization[115]
IQGAP1 (REF. 166)
PEBP4 (REF. 242)
Crystal structure of KSR2–MEK1 complex[64]
RAF kinase domain closure promotes dimerization[121]
Crystal structure of monomeric BRAF[122]
Development of sorafenib, the first RAF kinase inhibitor[236]
Delineation of the RAF dimerization interface[60]
• RAF dimers are functionally asymmetrical[149]
• KSR2 mutations are associated with severe obesity in humans[223]
Ras induces BRAF–CRAF heterodimerization[112]
BRAF-V600E promotes nevus formation and melanoma in vertebrates[225]
• RAF dimerization interface is a relevant therapeutic target[125]
• Development of 'paradox-breaking' RAF inhibitors[206]
Prohibitin modulates CRAF activation[239]
Ser29, Ser43, Ser289, Ser296, Ser301, Ser642 (ERK, 1992)[179,240]
Ser465, Ser467 (RAF, 2013)[207]

Viral oncoproteins have N-terminal truncations and are fused to the *N*-myristoylated (N-myr) viral Gag protein



Kinase suppressor of RAS

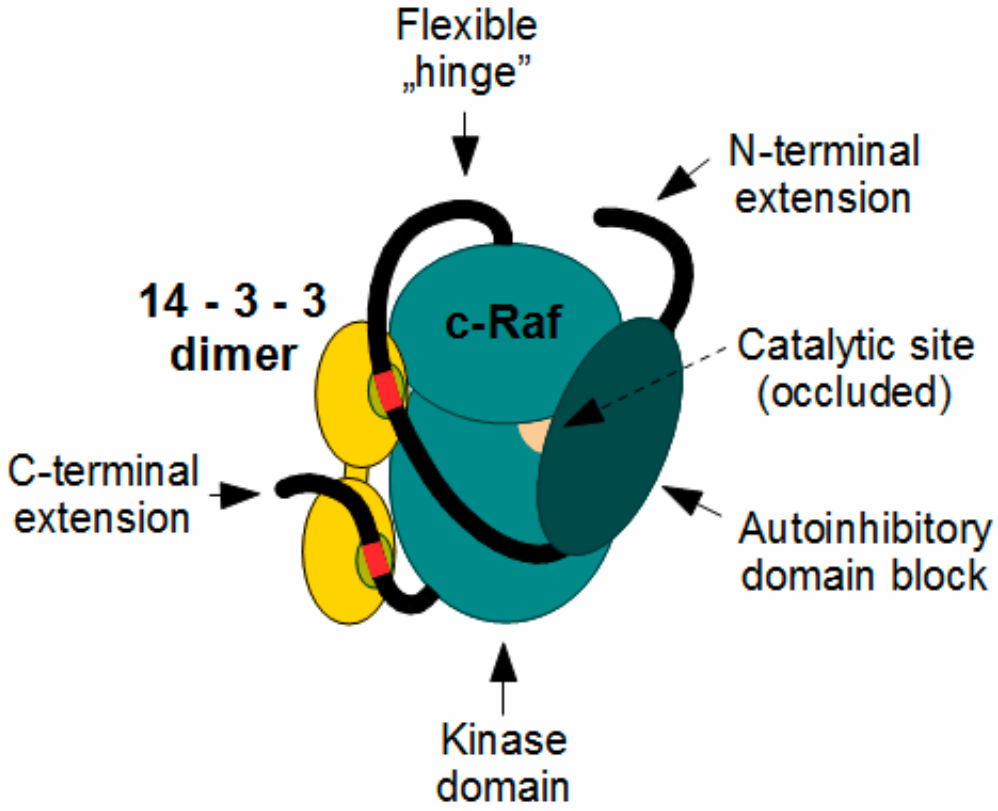


- Ras-binding domain: it binds GTP-Ras
- C1 domain: it is a special zinc finger, rich in cysteines and stabilized by two zinc ions. It interacts with lipids and aids in the recognition of GTP-Ras. The close proximity of these two domains allows them to act as a single unit to negatively regulate the activity of the protein kinase domain, by direct physical interaction.

Between the auto-inhibitory domain block and the catalytic kinase domain, a long and very flexible region acts as a natural "hinge" between the rigidly folded autoinhibitory and catalytic domains.

- The C-terminal half of c-Raf folds into a single protein domain, responsible for catalytic activity.

