The Role of Protein-Protein Interactions in the Activation Cycle of RAF Kinases

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Summary

Members of the RAF protein kinase family are key regulators of diverse cellular processes. The need for isoform-specific regulation is reflected by the fact that all RAFs not only display a different degree of activity but also perform isoform-specific functions at diverse cellular compartments. Protein-protein-interactions and phosphorylation events are essential for the signal propagation along the Ras-RAF-MEK-ERK cascade. More than 40 interaction partners of RAF kinases have been described so far. Two of the most important regulators of RAF activity, namely Ras and 14-3-3 proteins, are subject of this work. So far, coupling of RAF with its upstream modulator protein Ras has only been investigated using truncated versions of RAF and regardless of the lipidation status of Ras. We quantitatively analyzed the binding properties of full-length B- and C-RAF to farnesylated H-Ras in presence and absence of membrane lipids. While the isolated Ras-binding domain of RAF exhibit a high binding affinity to both, farnesylated and nonfarnesylated H-Ras, the full-length RAF kinases demonstrate crucial differences in their affinity to Ras. In contrast to C-RAF that requires carboxyterminal farnesylated H-Ras for interaction at the plasma membrane, B-RAF also binds to nonfarnesylated H-Ras in the cytosol. For identification of the potential farnesyl binding site we used several fragments of the regulatory domain of C-RAF and found that the binding of farnesylated H-Ras is considerably increased in the presence of the cysteine-rich domain of RAF. In B-RAF a sequence of 98 amino acids at the extreme N terminus enables binding of Ras independent of its farnesylation status. The deletion of this region altered Ras binding as well as kinase properties of B-RAF to resemble C-RAF. Immunofluorescence studies in mammalian cells revealed essential differences between B- and C-RAF regarding the colocalization with Ras. In conclusion, our data suggest that that B-RAF, in contrast to C-RAF, is also accessible for nonfarnesylated Ras in the cytosolic environment due to its prolonged N terminus. Therefore, the activation of B-RAF may take place both at the plasma membrane and in the cytosolic environment.

Furthermore, the interaction of RAF isoforms with Ras at different subcellular sites may also be governed by the complex formation with 14-3-3 proteins. 14-3-3 adapter proteins play a crucial role in the activation of RAF kinases, but so far no information about the selectivity of the seven mammalian isoforms concerning RAF association and activation is available. We analyzed the composition of *in vivo* RAF/14-3-3 complexes isolated from mammalian cells with mass spectrometry and found that B-RAF associates with a greater variety of 14-3-3 proteins than C- and A-RAF. *In vitro* binding assays with purified proteins supported this observation since B-RAF showed highest affinity to all seven 14-3-3

isoforms, whereas C-RAF exhibited reduced affinity to some and A-RAF did not bind to the 14-3-3 isoforms ε, σ, and τ. To further examine this isoform specificity we addressed the question of whether both homo- and heterodimeric forms of 14-3-3 proteins participate in RAF signaling. By deleting one of the two 14-3-3 isoforms in *Saccharomyces cerevisiae* we were able to show that homodimeric 14-3-3 proteins are sufficient for functional activation of B- and C-RAF. In this context, the diverging effect of the internal, inhibiting and the activating C-terminal 14-3-3 binding domain in RAF could be demonstrated. Furthermore, we unveil that prohibitin stimulates C-RAF activity by interfering with 14-3-3 at the internal binding site. This region of C-RAF is also target of phosphorylation as part of a negative feedback loop. Using tandem MS we were able to identify so far unknown phosphorylation sites at serines 296 and 301. Phosphorylation of these sites *in vivo*, mediated by activated ERK, leads to inhibition of C-RAF kinase activity. The relationship of prohibitin interference with 14-3-3 binding and phosphorylation of adjacent sites has to be further elucidated. Taken together, our results provide important new information on the isoform-specific regulation of RAF kinases by differential interaction with Ras and 14-3-3 proteins and shed more light on the complex mechanism of RAF kinase activation.

Zusammenfassung

RAF Protein Kinasen sind essentielle Regulatoren verschiedener zellulärer Prozesse. Unterschiedlich starke Aktivitäten und Lokalisation der drei RAF Isoformen erfordern eine isoform-spezifische Regulation. Der Einfluss von Protein-Protein Interaktionen und Phosphorylierungen ist dabei mitentscheidend für die Signalweiterleitung entlang der Ras-RAF-MEK-ERK Kaskade. Mehr als 40 Interaktionspartner der RAF Kinasen wurden bereits beschrieben von denen zwei der wichtigsten, Ras und 14-3-3 Proteine, Gegenstand der vorliegenden Arbeit sind. Die Interaktion von RAF mit seinem vorgeschaltetem Modulatorprotein Ras wurde bislang nur mit verkürzten RAF-Proteinen und ohne Rücksicht auf den Lipidierungsgrad von Ras untersucht. Wir haben die Bindeeigenschaften von B- und C-RAF in voller, nativer Länge zu farnesyliertem H-Ras in Gegenwart und Abwesenheit von Membranlipiden quantifiziert. Während die isolierte Ras-Bindungsdomäne eine hohe Affinität sowohl zu farnesyliertem als auch nicht-farnesyliertem H-Ras aufweist, zeigen die RAF Proteine in voller Länge entscheidende Unterschiede in ihrem Bindeverhalten zu Ras. C-RAF benötigt für eine effiziente Interaktion mit H-Ras dessen C-terminale Farnesylgruppe, wobei B-RAF auch an nicht-farnesyliertes H-Ras im Cytosol bindet.

Um die verantwortliche Farnesylbinderegion zu identifizieren haben wir verschiedene Fragmente der regulatorischen Domäne von C-RAF eingesetzt. Dadurch konnten wir zeigen, dass die Affinität zu farnesyliertem Ras in Gegenwart der sogenannten Cystein-reichen Domäne von RAF beträchtlich erhöht war. In B-RAF ist eine Sequenz von 98 Aminosäuren am N-Terminus verantwortlich für die Ras-Bindung unabhängig von dessen Farnesylierungszustand. Die Deletion dieser Sequenz von B-RAF veränderte die Ras-Bindungseigenschaften sowie die Kinaseaktivität vergleichbar mit C-RAF. Durch Immunfluoreszenzversuche in Säugerzellen konnten darüber hinaus Unterschiede in der Kolokalisation von B- und C-RAF mit Ras beobachtet werden. Zusammenfassend deuten unsere Ergebnisse darauf hin, dass B-RAF, im Gegensatz zu C-RAF, aufgrund seines verlängerten N-Terminus in der Lage ist bereits im Cytosol auch mit unfarnesyliertem Ras zu interagieren, wodurch die Aktivierung von B-RAF sowohl im Cytosol als auch an der Plasmamembran erfolgen kann.

Die Interaktion der RAF-Isoformen mit Ras in unterschiedlichen zellulären Kompartimenten kann aber auch durch die Komplexbildung mit 14-3-3 Proteinen beeinflusst werden. Die 14-3-3 Adapter Proteine spielen eine entscheidende Rolle im Aktivierungszyklus der RAF Proteine. Bislang waren jedoch keine Details bezüglich der Selektivität der sieben 14-3-3 Isoformen aus Säugerzellen hinsichtlich der Assoziation mit und Aktivierung der RAF

Kinasen bekannt. Wir haben RAF/14-3-3 Komplexe aus Säugerzellen isoliert und durch Massenspektrometrie analysiert. Dadurch konnten wir zeigen, dass B-RAF mit einer größeren Vielfalt an 14-3-3 Isoformen bindet als C- und A-RAF. *In vitro* Bindungsversuche mit gereinigten Proteinen bestätigten die höhere Affinität von B-RAF zu allen sieben Säuger-14-3-3 Proteinen. C-RAF dagegen zeigte eine deutlich reduzierte Affinität, während für A-RAF keine Bindung zu den 14-3-3 Isoformen ε, σ, und τ festgestellt wurde. Um diese Isoformspezifität weiter aufzuklären haben wir untersucht, ob sowohl Homo- als auch Heterodimere von 14-3-3 in der Lage sind die RAF-Signaltransduktion zu beeinflussen. Durch die Deletion einer der beiden 14-3-3 Isoformen aus *Saccharomyces cerevisiae* konnten wir zeigen, dass bereits ein 14-3-3 Homodimer für die korrekte Aktivierung von B- und C-RAF ausreichend ist. In diesem Zusammenhang konnte auch die Rolle der internen, inhibierenden 14-3-3 Bindestelle in RAF gegenüber der C-terminalen, aktivierenden Stelle dargelegt werden. Zusätzlich zeigen wir, dass Prohibitin seinen aktivierenden Einfluss gegenüber C-RAF durch die Beeinträchtigung der 14-3-3 Bindung an der internen Stelle in RAF ausübt. Diese Region in C-RAF ist das Ziel von Phosphorylierungen im Zuge eines negativen Rückkopplungsmechanismus. Durch den Einsatz von Tandem-Massenspektrometrie konnten wir bislang unbekannte Phosphorylierungsstellen an den Serinen 296 und 301 identifizieren deren ERK-vermittelte Phosphorylierung *in vivo* eine Inaktivierung der C-RAF bewirkt. Der Zusammenhang zwischen der Behinderung der 14-3-3 Anlagerung durch Prohibitin und die Phosphorylierung in unmittelbarer Nachbarschaft bedarf weiterer Untersuchungen. Zusammengefasst liefern unsere Ergebnisse wichtige Informationen bezüglich der isoform-spezifischen Regulation der RAF Kinasen durch die Interaktion mit Ras und 14-3-3 Proteinen und helfen die komplexen Mechanismen der RAF Aktivierung weiter aufzuklären.

1. Introduction

Multi-cellular organisms are dependent on the ability to respond to changes in the environment. Various stimuli like hormones, growth factors, cytokines, or electric pulses are able to induce complex cellular processes that are responsible for the fate of the cell. But also cell-cell contacts or changes in the surrounding conditions, e.g. pH, temperature, mechanical stress, or radiation can trigger the switch between proliferation and differentiation or survival and apoptosis. Interference within these activities can lead to detrimental effects and, in the worst case, threatens survival of the organism. Effective communication of single cells with their environment is therefore indispensable for the survival of the collective.

One characteristic feature of signal transduction is the amplification of a rather simple stimulus to a complex response. The binding of a single extracellular molecule to its receptor on the cell surface can initiate the transmission of this signal along a predefined pathway. These pathways usually consist of numerous proteins or second messengers and allow integration of diverse cellular functions including transcriptional activity in the nucleus. An extensively studied example for these mechanisms of signal transduction is the Ras/RAF/MEK/ERK pathway, which plays a key role in diverse cellular functions.

The focus of this work lies on the central element of this cascade, the proto-oncogene RAF. This family of serine/threonine-kinases consists of three members, A-, B-, and C-RAF. The existence of three highly conserved isoforms with specific function implies isoform-specific regulation, ensured by sophisticated mechanisms including post-translational modification, formation of protein-protein complexes, and subcellular localization.

1.1 Protein Phosphorylation and Protein-Protein Interaction – Essential Tools for Signaling Networks

1.1.1 Protein Phosphorylation

Changing conditions in the environment require the possibility of a dynamic behavior of cellular responses. On protein level, posttranslational modifications play a key role for the cells to adapt to the new situation. The most intensely studied posttranslational protein modification is phosphorylation, described for the first time by Nobel laureates Fischer and Krebs more than 60 years ago [1,2]. This fundamental mechanism enables the cell to dynamically respond to a multitude of signals. It has been estimated that at least one third of all proteins in a typical mammalian cell are regulated by reversible addition of a phosphate and the corresponding enzymes, kinases and phosphatases, account for 2 – 4% of all genes in a eukaryotic cell [3,4]. Effects of phosphorylation of proteins include the induction or inhibition of enzyme activity, relocalization between subcellular compartments, protein interaction, as well as tagging for degradation. The most common targets for phosphate transfer are the side chains of amino acids residues serine, threonine and tyrosine. In fact, serine/threonine kinases regulating glycogen metabolism were the first to be identified [1]. Very rarely phosphorylation of histidines and cysteines [5], and even less frequent glutamic and aspartic acids, as well as lysines and arginines occurs [4]. In general, protein kinases and phosphatases are specific for one target amino acid. However, so called dual-specificity kinases and phosphatases are able to act on serine/threonine and additionally on tyrosine. Substrate recognition is not only dependent on the single amino acid to be modified, but is also mediated by a surrounding consensus sequence. Commonly, secondary interaction motifs at a distinct site further increase specificity between kinase/phosphatase and their substrates.

