B-Raf signalling Entry 02143

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Synonyms

The proto-oncogene *BRAF* encoding the serine/threonine kinase B-Raf is also known under following synonyms:
- v-*raf* murine sarcoma viral oncogene homolog B1
- B-*raf*-1
- *BRAF*1
- EC 2.7.11.1
- MGC126806
- MGC138284
- *RAFB*1
- p94
- c-R*mil*

Definition

B-Raf signalling comprises the activation of the proto-oncogene product B-Raf and its downstream effectors and represents a key regulatory step in the activation of the canonical → MAP kinase pathway by various extracellular stimuli and oncogene products such as → RAS and activated receptor tyrosine kinases like → NTRK and → RET. Aberrant B-Raf activity as a result of somatic mutations is observed in 8 % of human cancers.

Characteristics

Physiological aspects of B-Raf signalling

B-Raf is a member of the → Raf kinase family and represents an important component of the Ras/Raf/MEK/ERK → MAP kinase signal transduction pathway, which plays a pivotal role in growth control and differentiation. Dysregulation of this pathway is observed in about 30 % of human tumours and represents an established mechanism for tumourigenesis. In their role as gatekeepers of this pathway, Raf-kinases appear as attractive targets for therapeutic intervention. The Raf-family contains three genes in vertebrates, A-Raf, B-Raf and Raf-1 as well as D-Raf and LIN-45 in *Drosophila* und *Caenorhabditis*, respectively. While the *RAF1* gene displays a ubiquitous and prominent expression pattern, B-Raf is predominantly expressed in neuro-ectoderm derived tissues, placenta, the hematopoietic system and the testis. However, gene targeting experiments in mice and **DT40 B cells** revealed that B-Raf represents the major ERK activator, even if it is expressed at barely detectable levels, whereas Raf-1 serves as an accessory ERK activator. Among the three mammalian isoforms, B-Raf displays the highest affinity towards its substrate MEK and has the highest activities in biological and ***in vitro* kinase assays**. In many cell types, B-Raf plays a non-redundant role in the maintenance of ERK signalling induced by various extracellular signals and thereby regulates directly, or in concert with other signalling pathways, the expression of important target gene products such as growth factors and cytokines. The importance of B-Raf for the efficient expression of ERK-regulated target gene products is most likely explained by the fact that ERK activation is not only required for the induction of **immediate early gene** transcription, but also for the stabilisation of the resulting proteins by phosphorylation through sustained ERK signalling**.** The correlation between B-Raf expression and sustained ERK signalling has been implicated in various physiological processes such as lymphocyte activation, myelopoiesis, angiogenesis, development of extra-embryonic tissues as well as for the growth-factor-mediated survival of neurons and their effector functions. The discovery of germ-line mutations with mostly slight to moderate gain-of-function character in the *SOS*, *KRAS*, *HRAS*, *SHP2/PTPN11*, *BRAF* and *MEK1/2* genes in patients suffering from the various **neuro-cardio-facial-cutaneous syndromes**

illustrates that tight control of this pathway upstream or at the level of the B-Raf/MEK interface is key to the normal development and homeostasis of many organs.

B-Raf signalling and tumour development

The high biological relevance of B-Raf is also reflected in the discovery that → somatic alterations of the *BRAF* gene occur in about 8 % of all human tumours with particular high frequencies in melanoma (70 %), ovarian (30 %), thyroid (27 %), colorectal and biliary tract carcinoma (both 15 %). Many of the resulting mutant B-Raf proteins cause chronic ERK activation and transform a variety of cell types *in vitro*. Furthermore, the B-Raf^{V600E} oncoprotein, which is the most frequently found mutant and occurs in 7 % of human tumours, induces neoplasms in transgenic mice and zebrafish. Apart from their established role as ERK activators, B-Raf^{V600E} and other oncogenic mutants have been shown to activate the → NF-κB pathway, although the exact mechanism for this oncologically relevant aspect of B-Raf sigalling remains elusive.