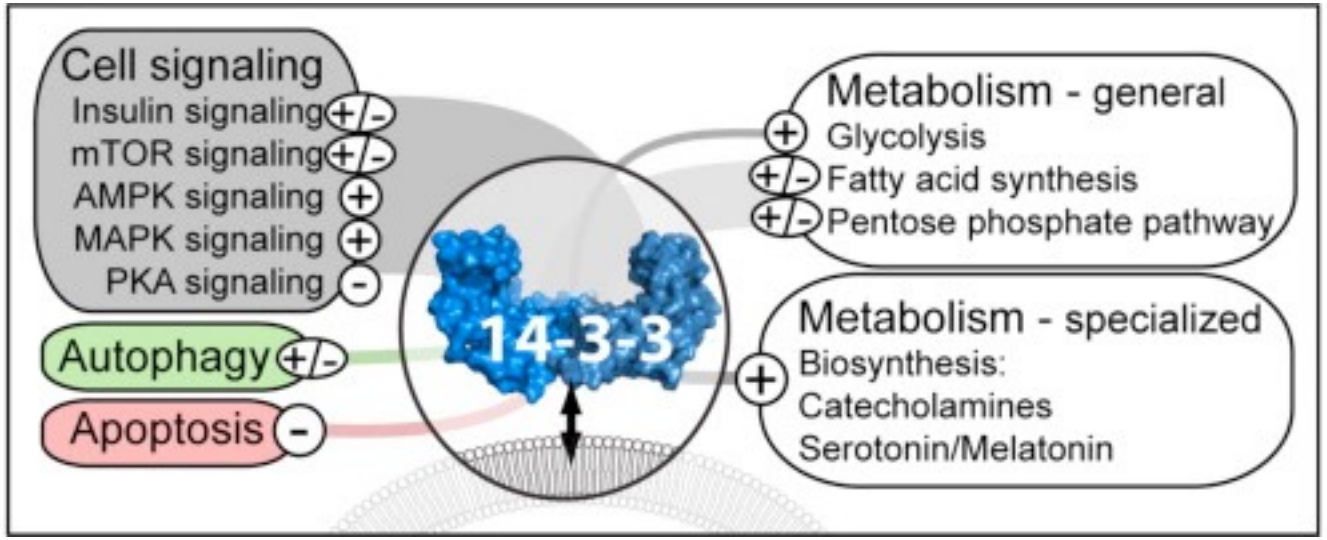
Regulation of c-Raf activity



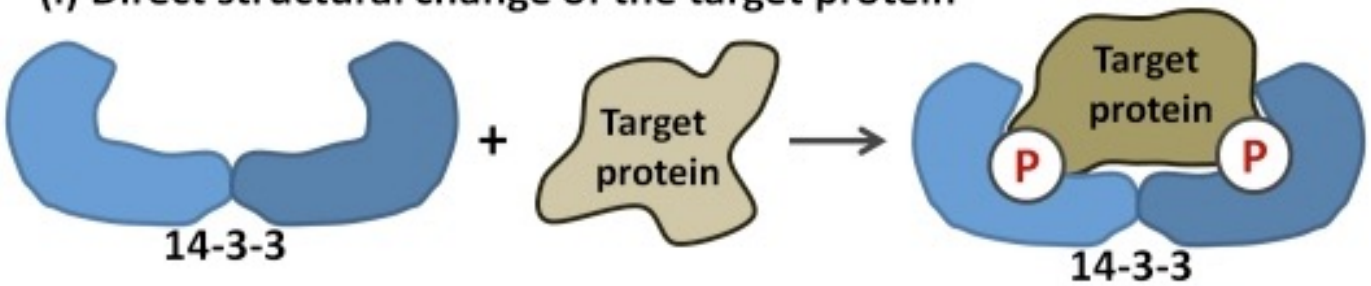
The most important regulatory mechanism involves the direct, physical association of the N-terminal autoinhibitory block to the kinase domain of c-Raf. It results in the occlusion of the catalytic site and full shutdown of kinase activity. This "closed" state can only be relieved if the autoinhibitory domain of Raf engages GTP-bound Ras.

14-3-3 proteins

- Very well conserved in mammals, as well as in plants: they are among the very few signaling elements that are shared by both animals and plants.
- Family of acidic brain proteins. The name was given based on particular elution pattern on chromatography (14th fraction)
- They usually work as dimers
- They bind to peptides, usually containing a phosphorylated serine or threonine residue
- 14-3-3 proteins are a major class of molecular chaperones, with more than 200 proteins that have been shown to be targeted.