The transfer of the γ–phosphate from ATP to the substrate is performed by a conserved catalytic domain shared by all protein kinases. Tight regulation of this kinase activity is critical. The importance of this mechanism for cellular processes becomes apparent especially when it is disrupted in the diseased state. With the discovery of the v-Src oncoprotein being a protein tyrosine kinase (PTK) [6], a myriad of diseases caused by dysregulated protein kinases has become the topic of subsequent work. Moreover, protein kinases have become prime targets for drug development, especially in cancer. Today, about ten different protein kinase inhibitors are approved for therapy with several more being tested in clinical trials [7].

The co-localization of kinases and phosphatases with their substrates in discrete cellular compartments is a prerequisite for this signal transfer mechanism. Since protein phosphorylation can also provide conditional protein interaction sites, its importance for signal transduction is evident.

1.1.2 Protein-Protein Interactions

Cells function as a system consisting of numerous interacting players rather than a simple collection of individual molecules. Since proteins do not act in isolation, their properties are influenced by neighboring polypeptides and other components of the cells, such as phospholipids, nucleic acids, carbohydrates, and small molecule second messengers. Continuous modulation of protein-protein interactions provides the prerequisite for many biological signaling networks. The physiological functions of protein interactions include localization and trafficking, recognition of posttranslational modifications (e.g. phosphorylation, ubiquitination), and arrangement of multiprotein complexes and "molecular machines", large complexes that undertake core cellular functions, such as DNA replication or translation.

Experimental identification of protein-protein interactions can be achieved by either large scale screening or analysis of specific interactions. While high-throughput assays like Yeast two hybrid [8,9], phage display [10] and affinity purification [11,12] indicate physical interactions, biophysical methods like NMR spectroscopy [13], X-ray crystallography [14,15], and Surface Plasmon Resonance (SPR) [16] allow studies of specific protein interactions and provide structural information, dynamic and kinetic characterization. However, the above mentioned techniques are not capable of identifying protein interactions in living cells. This can be achieved by imaging techniques like the Fluorescence Resonance Energy Transfer (FRET [17]), Bimolecular Fluorescence Complementation (BiFC [18]), as well as non-invasive imaging technologies using bioluminescence or γ-ray transmission through small living animals [19,20]. But only the combination of distinct approaches yields orthogonal data sets, complementing one another. With the help of sophisticated software tools it is possible to generate protein interaction networks based on experimentally verified interactions.

In Figure 1, an example for the complexity of a protein interaction network is shown for the Mitogen-activated protein kinase kinase kinase (MAP3K) C-RAF (from http://string.embl.de [21]).

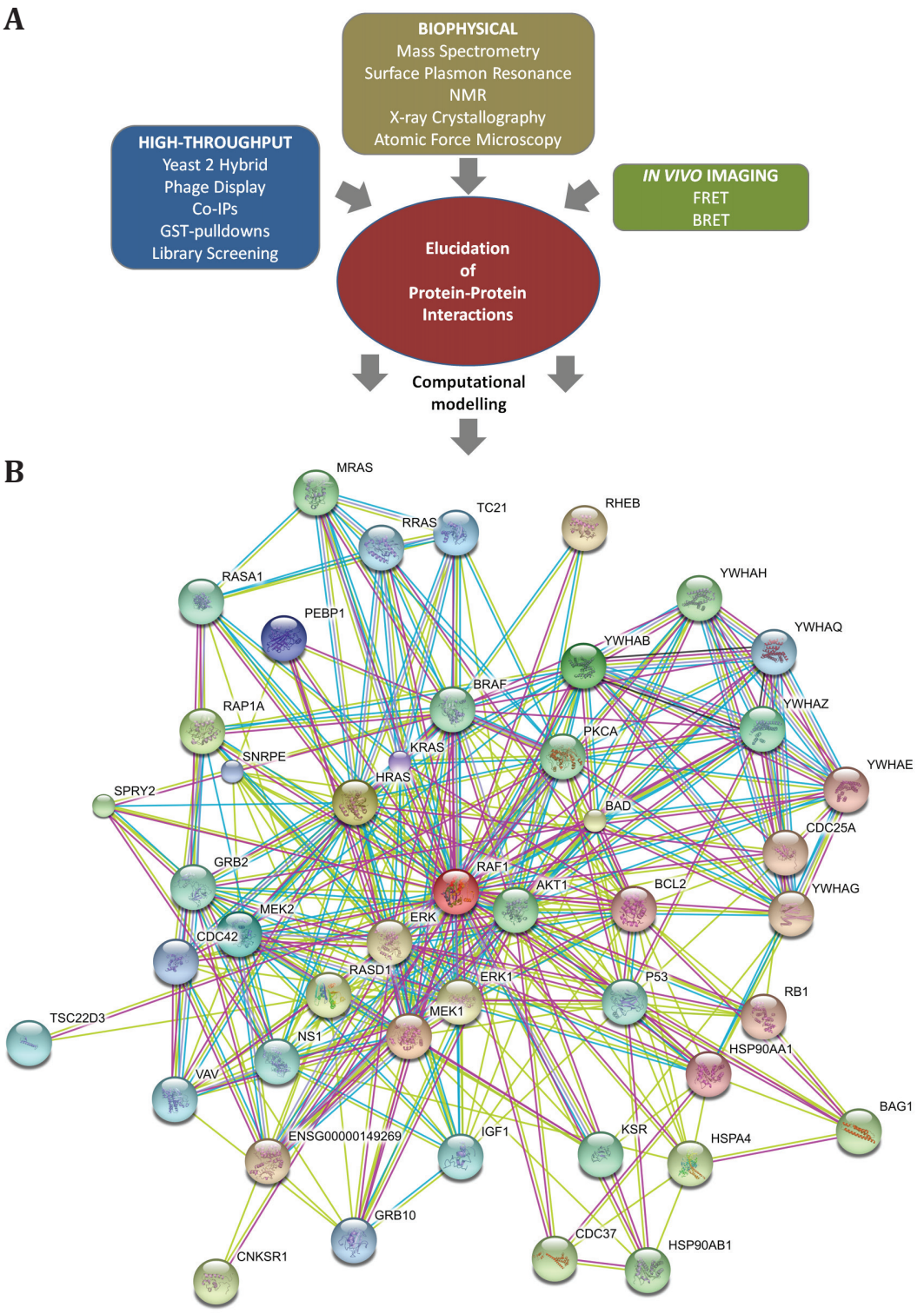

Figure 1: From experimental analysis to complex interaction maps. (A) The combination of diverse techniques leads to identification of single protein-protein interactions. Computational analysis of these data allows construction of extensive interaction networks. (B) The interaction network with 45 direct or functional interactors (confidence score >0.95) of C-RAF (RAF1) was visualized using STRING (http://string.embl.de). The color of the connecting lines displays the source of interaction data (red: experimental data, blue: databases, green: text mining).

The peptide sequence of every protein contains characteristic binding domains or properties that distinguish protein interaction domains, so called interfaces, from the rest of the protein surface. Usually, sequence conservation within these interfaces is very high. The composition of residues differs between obligate and transient complexes: the former relying on hydrophobic interactions, the latter more on salt bridges and hydrogen bonds. Furthermore, electrostatic interactions and van-der-Waals forces can also influence the specificity and stability of interacting proteins. Until now, dozens of such interfaces could be identified in numerous proteins [22,23]. This collection comprises SH2 domains (binding to phosphotyrosyl residues, e.g. sequence pYxNx for Grb2) and SH3 domains (recognizes proline rich sequences such as xPxxP), WW domains (also proline rich, consensus sequences are PPxY or PPxP), or the PDZ module (hydrophobic C-termini, e.g. Val-COO-) and 14-3-3 domains (see chapter 1.2.5) and many more.
All major signaling pathways involve a vast array of enzymes, anchoring, scaffolding, adaptor and other regulatory proteins that interact with each other and itself are influenced by other proteins as well as external and internal conditions, e.g. cell stage, subcellular localization. The MAP kinase cascade is one of the best studied signaling pathways with numerous proteins as supporting actors for the key players Ras, RAF, MEK, and ERK.

1.2 The MAP Kinase Signaling Cascade

The Mitogen-Activated Protein Kinase signaling pathway family (MAPK pathway, or MAP Kinase pathway) consists of related cascades that couple different types of cell surface receptors to at least six groups of MAP kinases [24]. Some of them are involved in cell differentiation and apoptosis and are mainly activated in response to cellular stress and cytokines (e.g. JNK and p38). Others can be induced by stress stimuli as well as growth factors (ERK5). The so called “classical” MAP kinases, ERK1 and ERK2 (extracellular signal-regulated kinases), are activated by mitogens that bind to receptor tyrosine kinases (RTK) at the plasma membrane. After dimerization of the receptor monomers the intrinsic tyrosine kinase activity phosphorylates the intracellular domain where guanine nucleotide exchange factors for a small G-protein subsequently bind.
Between corresponding receptor and final response all MAPK cascades comprise a core of three sequentially activated kinases responsible for signal transmission and amplification: the serine/threonine specific MAPK kinase kinase (MAP3K, “membrane shuttle kinase”) that activates a MAPK kinase (MAP2K, MKK or MEK, “dual-specificity kinase”) which, in

turn, regulates the MAPK ("nuclear shuttle kinase") by concomitant Tyrosine and Serine/ Threonine phosphorylations. This stepwise signal propagation, in comparison to systems consisting of only one kinase (e.g. cAMP pathway), enables the cell to precisely regulate signal strength and duration.

The Ras/RAF/MEK/ERK cascade is the best described MAP kinase cascade (for reviews see [25] and [26]). The model in figure 2 displays a simplified overview of this pathway with a universal growth factor (GF) as the stimulating agent. Binding of growth factor ligands to the receptor tyrosine kinase (RTK) induces dimerization and autophosphorylation of tyrosines at the intracellular domain. The phosphorylated tyrosines serve as docking sites for the adaptor protein Grb2 (Growth factor receptor-bound protein 2) which in turn via its SH3 domain binds to the guanine nucleotide exchange factor SOS (Son Of Sevenless) [27]. By exchanging GDP (guanosine diphosphate) for GTP (guanosine triphosphate) in Ras, the resulting conformational change of Ras allows binding and activation of effector proteins like RAF, RalGDS (Ral guanine nucleotide dissociation stimulator) or PI3K (Phosphatidylinositol 3-kinase) [28] and subsequent signal propagation.

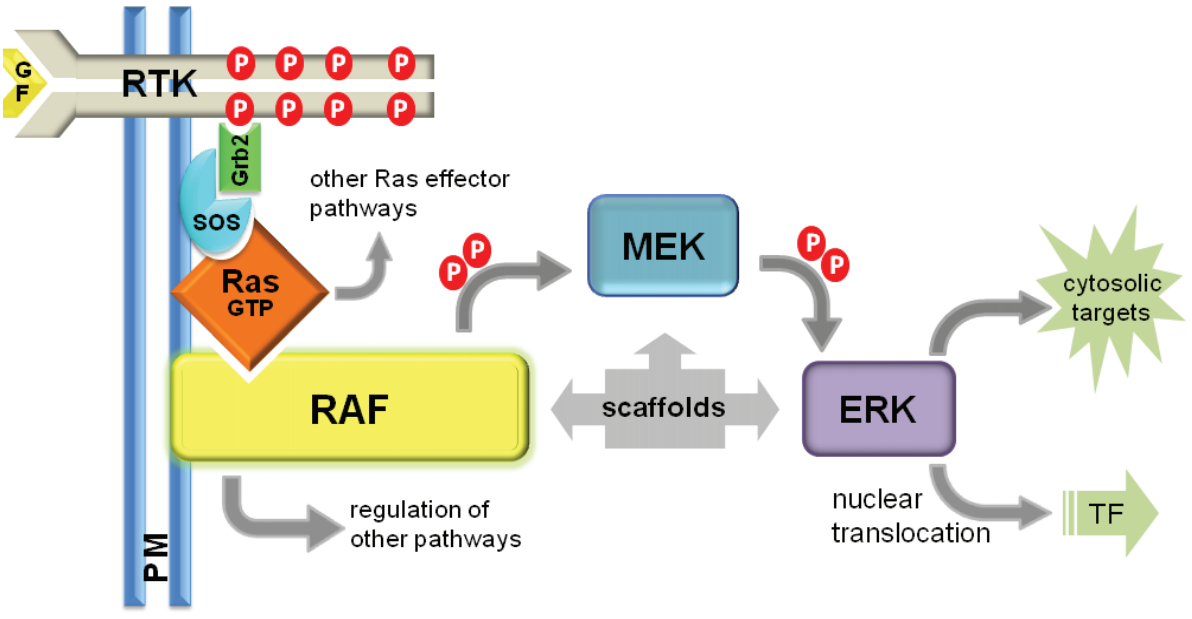

Figure 2: The Ras–RAF–MEK–ERK pathway. Growth factor (GF) binding to receptor tyrosine kinases (RTK) spanning the plasma membrane (PM) recruits adaptor proteins (Grb2 and SOS) and activation of Ras by GTP loading follows. Ras can signal to different pathways, e.g. activates RAF, which not only regulates the MAPK cascade. With support of scaffolding proteins RAF phosphorylates MEK which in turn phosphorylates ERK. ERK translocates to the nucleus where it activates various transcription factors (TF) but has also cytoplasmic substrates.