Dysregulated B-Raf signalling in the absence of any *BRAF* mutations has been also implicated in various neoplastic diseases. For example, hyper-activation of wildtype B-Raf has been observed in → Polycystic Kidney Disease. Similarly, over-expression and de-regulation of B-Raf have been implicated in → Kaposi Sarcoma. Likewise, amplification and/or overexpression of the *BRAF* gene were described as alternative events to *BRAF* mutations in → Melanoma. Furthermore, B-Raf serves as an important signal transducer of upstream oncogene products such as → RAS or activated receptor tyrosine kinases (RTKs) such as → RET, → NTRK, → Epidermal Growth Factor Receptor family members or the → Kit/Stem cell factor receptor. Indeed, in many cell types where the chronic activation of the RAF/MEK/ERK effector arm by these oncoproteins represents a major mechanism of cellular transformation, a mutual exclusivity is observed between mutations in *BRAF* or genes encoding its upstream activators. For example, gain-of-function mutations in either the → *RET*, → NTRK, → *RAS* or *BRAF* proto-oncogenes account for 70 % of papillary thyroid carcinoma and provoke similar transformed phenotypes indicating that the activation of B-Raf effectors such as ERK and NF-κB is a major driving force in thyrocyte transformation. Similar constellations have been described for → *RAS* and *BRAF* in

melanoma, colorectal and ovarian carcinoma. However, it should be noted that Ras and B-Raf transformed cells differ in their responsiveness to MEK-inhibitors showing that both oncoproteins, while having a large group of effectors in common, also trigger **oncogene addiction** through distinct mechanisms. Oncogenic B-Raf not only mimics growth factor signalling, but also induces a variety of auto- and paracrine acting growth factors itself, e.g. → Heparin-Binding Epidermal Growth factor (EGF)-Like Growth Factor, chemokines and pro-inflammatory and angiogenic cytokines like → Vascular Endothelial Growth Factor A. Apart from tumour initiation, tissue culture experiments suggest that oncogenic B-Raf also contributes to tumour progression by inducing two additional key events in metastasis: the → Epithelial to Mesenchymal Transition of the oncogene-bearing cell and the **angiogenic switch** in its environment through the aforementioned growth factors and cytokines.

It should be noted, however, that aberrant B-Raf activity does not necessarily result in tumourigenesis unless profound changes in the regulatory network underlying cell cycle control have occurred. Through the ERK and NF-κB pathways, oncogenic B-Raf stimulates not only the production of positive cell cycle regulators such as Cyclin D1, but also induces negative regulators such as cyclin-dependent kinase inhibitors like $p16^{INK4A}$. Consequently, chronic B-Raf/ERK signalling ultimately results in cell cycle arrest and cellular → senescence. For example, melanocytes with an intact cell cycle control program become growth arrested by chronic B-Raf signalling and develop only into benign nevi. However, if important negative cell cycle regulators and tumour suppressor genes like → INK4A and → p53 are lost, oncogenic B-Raf signalling will trigger cell cycle progression and drive tumour development.

B-Raf structure and regulation

Like many other protein kinases, B-Raf is part of a large multi-protein complex or **signalosome** in which the individual components regulate B-Raf conformation and activity through various protein-protein interactions in a dynamic spatio-temporal manner. Key to the understanding of the (dys-)regulation of B-Raf is the knowledge of its modular structure. B-Raf shares three highly conserved regions (CR) with the other members of the Raf-family (**Fig. 1**): the N-terminal CR1 contains the Ras-GTP binding

domain, which initiates the interaction with activated Ras, and the Cystein-rich domain involved in the stabilisation of Ras/Raf interaction. The CR2 contains a negative regulatory serine residue (S365) that serves as a binding site for **14-3-3 proteins** upon phosphorylation by → Akt and other kinases. The catalytic domain (CR3) harbours phosphorylation sites for Raf-regulating enzymes within two segments, the N-region and the **activation loop**. B-Raf carries a second 14-3-3 binding motif around S729 at the C-terminal end of the CR3 domain, which is essential to couple B-Raf to its downstream effector MEK.