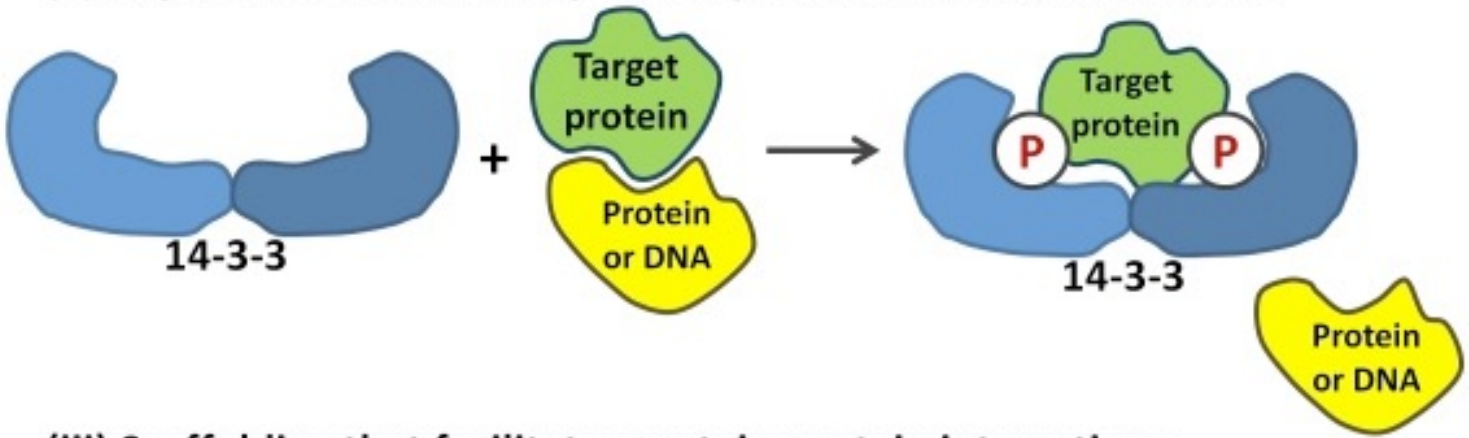


(i) Direct structural change of the target protein



Regulation of enzymatic activity

(ii) Physical occlusion of sequence-specific or structural features

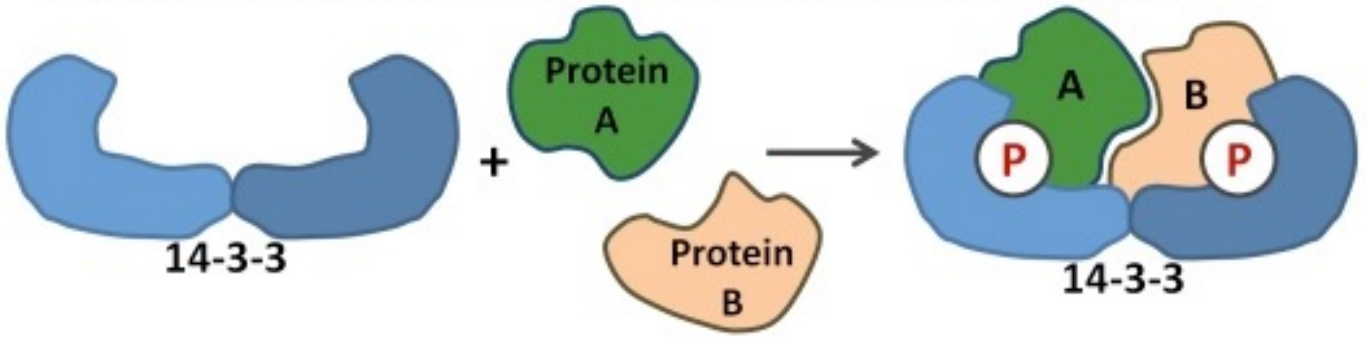


Regulation of subcellular localization

Inhibition of protein-protein or protein-DNA interactions

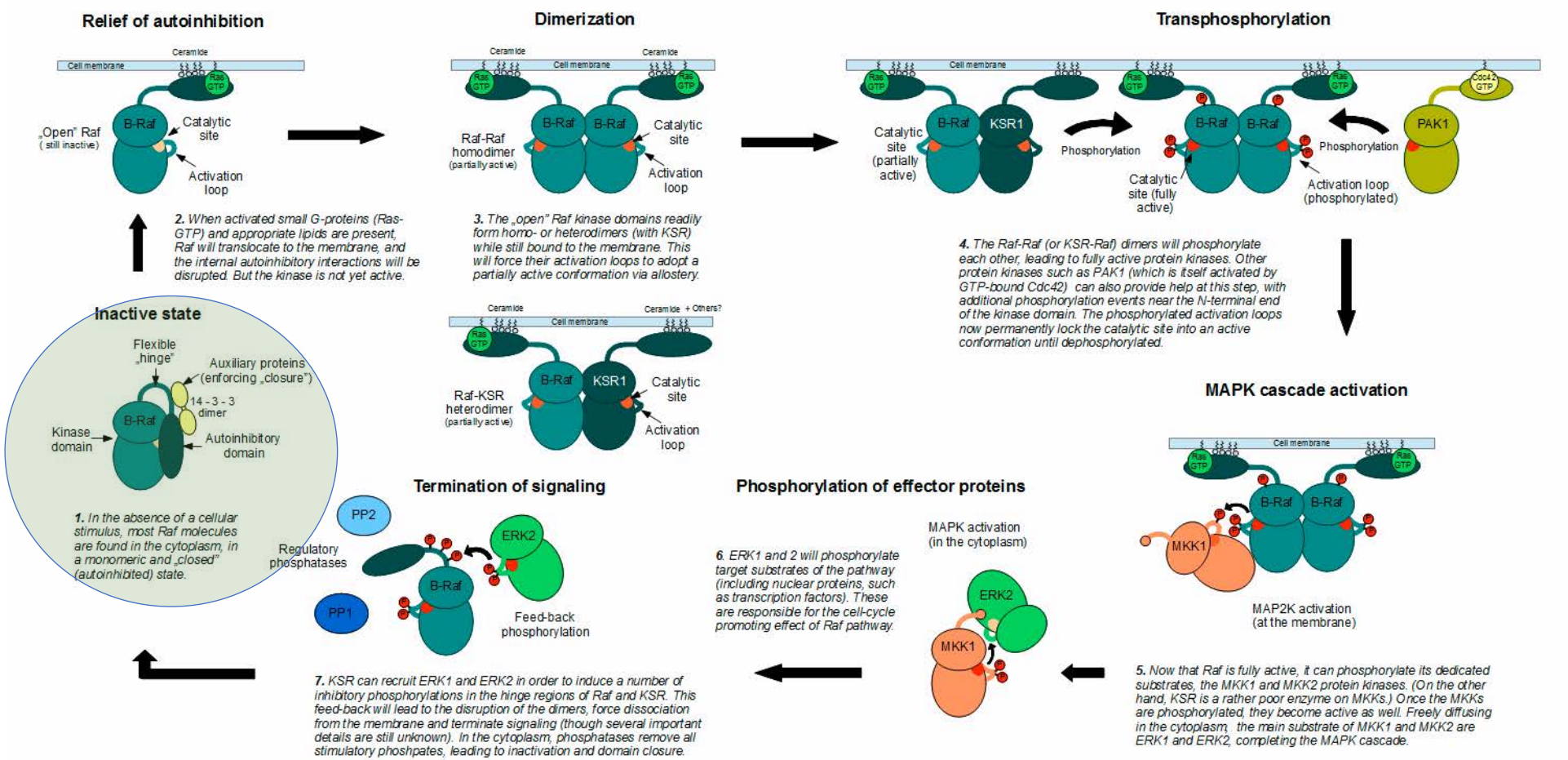
Protection against dephosphorylation or proteolytic degradation