1.2.1 RAF Kinases

The first description of the oncogene v-raf (rapidly growing fibrosarcoma) from the transforming murine retrovirus 3611-MSV in 1983 [29] was followed by the discovery of a cellular homolog, c-raf, in 1985 [30]. Subsequently, two paralogs in vertebrates, namely a-raf and b-raf were described [31,32]. Several homologs could be found in other eukaryotes like *Drosophila* (Draf [33]), *Caenorhabditis* (lin-45 [34]), and also *Arabidopsis* (CTR1 [35]). The mammalian gene products A-, B-, and C-RAF are serine/threonine kinases that act as "membrane shuttle kinases" due to their cytosolic but membrane-associated localization. All RAF isoforms share highly conserved regions at the N-terminus (CR1 and CR2) and a third one (CR3) that accounts for the catalytical active part, the kinase domain, at the C-terminus. A summary of the domain structure and some of the large number of phosphorylation sites is depicted in figure 3.

CR1 contains two important regions for interaction with Ras proteins (Ras binding domain, RBD, see chapter 1.2.3) and membrane association (cysteine rich domain, CRD) [36,37]. The serine/threonine rich conserved region 2 is mainly involved in phosphorylation-dependent activation of RAF [38,39]. This part of RAF comprises a flexible hinge region between regulatory N-terminus and catalytic C-terminus that influences the conformation of the active and inactive kinase through phosphorylation as has been recently shown for A-RAF [40]. Especially the phosphorylated serine residue at position 259 in C-RAF (and corresponding sites in A- and B-RAF) plays an essential role in the RAF activation cycle as it provides the internal binding site for 14-3-3 adaptor proteins [41]. Not far from the internal 14-3-3 interaction motif, between amino acids 297 and 335, a binding site for the membrane associated chaperone prohibitin was defined (see chapter 1.2.4 and [42]).

Several lipid binding domains have been described in RAF kinases. Binding to phosphatidylserine (PS) via the CRD and to phosphatidic acid (PA) via a RKTR motif located in the kinase domain is known for RAF [43,44]. A distinct affinity for cholesterol, found in high concentrations in membrane microdomains, so called lipid rafts, could also be determined [45]. Although the mechanisms of lipid-mediated RAF regulation are not yet completely resolved, it is most likely, that specific lipid affinities may govern the targeting of RAF kinases to distinct cellular compartments [46,47,48].

CR1 and CR2 are considered to be part of the regulatory N-terminal half of the protein. However, CR3 with the kinase domain also harbors several phosphorylation sites necessary for the proper function of the protein. The kinase domain yields the catalytic activity and transfers the γ-phosphate from ATP to a hydroxyl moiety on a protein substrate.

Amongst all RAF kinases this domain is highly conserved. A low resolution structure of the B-RAF kinase domain was solved in 2004 by Wan *et al*. and provides important insights into oncogenic activation by comparison of the wild type and the V600E mutant form [49]. The overall bilobal structure with the DFG motif (aa 593-595), that forms polar contacts with all three phosphates, resembles that of other serine/threonine kinases and conforms to expectations based on modelling [50]. The inactive conformation is sustained by an atypical interaction between a glycine-rich loop and the activation segment. For activation, phosphorylation of a regulatory threonine in position 599 (corresponding to positions 452 in A-RAF and 491 in C-RAF) within the activation segment is necessary and enables the active conformation. The oncogenic mutation V600E, accounting for ~90 % of oncogenic B-RAF and therefore ~70 % of human melanoma cases, destabilizes the inactive conformation leading to a hyperactivated B-RAF kinase.

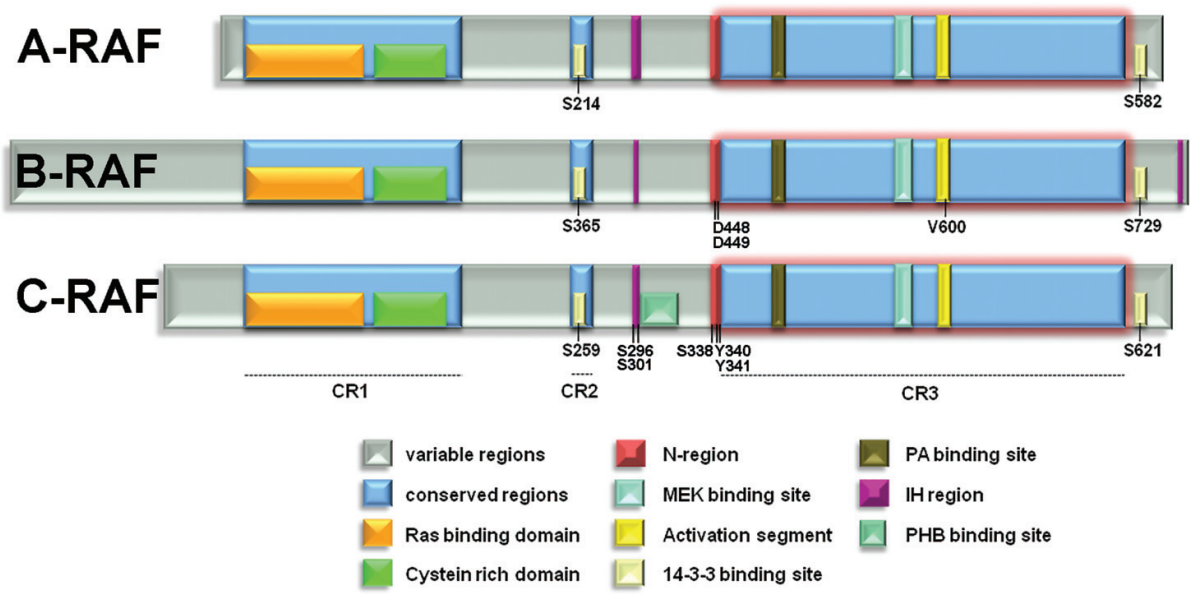

Figure 3: Domain structure of the mammalian A-RAF (606aa), B-RAF (766aa), and C-RAF kinase (648aa). For clarity, only some important phosphorylation sites are marked. RBD stretches from amino acids 13-91 in A-RAF, from 150-227 in B-RAF, and from 51-131 in C-RAF. The kinase domain is highlighted by a red glowing around CR3. For color coding of the domains see legend.

Since C-RAF was the first isoform to be described, most work on regulatory phosphorylation sites and binding partners has been carried out for this isoform. During the RAF activation process phosphorylation events are crucial and tightly regulated. Most phosphorylation sites in RAF are well established, but phosphorylation still is a controversial aspect of RAF research since the discovery of growth factor-induced tyrosine phosphorylation of C-RAF [51]. Three classes of regulatory phosphorylation sites can be distinguished:

14-3-3 proteins binding motifs [41], targeting sites [52], and conformation-relevant sites [49]. Three basal phosphorylation sites, the serine residues at positions 43, 259, and 621 have been identified in C-RAF by Morrison *et al*. [38]. Serines 259 and 621 are involved in binding of 14-3-3 proteins with phosphorylation of serine 621 being essential for C-RAF activation, since mutation to alanine results in a RAF protein unresponsive to growth factor stimulation [53,54]. In contrast, exchange of serine 259 to alanine or aspartic acid enhances the kinase activity, indicating an inhibitory effect by phosphorylation of the serine [38,55,56,57,58]. Furthermore, the binding of 14-3-3 proteins to the C-terminal conserved site has been found to be differentially and dynamically regulated [54]. While phosphorylation of serines 43, 233 and also 259 results in negative regulation of C-RAF function [59], phosphorylation of C-RAF serine 338 correlates with Ras mediated stimulation and is required for RAF activation [60]. The origin of the activating tyrosine 340/341 phosphorylation is still unclear. Mutation of these residues to aspartic acid results in constitutively active C-RAF [60,61]. It has been ascribed to phosphorylation by receptor tyrosine kinases and the Src kinase family and to be coincident with RAF activation, at least in some cell types [62,63]. Since activated RAF doesn't exhibit phosporylated tyrosines, a receptor- and/or cell-type dependency for RAF tyrosine phosphorylation has been concluded. Thus, the possibility of direct tyrosine phosphorylation of RAF by tyrosine receptor kinases remains [62]. The fact that these position in B-RAF are naturally taken by two aspartic acids (D448 and D449) may partially account for the high basal activity of B-RAF. In a recent study Xia *et al*. demonstrated the regulatory interaction between N-terminus and C-terminus of D-RAF, the B-RAF ortholog from *Drosophila* [64]. They suggest the participation of tyrosine 510 in this autoinhibitory interaction. After Ras binding the N-terminus dissociates from the C-terminal region and the subsequent Src mediated phosphorylation of tyrosine 510 prevents reassociation.

C-RAF residues T268 and T269 have been reported to serve as autophosphorylation sites and targets for KSR [38,65]. Furthermore, protein kinase C (PKC) was described to phosphorylate serines 479 and 499 [39,66]. Phosphorylation of threonine 491 and serine 494, positioned in the activation loop of C-RAF, have been demonstrated to be necessary but not sufficient for C-RAF activation [67]. They probably cooperate with serine 338 and tyrosine 341 and further augment C-RAF activation.

The situation changed and RAF research focused on B-RAF after the description of several activating mutations in human cancer [49,68]. All together, B-RAF is mutated in 20% of all human cancers (www.sanger.ac.uk/genetics/CGP/cosmic). Amongst more than 40 different mutations on the B-RAF gene, a single one accounts for almost 90%

[68]. This substitution of a valine residue at position 600 for glutamic acid generates a constitutively active kinase [69]. Interestingly, some germline mutations in B-RAF have also been identified. Patients with the sporadic developmental disorder CFC (cardio-facio-cutaneous) syndrome bear a variety of B-RAF mutations except for the most frequent cancer associated mutation V600E [70,71]. For the first time, disease-associated mutations in RAF were described that are involved in membrane association, i.e. mutations in the CRD (A246P and Q257R) and PA binding domain (K499E). Hekman *et al*. demonstrated previously, that the ability of binding to lipids is essential for the reduction of the high basal B-RAF activity in unstimulated cells [45], indicating permanent B-RAF kinase activity associated with impaired lipid binding capacity. In fact, all CFC mutants deficient for lipid binding revealed significantly higher kinase activity compared with the wild type B-RAF. Very recently, Ritt *et al*. ascribed this effect to the constitutive heterodimerization of B-RAF with C-RAF [72], a complex that was already shown to have high kinase activity towards MEK [73]. Surprisingly, activity of the oncogenic B-RAF-V600E is inhibited by C-RAF in the dimer [74], maybe due to conformational restrictions.

The smallest and in terms of activity weakest member of the RAF kinase family is A-RAF. This isoform is so far only poorly investigated but recent work revealed a rather atypical behavior compared to B- and C-RAF. Nekhoroshkova *et al*. described A-RAF working upstream of ARF6 as a regulator of endocytosis and endocytic trafficking [48]. Furthermore, another study from our group identified several regulatory phosphorylation sites in the so called isoform-specific hinge region (IH region) between CR2 and the negatively charged region (N-region). Phosphorylation of serines 257, 262, and 264 lead to a charge switch that causes the detachment of A-RAF from the membrane [40].