Similar to the better-characterised Raf-1 isoform, B-Raf is activated by its interaction with small GTPases of the → RAS family and, in certain cell types, by the related → Rap1 GTPase. Although no crystal structure for any of the full-length Raf-proteins is available, various experimental approaches imply that Raf activation is accompanied by a transition from a closed, auto-inhibited into an open, active conformation in which the N-terminal lobe consisting of the CR1 and CR2 domains is displaced from the C-terminal lobe encompassing the CR3 (**Fig. 1**). The degree of auto-inhibition of B-Raf is influenced by the inclusion/exclusion of amino acid sequences within the linker region between N- and C-terminal lobe, which are encoded by alternatively spliced, tissue-specific exons and various phosphorylation events. Among the latter, two phosphorylation sites within the CR3, the N-region and the **activation loop**, are of particular importance (**Fig. 1**). The introduction of negative charges into the N-region, which is located at the N-terminal end of the CR3 domain, plays a critical, multi-faceted role in Raf activation. While the N-region of Raf-1 is charged through phosphorylation of its $S^{338}SYY^{341}$-sequence in a RAS-dependent manner by Ser/Thr- and Tyr-kinases, the equivalent serine residues within the N-region of B-Raf ($S^{446}SDD^{449}$-motif) are phosphorylated in a constitutive and RAS-independent manner (**Fig. 1**). Although structural data are still missing, several lines of evidence propose that N-region phosphorylation primes B-Raf for activation at the membrane by reducing the affinity between N-terminal and C-terminal lobe. The significance of the aspartate residues, which are the functional equivalents of the phospho-tyrosine residues in the SSYY-sequence of Raf-1, is two-fold: firstly the negative charge of the aspartate residues primes B-Raf for N-region phosphorylation by Casein Kinase 2

(CK2). Secondly, the D448 residue stabilises the conformation of activated B-Raf through the formation of a salt-bridge with R506 within the αC-helix within the CR3. The important role of the SSDD-sequence is highlighted by the fact that mutation of the serine and/or aspartate residues results in drastic reduction of the basal *in vitro* kinase and biological activities. Furthermore, it has been suggested that the different mechanisms that supply the N-region of B-Raf and Raf-1 with negative charges, account not only for the aforementioned isoform-specific differences in the enzymatic, biological and transforming activities, but also predispose the *BRAF* gene for oncogenic hits. However, while tissue culture experiments demonstrated that the rare B-Raf^{E586K} mutant indeed requires an intact SSDD-sequence to induce MEK/ERK activation and oncogenic transformation, the biological activity of the most frequently found mutant, B-Raf^{V600E}, is not affected by N-region neutralisation, at least not in experimental approaches involving the ectopic expression of this oncoprotein.

The interaction with Ras recruits B-Raf to the plasma membrane followed by the phosphorylation of the activation loop residues T599 and S602 (**Fig. 2**). This phosphorylation event leads to the dislocation of the activation loop relative to the overall catalytic domain thereby resulting in full B-Raf activity. The importance of the activation segment phosphorylation is established by the fact that mutation of these phosphorylation sites renders B-Raf resistant to extracellular signals and even to strong activators like oncogenic Ras^{V12}. Conversely, mutations that mimic the phosphorylation-induced dislocation of the activation segment, such as *BRAF*^{V600E}, lock B-Raf in an active conformation and confer high constitutive enzymatic and transforming activities to B-Raf independent of RAS. Consequently, these activation loop mutations are frequently found as → somatic alterations of the *BRAF* gene in human tumours.