(iii) Scaffolding that facilitates protein-protein interactions

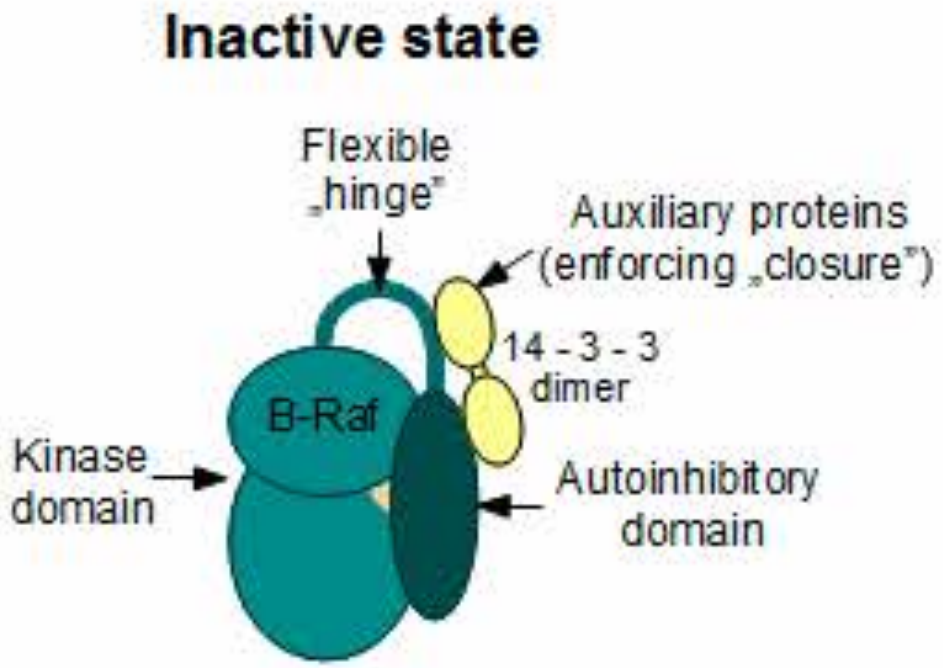


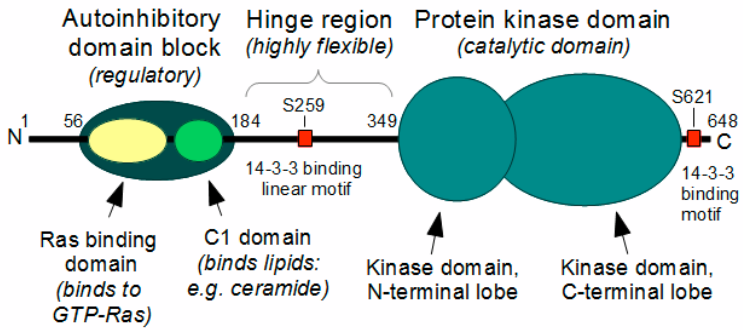
Stabilization of multiprotein complexes

The activation cycle of mammalian c-Raf



The activation cycle of mammalian c-Raf



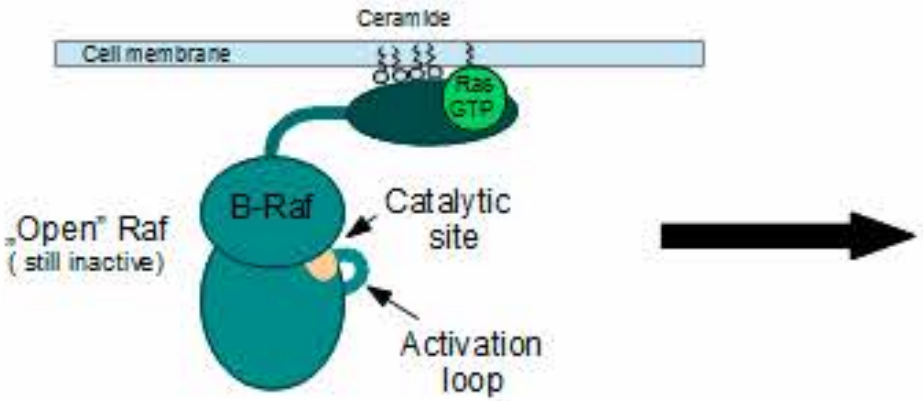


1. *In the absence of a cellular stimulus, most Raf molecules are found in the cytoplasm, in a monomeric and „closed" (autoinhibited) state.*

In quiescent cells, Raf-1 is phosphorylated on both 14-3-3 binding sites (by PKA?) and 14-3-3 maintains the Raf closed inactive conformation

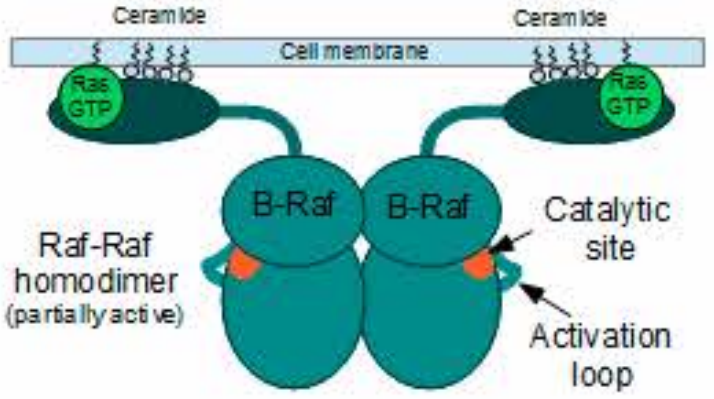
Regulation and function of Raf kinases / *Matallanas et al.* *Genes & Cancer* / vol 2 no 3 (2011)

Relief of autoinhibition

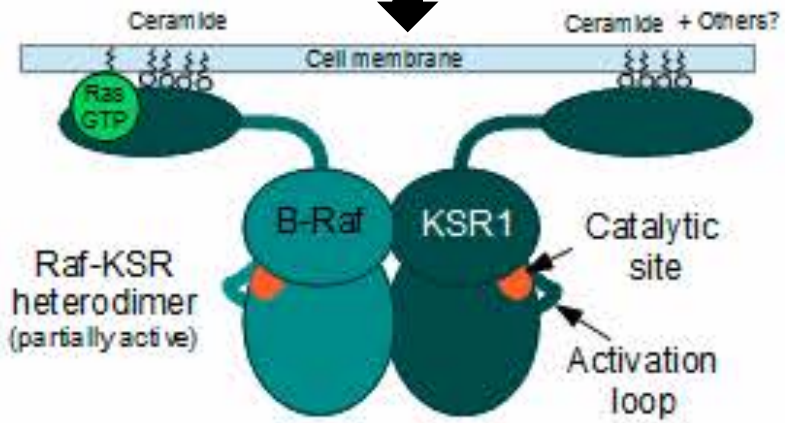


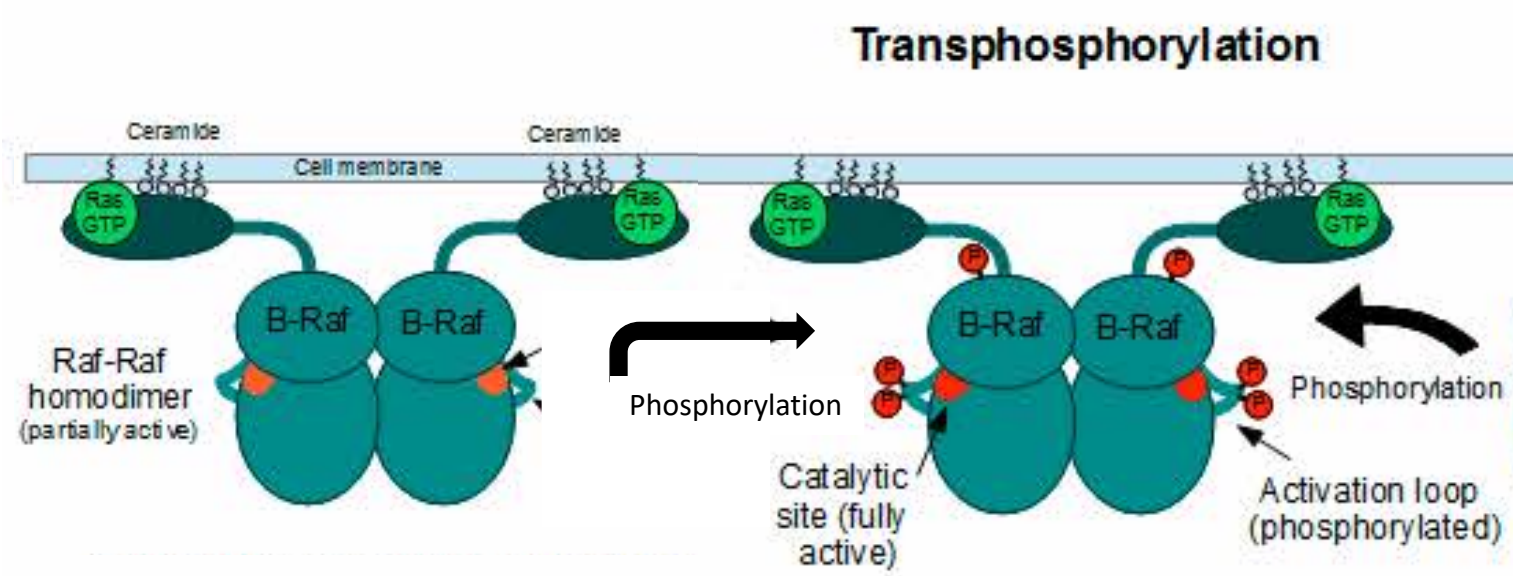
2. *When activated small G-proteins (Ras-GTP) and appropriate lipids are present, Raf will translocate to the membrane, and the internal autoinhibitory interactions will be disrupted. But the kinase is not yet active. Phosphatases PP1 or PP2A are co-recruited to the plasma membrane and de-phosphorylate the inhibitory residues; 14-3-3 proteins are released in the cytoplasm.*