The distinct cellular function of the three mammalian RAF isoforms is represented best by their differences in development of knock-out mouse models. Whereas A-RAF-/- animals die perinatally [75], C-RAF-/- and BRAF-/- mice die already during embryonic development [76,77]. Although, having an ubiquitous expression pattern in common, the expression levels differ between the isoforms. While A-RAF can be found in high amounts in urogenital tissue [78], B-RAF is primarily expressed in the nervous system but also to a lower extent in other tissues. C-RAF shows highest expression in striated muscle, cerebellum and fetal brain [79].

Another distinguishing feature of RAF isoforms is their level of basal and growth factor induced activity. C-RAF is slightly active in unstimulated cells but answers to growth factor stimulation with a strong increase in kinase activity, whereas B-RAF displays a high basal activity *in vitro* and only weak response to further stimulation. In contrast, A-RAF

activation reaches in maximum 20% of the C-RAF level [80]. RAF activation is not only achieved by phosphorylation, but is also influenced by the correct sequence of interactions with diverse other players along the signaling cascade.

1.2.2 Ras – The Path is the Goal

Owed to their role in human cancer (~30%, COSMIC database on www.sanger.ac.uk) Ras proteins like RAF are subject of studies for more than a quarter century now. More than 170 evolutionarily conserved proteins related to Ras constitute a large super family of GTP-hydrolyzing proteins (GTPases). It can be further classified in at least five subfamilies Ras, Rho, Rab, Arf, Ran [81,82]. All members, as well as the closely related Gα family, share the basic biochemical activity of GTP binding and hydrolysis (see figure 4).
The Ras subfamily consists of about 35 members, in which the oncoproteins H-, N-, and K-Ras represent the best studied members [28,82]. They are the gene products of three human genes, namely Ha(rvey)-, N(euroblastoma)-, and Ki(rsten)-ras. The K-ras gene is translated in two splice variants, K-Ras4A and -4B. All isoforms are almost identical and (except K-Ras4A) ubiquitously expressed. Nevertheless, their functions do not exhibit complete redundancy [83]. This is also evident from work that identified only K-Ras as embryonic lethal [84] in contrast to RAFs where all three isoforms are required for adult life [85].

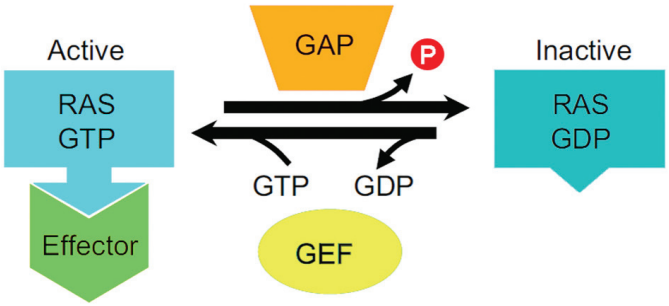

Figure 4: RAS proteins exist in equilibrium between GTP- and GDP bound forms. GEFs (Guanine nucleotide exchange factors) promote replacement of GDP by GTP, whereas GAPs (GTPase activating proteins) catalyze the intrinsic GTPase activity of Ras. Thereby the relative amount of each form is regulated. The GTP-bound conformation of RAS shows high affinity interactions with effector proteins that propagate downstream signaling (modified from [28]).

Ras proteins are post-translationally modified within their C-terminal hypervariable region (HVR) that contains two motifs responsible for targeting the fully processed proteins to the plasma membrane [82]. After synthesis in the cytosol a 15-carbon farnesyl isoprenoid is covalently added to the cysteine residue in the so called CAAX sequence (C = cysteine, A = aliphatic, X = any amino acid). This task is assigned to farnesyltransferase (FTase) unless the amino acid at the X position is leucine or phenylalanine (as in H-, K-, and N-Ras). In the next steps, endoplasmic reticulum-associated enzymes remove the residues AAX (Ras converting enzyme 1) and catalyze the carboxyl methylation (Isoprenylcysteine carboxyl methyltransferase) of the now farnesylated and terminal cysteine. These modifications render the previously hydrophilic into a hydrophobic protein region and are essential for the biological function of all Ras proteins [86]. However, a second signal is necessary for translocation to the correct subcellular compartment, in principal the inner surface of the plasma membrane. Immediately upstream of the CAAX domain a second signal was described. K-Ras4B bears the so called polybasic box, a stretch of six lysine residues and thereby can complete its transit to interact with negatively charged lipids in the plasma membrane. In contrast, K-Ras4A shares the covalent attachment of a palmitoyl moiety by a RAS palmitoyl transferase at cysteine 180 with H-Ras (two sites at C181 and C184) and N-Ras (C181). Whereas K-Ras4B with its polybasic sequence translocates directly to the plasma membrane via a yet uncharacterized pathway, the palmitoylated Ras isoforms trafficked through a vesicular pathway along the ER – Golgi route to the plasma membrane. In case of H-Ras it was shown that the two palmitoyl groups are not equally necessary for translocation. While palmitoylation of cysteine 181 directly targets H-Ras to the plasma membrane, the single palmitoyl group at residue C184 restricts the protein to Golgi localization [87].

In 2005, Rocks *et al*. [88] described a continuous cycle of de- and reacylation reactions as a mechanism for proper membrane localization and initiation of Ras activation. Using FRAP (fluorescence recovery after photobleaching) technology, they were able to determine the exchange rates of Ras between plasma membrane and Golgi localization. After depalmitoylation at the plasma membrane, the Ras pool at the Golgi membrane is refilled with a half-life of approximately 11 minutes and 2 minutes for H- and N-Ras, respectively. Considering the 21 hours half-life of N-Ras protein [89], this activation-status independent acylation/deacylation cycle is obviously an important regulator for the biological function of Ras.

Distinct plasma membrane microdomains and endomembranes like ER/Golgi, endosomes, and even mitochondria or nuclear membranes have been shown to provide platforms for

Ras signaling [90,91,92,93,94,95]. Further, a number of small G-proteins are not post-translationally lipidated and transform cells in their constitutively active form or induce differentiation events, such as neurite outgrowth [96]. Taken together, differences in lipidation along with compartmentalization allow some degree of signaling specificity even within a highly homologous protein family like the Ras proteins. But also differences in their activation mechanism are a distinguishing feature of Ras proteins.

The exchange of bound GDP in the basal state to GTP in the active state represents the rate-limiting step in Ras activation (Fig. 4). Several different Guanine nucleotide exchange factors (GEFs) catalyze the release of GDP and activation of Ras [97]. In contrast to GEFs, Guanine nucleotide dissociation inhibitors (GDIs) block the GDP release, thus stabilizing the inactive conformation [98]. By exchange of GDP for GTP, Ras proteins perform a conformational change and display a binding surface with high affinity for their effectors. Especially two domains, the so called "switch I" (amino acids 30-38 in H-Ras) and "switch II" (H-Ras aa59-67) regions undergo a structural change and provide a GTP-dependent access to downstream interaction partners [99]. Active, GTP-bound Ras associates with more than ten different effectors, including RAF and other serine/threonine kinases (MEKK1, PKC-ζ), various PI3K lipid kinases (p110-α, -β, -γ, and δ), and GDP-GTP exchange factors from the RalGDS family (RalGDS, Rgl, Rgl2) [100,101]. The intrinsic GTPase activity of Ras proteins is rather slow (measured for H-Ras at 3.4×10^{-4} s^{-1}) but can be increased with the help of GAPs (GTPase-activating protein) [102,103]. A broad choice of selective GEF, GDI and GAP regulators enhances signaling specificity within this large family of small G proteins (for review see [104] and [105]).

1.2.3 Ras – RAF Interaction

Ras proteins play an important role in RAF kinase activation, but the mechanistic processes in Ras-RAF coupling are not completely understood. The Ras binding domain (RBD) in C-RAF stretches from residue 51 to 131 and directly interacts with the switch-I region of active Ras-GTP [106]. In addition to the RBD, the CRD (Cysteine rich domain, aa 139-184) of C-RAF appears to play an auxiliary role in Ras-RAF coupling and activation of RAF. While the Ras-RBD interaction is understood in great detail, there are conflicting data regarding the role of CRD. The mutation of the zinc binding cysteines to serines decreases the interaction of Ras with the N-terminal part of C-RAF [107,108,109,110]. Therefore it has been proposed that the farnesyl residue of Ras directly interacts with the

hydrophobic surface of CRD. To validate this hypothesis, the interaction of farnesylated and nonfarnesylated H-Ras with the isolated C-RAF-CRD have been investigated [111,112]. The authors observed that only farnesylated Ras binds to CRD, however, as previously demonstrated [113], independent of the guanine nucleotide state of Ras. The tight binding of Ras-GTP to RBD was suggested to compensate *in vivo* for the very weak affinity constant of farnesylated Ras to CRD (KD approx. 20 µM). Additionally, the CRD was also reported to bind to 14-3-3 proteins and phosphatidylserine [43,114] and therefore may contribute in several ways to the regulation of C-RAF activation. In summary, these findings support a dual role for Ras: high affinity coupling of Ras-GTP to the C-RAF RBD and additional weaker coupling of farnesylated Ras to the CRD that seems to be necessary for C-RAF activation.

As described above, lipidation is another characteristic feature of Ras, indispensable for association with membranes [115]. Physical Anchoring of Ras to membranes is mediated via palmitoyl residues [116], and the stability of Ras insertion into artificial membranes is closely linked to the degree of hydrophobic modification. Additionally, the de- and repalmitoylation is important for the subcellular distribution of H-Ras [88], promoting the relocation of Ras from endomembranes to the plasma membrane. In contrast to C-RAF, there is only little information available concerning the binding of B-RAF to Ras.

1.2.4 Prohibitin

Prohibitin (PHB) is an evolutionarily well conserved and ubiquitous protein that plays a role in many cellular processes such as energy metabolism, cell cycle control and proliferation [117,118]. It is most concentrated in the inner mitochondrial membrane where it acts as a chaperone but can also be found in the cytosol or the plasma membrane [119,120,121]. In 2005, Rajalingam *et al*. described PHB as a new player in the Ras-RAF pathway. By direct interaction with C-RAF, but not Ras, it modulates RAF plasma membrane localization and activation [122]. Most interestingly, after PHB-depletion using siRNA, the level of C-RAF phosphorylation at pS259 (the internal 14-3-3 binding site) as well as related 14-3-3 binding was increased. Further elucidation of the properties of PHB interaction with RAF was also part of this work [123].

1.2.5 The RAF Substrate MEK

The dual specificity mitogen-activated protein kinase kinases (also MAPK/ERK kinases or MEK) are the predominant substrates of RAF kinases. The two isoforms MEK1 and MEK2 are highly homologous and share the ability of phosphorylating a tyrosine, as well as a threonine residue in their effector ERK [124]. A third isoform, the splice variant MEK1b was considered to be inactive, but a recent study showed highly specific activity towards one of the nuclear ERK isoforms, namely ERK1c [125]. Activation of MEK occurs via phosphorylation of two serine residues within their activation loop (218/222 for MEK1, 222/226 in MEK2) by RAF [126] and replacement of these with acidic residues generates constitutively active variants. Furthermore, Tpl2 (Cot), MEKK1, and Mos are also able to phosphorylate and activate MEK [127,128,129]. MEK phosphorylation by RAF requires interaction at two distinct sites within the kinase domain and phosphorylation at serine 471 (in C-RAF) [130,131]. Knock out of MEK1 in mice leads to death at an early embryonic stage [132], in contrast to MEK2 that does not give rise to an overt phenotype [133]. Considering their high sequence homology of ~80% this lack of redundancy is surprising. In fact, the MEK members like RAF are some of the most specific kinases known. They have not been shown to accept any other substrate than the MAP kinases ERK1 and ERK2, except for BAD. Their structure contains a proline-rich and a nuclear export sequence as well as an ERK-interaction site at the N-terminus [134]. A key player for assembling the RAF-MEK-ERK signaling cascade at the plasma membrane seems to be the scaffold protein KSR (kinase suppressor of ras, [135]). The interaction of MEK with KSR is constitutive [136] and mediated via the proline-rich sequence (aa 270-307 in human MEK1) that is also required for binding to RAF kinases and another complex mediator called MP1 [137,138].

