Analysis of the binding properties of the kinase C-RAF to mitochondria and characterisation of its effects on the cellular and molecular level

Untersuchung der Bindungseigenschaften der Kinase C-RAF an Mitochondrien und Charakterisierung der Effekte auf zellulärer und molekulare Ebene

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Contents

I dedicate this work to my parents

Summary

The proteins of the RAF family (A-RAF, B-RAF, and C-RAF) are serine/threonine-kinases that play important roles in development, mature cell regulation and cancer. Although it is widely held that their localization on membranes is an important aspect of their function, there are few data addressing this aspect of their mode of action. Here, we report that each member of the RAF family exhibits a specific distribution at the level of cellular membranes, and that C-RAF is the only isoform that directly targets mitochondria. We find that the RAF kinases exhibit intrinsic differences in terms of mitochondrial affinity, and that C-RAF is the only isoform that binds this organelle efficiently. This affinity is conferred by the C-RAF amino-terminal domain, and does not depend on the presence of RAS GTPases on the surface of mitochondria. Furthermore, we analyze the consequences of C-RAF activation on the cellular and molecular level. C-RAF activation on mitochondria dramatically changes their morphology and their subcellular distribution.

On the molecular level, we examine the role of C-RAF in the regulation of the pro-apoptotic Bcl-2 family member BAD. This protein exhibits the original mode of regulation by phosphorylation. Although several reports addressed the regulation of BAD by C-RAF, the exact mode of action as well as the consequences of C-RAF activation on BAD are still not completely understood. We show that the inducible activation of C-RAF promotes the rapid phosphorylation of BAD on Serine-112 (Ser-75 in the human protein), through a cascade involving the kinases MEK and RSK. Our findings reveal a new aspect of the regulation of BAD protein and its control by the RAF pathway: we find that C-RAF activation promotes BAD poly-ubiquitylation in a phosphorylation-dependent fashion, and increases the turn-over of this protein through proteasomal degradation.

Zusammenfassung

Die Proteine der RAF-Familie (A-RAF, B-RAF, C-RAF) sind Serin/Threonin-Kinasen, die eine wichtige Rolle in der Entwicklung, in der Zellregulation und in Krebs spielen. Obwohl es weitgehend anerkannt ist, dass die Lokalisation an Membranen ein wichtiger Aspekt ihrer Funktion ist, gibt es nur wenige Daten, die diesen Punkt ihrer Wirkungsweise beschreiben. Wir zeigen hier, dass jedes Mitglied der RAF-Familie eine spezifische Verteilung an zellulären Membranen besitzt, und dass C-RAF die einzige Isoform ist, die direkt an Mitochondrien bindet. Weiterhin zeigen wir, dass RAF-Kinasen intrinsische Unterschiede in ihrer mitochondrialen Affinität aufweisen, wobei C-RAF die einzige Isoform ist, die an dieses Organell effizient bindet. Diese Affinität wird von der amino-terminalen Domäne von C-RAF vermittelt und ist unabhängig vom Vorkommen der RAS-GTPasen auf der Oberfläche von Mitochondrien. Des Weiteren haben wir die Konsequenzen der Aktivierung von C-RAF auf zellulärer als auch auf molekularer Ebene untersucht. C-RAF Aktivierung an Mitochondrien hat drastische Änderungen in deren Morphologie und subzellulärer Verteilung zur Folge. Auf molekularer Ebene haben wir die Rolle von C-RAF in der Regulation des pro-apoptotischen Bcl-2 Familien Proteins BAD untersucht. Dieses Protein wird über Phosphorylierung reguliert. Obwohl eine Vielzahl von Arbeiten die Regulation von BAD durch C-RAF untersuchten, sind die exakte Wirkungsweise wie auch die Konsequenzen der C-RAF Aktivierung im Hinblick auf BAD immer noch unklar. Wir zeigen, dass die induzierbare Aktivierung von C-RAF die rapide Phosphorylierung von BAD an Serin 112 (Serin 75 im humanen Protein) zur Folge hat. Dieser Prozess wird durch eine Kaskade vermittelt, welche die Kinasen MEK und RSK einbezieht. Des Weiteren decken unsere Ergebnisse einen neuen Aspekt der Regulation des BAD Proteins auf und dessen Kontrolle durch den RAF-Signalweg: Wir zeigen, dass die Aktivierung von C-RAF die

phosphorylierungs-abhängige Poly-ubiquitylierung von BAD zur Folge hat. Weiterhin beschleunigt die Aktivierung von C-RAF den Umsatz des BAD Proteins durch proteasomalen Abbau.

Introduction

1.1. RAF kinases as protooncogenes and signaling through the RAS-RAF-MEK-ERK cascade

The family of RAF kinases are serine/threonine kinases that exhibit important roles in the regulation of the highly conserved RAS-RAF-MEK-ERK cascade. This cascade is crucial for cellular processes like proliferation, development and differentiation, but the deregulation of this cascade can also lead to cell transformation (Leicht et al. 2007). Homologues for RAF kinases are found in species from *C. elegans* to flies and mammals. In mammalian cells three isoforms of RAF kinases exist, called A-RAF, B-RAF and C-RAF. The first isoform of the RAF kinase family, C-RAF, was discovered in 1983 by Ulf Rapp and coworkers as a retrovirus transduced oncogene that was able to induce tumors in previously healthy organisms (Rapp UR et al., 1983). Since this first discovery RAF kinases gained much more importance. Recently, it turned out that the B-RAF isoform plays also an important role in cancer progression. This is underlined by the fact that in several cancer types, like melanoma or colorectal cancer, a single amino-acid exchange in B-RAF (a Valine to Glutamic acid exchange in position 600) is one of the most commonly found mutations (Davies et al., 2002). The third isoform of these kinases, A-RAF, is less well documented and its contribution in cancer development is less clear.

The isoforms of RAF kinases are ubiquitously expressed within an organism, but at different expression levels (Storm et al., 1990). While C-RAF is expressed in highest levels in striated muscle, cerebellum and fetal brain, B-RAF is expressed mainly in the nervous system, but also in other tissues in considerably lower amounts. A-RAF on the other hand is expressed in particularly high amounts in urogenital organs (Luckett et al., 2000). Although all RAF isoforms act mainly through the activation of the downstream cascade, there are indications

that the individual isoforms diverge in their function. Knockout experiments in mice showed that each RAF plays a distinct role in development (Pritchard et al. 1996; Wojnowski et al. 1997; Mikula et al. 2001; Huser et al. 2001). Whereas embryos of C-RAF-/- as well as B-RAF-/- die early in development, A-RAF-/- animals are viable, although they display neurological and intestinal abnormalities (Pritchard et al., 1996). B-RAF-/- embryos show overall growth retardation and increased apoptosis in endothelial tissues, which leads to vascular defects and death from vascular haemorrhage between day 10.5 and 12.5 (Wojnowski et al., 1997). A complete knockout of C-RAF in mice showed embryonic lethality in midgestation, poor development of the placenta and embryonic organs, in particular of the liver and the haematopoietic compartment (Mikula et al., 2001; Huser et al., 2001). These embryos were small in size and the lethality was essentially due to a high apoptosis rate in the liver. However, it remains unclear to which extent the individual functions of RAF can be attributed to their different expression levels or to their specific roles on the cellular level (Pelkmans et al., 2005).