Intracellular B-Raf activity is also regulated by the phosphorylation-dependent recruitment of **14-3-3 proteins** in an opposing manner (**Fig. 1**). Binding of 14-3-3 proteins to phospho-S729 at the C-Terminus of B-Raf is essential to couple B-Raf to the MEK/ERK pathway. In contrast, phosphorylation of S365 within the CR2 by Protein kinases A, → Akt or Serum-and-Glucocorticoid-induced kinase (SGK) generates a second binding site for 14-3-3 proteins, which negatively regulates B-Raf

activity, most likely through the stabilisation of the auto-inhibited conformation through the simultaneous binding of the 14-3-3 dimer to S365 and S729 (**Fig. 1** and **2**). 14-3-3 proteins are also involved in the RAS-stimulated formation of homo-dimers of B-Raf and its hetero-dimerisation with Raf-1 (**Fig. 2**). Indeed, B-Raf/Raf-1 hetero-dimers represent the most potent form of Raf-activity within the cell. Interestingly, *via* a negative feedback loop, activated ERK limits the longevity of these dimers by targeting an evolutionary conserved phosphorylation motif at the C-terminus of B-Raf (**Fig. 2**). In addition, B-Raf activity is modulated by other components of the **signalosome** such as the → HSP90/Cdc37 chaperone complex and **scaffold proteins** like Kinase-suppresor-of-Ras (KSR) and Connector-and-enhancer-of-KSR (CNK). Membrane phospholipids such as phosphatidylserine (PS) and phosphatidic acid (PA) are also discussed as important regulators of Raf activation. B-Raf is also negatively regulated by Sprouty-2 and Raf-kinase-inhibitory protein (RKIP), two proteins, which are both often down-regulated in human cancer raising the possibility that their epigenetic silencing represents an alternative mechanism to gain-of-function mutations in genes linked to the Ras/Raf/MEK/ERK pathway in human cancer. Similarly, B-Raf^{V600E} and other activation loop mutants are incapable of interacting with Sprouty demonstrating that the V600E mutation not only uncouples B-Raf from positive (interaction with Ras, N-region and activation loop phosphorylation) but also negative regulatory mechanisms.

B-Raf as a therapeutic target

The growing importance of B-Raf in tumour biology has fostered the development of therapeutic strategies aiming at either reducing the expression or activity of B-Raf or its downstream effector MEK. Various MEK inhibitors are currently in clinical trials and experiments in tissue culture and xenograft models indicate that tumour cells harbouring the *BRAF*^{V600E} mutation, but not those with RAS mutations, are highly "addicted" to ERK activity and are consequently particularly sensitive towards MEK inhibition. Similar results have been obtained in experiments in which the expression of B-Raf^{V600E} but not of wildtype B-Raf was specifically abolished by allele-specific → RNA interference illustrating the importance of this oncoprotein for the maintenance of

the tumour phenotype. Recent strategies also target B-Raf directly. The orally available multi-kinase inhibitor BAY 43-9006 (also known as Sorafenib or Nexavar), which was originally designed to block Raf-1, inhibits B-Raf as well as several receptor tyrosine kinases (RTKs) involved in neo-angiogenesis and tumour progression. However, it is assumed that the inhibition of the latter kinase class or the simultaneous inhibition of several kinases, rather than the inhibition of Raf itself, is responsible for the anti-tumour activity of BAY 43-9006, in particular in renal cell carcinoma. A third approach employs the requirement of the → HSP90/Cdc37 chaperone complex for the stability of B-Raf. In this regard, the HSP90 inhibitor **Geldanamycin** was shown to trigger the degradation of B-Raf by disrupting its association with the HSP90/Cdc37 chaperone complex. Interestingly, the stability of most activated B-Raf mutants, including B-Raf^{V600E}, appears to be more reliant on the chaperone complex than those of wildtype B-Raf suggesting that tumour cells driven by *BRAF* mutations will be particularly sensitive to **Geldanamycin**.