1.2.6 KSR – more than a scaffold

Kinase suppressor of Ras was identified in a genetic screen as a positive regulator of the MAP kinase cascade [139] and homologs can be found in all multicellular organisms [140]. Due to its high similarity with C-RAF it initially was thought to be a kinase, but compared to RAF, KSR lacks the essential lysine residue in the ATP-binding pocket. Consistently, kinase activity couldn't be proven unequivocally [140]. What could be demonstrated was its role as a MAPK scaffold protein binding to MEK1/2, ERK1/2, C-RAF and also B-RAF

[141]. While MEK interaction is constitutive, ERK binding occurs only after growth factor stimulation. KSR further associates with a whole array of signaling regulators of the Ras-RAF cascade, namely the 14-3-3 adaptor proteins, HSP70 and 90, C-TAK1, PP2A, CK2 and many more proving its important role in the assembly a signaling complex for RAF kinase. After RTK-mediated Ras activation, KSR with bound MEK translocates to the plasma membrane where it colocalizes with RAF. The following recruitment of ERK to the KSR mediated complex allows stepwise phosphorylation along the cascade [142,143]. Knock-out studies for KSR in mice revealed that KSR may be dispensable for but clearly enhances signaling from Ras [144]. Indeed, the group of Therrien described a novel function of KSR as an allosteric activator of RAF by forming a side-to-side heterodimer with RAF via their N-lobes in the kinase domain [145]. Furthermore, McKay *et al*. demonstrated the possibility of a negative feedback regulation and subsequent disruption of the complex due to phosphorylation of B-RAF and KSR by activated ERK [135]. This hypothesis was backed by Ritt *et al*. who identified S/TP sites in B-RAF that inhibit dimerization with C-RAF after feedback phosphorylation by active ERK [72].
Other scaffold proteins for the RAF module have also been described, namely CNK (connector enhancer of KSR), SUR-8 (suppressor of Ras-8), β-Arrestins, or MP-1 (MEK-Partner-1) [146]. The latter one, for instance, binds MEK1 and ERK1 (not MEK2 or ERK2) and localizes to late endosomes via interaction with the adaptor protein p14 [138,147]. In general, all scaffolds seem to guide the complex in a spatial way, depending on the activation signal from the respective stimulus.

1.2.7 The MAP Kinase ERK

ERK (Extracellular signal-regulated kinase) MAPKs are the key effectors at the end of the Ras-RAF cascade since their activity is responsible for the serine/threonine phosphorylation of many proteins in the cytosol and directly impact transcription from within the nucleus [24]. The two most important splice variants ERK1 and ERK2 share 83% sequence similarity and are expressed in all tissues but to a varying extend [148]. ERK is phosphorylated and activated by MEK (and only by MEK) on a tyrosine (Tyr204 and Tyr185 for ERK1 and 2, respectively) and subsequently on a threonine residue (Thr202 and Thr183), with the latter one being necessary for full activation and of cellular functions [124,149]. In contrast to MEK, the exchange of these activating phosphorylation sites with acidic residues is not sufficient for constitutive activation [150]. The consensus

sequence for ERK phosphorylation was described as P-X-S/T-P but a minimal sequence consisting of serine/threonine followed by a proline was shown to be sufficient [151,152]. The long list of ERK substrates includes transcription factors like SAP-1, c-Jun, c-Fos, p53, and Elk1, but also the cytosolic localized proteins MK2, p90/RSK1 or Phospolipase A2 (for review see [153] and [124]). Most interestingly, ERK is also able to phosphorylate RAF and KSR thereby generating a negative feedback loop that fine-tunes the signal output of the Ras-RAF cascade ([154,155] and this work)

1.2.8 14-3-3 Proteins

14-3-3 proteins are a class of highly conserved proteins found in all eukaryotic organisms indicating an early evolutionary origin (for review see [156]). They are small (~30 kDa) ubiquitously expressed acidic proteins that are involved in central physiological pathways, such as signal transduction, cell cycle regulation, apoptosis, metabolism, or protein trafficking [157,158,159]. With their first description in 1967, they acquired their name from the chromatography position and migration pattern in gel electrophoresis in the course of classifying all bovine brain proteins [160]. This protein family gained increasing interest with the identification of their role as an activator of the tyrosine and tryptophan hydroxylases [161], as well as for the C-RAF kinase [162]. The number of isoforms ranges in different eukaryotes between 12 in Arabidopsis [163], and seven in human to two isoforms in *Drosophila melanogaster*, *Caenorhabditis elegans* and the budding Yeast (BMH1 and BMH2) [164]. In mammalian cells seven isoforms are expressed from seven distinct genes denoted β, γ, ε, η, σ, τ, and ζ. From their native size of approx. 60 kDa and even more after structural analysis it became obvious that 14-3-3 proteins exclusively function as homo- and heterodimers [165]. The crystal structures revealed that 14-3-3 dimers form a flattened horseshoe-like conformation with each monomer consisting of nine anti-parallel α-helices (α-A to α-I). For example, at the connection interface of the ζ-homodimer, α-A and α-B from one monomers interact with α-C' and α-D' from the second monomer via three salt bridges and several polar residues (see figure 5 and [166]). 14-3-3 protein dimers self-assemble to homo- or heterodimers. The dimer formation pattern for the two isoforms ε and γ has been investigated by Chaudhri *et al*. [167]. 14-3-3ε formed heterodimers with β, γ, ζ and η, but no ε homodimers were detected. In contrast, the σ isoform exists exclusively as homodimers [168]. A comparison of 14-3-3 dimer crystals with and without bound ligands revealed a highly rigid shape [165,169]

that is able to induce conformational changes within the ligands. Thereby, 14-3-3 binding can alter substrate properties such as catalytic activity or protein stability. The possibility of constituting 21 different combinations of 14-3-3 homo- and heterodimers *in vivo* may have important implications for function of 14-3-3 proteins and their client specificity. However, the specificity of the possible dimer combinations is still poorly understood.
To date, more than 150 binding partners of 14-3-3 proteins have been found and the number is still rising [170]. Interaction of ligands occurs primarily within the binding cleft presented by each monomer (see figure 5). As a consequence, each dimer can interact with two ligands simultaneously. This association is guided by defined peptide motifs with the consensus sequences RSXpSXP (mode-1) and RXXXpSXP (mode-2, pS means phosphorylated serine) in the client peptide sequence. Of note, also binding to non-phosphorylated peptide sequences has been observed (e.g. LDL) but is rather uncommon [171].

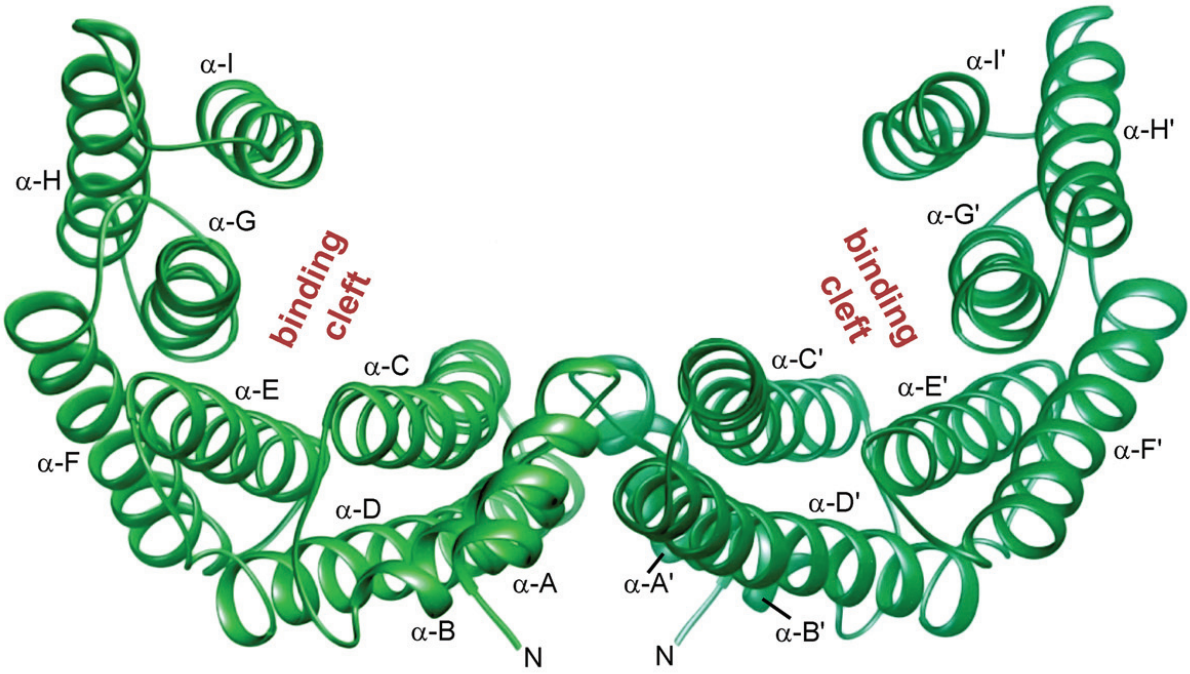

Figure 5: Crystal structure of the 14-3-3 ζ homodimer. Nine anti-parallel α-helices form one monomeric subunit. The dimer interface consists of residues within the first four helices (α-A to α-D). Roof and wall of the binding cleft is comprised by residues in helices H and E. The approximate dimension of the central channel is 35Å x 35Å x 20Å (broad, wide and deep) ([165], structure modified from [172]).

Instead of classifying 14-3-3 proteins in the context of their targets, Bridges and Moorhead suggest another approach for classification related to three diverse modes of action (see figure 6 and [173]). One possible effect of 14-3-3 binding is to induce a reshaping of the

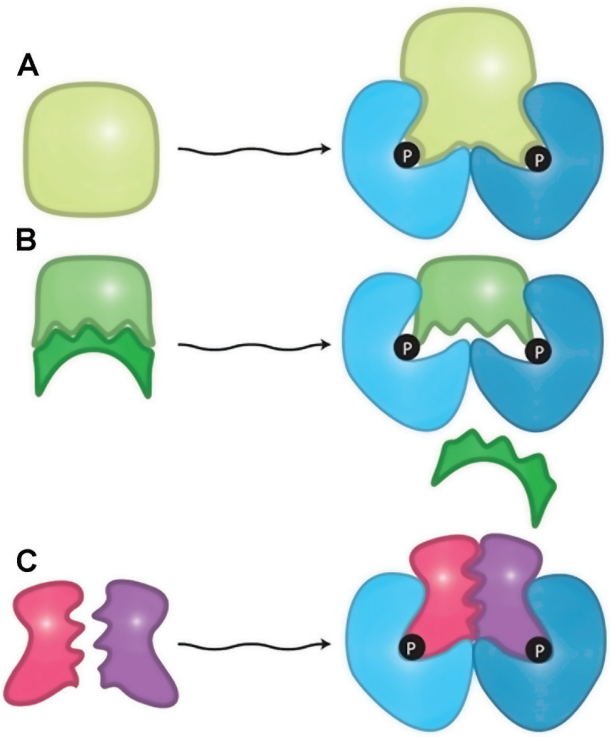

Figure 6: Effects of 14-3-3 binding on target structure. 14-3-3 monomers are shown in different shades of blue. (A) Induction of a conformational change, at binding site or at distant ligand sites. (B) Masking of interaction sites within the target. (C) Cross-linking of two proteins via simultaneous 14-3-3 interaction. (modified from [173])

ligand structure (Fig. 6A). This "molecular anvil hypothesis", described by Michael Yaffe [169], not only allows structural rearrangements at the 14-3-3/ligand interaction site but may also result in alteration of distant steric arrangements within the ligand. Another, more spatially restricted mode of action would be the blocking or direct occlusion of important features in the ligand (Fig. 6B). This may be especially important for phosphorylation regulated enzymes such as RAF that in case of 14-3-3 interaction could not be dephosphorylated at relevant sites. The third 14-3-3 effect involves two separate proteins that are linked through simultaneous binding by 14-3-3 (Fig. 6C). A more general outcome may be the translocation of target proteins from membranes into the cytosol by sequestering through 14-3-3.

14-3-3 proteins are known to interact with a large number of signaling proteins including Cdc25 phosphatases, PKC, RAF kinases, KSR, and BAD protein [174]. In plants regulatory interactions with ion-channels are also known [175].

All RAF kinases possess two mode-1 14-3-3 binding sites surrounding serines 214/582, 365/729, and 259/621 in A-, B-, and C-RAF, respectively. While the C-terminal 14-3-3 protein binding sequence is identical in all mammalian RAFs (RSApSEP), the motif surrounding serine 365 in B-RAF (RSSpSAP) differs from the corresponding sites in A- and C-RAF (RSTpSTP). A third 14-3-3 binding site surrounding serine 233 (RYpSTP) in C-RAF has also been reported [176]. Further, a rather atypical 14-3-3 binding site (RTK) at the C-RAF-CRD has been proposed [114]. However, involvement of these two sites in RAF

regulation is not completely understood.