Signaling of the canonical RAS-RAF-MEK-ERK cascade comprises subsequent events of phosphorylations (Shaul, Seger, 2007). Treatment of mammalian cells with mitogens, like growth factors, cytokines and hormones, initiates this mitogenic cascade (Fig.1). For instance, upon binding of epidermal growth factor (EGF) to the EGF-receptor at the plasma membrane, several phosphorylation events are initiated, starting with trans-phosphorylations of the intracellular Receptor-Tyrosine-Kinase (RTK) subunits of the EGF receptor family. Several adaptor proteins bind to the intracellular domains of the RTKs, which finally results in the activation (conversion from the GDP-bound into the GTP-bound state) of the small membrane bound GTPases of the RAS family (Hancock, 2003). This leads to an increase in intracellular levels of GTP-loaded RAS followed by the sequential activation of RAF-MEK-ERK (Marais, Marshal, 1996). The RAF kinases bind to RAS with their conserved structural determinant,

the RAS-binding domain (RBD) and the cysteine-rich domain (CRD) (Fig. 2) (Wittinghofer and Nassar, 1996), and several phosphorylations combined with structural changes in RAF lead to activation of the kinase (see below).

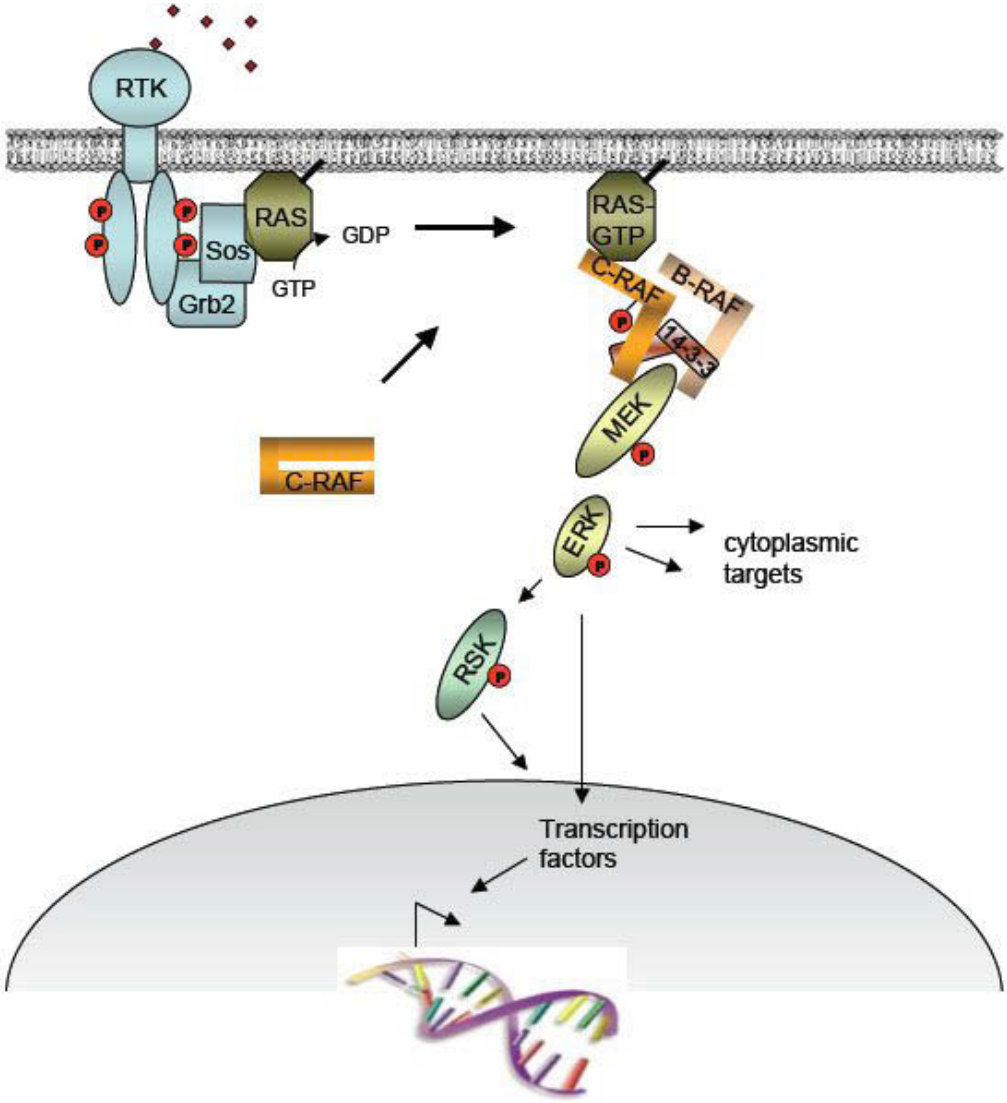


Figure 1: **Schematic representation of RAF activation**

Binding of epidermal growth factor (EGF) to its receptor at the plasma membrane leads to the trans-phosphorylation of the EGF-receptor tyrosine kinase (RTK) subunits. A complex formation is settled including the adapter proteins growth factor receptor bound 2 (Grb2), son of sevenless (SOS) and proteins of the RAS family of small GTPases. This results in the conversion of RAS from its GDP-bound (inactive) into its GTP-bound (active) state. RAS proteins bind via their lipid modifications to the membrane. Activated RAS associates with RAF kinases at the level of the membrane. Complex events of phosphorylations together with changes in the molecular binding partners result in the allosteric opening of the RAF conformation. The subsequent progression of the kinase cascade can include the formation of heterodimers between C-RAF and B-RAF. Proteins of the 14-3-3 family are probably important in this binding. The activated RAF kinases phosphorylate the downstream effectors MEK 1 and 2 (mitogen activated kinase), which in turn phosphorylates ERK 1 and 2 (extracellular regulated kinase). Active ERK can target many cytosolic proteins, like the downstream effector kinases of the p90 ribosomal S6 kinase family (RSK). ERK is also able to shuttle inside the nucleus and target several transcription factors to regulate gene expression.

At the same time a further complex organization is settled, where the other components of the cascade (MEK and ERK) are brought into close proximity to RAF and each other (Shaul, Seger, 2007). The cascade proceeds in the RAF-mediated activation and phosphorylation of the kinases MEK 1 and 2 (mitogen-activated protein kinase kinase MAPKK) at two sites, which in turn progresses through the MEK-mediated phosphorylation of ERK 1 and 2 (Extracellular Regulated Kinase or MAPK). Finally, activated ERK is able to mediate this extracellular response and targets multiple cytosolic, but also nuclear effector molecules. ERK can shuttle inside the nucleus, where it phophorylates and regulates several transcription factors, particularly myc, Elk1, p53, Ets1/2 and the AP-1 complex that comprises various heterodimers of c-fos and c-jun family members (Treisman, 1996; Adhikary and Eilers, 2007; Gille et al., 1992; Milne et al., 1994; Yang et al., 1996; Buchwalter et al. 2004; Balmanno and Cook, 1999; Shaulian and Karin, 2002).

1.2. Structure of RAF Kinases

Most of the structural determinants of RAF kinases are well conserved between the different isoforms (Fig. 2). While the amino-terminal region represents the regulatory domain of these molecules, the carboxy-terminal region harbours the catalytic activity (Wellbrock et al. 2004). All RAF- isoforms contain three conserved regions (CR1-3), with CR1 including the Ras-binding domain (RBD) and the cystein-rich domain (CRD), both important for the binding to activated RAS-GTPases (Wittinghofer and Nassar, 1996). The CR2 includes a negative regulatory site, *i.e.* one of the 14-3-3 binding sites (Serin 259 in C-RAF) and the CR3 at the carboxy-terminus represents the catalytic domain. Interestingly, most of the divergences between the RAF-isoforms are concentrated in the amino-terminal part.