Dimerization



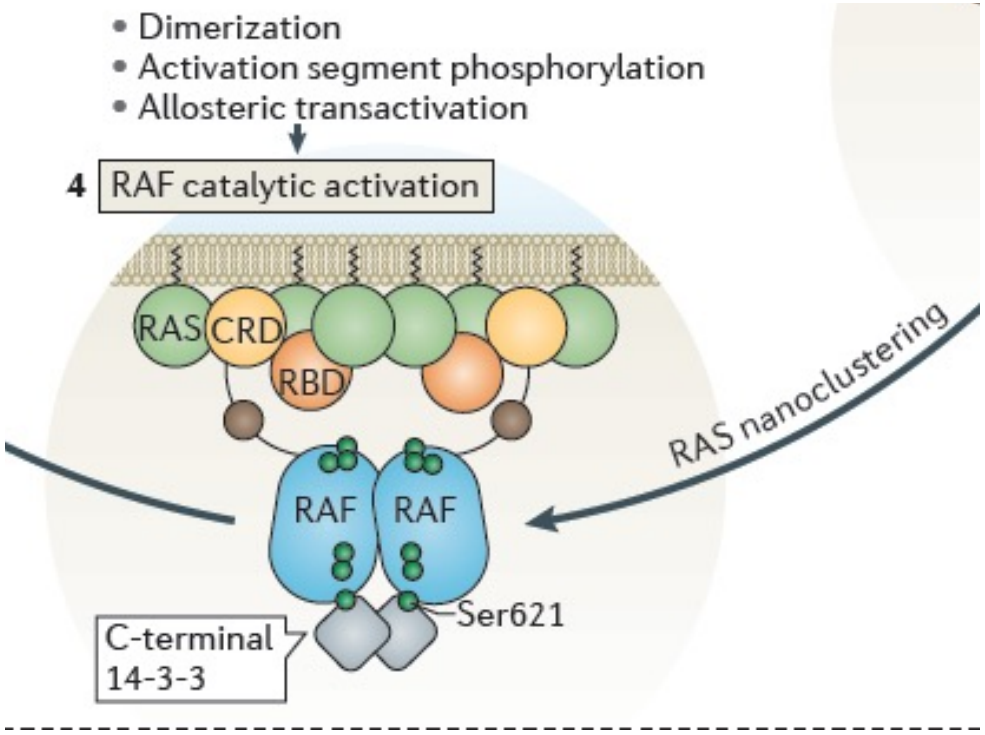
3. *The „open" Raf kinase domains readily form homo- or heterodimers (with KSR) while still bound to the membrane. This will force their activation loops to adopt a partially active conformation via allostery.*





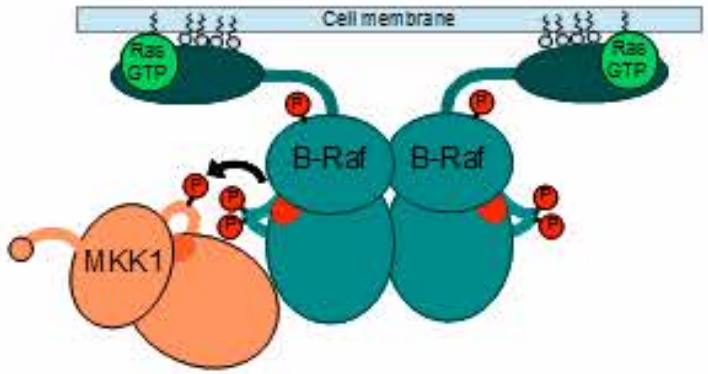
4. *The Raf-Raf (or KSR-Raf) dimers will phosphorylate each other, leading to fully active protein kinases.*

The phosphorylated activation loops now permanently lock the catalytic site into an active conformation until dephosphorylated.



Membrane binding and RAS nanoclustering augment the effective concentration of RAF and thereby contribute to RAF dimerization.

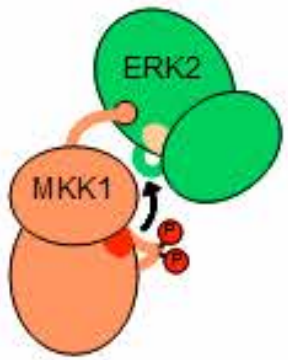
MAPK cascade activation



MAP2K activation
(at the membrane)

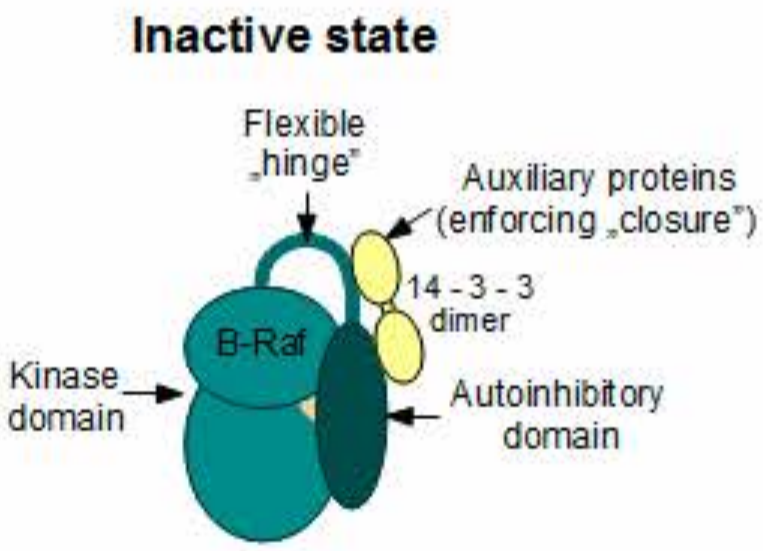
5. *Now that Raf is fully active, it can phosphorylate its dedicated substrates, the MKK1 and MKK2 protein kinases. (On the other hand, KSR is a rather poor enzyme on MKKs.) Once the MKKs are phosphorylated, they become active as well. Freely diffusing in the cytoplasm, the main substrate of MKK1 and MKK2 are ERK1 and ERK2, completing the MAPK cascade.*