1.2.9 Current Model of RAF Activation

The precise course of events from the inactive RAF in the cytosol to the phosphorylation of MEK is still controversial, not to speak about the mechanisms of inactivation. A large number of proteins ranging from kinases and phosphatases to chaperones and scaffolds, as well as lipid interaction and correct localization have to act in concert (see figure 7). Although direct experimental support is missing, the regulatory part at the N-terminus of RAF may interact in the unstimulated cell with the catalytic domain. This gives rise to a sandwich-like, inactive conformation further stabilized by association with 14-3-3 proteins [177]. Rapp *et al*. [25] suggested that 14-3-3 proteins are necessary for the stabilization of inactive and growth factor-mediated active conformations of RAF. The association of RAF with plasma membrane microdomains called “rafts” is the initial step in the activation process [45]. Due to its inherent lipid binding properties governed in part by the C-terminal PA-binding site, a pool of inactive RAF may also exist already localized at the plasma membrane [25,45]. The attachment to the membrane is further promoted by the high affinity interaction of the RAF-RBD with GTP-loaded and thereby activated Ras [46]. This complex then migrates to the non-rafts microdomains [178]. In contrast to previous views, binding of PHB but not Ras is responsible for the displacement of 14-3-3 from the internal binding site on RAF [122,123]. As a consequence, phosphatases get access to the inhibitory phospho-serine 259 [58,179]. It is controversially discussed whether the semi-occupied 14-3-3 dimer binds to another yet unknown motif in the C-terminal part of RAF for stabilization of the active conformation or cross-links the scaffold KSR, thereby guiding the signaling complex [53,136]. Recently, the group of Therrien described the allosteric activation of RAF by interaction of KSR via the N-lobe of the kinase domains [145]. This interaction not only directly activates C-RAF but can also provide easy access to its substrate MEK that comes constitutively bound with KSR. Another possibility is the formation of a RAF-heterodimer as already suggested by our group. This option is supported by its dependence of the C-terminal 14-3-3 binding site surrounding pS621 of C-RAF [180] and pS729 of B-RAF [72]. As a result, the possibility of cross-activation by phosphorylation from B- to C-RAF arises [181]. For full activation of C-RAF subsequent phosphorylation of serine 338 and tyrosine 341 is necessary. With KSR and its constitutive binding partner MEK located in close vicinity to RAF the signaling road is paved for downstream signal propagation.

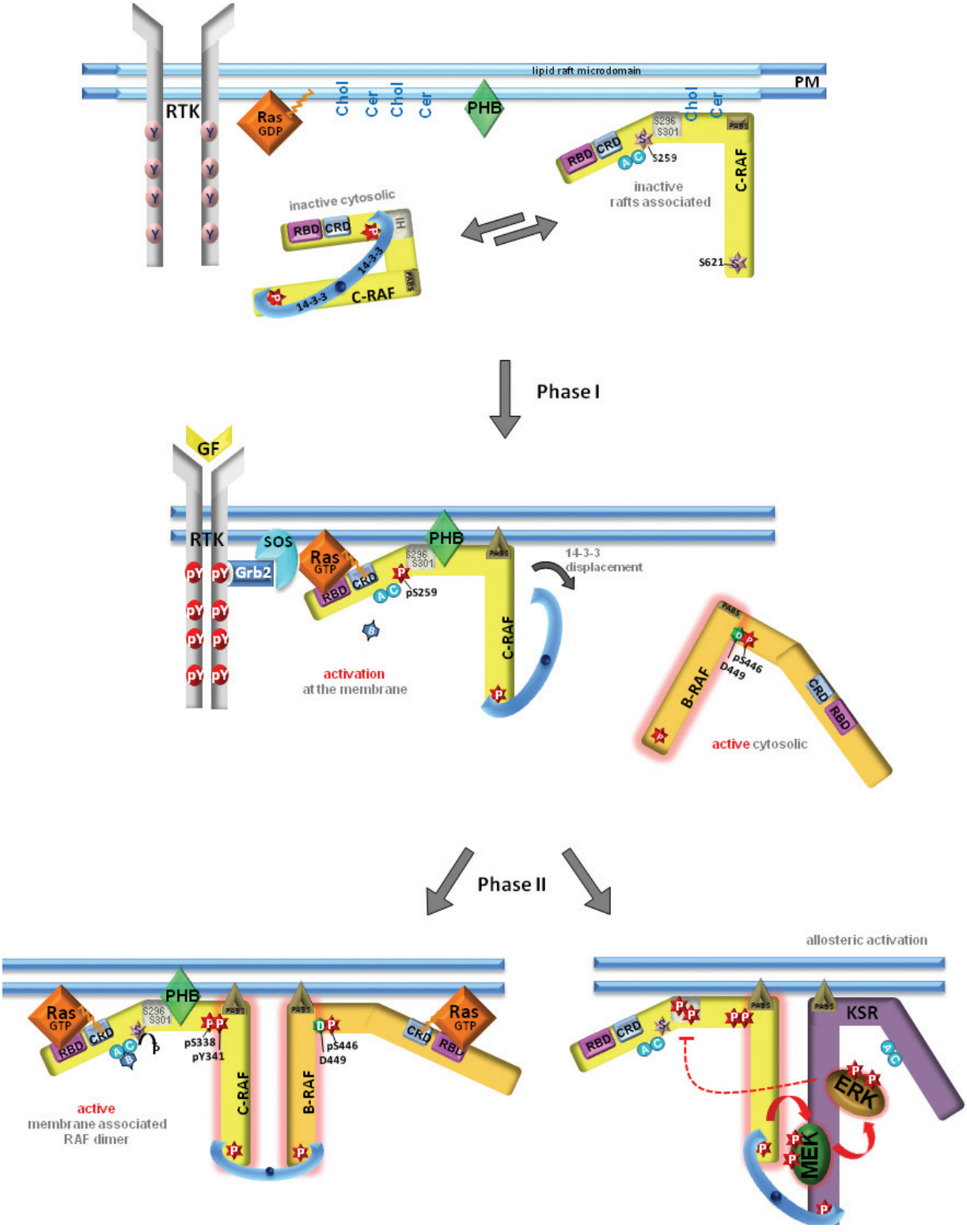

Figure 7: Simplified Model for the activation mechanism of RAF. Locked cytosolic C-RAF is recruited to the plasma membrane (PM) by Ras-GTP followed by PHB mediated displacement of 14-3-3 from the internal binding site. Since B-RAF may already be activated in the cytosol, C-RAF requires further phosphorylation and dimerization with B-RAF and/or KSR. For details see 1.2.9.

2. Scientific work

2.1 *B- and C-RAF display essential differences in their binding to Ras: The Isotype Specific N-terminus of B-RAF Facilitates Ras Binding.*

Andreas Fischer, Mirko Hekman, Jürgen Kuhlmann, Ignacio Rubio, Stefan Wiese, and Ulf R. Rapp.

Journal of Biological Chemistry. 2007 Sep 7;282(36):26503-16. Epub 2007 Jul 16. doi:10.1074/jbc.M607458200

2.2 *Regulation of RAF activity by 14-3-3 proteins: RAF kinases associate functionally with both homo- and heterodimeric forms of 14-3-3 proteins.*

Andreas Fischer, Angela Baljuls, Jörg Reinders, Elena Nekhoroshkova, Claudia Sibilski, Renate Metz, Stefan Albert, Krishnaraj Rajalingam, Mirko Hekman, and Ulf R. Rapp.

Journal of Biological Chemistry. 2009 Jan 30;284(5):3183-94. Epub 2008 Dec 2. doi:10.1074/jbc.M804795200

2.3 *Novel C-Raf phosphorylation sites: serine 296 and 301 participate in Raf regulation.*

Mirko Hekman, **Andreas Fischer**, Lawrence P. Wennogle, Y. Karen Wang, Sharon L. Campbell, and Ulf R. Rapp

FEBS Letters. 2005 Jan 17;579(2):464-8. Epub 2004 Dec 18. doi:10.1016/j.febslet.2004.11.105

3. Discussion

The complexity of RAF kinase signaling and especially RAF kinase activation is best reflected by the fact that more than 25 year since their first description passed and there are still enough open questions for many PhD theses like this one.

The most prevalent method for the regulation of cell signaling is phosphorylation, a mechanism already known for almost 60 years [182]. The discovery that tyrosine phosphorylation of proteins by a Src kinase is implicated in transformation and oncogenesis push-started this research area in the 1970s [183,184,185]. In this context it has to be mentioned, that protein kinases and phosphatases are not merely counteracting each other on the same target. In fact, instead of simply assuming a kinase-"on" – phosphatase-"off" cycle, their performance can also have inverted or cooperative effects. Best example in the context of this work is the outcome of Ser259 phosphorylation and dephosphorylation in C-RAF. The activation cycle of RAF includes the alteration of its conformation from inactive to active. This event is mediated by 14-3-3 proteins binding to two phosphorylated motifs surrounding serine 259 and 621. The inactive conformation is stabilized by 14-3-3 interacting with both sites, whereas the active form of C-RAF is dependent on the release of 14-3-3 from the internal S259 site [25]. In this case the phosphatase ensures that a reassociation of 14-3-3 is not possible.

Phosphorylation and dephosphorylation events require the contact of the enzyme with its substrate. Moreover, phosphorylated residues can also be the basis for protein-protein interaction sites. This mechanism was first described for SH2 domains binding to phosphorylated tyrosine motifs [186,187]. The work at hand may also serve as an excellent example for phosphorylation dependent protein-protein interaction regarding the above mentioned association of 14-3-3 proteins with phosphorylated RAF sites.

The broad range of signaling effects emerging from an active Ras-RAF cascade calls for an efficient control of substrate specificity, signal strength, and shutdown mechanisms. This is partially achieved by defined protein-protein interactions and formation of signaling complexes in a spatial and temporal manner [188,189]. For example, C-RAF interacts *in vivo* with more than 40 proteins in a direct or at least functional manner (see Fig. 1). Some of them are kinases or phosphatases for direct activation (PKA, PP2A) others provide a platform for bringing the substrate into position (KSR). With this vast amount of involved proteins in mind it is obvious that only the slightest misadjustment may lead to severe effects that end up in a disease state.

Two of the most important regulators of RAF kinase activity, Ras and 14-3-3 proteins, are the main subject of this work. Attention was focused on the binding specificity and the quantification of affinities using various experimental methods.

3.1 Ras and RAF – Tying up for Activation

The interaction of Ras proteins with isolated RAF-RBDs has already been reported, however, without taking into account the farnesylation of Ras [190,191,192]. In addition, the binding properties of full length RAF towards Ras are not known so far and may differ drastically from the isolated RBD.

Unfortunately, the isolation of natively lipidated H-Ras from cells does not yield sufficient material and is accompanied by a loss of lipid residues. Collaboration with the group of Jürgen Kuhlmann (MPI Dortmund, Germany) provided us access to recombinant H-Ras protein preparations that were lipidated *in vitro* by Farnesyltransferase and subsequently loaded with GTP to mimic the activated state. These preparations exhibited properties of native proteins and were still capable of transforming PC12 cells [116]. Using the surface plasmon resonance (SPR) method, the association and dissociation constants for interaction of purified native H-Ras with full length RAF kinases (purified from Sf9 insect cells) were determined. While the isolated RBD fragments did not discriminate between farnesylated and nonfarnesylated Ras, full length RAF proteins demonstrated differences in binding affinities (see chapter 2.1, table 1). In summary, C-RAF exclusively bound to farnesylated and activated H-Ras-GTP with a KD-value of 460 nM. Compared to B-RAF (KD ≈ 60nM), this is an almost 8 times weaker affinity but still in a meaningful range considering the cellular environment. Furthermore, C-RAF-R/L that was activated *in vivo* by coexpressing constitutively active H-Ras-G12V and Lck-T505F, bound with a markedly reduced affinity (KD = 860 nM) to farnesylated Ras. This observation is in accordance with another study where we showed an increase in phosphorylation of C-RAF-Y340D/Y341D at MAP kinase sites (see chapter 2.3, table 1). These sites have been shown to render the activated RAF resistant to Ras interaction [154].