A crucial determinant in reaching the full kinase activation of RAF is the conserved N-region (Mason et al., 1999). This region is located just upstream in the catalytic domain and is characterized by its highly negatively charged aminoacid composition. It buries several

phosphorylatable residues that are targeted by different kinases (Jin et al., 2003; Mason et al., 1999), thereby increasing the negative charge of this region. Other important structural determinants are the activation segment and the glycine-rich loop (or P-loop) within the kinase domain (Wan et al., 2004). The direct contribution of these regions in the kinase activation is discussed in a later chapter. The activation segment of all RAF isoforms contains also phosphorylatable residues, which results in a conformational change of the catalytic domain. In this context it is important to mention that the most commonly found mutation in B-RAF (V600E) lies within this segment, rendering this kinase constitutively active.

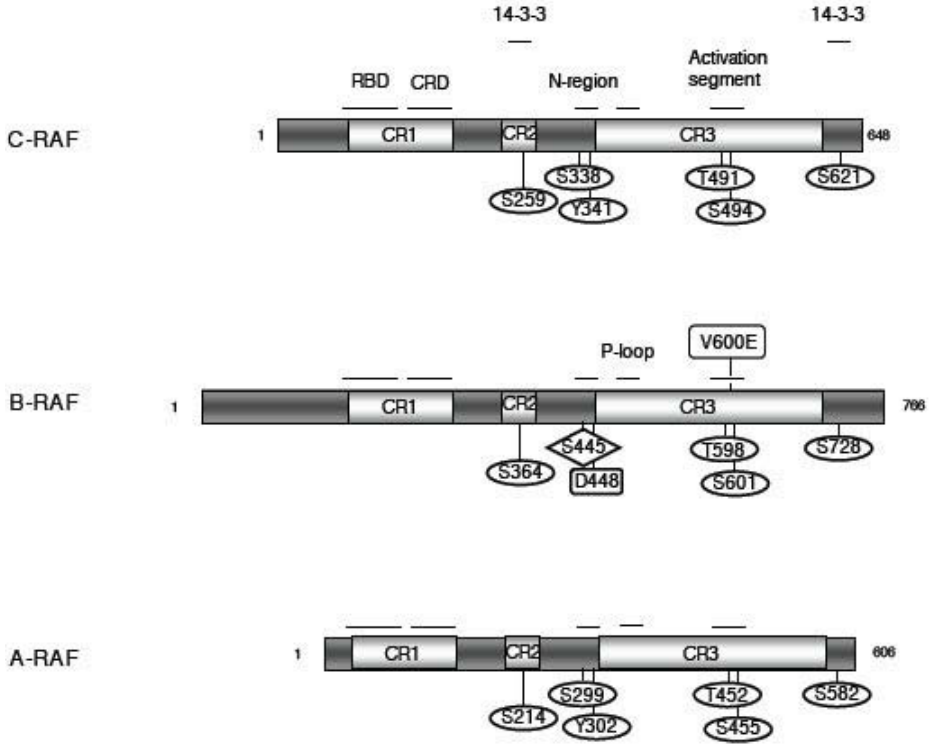


Figure 2: Structural determinants of the RAF isoforms

Representation of A-, B-, C-RAF with the respective regulatory sites highlighted. CR1, 2, 3 illustrate the conserved regions between the isoforms. The amino acids that are highlighted below the individual isoforms refer to respective phosphorylation sites. The CR1 contains the RAS-binding domain (RBD) and the cystein-rich domain (CRD), both required for the recruitment to the plasma membrane. CR2 includes an internal 14-3-3 binding site and CR3 represents the catalytic domain. S259 and S621 of C-RAF are in the phosphorylated state binding sites for the scaffold proteins of the 14-3-3 family. Shortly upstream of CR3 is the N-region, which represents a crucial regulatory domain to achieve full kinase activity. This region is characterized by its high content in negative charges due to several phosphorylatable residues. Among this residues are Y340 and Y341 in C-RAF. The latter is conserved in A-RAF (Y302), but is replaced with an aspartic acid (D448) in B-RAF. S338 is conserved in all RAF proteins (S299 in A-RAF and S445 in B-RAF), but it is constitutively phosphorylated in B-RAF. The activation-segment and the glycine-rich loop (or P-loop) within the CR3 are further regulatory regions (details see text). The two activation-segment phosphorylation sites of C-RAF comprise T491 and S494, which are conserved in A-RAF (T452 and T455) and B-RAF (T598 and S601).

1.3. Regulation of RAF kinases

The regulation of RAF-kinases is highly complex and still not completely understood. RAF kinases can be regulated in several ways, which include membrane recruitment, phosphorylations, heterodimer formation and complex formation with other proteins. Further possible regulatory mechanisms, like the control of the RAF protein level (Stancato et al., 1997) and the regulation through microRNAs (http://microrna.sanger.ac.uk/) are not presented here.

1.3.1. Membrane recruitment

Mammalian cells contain four RAS-isoforms (H-RAS, N-RAS, K-RASa, K-RASb) that bind RAF-kinases with different affinities (Weber et al., 2000). The binding to RAS proteins represents one important step in the membrane recruitment of RAF to the inner leaflet of the plasma membrane (Hancock, 2003). Although the interaction between RAS-GTPases and RAF is well established, the exact mode of action is still not completely understood. On the one hand RAS-GTPases can work as allosteric openers of the RAF conformation (Terai, Matsuda, 2005), but on the other hand RAS-GTPases might also actively recruit RAF to the membrane (Hancock, 2003). The binding to RAS-GTPases at the plasma membrane might even be sufficient to activate RAF kinases, since the artificially induced recruitment of C-RAF to the plasma membrane, achieved through the fusion of C-RAF to a CAAX-box, is sufficient to render the kinase active (Stokoe et al., 1994).

The individual RAS proteins are also located in distinct membrane microdomains, so by engaging with different RAS isoforms, the RAF proteins will be exposed to distinct environments, which might affect their activity. The localization of RAF to specialized microdomains in the plasma membrane, characterized by their specific lipid compositions, especially cholesterol-enriched microdomains (so-called lipid rafts), play important roles in signaling processes (Allen et al., 2007). It was shown, that the localization of H-RAS to

cholesterol-enriched microdomains is important in the activation process of RAF kinases *in vivo* (Roy et al., 1999). In addition, C-RAF can bind, at least *in vitro*, to distinct sets of phospholipids, like phosphatidylserin, and cholesterol-enriched lipid compositions (Hekman et al., 2002), which might further contribute to its membrane localization and regulation.

1.3.2. Phosphorylation

Phosphorylations are important events for RAF-kinase regulation. Multiple phosphorylation sites are reported: some regulate RAF kinase activity indirectly, by changing its interactions with binding partners, while others play a direct activating role, mainly by changing the protein conformation (see Fig. 2).

1.3.2.1. Direct regulatory role

Unfortunately, there is no structural data available for the full length forms of RAF kinases. However, insights into the conformational changes resulting from phosphorylations were derived from the crystallographic structure of the catalytic domain of B-RAF (Wan et al., 2004). Internal interactions between the activation segment and the Glycine-rich loop account directly for the activation process. These direct interactions displace the activation segment and traps B-RAF in an inactive conformation. Phosphorylations within these regions (T598, S601 in B-RAF) directly regulate the protein fold leading to a disruption of this interaction. The activation segment becomes liberated and the kinase can fold into its active conformation. This mechanism was described for B-RAF, but similar mechanisms are likely to account also for the kinase activation of the other isoforms. Mutations, like the V600E substitution in the activation segment of B-RAF, might mimick a phosphorylation event, which probably disrupts the interaction of the mentioned regions and renders the mutant kinase constitutively active.