MAPK activation
(in the cytoplasm)

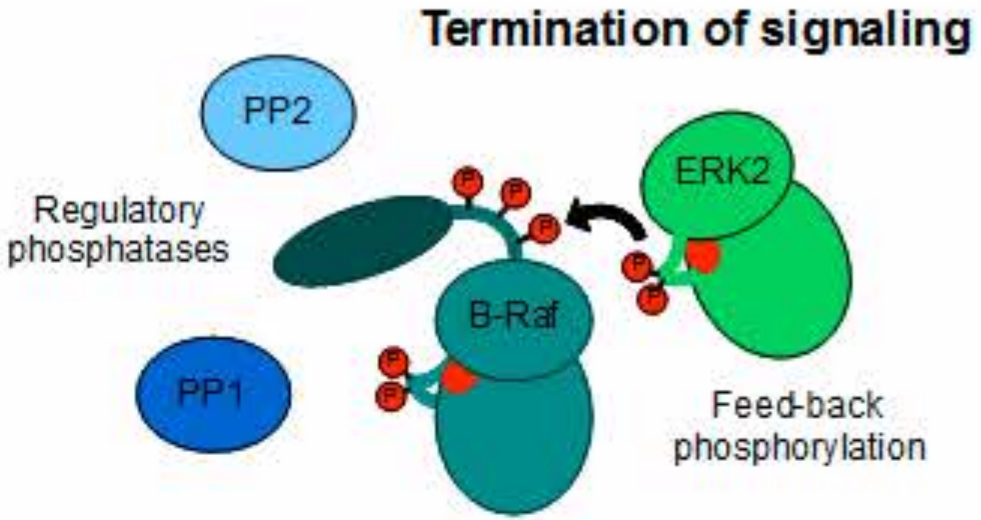


Phosphorylation of effector proteins

6*. ERK1 and 2 will phosphorylate target substrates of the pathway (including nuclear proteins, such as transcription factors). These are responsible for the cell-cycle promoting effect of Raf pathway.*



1. *In the absence of a cellular stimulus, most Raf molecules are found in the cytoplasm, in a monomeric and „closed" (autoinhibited) state.*



ERK signalling implements a **negative feedback loop** in which ERK phosphorylates several inhibitory sites in distinct regions of activated RAF, causing a release from activated RAS and the disruption of RAF dimers. In the cytoplasm, phosphatases remove stimulatory phosphates leading to inactivation and domain closure

Come e’ possibile che le MAPKinasi, solubili nel citoplasma, riescano a trovarsi esattamente nei paraggi di Raf attivato in membrana e subirne la fosforilazione?

Non puo’ essere un evento regolato dal caso!!

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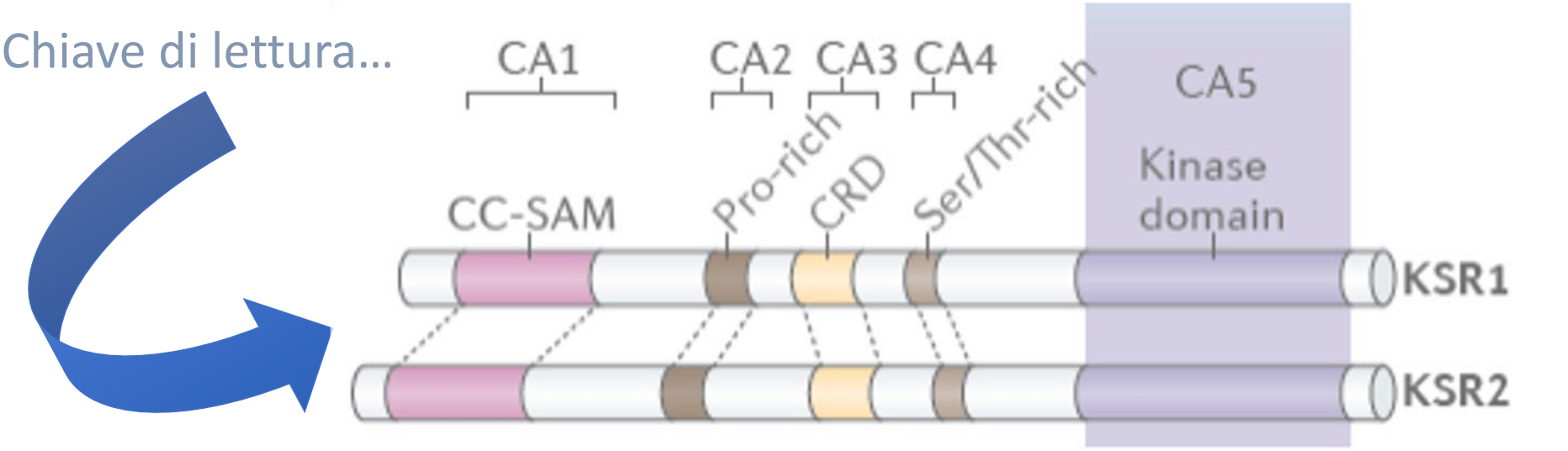
Chiave di lettura…