Interestingly, B-RAF also bound to nonfarnesylated Ras with high affinity (approx. 80 nM). To test, whether the reason for this difference compared to C-RAF lies within the elongated N-terminus of B-RAF (see sequence alignment in chapter 2.1, Fig. 5), a truncated form of B-RAF was tested in the same experimental setup. The shorter version called B-RAF-ΔN98 indeed displayed a 3-fold reduced affinity towards nonfarnesylated Ras, indicating a

regulatory role for the first 98 amino acids of B-RAF. Furthermore, deletion of this fragment also rendered B-RAF sensitive for activation by EGF in a cell culture system (chapter 2.1, Fig. 6A).

So far, no particular function of the first 98 amino acids of B-RAF has been described. However, these results imply that the extreme N-terminus of B-RAF governs the accessibility to Ras and may be jointly responsible for the extraordinarily high basal kinase activity *in vitro*. In contrast to another study [193], we did not detect any influence of the B-RAF N-terminus on heterodimerization with C-RAF (chapter 2.1, Fig. 6B, and [180,181]). This is consistent with results from Rushworth *et al*. who demonstrated that increased amounts of 14-3-3 proteins enhance RAF heterodimerization. Furthermore, this study shows that the C-terminal part of B-RAF is responsible for heterodimerization since the phosphorylation of T753 in B-RAF promoted the disassembly of heterodimers with C-RAF [73].

Another possible explanation for this unexpected Ras binding behavior of B-RAF would be the influence of the N-terminus on 14-3-3 interaction. B- and C-RAF share two conserved 14-3-3 binding sites surrounding the phosphorylated serines at positions 365/729 (B-RAF) and 259/621 (C-RAF). However, the internal motif in B-RAF (RSSpSAP) differs from that in C-RAF (RSTpSTP) and therefore alters the binding affinity towards 14-3-3 proteins, as was determined by a peptide inhibition assay (see chapter 2.2, Fig. 3). Thus, the modified internal 14-3-3 binding motif together with the extended N-terminus of B-RAF may cause an open conformation, accessible for incomplete processed Ras located in the cytosol and stabilized by 14-3-3 binding.

In contrast, the closed C-RAF structure with weak kinase activity (as proposed in [45] and [25]) would have to be freed from 14-3-3 for Ras interaction at the plasma membrane. This could be achieved by interaction of C-RAF with the plasma membrane where prohibitin displaces 14-3-3 from the internal binding site. After a subsequent conformational change, the CRD of C-RAF is available for the farnesyl group as well as plasma membrane lipids and guides Ras-GTP interaction with the RBD (see introductory figure 7).

The fact that B-RAF can interact with unprocessed H-Ras in the cytosol could be further supported by *in vivo* imaging analysis (chapter 2.1, Fig. 8). Whereas C-RAF predominantly localized to the plasma membrane, B-RAF demonstrated pronounced colocalization with an active Ras mutant (H-Ras-G12V/C186S) that cannot be farnesylated and thereby resides in the cytosol. To determine the physiological consequence, the potential of Δ-N-B-RAF to transform PC12 cells was determined by measuring the length of neurite outgrowth of PC12 cells after stimulation with NGF. This experiment revealed an unchanged

transformation capacity of B-RAF in combination with the wild typic Ras mutant compared to the cytosolic Ras mutant C186S. C-RAF, in contrast, could not be activated in the cytosol by the nonfarnesylated H-Ras-G12V/C186S.

These data demonstrate that farnesylation of Ras proteins may not be necessary for effective association with B-RAF and induction of its activation. The possibility of B-RAF getting primed for activation by newly synthesized or incomplete lipidated Ras in the cytosol remains elusive. However, it is well possible that oncogenic and lipid binding deficient B-RAF mutants [49,68,70,71] remain constitutively active due to uncontrolled association with unprocessed Ras proteins. Some members of the large Ras protein family have been described that lack lipid modifications, e.g. Rit1/Rit2, Rap1, and RASL11/RASL12. Interestingly, the Rit family also reveals similar effector binding surfaces compared to H-, N- and K-Ras. Additionally, they are required for neurite outgrowth in PC12 cells and stimulate initiation, elongation via B-RAF [96,194].

Taken together, these data support the current RAF activation model shown in the introductory figure 7 and suggest a difference in complex formation for B- and C-RAF with Ras and 14-3-3 proteins. This difference is due to an extended N-terminus in B-RAF that not only governs the accessibility towards Ras but also may in part be responsible for the high basal activity of cytosolic B-RAF. The influence of 14-3-3 binding on conformational changes in RAF can of course not definitely be answered without knowing the precise crystal structure of RAF or, in the best case, the structure of a co-crystal with a bound 14-3-3 dimer.

3.2 14-3-3 proteins – Adaptors for the (conformational) change we need

Due to their ubiquitous expression in eukaryotic cells and their known involvement in processes ranging from proliferation to apoptosis, 14-3-3 proteins are key regulators of cellular signaling [159]. In 1995, the participation of 14-3-3 proteins in RAF activation has been described before the exact binding motifs were identified two years later [41,195]. Since then, the research on RAF and 14-3-3 proteins revealed more binding partners and details on the mechanisms of complex formation. However, studies on isoform specificity between RAF and 14-3-3 proteins were restricted to single RAFs or 14-3-3 isoforms and no quantitative data on affinities were available.

Using mass spectrometry (MS) analysis we first identified 14-3-3 proteins associated with RAF in HEK 293 cells. To our surprise, the three RAF isoforms displayed differences

in the composition of associated 14-3-3 proteins. While A-RAF only precipitated with the τ and ε isoform, C-RAF was bound to ζ and ε (see chapter 2.2, Fig. 1A). In contrast, B-RAF was associated with all seven mammalian 14-3-3 isoforms, except σ. This could be due to the fact that the σ isoform exclusively exists as a homodimer and may thereby be restricted to a smaller number of binding partners [168]. Furthermore, 14-3-3 σ exhibits a rather restricted expression pattern compared to the other six isoforms (http://www.proteinatlas.org/index.php). Strikingly, the ε isoform was present in all three samples, which reflects its exceptional status as the one isoform that cannot form homodimers, but is able to dimerize with all other isoforms (except σ). These results are consistent with the finding that all three RAF isoforms perform their function in different subcellular locations and for which they require differential regulatory mechanisms [26,46,48].

The biosensor measurements using the Biacore setup revealed more detailed information about specific interaction preferences. For this assay, homodimers of all seven mammalian 14-3-3 isoforms were purified as GST fusion proteins and their specificity towards A-, B-, and C-RAF was determined. Of note, the ε isoform also purified as a homodimer. This was confirmed by determining the native elution size by exclusion chromatography. The lack of suitable binding partners presumably forced the monomers to dimerization since the monomeric 14-3-3 proteins are thermodynamically unstable [167]. While B-RAF exhibited good binding values with all seven isoforms, C-RAF already displayed decreased affinity especially to the σ, η, and τ homodimers. Considering the MS data, this implies that C-RAF prefers a ζ homodimer or a ζ/ε heterodimer, at least in our HEK 293 system. The possibility that two 14-3-3 isoforms are sufficient is also known from other organisms like *Drosophila* or *Caenorhabditis*. With the MS data in mind, the biosensor results for A-RAF are of particular interest. Neither the ε nor the τ homodimer showed significant binding to A-RAF on the Biacore chip. For the *in vivo* data, where only those two isoforms could be detected this suggests, that only a heterodimer consisting of τ and ε was able to complex with A-RAF in HEK 293 cells.

The pseudokinase KSR was also shown to interact with a larger number of 14-3-3 isoforms [179]. Predominant cytosolic localization of B-RAF and KSR in quiescent cells may be one reason for this observation [143]. Different 14-3-3 dimers associated with its clients like KSR and RAF could perform the diverse functions described in 1.2.8. The necessity of 14-3-3 binding for RAF activity is apparent from an *in vitro* kinase assay. The mutation of the C-terminal binding motif in B- and C-RAF and the resulting loss of 14-3-3 interaction led to a complete loss of activity (see chapter 2.2, Fig. 4A and B, as well as [54]). It is very likely, that this function as well as the role of 14-3-3 as a cross-linker for the dimerization of B-

and C-RAF [180] and the complex formation of RAF with KSR [196] requires a different set of 14-3-3 proteins.

The question if one single isoform could be sufficient for activation of RAF *in vivo* was addressed by using the budding yeast as a test system. *Saccharomyces cerevisiae*, possesses only two 14-3-3 isoforms, designated BMH1 and 2. The knock-out of the *bmh2* gene and the residual BMH1 protein still resulted in perfectly active B- and C-RAF (see chapter 2.2, Fig. 2). This approach can only serve as a proof of principle since in the mammalian situation a knock-down of all but one isoform may interfere with essential cellular processes.

A differential influence of 14-3-3 binding to the internal and C-terminal motif on RAF activation had been described by this laboratory and could be confirmed in this study (chapter 2.2, Fig. 4 and [54]). Briefly, substitution of the serine at the internal 14-3-3 binding site with alanine (C-RAF-S259A and B-RAF-S365A) leads to a pronounced increase in RAF activity, whereas the serine to alanine exchange at position 621 in C-RAF and 729 in B-RAF completely abolishes kinase activity. However, the analogous A-RAF mutant displayed an inverted activity pattern. In this case, A-RAF-S214A revealed a reduction of activity, whereas the C-terminal mutant displayed activity on the same level as the wild typic A-RAF. Recently, our group was able to show that the activation mechanism for A-RAF differs considerably from B- and C-RAF. Especially distinct phosphorylations within the N-region and in the newly defined IH-region seem to play a leading role in this unique characteristic [40,197].

A critical role of 14-3-3 binding for RAF activation is further supported by two recent studies [198,199] that described activating C-RAF mutations in the vicinity of the internal binding motif surrounding pS259. As the reason for this pathological effect lies in an impaired 14-3-3 binding to C-RAF, these data support our model of an 14-3-3-stabilized inactive C-RAF that requires the release of 14-3-3 from the internal site for activation (see model in Fig. 7 and [25]).

In contrast, regulation of kinase activity by 14-3-3 in the cancer-associated B-RAF-V600E mutant seems to be lost. This hyperactive form of B-RAF can be found in approx. 70% of all human malignant melanoma [68]. An exchange of serines 365 and 729 to alanine in this V600E background neither led to an increase nor a decrease in kinase activity, respectively, indicating the independence from any regulatory mechanism by interacting with 14-3-3 proteins. Very recently, this hypothesis was reconfirmed by Ritt *et al*. [72]. A similar effect could be observed by expressing B-RAF in yeast (chapter 2.2, Fig. 2). A possible explanation may be a threonine phosphorylation at position 599 within the activation loop by an unknown kinase that may induce the active conformation of the

V600E mutant [49].

The activating effect that mutation of the internal 14-3-3 binding motif has is consistent with the proposed mechanism where the membrane localized prohibitin is supposed to directly displace 14-3-3 during activation of C-RAF. To further test this hypothesis, an *in vitro* competition assay based on the SPR methodology was performed. In this approach, PHB proved capable of interfering with 14-3-3 binding to the internal motif in C-RAF (see chapter 2.2, Fig. 5A). Furthermore, this effect exhibited a pronounced 14-3-3 isoform specificity. While the binding of 14-3-3 isoforms σ and ε to C-RAF was completely inhibited, interaction with τ was only weakly reduced but η could barely be blocked by PHB. This result is quite surprising since the η and also τ isoform showed only weak affinity to C-RAF compared to β, ζ, and γ (Fig. 1B, chapter 2.2). This effect may be due to a possible preference of 14-3-3 isoforms η and τ towards the C-terminal 14-3-3 interaction motif that may still be accessible during PHB binding next to the internal site.

Taken together, these results support the working model for C-RAF activation where PHB is responsible for the displacement of 14-3-3 from the inhibitory internal binding motif, thus contributing to the activation of RAF. Furthermore, the influence of 14-3-3 proteins on RAF activation is isoform-specific. The presence of seven isoforms in mammalian cells and up to 21 different possibilities for dimer formation enables a broad variety of effects by 14-3-3 interaction throughout the organism. Our results suggest that these slightly different dimers carry out distinct functions and display certain substrate specificity.

3.3 Fine tuning by feedback

After the first activating steps at the membrane through interaction with Ras and PHB, several phosphorylations are necessary to get RAF proteins fully activated [25,38,60,80]. However, a quantitative analysis of the degree of phosphorylation in C-RAF has not been carried out so far. As part of this work, comparison of the phosphorylation status of C-RAF wt and the constitutively active mutant C-RAF-Y340D/Y341D (C-RAF-DD) was performed using mass spectrometry.