A crucial event in RAF activation seems to be the liberation of the catalytic domain from the hindrance of the amino-terminal regulatory domain. This inhibiton is probably overcome by binding to partner molecules, but also through phosphorylations. Phosphorylation of several residues in the N-region and the kinase domain seems to be important in this respect (Chong, Guan, 2003). Kinases of the Src family phosphorylate Y340, Y341 of C-RAF (Marais et al., 1997; Fabian et al 1993). Apparently, this reduces the inhibitory effect of the N-terminus on the kinase domain (Tran, Frost, 2003). S338 seems to play a similar role in this respect, but its contribution is less clear (Alavi et al., 2003; Jin et al 2005; Ritt et al., 2007). The negative charge of this region is supposed to be essential for C-RAF´s kinase activity (Mason et al., 1999). This could also partially explain why B-RAF has an elevated kinase activity in basal conditons compared to the other RAF-isoforms. The N-region of B-RAF harbours an aspartic acid residue in position 448 (corresponding to C-RAF´s position Y341), probably mimicking a phosphorylation. In addition, the corresponding residue in B-RAF to S338 of C-RAF is constitutively phosphorylated (Mason et al., 1999). Therefore, the N-region of B-RAF shows already a higher negative charge compared to its other isoforms, which correlates with an elevated kinase activity.

There are several other phosphorylation sites, which negatively regulate RAF kinase activity. These sites are targeted by ERK and function in a feedback mechanism (Dougherty et al. 2005; Hekman et al., 2005). However, the exact contribution of these phosphorylation sites remains unclear.

1.3.2.2. Indirect regulatory role

The phosphorylation of RAF also creates binding sites for regulatory protein interactions. In this respect the best documented regulation is the binding to the scaffold/adapter proteins of the 14-3-3 family (Muslin et al 1996, Tzivion et al 1998). The family of 14-3-3 proteins comprises several isoforms that are ubiquitously expressed and highly abundant within all

eukaryotic cells. They bind to and modulate the activity of multiple interaction partners. Therefore, these proteins are crucial in various physiologic situations, like cell cycle control, apoptosis or mitogenic signal transduction (Tzivion and Avruch, 2002). 14-3-3 proteins recognize a conserved binding motif only in the phosphorylated state (Yaffe et al., 1997). Each RAF isoform contains two such binding motives. One site lies within the CR2, while the second site is located downstream of the catalytic domain. Binding to 14-3-3, as well as to other proteins, such as the chaperones cdc37, Hsp70, Hsp90 (Zhu J et al., 2005; Stancato et al., 1997; Grammatikakis et al. 1999), probably also participates in changing the conformation of RAF, thereby regulating the activity status. Serine 259 of C-RAF represents the first 14-3-3 binding site. It can be phosphorylated by PKA, when intracellular cAMP levels are high (Dhillon et al. 2002; Light et al., 2002), but also by AKT/PKB (Zimmermann and Moelling, 1999). When 14-3-3 is bound to this site the C-RAF kinase activity is reduced, probably through an interference with the binding to RAS. Although RAS–GTP can disrupt 14-3-3 binding to CR2 (Kubicek et al. 2002; Light et al., 2002), 14-3-3 displacement seems to occur in cells through an active process that involves dephosphorylation of S259 and binding to other partners, like Prohibitin (Abraham et al., 2000; Rajalingam et al. 2006).

The phosphorylation of the carboxy-terminal 14-3-3 binding site (S621 in C-RAF) is important to reach an active kinase (Light et al. 2002; Tzivion et al., 1998). The binding of 14-3-3 to this site seems to be the crucial event, rather than phosphorylation alone (Light et al., 2002). The underlying mechanism is not entirely clear, but it might include a change in the RAF conformation, but also the formation of heterodimers between RAF-kinases.

The first evidence for heterodimer formation of RAF kinases was obtained in cells that overexpressed a constitutively active H-RAS (Weber et al., 2001). There, it was possible to co-immunoprecipitate B-RAF with C-RAF, but only when the C-terminal 14-3-3 binding site of C-RAF was intact. This points to a crucial role for 14-3-3 in heterodimer formation. An important characteristic of the RAF heterodimers is constituted by the fact that the kinase

activity of both RAF-molecules in the heterodimer is enhanced (Garnett et al, 2005). This also explains how several B-RAF mutants with impaired kinase activity still mediate a high ERK phosphorylation. The high signaling properties of these B-RAF mutants work through bypassing the signaling via C-RAF (Garnett et al., 2005).

1.3.3. Oligomerization and complex formation with other proteins

RAF-kinases act in multimolecular complexes (Kyriakis, 2007). The signaling output is therefore strongly regulated by assembling the right complex in a temporal and spatial fashion. Components of the RAF cascade can be present with different scaffold proteins, such as KSR (kinase suppressor of RAS) or CNK (connector enhancer of KSR), MEK-Partner 1 (MP-1) or SUR-8 to form a functional active cascade (Claperon and Therrien, 2007; Morrison, Davis, 2003).

The pseudokinase KSR shares high homology to RAF kinases and establishes interactions with the same binding partners as RAF, like 14-3-3, Hsp90 and Cdc37 (Claperon and Therrien, 2007), which facilitates the interaction to each other. Even the membrane localization seems to be analogous to RAF, since phosphorylations lead to a translocation to the plasma membrane (Muller et al., 2001).

These scaffold proteins can stabilize, localize and coordinate interactions between the individual components of the pathway. This increases the efficiency of the signaling through them and maintains fidelity by restricting interactions between the different components of the cascade.

1.4. Anti-apoptotic functions of RAF

1.4.1. General aspects on apoptosis

RAF kinases have a crucial impact on cellular survival, mainly by regulating the apoptotic pathway, a cell suicidal programm initiated upon different cytotoxic insults (see Fig. 3).

Apoptosis or programmed cell death is an evolutionary highly conserved cell suicidal mechanism, which is important for multicellular organisms in processes like development and tissue homeostasis. Two major pathways can initiate apoptosis: we distinguish the extrinsic and the intrinsic pathway (Fig. 3). On the one hand ligand binding to death receptors (for example members of the tumor necrosis factor (TNF) receptor family) results in the activation of the extrinsic pathway, which typically leads to the direct activation of caspases, mainly caspase 8 and 10. The intrinsic pathway is induced by UV- or γ-irradiation or through trophic factor deprivation. A cell needs the constitutive presence of trophic factors (growth factors and cytokines) to keep the underlying survival pathways active, which in turn blocks cell death (Letai, 2006). The Bcl-2 family of proteins are key regulators in apoptosis progression of the intrinsic pathway. These two major ways of apoptosis induction are largely independent, but in certain cell types the extrinsic pathway can intersect with the intrinsic through the activation of one Bcl-2 family member (BID) (Fig.3).

Mitochondria constitute a convergence point in apoptosis progression (Youle and Strasser, 2008). In the intrinsic apoptotic pathway, but also in the extrinsic pathway through the cleavage of BID, the mitochondrial outer membrane (MOM) becomes permeabilized, which leads to the release of apoptogenic factors, such as cytochrome c (Fig.3). Cytochrome c is a crucial component in the mitochondrial respiratory chain, but in the context of apoptosis it constitutes a co-factor to establish a complex for caspase activation (Newmeyer, Ferguson-Miller, 2003). This so-called apoptosome is a heptameric complex comprising the molecule Apaf-1 in conjunction with cytochrome c. Once this complex is formed it leads to the conversion of the inactive to the active forms of initiator caspases (caspase 2, 8, 9). This initiator caspases in turn convert the executioner caspases (caspase 3, 6, 7) through cleavage into their active state. These activated enzymes cleave a series of crucial intracellular proteins, such as the polyADP ribose polymerase (PARP), activate DNAses and orchestrate the destruction of the cell. Typically, apoptotic cells show characteristics like DNA-laddering,

fragmentation of the nucleus and breaking up a cell into vesicles that are rapidly engulfed by other cells.