Figure legends:

B-Raf signalling. Fig. 1 - **Model of the B-Raf activation cycle**. B-Raf contains three conserved regions: CR1 (blue) consisting of the Ras-binding domain (RBD) and the Cystein-rich domain (CRD), CR2 (green) and the kinase domain CR3 (blue). Inactive B-Raf resides in the cytoplasm in a closed, inactive conformation stabilised by 14-3-3. Interaction of B-Raf with a complex consisting of CK2 and the **scaffold protein** KSR results in phosphorylation of S446 (and perhaps S447) in the N-region thereby transferring B-Raf into a more open conformation. The constitutive basal phosphorylation of B-Raf at S446 suggests that a large fraction of B-Raf resides in this primed state. Interaction with activated Ras (Ras-GTP) leads to phosphorylation of T599 and S602 within the **activation loop**, which induces a conformational change within the CR3 and renders B-Raf active. B-Raf is supposedly inactivated by phosphatases, re-phosphorylation of the inhibitory residue S365 and transition into the closed conformation.

B-Raf signalling. Fig. 2 – **Modulation of B-Raf signalling**. Extracellular signals received by various receptor classes trigger the activation of Ras-GTPases by stimulating their loading with GTP. Activated Ras not only recruits B-Raf and promotes its phosphorylation by unknown activation loop kinases, but also stimulates its homo- and hetero-dimerisation. The activity of B-Raf (and Raf-1) is fine tuned by a multitude of positive and negative modulators. The longevity of B-Raf/Raf-1 heterodimers is determined by a rapid negative feedback loop from ERK. In a delayed negative feedback loop, sustained B-Raf/ERK signalling also induces the transcription of Sprouty-2, a negative regulator of B-Raf.

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Keyword definitions

- **14-3-3 proteins**

  14-3-3 proteins are abundant, highly conserved adaptor proteins of approx. 30 kDa found in all eukaryotes. Similar to the binding of → SH2 domains to phosphorylated tyrosine residues, 14-3-3 proteins usually bind to their client protein *via* a phosphorylated Ser- or Thr-residue in a sequence-specific context (mode I: RSXpS/TP; mode II: RXXXpS/TXP with p indicating the phosphorylated residue and X any amino acid). 14-3-3 proteins assist in the stabilisation of the client protein in either an active or inactive conformation. 14-3-3 proteins regulate a multitude of biological processes ranging from metabolic control to the regulation of apoptosis, cell cycle progression and mitogenic signalling. One of the seven of human 14-3-3 genes, 14-3-3σ/Stratifin, is frequently epigenetically silenced in breast, lung and prostate cancer and is discussed as a → tumour suppressor gene.

- **Activation loop**

  A 20- 25-residue segment within the catalytic domain of protein kinases that functions to regulate their kinase activity.

- **Angiogenic switch**

  Angiogenic switch describes the shifting of the equilibrium between pro- and anti-angiogenic factors in favour of the pro-angiogenic factors resulting in neo-angiogenesis, an essential prerequisite for tumour growth and metastasis.

- **DT40**

  Due to its extraordinary high rate of homologous recombination and gene targeting activities, the chicken B cell lymphoma line DT40 has become an important experimental system to address various areas in cell biology by combining cellular and biochemical read-outs with gene targeting

approaches. Research areas using DT40 cells as an important model system include DNA repair, somatic hypermutation, signal transduction, mitosis, cell cycle regulation, chromatin dynamics, RNA processing etc. DT40 cells are regarded as immortalised immature B cells (bursal stem cells), which are prevented from further differentiation. The transformation event, which gave rise to this cell line, was caused by insertion of the Avian Leukosis Virus into the → MYC locus thereby causing overexpression of the c-Myc protein, which in turn mediates the differentiation block of DT40. The exact molecular basis for the high rate of homologous recombination between exogenous DNA and the genome is still elusive, but is probably linked to the retained capability of DT40 lymphoma cells to continue immunoglobuline gene diversification by gene conversion. DT40 cells are easy to transfect, which suits them for transient and stable expressions of transgenes in order to perform rapid complementation analyses of loss-of-function phenotypes.