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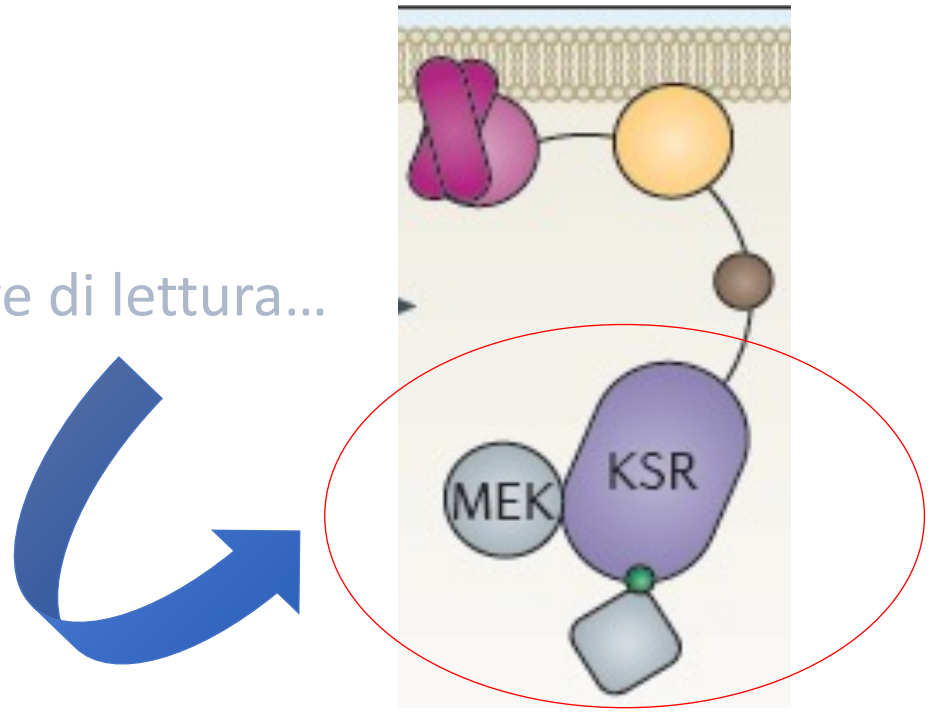


(KSR-1)

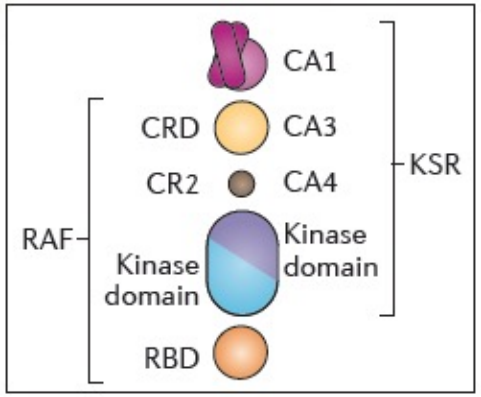
The Kinase Suppressor of RAS (KSR-1) was originally identified in genetic screens in Drosophila and Caenorhabditis elegans designed to isolate mutations in genes that modify the phenotypes associated with oncogenic RAS alleles.

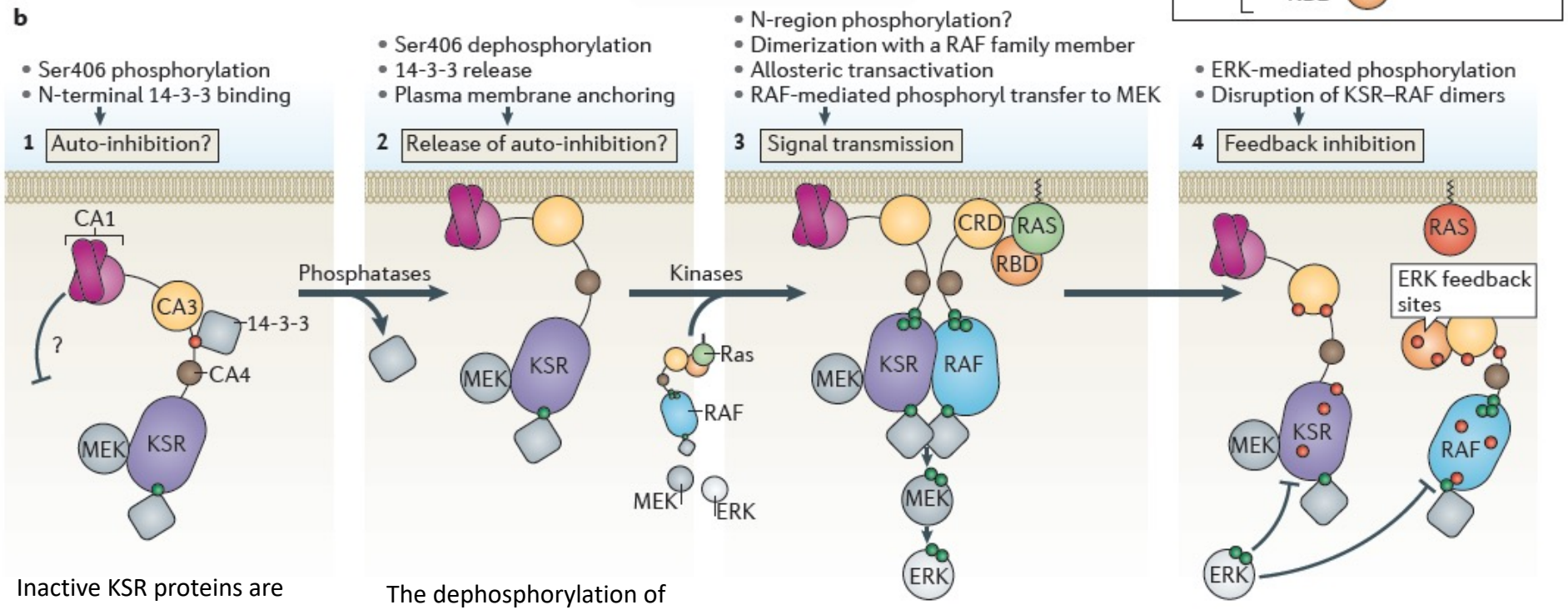
In mammalian cells, KSR-1 acts as a molecular scaffold to assemble a macromolecular complex of MAPK pathway components to facilitate efficient signal transmission and is required for mutant RAS-mediated cellular transformation.

In quiescent cells, KSR-1 is phosphorylated on S297 and S392 by C-TAK1 and held in an inactive state in the cytosol by 14-3- 3 proteins.

RAS activation stimulates the dephosphorylation of KSR-1 on S392, resulting in its translocation to the plasma membrane where it potentiates MAPK signaling (Ory et al., 2003).