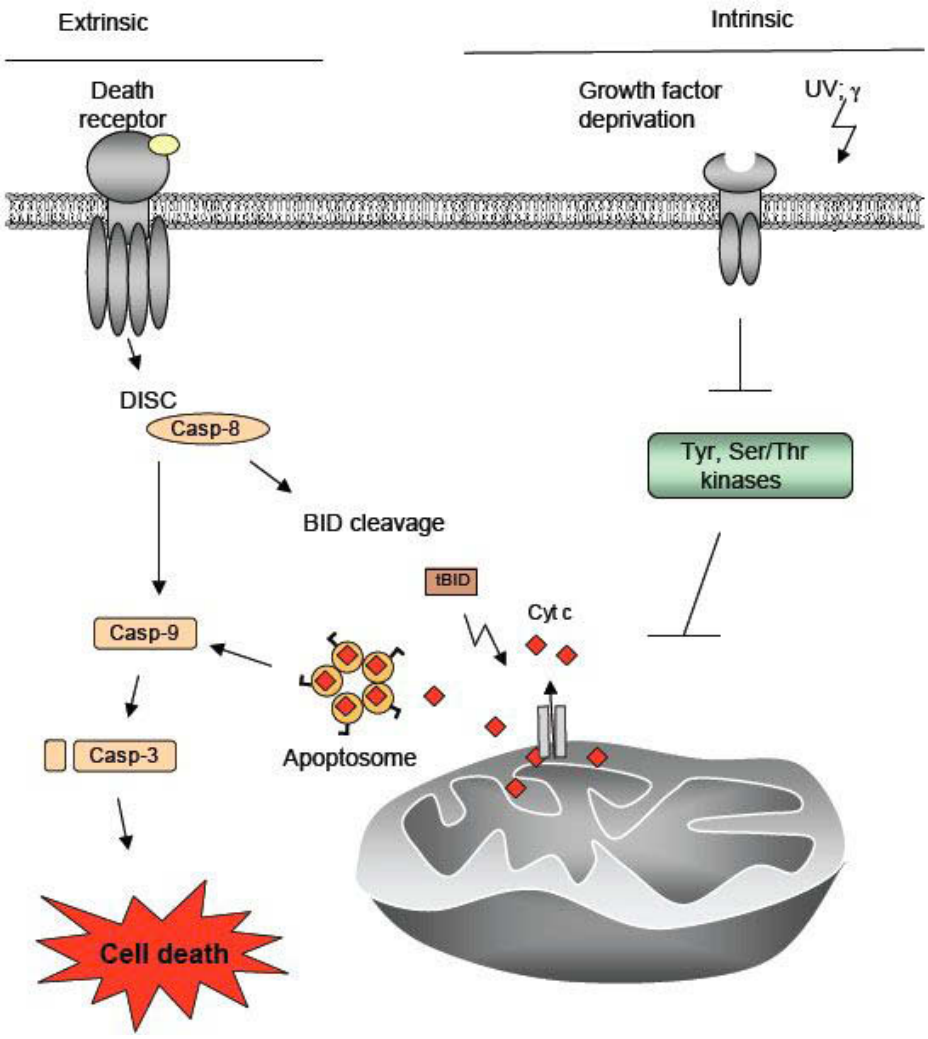


Figure 3: Mitochondrial regulation of apoptosis

Apoptosis is initiated either by the extrinsic or the intrinsic pathway. The extrinsic pathway is triggered upon binding of a ligand, like FasL, to the respective death receptors. The death receptor induced signaling complex (DISC) is formed, which leads subsequently to the activation of caspase-8 that activates the effector caspases. These cystein and aspartate specific enzymes cleave several cellular proteins, which finally leads to cell death. The intrinsic apoptotic pathway is initiated upon various cytotoxic stresses, like UV-irradiation, or is initiated by the lack of survival/growth factors. This interferes with the survival pathway signaling, that involves Ser/Thr- and Tyr-kinases. Upon apoptotic insults a complex interplay between apoptosis regulating proteins is initiated, which finally leads to a change in the mitochondrial membrane potential and to the permeabilization of the mitochondrial outer membrane (MOM). This permeabilization process is mediated either directly by pro-apoptotic members of the Bcl-2 family or by modulating existing pores, like VDAC (voltage dependent anion channel). However, this step is important to release cytochrome c from the mitochondrial intermembrane space. Cytochrome c constitutes a crucial component of a heptameric complex formed in the cytosol. This so-called apoptosome comprises an oligomer of APAF-1 together with cytochrome c. The formation of this complex converts the initiator-caspases (e.g. Casp-9) from their inactive into their active state. The active initiator caspases cleave the executioner pro-caspases to reach the activated enzymes (e.g. Casp-3) leading to the destruction of the cell. Proteins of the Bcl-2 family are crucial regulators of the onset of apoptosis at the level of mitochondria mainly in the intrinsic pathway. There, apoptosis proceeds through a complex interplay between anti- and pro-apoptotic proteins of Bcl-2 proteins. In some cell types the extrinsic pathway can interfere with the intrinsic apoptotic machinery through the caspase 8 mediated cleavage of the BH3 only protein BID leading to MOM permeabilization.

1.4.2. Role of the Bcl-2 family in apoptosis

The Bcl-2 family of proteins controls the mitochondrial phase of apoptosis progression, although their exact mode of action is still not completely understood (Youle, Strasser, 2008) (Fig. 4). Additionally, several proteins of the Bcl-2 family contribute in the regulation of various other important cellular processes that are non-related to apoptosis, like autophagy (Pattingre et al., 2005, Maiuri et al., 2007), mitochondrial fission and fusion (Karbowski et al., 2006), and the glucose metabolism (Danial NN et al., 2003 and 2008).

The first member of this family identified was Bcl-2, an oncoprotein activated via chromosomal translocation in human follicular lymphomas (Youle and Strasser, 2008). The increased expression of Bcl-2 protects cells by blocking apoptosis rather than actively promoting cell survival. Since this first discovery many other Bcl-2 family proteins were identified. The Bcl-2 family consists of pro-apoptotic (e.g. Bax, Bak) as well as anti-apoptotic members (e.g. Bcl-2, Bcl-xL). All of these core Bcl-2 proteins contain at least one of the four so-called Bcl-2 homology domains (BH-domains 1-4) (Fig.4). These α-helical BH-domains enable those proteins to form homo- as well as hetero-oligomers. Oligomerization between pro- and anti-apoptotic Bcl-2 proteins represents one important mechanism of their regulation, since anti-apoptotic members can keep their pro-apoptotic counterparts in check.
A further regulatory mechanism includes the change in the intracellular localization pattern of Bcl-2 proteins. Many Bcl-2 proteins exist in an equilibrium between the cytoplasm and intracellular membranes (Jeong et al., 2004; Wolter et al., 1997). In apoptotic conditions these proteins translocate then to the mitochondria, where they establish interactions and participate in the progression of apoptosis. Although the localization to mitochondrial membranes is widely acknowledged, other membrane compartments, like the endoplasmic reticulum (Zhu W et al., 1996), are also targeted by Bcl-2 proteins. Apoptosis induction from these

compartments can also be regulated by members of this protein family (Pinton and Rizutto, 2006).