- **Geldanamycin**

  A benzoquinone ansamycin antibiotic from *Streptomyces hygroscopicus* var. Geldanus that binds to Heat Shock Protein 90 (Hsp90) and thereby blocks its function as an important chaperone. From a tumour biology perspective, the more than 50 → HSP90 client proteins include → TP53, the kinases → SRC, → Raf-1, → B-Raf, → HER-2/neu/ErbB2 and → Bcr-Abl as well as several members of the steroid hormone receptor family, which become de-stabilised and subsequently degraded upon treatment with Geldanamycin or its clinically more relevant derivatives 17-AAG (17-allylamino-17-demethoxygeldanamycin) and 17-DMAG (17-(Dimethylaminoethylamino)-17-demethoxygeldanamycin).

- **Immediate early genes**

  A class of approximately 100 structurally and functionally unrelated genes, whose transcription is induced through the phosphorylation and activation of pre-existing transcription factors such as ELK-1 by intracellular signalling pathways like → MAP kinases. A hallmark of immediate early genes (IEGs) is that their transcription is independent from *de novo* protein synthesis, which distinguishes them from delayed early genes. Examples for IEG products include c-FOS, a component of the → AP-1 transcription factor complex, and several members of dual specificity phosphatases, which act as negative feedback regulators of → MAP kinases.

  ***in vitro* kinase assay**

  Analytical method to measure the activity of protein or lipid kinases *in vitro*. To this end, kinases are usually purified from cell extracts by immunoprecipitation and then mixed with ATP and an appropriate substrate. Kinase activity is then analysed by measuring the phosphorylation status of the substrate, either by immunochemical methods (ELISA or Western Blotting) using phospho-specific antibodies or by its labelling with radioactive phosphorus.

- **Neuro-cardio-facial-cutaneous syndromes**

  The neuro-cardio-facial-cutaneous syndrome complex compromises the congenital disorders Noonan syndrome, Costello syndrome, LEOPARD syndrome and cardio-facious-cutaneous syndrome. These syndromes share a constellation of similar phenotypic features (neural, cardial, skeletal and ectodermal defects) and a pathogenesis caused de-regulation of Ras/ERK pathway by germ-line mutations in genes encoding its core elements (*RAS*, *BRAF*, *MEK1,2*) or important modulators of Ras activation such as *NF*1, *SOS* or *PTPN11/SHP2*. Some of the syndromes, e.g. neurofibromatosis, Noonan and Costello syndrome, are associated with several neoplastic diseases.