While some of the described phosphorylation sites (T268, T269, S338, T491, S494) could not be found, we detected an identical degree of phosphorylation for the two 14-3-3 binding motifs (S259 and S621) in wild type and mutationally activated C-RAF. Most intriguingly, two unknown phosphorylation sites were observed, namely serine 296 and 301. These sites reside in close vicinity to the CR2 in RAF where the internal 14-3-

3 binding motif is located. Interestingly, the degree of phosphorylation differs for both sites if compared between C-RAF wild type and C-RAF-DD. While phosphorylation of serine 296 moderately increases from 55% to 65% in the activated state, an almost 3-fold increase can be observed for serine 301 (15% to 40%). Both positions resemble putative MAPK targeting sequences as the phosphorylated serine is followed by a proline residue. While the SP site at position 296 from C-RAF is conserved in A-RAF (S257), the second site in A-RAF (S262) lacks the proline residue following after serine. In B-RAF, these sites are not conserved but a C-terminal sequence SPKTP (aa750-754) has been described as a site for negative feedback phosphorylation mediated by ERK [200]. This sequence is exclusively found in B-RAF.

Our group recently defined several MAPK feedback sites in the variable region between CR2 and the kinase domain of A-RAF that positively regulate A-RAF kinase activity. Phosphorylation in this so-called IH-Region of A-RAF was suggested to induce a charge switch and thereby enables dissociation of A-RAF from the plasma membrane [40].

To test, whether the newly identified phosphorylation sites in C-RAF also display any influence on activity, we mutated these residues and determined the enzymatic activity in a kinase assay. Contrary to the results obtained from A-RAF, the substitution of serines in position 296 and 301 of C-RAF with alanine did not inhibit but further increased kinase activity in both unstimulated and EGF treated cells (see chapter 2.3, Fig. 4). At the same time, the group of Deborah Morrison also described phosphorylation sites in C-RAF that were directly targeted by activated ERK and negatively regulate C-RAF activity [154]. Guri Tzivion's group also examined these ERK feedback phosphorylation sites, however, reported a positive effect on RAF activity [201]. The study of Dougherty and Morrison newly identified a total of five feedback sites, S29, S289, S296, S301, and S642. However, since they only investigated differences between the wild typic version of C-RAF and the combination of all five sites mutated to alanine, the exact linkage of single sites to ERK as responsible kinase was not possible. Furthermore, a direct impact of hyperphosphorylation of C-RAF on its properties to interact with Ras could be determined. Additionally, Dougherty *et al*. [154] were able to show the involvement of the phosphatase PP2A in the recycling of this hyperactivated C-RAF. These results are in accordance with previous findings that identified PP2A as being responsible for dephosphorylation of KSR and C-RAF [179]. The described recycling mechanism for feedback phosphorylated and thereby attenuated C-RAF is especially interesting considering the delicate position of these sites within the C-RAF peptide sequence. In close vicinity of serines 289, 296 and 301 a positive regulator for C-RAF activity, prohibitin, has been shown to bind [118]. As

described previously, PHB displaces 14-3-3 from C-RAF during its activation at the plasma membrane. After signal propagation along the RAF cascade to MEK and ERK, activated ERK would phosphorylate these feedback sites. This feedback phosphorylation then enables binding of PIN-1. The prolyl isomerase PIN-1 isomerizes pSP and pTP bonds [202] similar to its role in PP2A mediated dephosphorylation of other proteins such as Myc and Cdc25C [203,204]. PIN-1 only binds to hyperphosphorylated RAF and prepares the feedback sites for dephosphorylation by PP2A [154]. This step is important for rendering RAF ready for another activation cycle. A model for the RAF activation cycle, including these data can be found in figure 7 of the introduction.

Most recently, the group of Morrison described a similar mechanism of ERK-mediated feedback phosphorylation on B-RAF [72]. They were able to attribute the inhibited binding to activated Ras and disrupted heterodimerization with C-RAF to the phosphorylation of four S/TP sites by activated ERK. Like in the case of C-RAF, dephosphorylation of these sites requires PP2A and PIN-1 activity and restores B-RAF for the next round of activation.

4. References

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5. Appendix

5.1 Abbreviations

aa	amino acid
ATP	Adenosine-5'-triphosphate
BMH1, 2	brain modulosignalin homolog 1, 2
cAMP	cyclic Adenosine monophosphate
CFC	cardio-facio-cutaneous
CNK	Connector-enhancer of KSR
CR1, 2, 3	conserved region 1, 2, 3
CRD	cysteine rich domain
C-terminal	carboxy-terminal
Da	Dalton
DNA	Deoxyribonucleic acid
E. coli	*Escherichia coli*
e. g.	exempli gratia („for example“)
EGF	epidermal growth factor
EGFR	Epidermal growth factor receptor
ER	Endoplasmic reticulum
ERK	extracellular signal-regulated kinase
et al.	et alii ("and others")
-farn	farnesylated
GAP	GTPase-activating proteins
GDI	guanosine nucleotide dissociation inhibitors
GDP	guanosine diphosphate
GEF	guanine nucleotide exchange factor
Grb2	growth factor receptor-bound protein 2
GTP	guanosine triphosphate
HVR	hypervariable region
i.e.	id est ("that is")
IH-region	isoform-specific hinge region
JNK	c-Jun N-terminal kinases
k_a	association constant
k_d	dissociation constant
KD	affinity constant (k_d/k_a)
kDa	kilodalton
KSR	kinase suppressor of Ras
Lck	leukocyte-specific protein tyrosine kinase
MAPK	mitogen-activated protein kinase
MAP2K	MAPK kinase
MAP3K	MAPK kinase kinase
MEK	mitogen-activated protein kinase kinase
MEKK	MAPK/ERK activating kinase kinase
MP1	MEK-partner 1
MS	mass spectrometry
N-terminal	amino-terminal
N-region	negative-charge regulatory region

NES	nuclear-export sequences
NLS	nuclear-localization sequences
PA	phosphatidic acid
PAK	p21-activated kinases
PC12	Rat pheochromocytoma cell
PHB	prohibitin
PI3K	phosphatidylinositol 3-kinase
PKA	protein kinase A
PKB	protein kinase B
PKC	protein kinase C
PMA	phorbol 12-myristate 13-acetate
PP2A	protein phosphatase 2A
PS	phosphatidyl serine
pS	phosphoserine (pSer)
pT	phosphothreonine (pThr)
PTK	protein tyrosine kinase
pY	phosphotyrosine (pTyr)
RAF	rapidly growing fibrosarcoma
RalGDS	Ral guanine nucleotide dissociation stimulator
Ras	Rat sarcoma
RBD	Ras binding domain
Rsk	ribosomal S6 kinase
RTK	receptor tyrosine kinase
Sf9	*Spodoptera frugiperda* insect cell line, clone 9
SH2	Src homology 2
siRNA	short interfering RNA
SOS	son of sevenless
SPR	surface plasmon resonance
Src	sarcoma
SUR-8	suppressor of Ras-8
vs.	versus
wt	wild type

5.2 List of Publications

(1) Polzien L, Baljuls A, Rennefahrt UE, **Fischer A**, Schmitz W, Zahedi RP, Sickmann A, Metz R, Albert S, Benz R, Hekman M, Rapp UR. *Identification of novel in vivo phosphorylation sites of the human pro-apoptotic protein bad: pore-forming activity of bad is regulated by phosphorylation*. J Biol Chem. 2009 Aug 10. [Epub ahead of print]

(2) **Fischer A**, Baljuls A, Reinders J, Nekhoroshkova E, Sibilski C, Metz R, Albert S, Rajalingam K, Hekman M, Rapp UR. *Regulation of RAF Activity by 14-3-3 Proteins - RAF Kinases Associate Functionally with both Homo- and Heterodimeric Forms of 14-3-3 Proteins*. J Biol Chem. 2009 Jan 30;284(5):3183-94

(3) Fueller J, Becker M, Sienerth A, **Fischer A**, Hotz C, Rapp UR, Galmiche A. *C-RAF Activation promotes BAD Poly-Ubiquitylation and Turnover by the Proteasome.* BBRC 2008 Jun 13;370(4):552-6.

(4) Latz A, Becker D, Hekman M, Muller T, Beyhl D, Marten I, Eing C, **Fischer A**, Dunkel M, Bertl A, Rapp UR, Hedrich R. *TPK1, a Ca(2+)-regulated Arabidopsis vacuole two-pore K(+) channel is activated by 14-3-3 proteins.* Plant J. 2007 Nov;52(3):449-59.

(5) **Fischer A**, Hekman M, Kuhlmann J, Rubio I, Wiese S, Rapp UR. *B- and C-RAF Display Essential Differences in Their Binding to Ras: The Isotype-Specific N Terminus of B-RAF Facilitates Ras Binding.* J Biol Chem. 2007 Sep 7;282(36):26503-26516.

(6) Rapp UR, **Fischer A**, Rennefahrt UE, Hekman M, Albert S. *BAD association with membranes is regulated by Raf kinases and association with 14-3-3 proteins.* Adv Enzyme Regul. 2007;47:281- 5.

(7) Hekman M, Albert S, Galmiche A, Rennefahrt UE, Fueller J, **Fischer A**, Puehringer D, Wiese S, Rapp UR. *Reversible membrane interaction of BAD requires two Cterminal lipid binding domains in conjunction with 14-3-3 protein binding.* J Biol Chem. 2006 Jun 23;281(25):17321- 36.

(8) Hekman M, **Fischer A**, Wennogle LP, Wang YK, Campbell SL, Rapp UR. *Novel C-Raf phosphorylation sites: serine 296 and 301 participate in Raf regulation.* FEBS Lett. 2005 Jan 17;579(2):464-8.

5.3 Poster Abstracts

(1) **Fischer A**, Hekman M, Reinders J, Nekhoroshkova E, Sibilski C, and Rapp URR. *Regulation of RAF Activity by 14-3-3 Proteins.*
EMBO workshop – "Can epigenetics influence reprogramming & metastatic progression?"
2008, October 6 – 9, Banz Monastery, Germany

(2) **Fischer A**, Hekman M, Reinders J, Nekhoroshkova E, Albert S, and Rapp UR. *Regulation of RAF Activity by 14-3-3 Proteins*.
Gordon Research Conference – "Biology of 14-3-3 Proteins"
2008, February 27 – 29, Ventura, CA, USA

(3) Hekman M, **Fischer A**, Rubio I, Wiese S, Kuhlmann J, Baljuls A, and Rapp UR. *Regulation of RAF Kinases by Ras and 14-3-3 Proteins.*
International Symposium of the SFB 487: Membrane Proteins and Diseases
2007, June 7 - 9, Würzburg, Germany

(4) Albert S, Hekman M, Galmiche A, Rennefahrt UEE, Fueller J, **Fischer A**, Wiese S, and Rapp URR. *The Role of 14-3-3 Proteins in membrane association of proapoptotic BAD Protein.*
Gordon Research Conference – "Biology of 14-3-3 Proteins"
2006, February 27 – 29, Ventura, CA, USA

(5) Robubi A, Hekman M, **Fischer A**, Albert S, Rapp UR, and Wiese S. *Regulation of Raf protein kinases by 14-3-3 proteins and KSR.*
ELSO meeting 2004, Sep 4 – 8 2004, Nice, France.

(6) Hekman M, **Fischer A**, Wiese S, Albert S, and Rapp URR. *Regulation of Raf and KSR Signalling by Phosphorylation, 14-3-3 Proteins and Lipid Associations.*
EMBO meeting – "Ras-dependent pathways in human cancer"
2004, Nov 28 – Dec 1, Banz Monastery, Germany

5.4 Erklärung

(gem. § 4 Abs. 3 S. 3, 5 und 8 der Promotionsordnung)

(1) Hiermit erkläre ich an Eides statt, dass ich die vorliegende Dissertation selbständig angefertigt und keine anderen als die von mir angegebenen Quellen und Hilfsmittel benutzt habe.

(2) Ich erkläre ausserdem, dass diese Dissertation weder in gleicher noch in anderer Form bereits in einem anderen Prüfungsverfahren vorgelegen hat.

(3) Ich habe früher ausser den mit dem Zulassungsgesuch urkundlich vorgelegten Graden keine weiteren akademischen Grade erworben oder zu erwerben versucht.

Petershausen, den 14.01.2010 (Andreas Fischer)