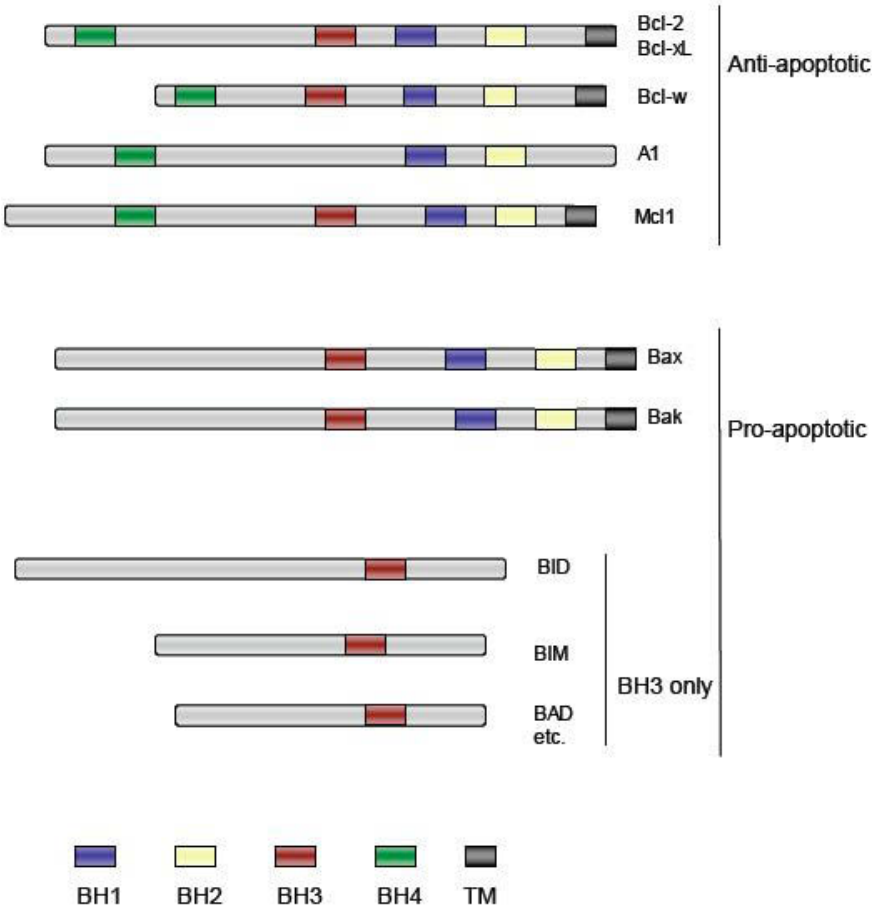


Figure 4: Representation of core Bcl-2 family proteins and BH3-only proteins.

The Bcl-2 family of proteins is subdivided into an anti-apoptotic (with the founding member Bcl-2) and a pro-apoptotic class. A further subdivision of the pro-apoptotic class is constituted by the BH3-only proteins. All Bcl-2 proteins contain at least one of the four so-called Bcl-2 homology domains: BH1 in blue, BH2 in yellow, BH3 in dark red, and BH4 in green. Several Bcl-2 family proteins contain an carboxy-terminal membrane anchor, that is involved in their binding to different intracellular membrane organelles (shown in black). The BH domains form α-helical structures that are important for the homo- and hetero-oligomerization between the proteins of this family. The BH1, 2, 3 domains form a hydrophobic pocket to bind BH3 domains. The BH3 domain, particularily from BH3 only proteins, mediates the interaction with the core Bcl-2 family proteins and thereby promotes apoptosis.

Permeabilization of the MOM results in the release of proapoptotic factors, which probably represents a point of no return in apoptosis induction. In this respect gene knock-out experiments indicated that the proteins Bax and Bak constitute a core component of the apoptotic machinery: in mouse embryonic fibroblasts (MEF) the lack of both proteins renders those cells resistant to a wide range of apoptotic stimuli (Wei et al., 2001). This observation

introduced the concept that the final step of apoptosis initiation on the level of mitochondria proceeds mainly through these two proteins. During apoptosis progression Bax and Bak proteins change their conformation, oligomerize and translocate to mitochondria at the same time scale when the permeabilization of the MOM occurs. How exactly this permeabilization occurs is still a matter of debate (Newmeyer and Ferguson-Miller, 2003). There are several mechanisms suggested: i) Bcl-2 proteins could modulate residential mitochondrial pores (such as VDAC) in their function (Kuwana et al., 2002). ii) More strikingly, pro- and anti-apoptotic Bcl-2 proteins resemble the protein structure of the membrane insertion domain of Diphteria Toxin (Muchmore et al., 1996; Reed et al., 2004). This structural relationship plus the carboxy-terminal transmembrane domain enables these proteins to insert deeply into membranes and potentially to form pores (Minn et al., 1997, Kuwana et al., 2002). However, the relevance of the pore forming properties of the Bcl-2 proteins is debated. iii) Another possibility is that the pro-apoptotic Bax and Bak also interfere with the regulation of mitochondrial fission and fusion (Karbowski et al., 2006), which might be an important mechanism in the fragmentation of mitochondria and the subsequent cytochrome c release. Whatever the mechanism is, Bax and Bak need to get activated for launching their pro-apoptotic properties. In this respect a subset of the pro-apoptotic Bcl-2 family, the BH3-only proteins, plays an important role.

1.4.3. BH3-only proteins regulate the apoptotic pathway

The BH3 only subclass of pro-apoptotic Bcl-2 proteins consists of at least eight members, namely BAD, BID, BIM, Bik, Bmf, Nrk, Noxa and Puma. These proteins only show homology to other Bcl-2 proteins in their BH3 domain, which is important for their killing function. The BH3 domain is the responsible determinant to bind to anti- as well as to pro-apoptotic Bcl-2 proteins. Structural studies of at least six multidomain Bcl-2 family members (pro- and anti-apoptotic) revealed that they build globular structures with their BH1, 2, 3

domains, forming a hydrophobic groove (Petros et al., 2004). The BH3-domain is an amphipathic α-helix consisting of about 24 residues, which can bind to this hydrophobic groove. However, subtle differences in the aminoacid composition of the BH3 domains result in a specific binding pattern of BH3 only proteins to other Bcl-2 family members (Chen et al. 2005; Certo et al. 2006).

Generally, BH3 only proteins are proposed to function as sentinels of the cellular health status. Therefore, BH3 only proteins have to react quickly to changes of cellular health and to reach their pro-apoptotic potential by several means. These mechanisms include the increase of the protein level either through transcriptional control or through regulation of the protein stability (Oda et al. 2000; Luciano et al., 2003; Domina et al., 2004), some post-translational protein modifications (Zha et al., 1996; Zha et al., 2000), or changes in their subcellular distribution (Puthalakath et al., 1999) and interaction partners (Zha et al., 1996; Datta et al., 2000).

There is general agreement about which pairs of Bcl-2 family proteins can interact, but the order of these binding events, and their relative importance for cell death versus cell survival, has been difficult to resolve. BH3 only proteins can bind to anti-apoptotic Bcl-2 family members, however with preferred specificities. Whereas BIM, truncated BID (tBID) and PUMA bind all anti-apoptotic proteins, BAD and Noxa show a more restricted binding pattern (Kim et al., 2006) (Fig.5). The latter proteins bind only to some of the anti-apoptotic members, which might partially explain their less pronounced killing abilities compared to BIM, tBID and PUMA. But this defined binding pattern probably allows a tissue-specific regulation and stress specific cellular responses.

Somehow the BH3-only proteins activate the pro-apoptotic proteins Bax and Bak, but the route to this activation is still unclear. A major controversial point is, whether some BH3 only proteins can bind to Bax and Bak directly. Cell-free models of Bax activation, which do not fully reflect the *in vivo* situation, show that pro-apoptotic Bax acts synergistically with

peptides corresponding to the BH3 domain of some BH3-only proteins, notably those from BID and BIM, to induce cytochrome c release from isolated mitochondria (Kuwana et al., 2005; Certo et al. 2006). These proteins are therefore referred to as "activators" (Letai et al. 2002) (Fig. 5). However, other reports state that the binding of these BH3-only proteins to Bax/Bak is very weak under physiological conditions (Willis et al., 2005). At least, there is a consensus that the other BH3-only proteins act as "sensitizers", reacting to changes of the cellular health status, but conferring their apoptotic potential indirectly.