- **Oncogene addiction**

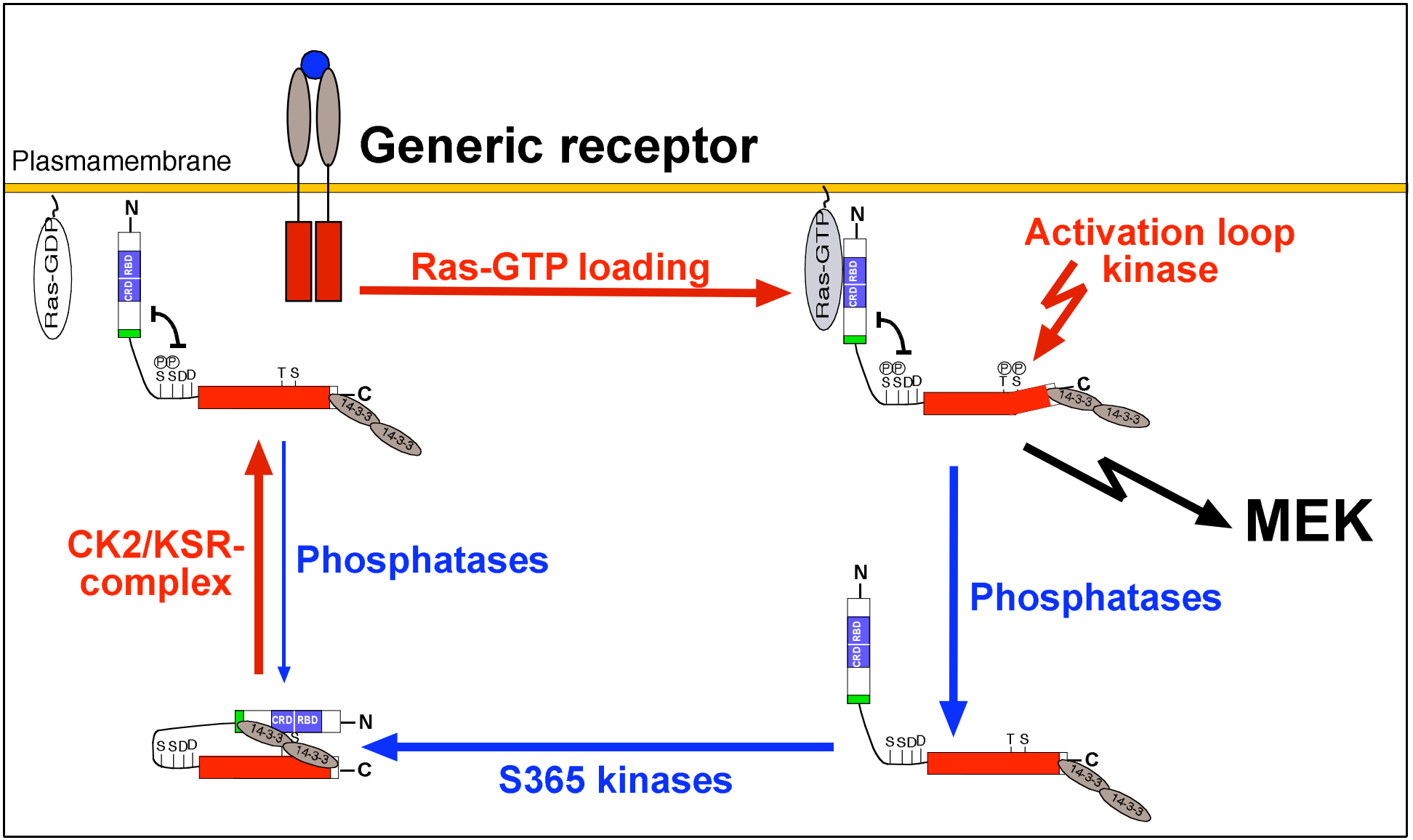
  Oncogene addiction represents a phenomenon in which the survival and proliferation of tumour cells become dependent on the presence of one or few oncogene product(s) and its/their effector functions. For example, an oncoprotein acting as a signalling element downstream of growth factor receptors can confer growth factor independence by mimicking the presence of growth factors. Removal of this oncogene product, e.g. by siRNA, or interference with its function, e.g. by inhibition of the oncoprotein itself or its downstream effectors, results in growth arrest, differentiation or even death of the tumour cell. Oncogene addiction is often regarded as a potential *Achilles heel* for tumour therapy, however, it should be considered that the degree of oncogene dependence can be influenced by the environmental setting, e.g. *in vitro* vs. *in vivo* conditions.

- **Scaffold proteins**

  Scaffold or docking proteins coordinate the spatio-temporal activation of signalling pathways by assembling their individual components to a multi-protein complex or **signalosome**.

  Signalosome

  Functional definition of a multi-protein complex of various signalling elements, whose association and activities is regulated by scaffold proteins. The assembly of the signalosome is modulated in a complex spatio-temporal manner and ensures the specificity and speed of intracellular signal transduction.