Steps involved in KSR regulation often parallel those defined for RAF proteins





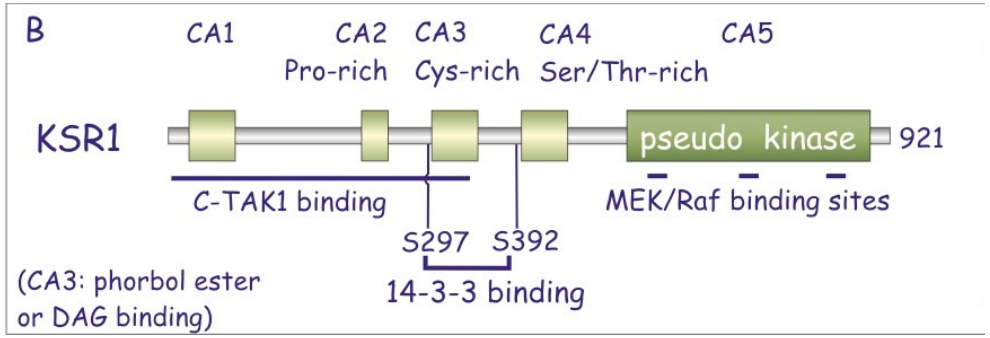
Inactive KSR proteins are kept in the cytosol through interaction with inhibitory 14-3-3 proteins in their N-terminal region. KSR and MEK proteins form constitutive complexes

The dephosphorylation of Ser406 allows 14-3-3 release and plasma membrane anchoring of KSR proteins via conserved area 1 (CA1) and CA3.

KSR proteins heterodimerize with other RAF proteins, leading to RAF transactivation and MEK–ERK signalling.

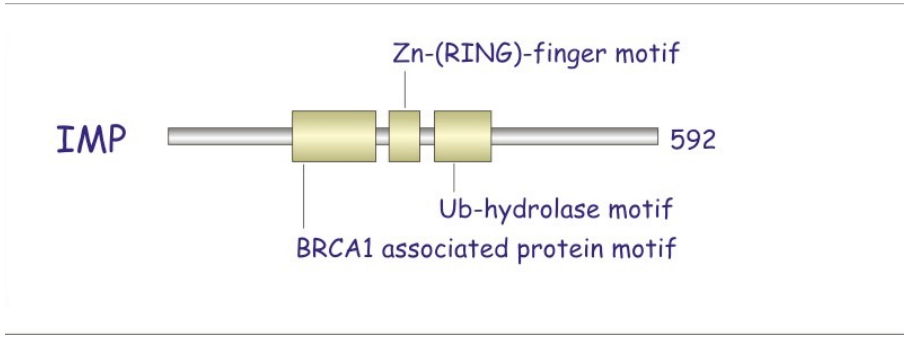
ERK-mediated negative feedback phosphorylation of several sites in RAF and KSR disrupts RAF–KSR dimers, leading to signal attenuation.

REGULATION OF THE MAPK SIGNALING CASCADE



KSR1 has a number of conserved domains, and a kinase domain that resembles that of Raf, but lacks an essential lysine and is therefore inactive.

IMP (impedes mitogenic signal propagation) is an E3-ligase and binds E2-ubiquitin. The E3-ligase activity is activated by binding to RasGTP and this results in the auto-ubiquitylation of IMP, followed by its destruction.



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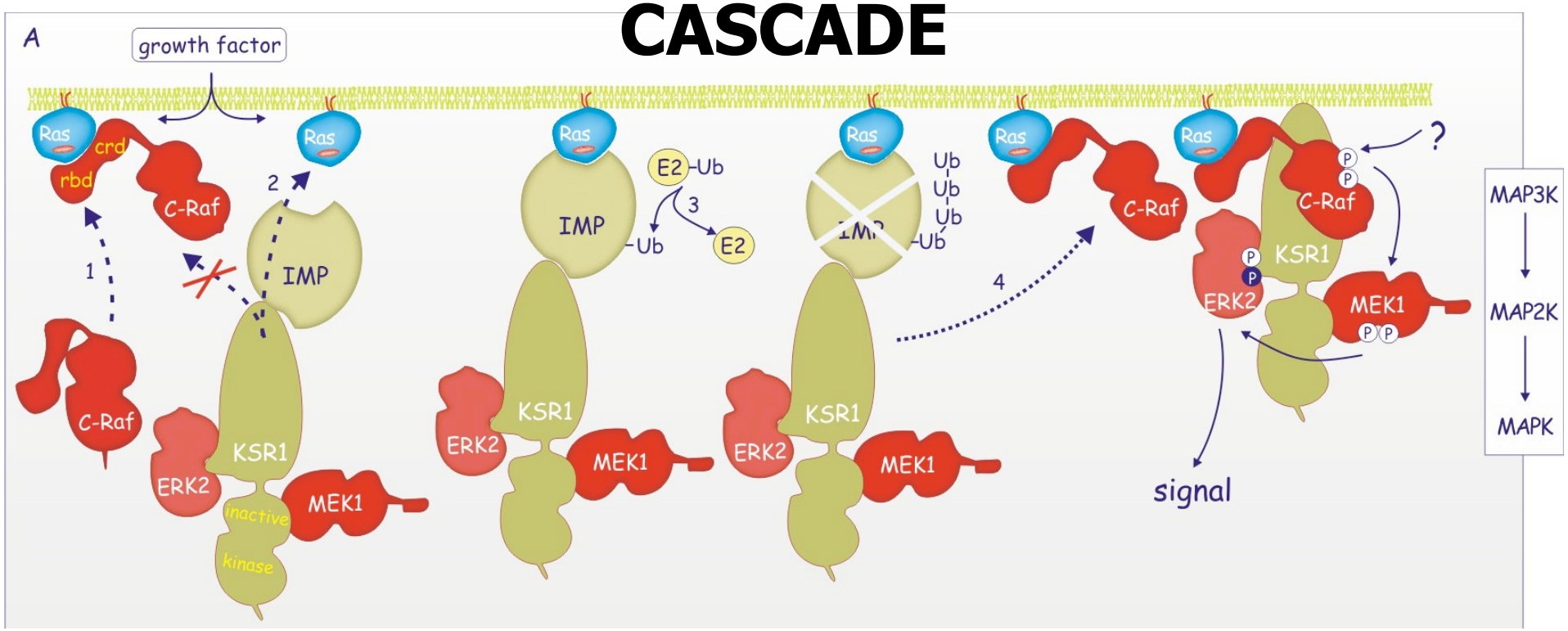

In order to activate the ERK pathway, Ras has not only to recruit C-Raf, but also to remove **IMP**, the inhibitor which prevents formation of the Raf-MEK-ERK signalling cassette.
RasGTP binds IMP and this initiates a series of autoubiquitylations that mark the protein for destruction by the proteasome.
MEK1 and ERK2, linked to the scaffold protein KSR1, are now able to join C-Raf, enabling the signal to pass from one kinase to another