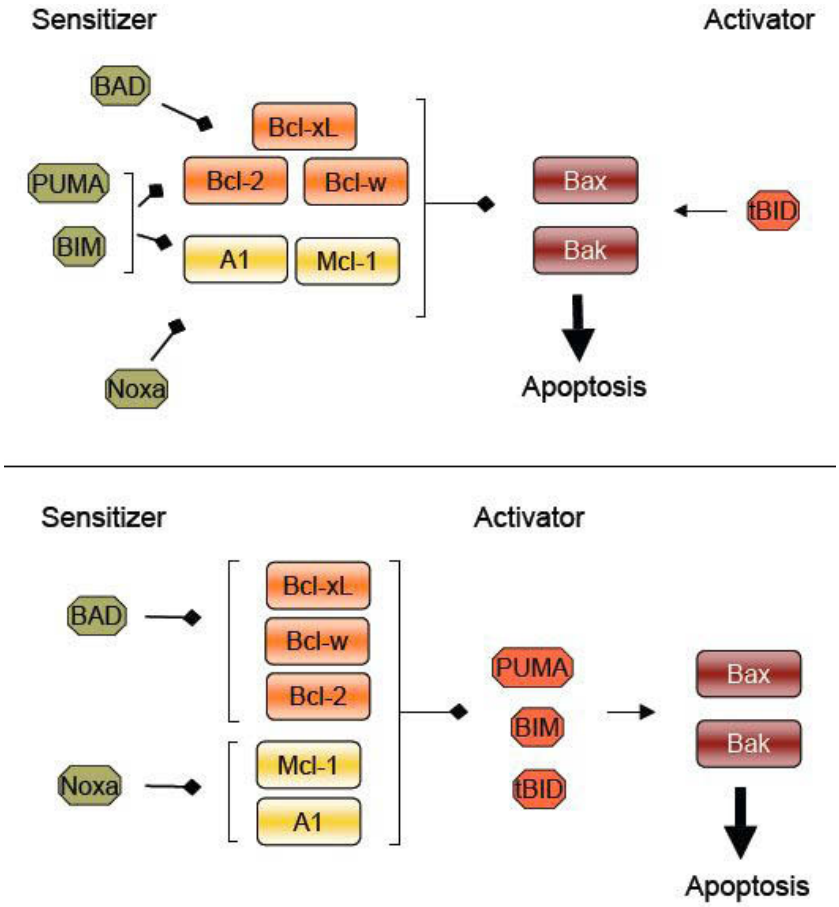


Figure 5: Suggested models of the mode of action of BH3 only proteins

A: BH3 only proteins activate the core pro-apoptotic Bax/Bak indirectly through derepression. Some BH3 only proteins (BAD, Noxa and others that are not depicted here) only bind to a subset of anti-apoptotic Bcl-2 proteins, whereas other BH3 only (BIM, PUMA) bind to all of them. In this model the BH3 only proteins do not activate Bax/Bak directly, but act as sensitizers, with the exception of tBID that might activate Bax/Bak directly. Bax/Bak are kept in check by their anti-apoptotic counterparts until sensitizer BH3 only proteins are activated and compete with the binding to their preferred anti-apoptotic binding partners. Liberated Bax/Bak induce permeabilization of the MOM. B: Hierarchy model of BH3 only proteins. Some BH3 only proteins (BAD, Noxa and others, that are not depicted here) only show binding to some anti-apoptotic Bcl-2 proteins, while BIM, tBID and PUMA can bind to all anti-apoptotic Bcl-2 proteins, but also directly to Bax/Bak. This activator BH3 only proteins are kept in check through the anti-apoptotic Bcl-2 proteins. Sensitizer BH3 only react to apoptotic stimuli and bind to their preferred partner Bcl-2 proteins. This liberates the activator BH3 only proteins, which in turn associate with and activate Bax/Bak, thereby activating them, which leads to apoptosis.

Recently, two major models are suggested, how BH3-only proteins induce apoptosis. The first model suggests that there is a hierarchy of BH3-only proteins, where some proteins (sensitizers) act upstream of others (activators) (Kim et al., 2006; Galonek and Hardwick, 2006 and Fig.5): A "sensitizer" BH3-only protein, like BAD, Noxa and some others, responds directly to survival factors resulting in suppression of their pro-apoptotic function. In the absence of growth factors, these proteins engage specifically their preferred anti-apoptotic Bcl-2 proteins. The targeted Bcl-2 proteins then release the "activator" BH3-only proteins that in turn bind to and activate the Bax/Bak directly. The second model suggests that the binding of BH3-only proteins to Bax/Bak is very weak under physiological conditions, neglecting the direct activatory role of BH3-only proteins on the core pro-apoptotic proteins (maybe with the exception of tBID). Activation of Bax/Bak is rather mediated through the release of them from their anti-apoptotic counterparts. There, the differential binding abilities of BH3-only to anti-apoptotic proteins regulate the release of Bax and Bak, resulting in apoptosis (Willis et al., 2007; Youle, 2007).

1.4.4. Control of the apoptotic machinery by RAF kinases

Cellular survival requires the continous presence of growth factors. This effect of growth factors is achieved through the coordinated action of different signaling pathways, such as the RAF cascade, that inactivates the apopotic machinery (Letai, 2006). Blocking the RAF pathway, for instance with chemical inhibitors, results in decreased cellular survival. On the other hand the constitutive activation of the RAF-cascade increases the survival rate, even in the absence of growth factors (von Gise et al., 2001). This clearly points to the importance of this pathway in the mediation of the prosurvival and anti-apoptotic effects of trophic factors. Furthermore, knock-out experiments of B-RAF and C-RAF indicated that the pro-survival function of these kinases is also important in primary, non-transformed cells (Wojnowski et al. 1997; Huser et al., 2001; Mikula et al., 2001).

C-RAF modulates cell survival on several levels (Fig. 6). The mechanisms include: i) a direct regulation of the proteins that are important for apoptosis, ii) transcriptional regulation of such proteins, iii) transformation, including changes in cell physiology and metabolism, and appearance of multiple autocrine/paracrine loops.