Plasmamembrane
Generic receptor
Ras-GDP
N
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S S D D
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14-3-3
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Phosphatases
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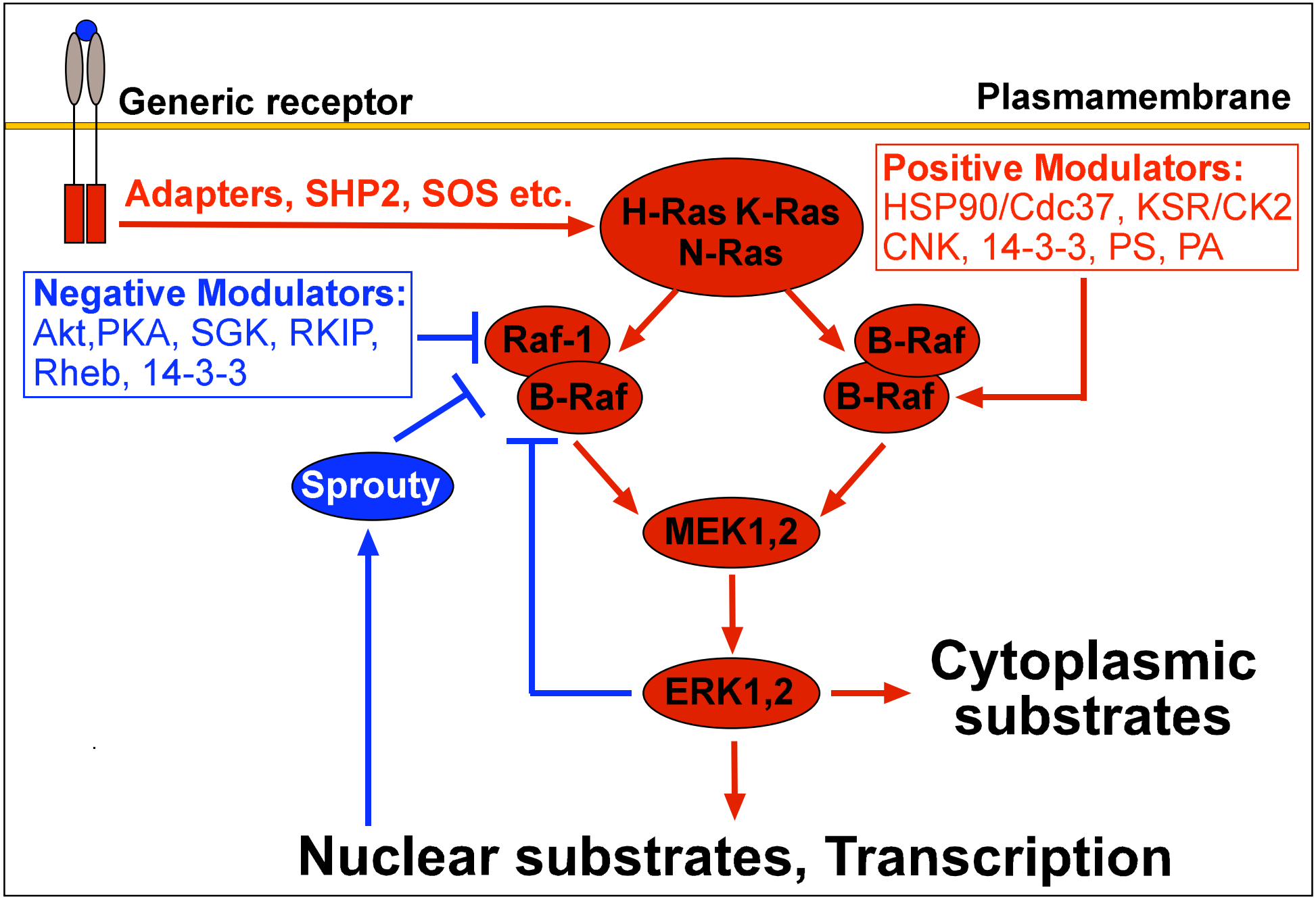
S365 kinases


Generic receptor
Plasmamembrane
Adapters, SHP2, SOS etc.
H-Ras K-Ras
N-Ras
Positive Modulators:
HSP90/Cdc37, KSR/CK2
CNK, 14-3-3, PS, PA
Negative Modulators:
Akt,PKA, SGK, RKIP,
Rheb, 14-3-3
Raf-1
B-Raf
B-Raf
B-Raf
Sprouty
MEK1,2
ERK1,2
Cytoplasmic
substrates
Nuclear substrates, Transcription