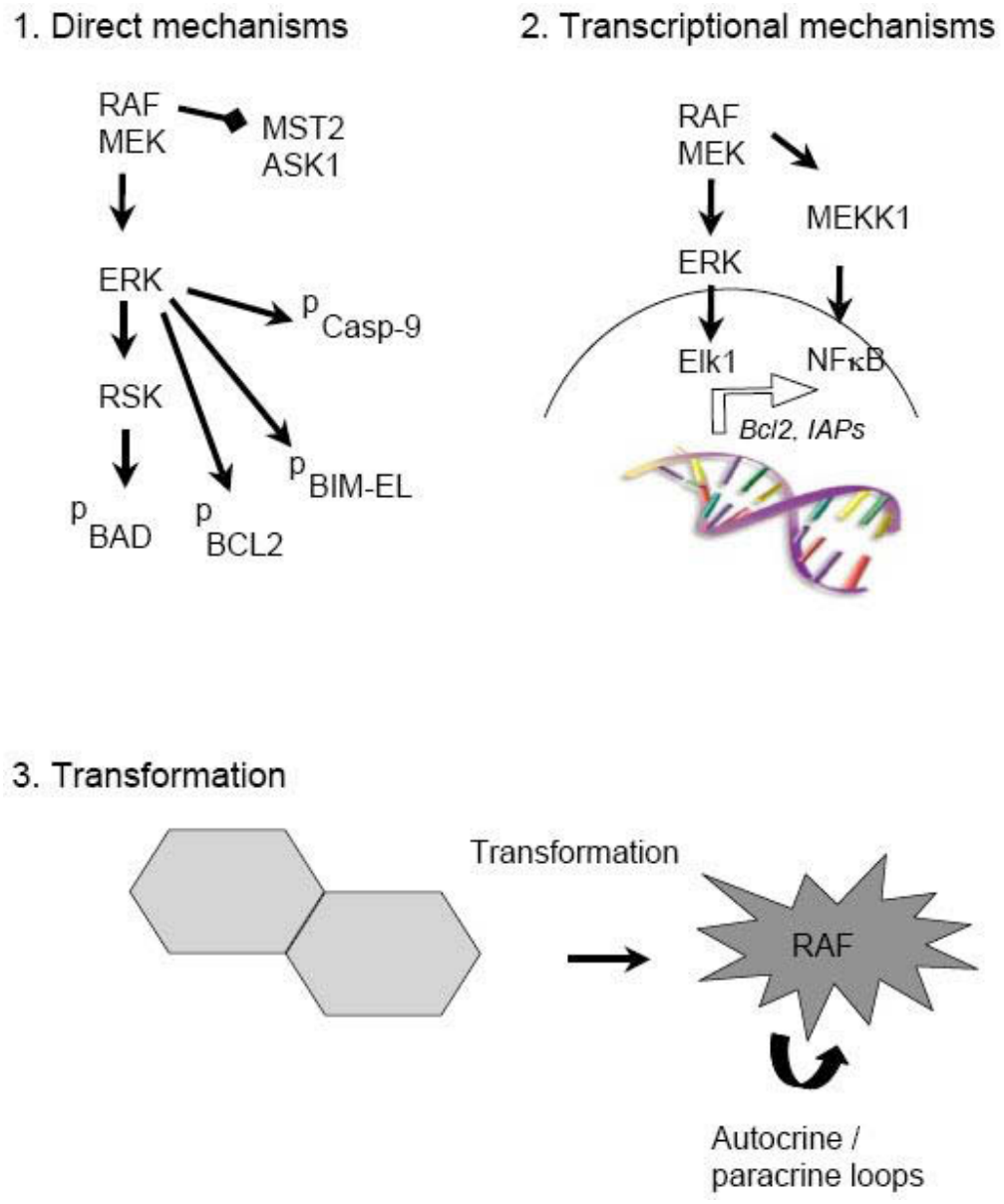


Figure 6: RAF controls apoptosis in multiple ways
The RAF cascade interferes with the regulation of apoptosis through several mechanisms. Several proteins are targets of the RAF cascade. The phosphorylation of those proteins interferes with their respective functions in apoptosis regulation. But also indirect mechanisms account for the anti-apoptotic effects of (C-)RAF (MST2, ASK1). Additionally, the RAF cascade controls apoptosis through regulating the expression of proteins via transcriptional as well as translational mechanisms. As a result of these regulations RAF can lead to transformation, which creates the appearance of multiple autocrine and paracrine loops, rendering those cells less sensitive to apoptotic triggers.

1.4.4.1. Direct regulation

Important targets of the RAF pathway are proteins of the Bcl-2 family. The RAF-ERK module phosphorylates directly the anti-apoptotic protein Bcl-2 and enhances its apoptosis suppressing properties (Deng et al., 2000). However, the direct role of this phosphorylation in cell survival is still not completely determined. Additionally, the RAF pathway also targets pro-apoptotic Bcl-2 proteins, like BIM and BAD. The phosphorylation under prosurvival conditions of these proteins leads to an inactivation of their pro-apoptotic properties. However, the reported consequences of these phosphorylations are different. The phosphorylation of BIM finally results in the degradation of the protein (Luciano et al., 2003; Ley et al., 2003; Ewings et al. 2007). In contrast, the phosphorylation of BAD leads to its sequestration into the cytosol (Zha et al., 1996; Scheid et al., 1999). We will go into more detail on the regulation of BAD protein by RAF kinases in a later chapter.
Furthermore, the RAF cascade interferes with the progression of apoptosis downstream of mitochondria. The ERK-mediated phosphorylation of the initiator caspase-9 blocks its activity, thereby interfering with the execution of apoptosis (Allan et al., 2003). But C-RAF does not only rely on its kinase activity to counteract apoptosis. C-RAF can modulate the activity of crucial apoptosis regulating kinases through its binding, like the mammalian sterile20-like kinase (MST2) (O´Neill et al., 2004) or apoptosis stimulating kinase 1 (ASK1) (Chen et al., 2001). So the RAF cascade can protect cells from apoptosis by interfering with apoptosis progression upstream but also downstream of mitochondria.

1.4.4.2. Transcriptional regulation

An important consequence of the activation of the RAF cascade is the transcriptional regulation of various genes. The kinase ERK is able to shuttle inside the nucleus and to regulate several transcription factors directly by phosphorylation (Treisman 1996). Probably the most crucial target transcription factor families are Ets and AP-1, where the latter actually

comprises various combinations of the factors c-jun and c-fos. The transciption and the stability of c-fos is directly regulated by ERK (Whitmarsh, 2007). The RAF cascade in turn regulates through these transcription factors the expression of various genes that are important for many cellular processes like cell cycle, differentiation and cell survival (Shaulian and Karin, 2002).

In the control of apoptosis the RAF pathway suppresses apoptosis induction through the direct transcriptional regulation of pro- and anti-apoptotic Bcl-2 family proteins (Townsend et al., 1998; Shinjyo et al., 2001), as well as the inhibitor of apoptosis proteins (IAPs) (Gotz et al., 2005). Futhermore, the RAF pathway can also activate NF-κB, another transcription factor central to cell transformation and survival through the activation of the kinase MEKK1 (Baumann et al., 2000).

Furthermore, the RAF pathway might regulate the transcription by interfering with the epigenetic status of the chromatin. Epigenetic control means that the accessibility of the chromatin is modulated by several means, achieved by post-translational modifications, including methylation, acetylation and phosphorylation of the DNA-packaging histone proteins (Dyson et al., 2005).

1.4.4.3. Transformation, regulation of cell differentiation, and survival regulation

As a consequence of the direct and the transcriptional mechanisms, RAF kinases deeply interfere with cell physiology. The regulation of the signaling strength and duration of the signaling output are important parameters, first revealed in the differentiation process of the pheochromocytoma cell line PC12 (Cowley et al., 1994; Traverse et al., 1992; Marshall, 1995).

In the context of cancer, RAF produces a broad range of changes that are described as transformation. An important characteristic of transformed cells is that they are notoriously resistant to apoptosis (Schulze et al., 2001). This might be due to the fact that such cells are

constantly activating their survival pathways in that they produce their own growth factors, establishing multiple autocrine or paracrine loops (Schulze et al. 2004). The presence of autocrine and paracrine loops renders those cells almost independent of regulations from the outside trophic control.

It is also likely that the constant activation of the RAF pathway affects the metabolism of cells. In cancer, cells often exhibit a modified metabolism state, which is commonly called the "Warburg effect". These cells use then glycolysis as major energy supply rather than the oxidative phosphorylation of mitochondria (Gottlieb et al., 2005). There are indications that RAF-kinases might account for some of these metabolic features present in cancer cells, since the introduction of an activated version of C-RAF into NIH3T3 cells is sufficient to increase the content of glycolytic metabolites (LeMellay et al. 2002).

Taken together RAF regulates cell survival at multiple levels. One crucial aspect of this function probably is the their intracellular localization, such as at the mitochondrial level (Galmiche, Fueller, 2007).

1.5. RAF kinases at the level of mitochondria

The first report detecting a RAF-isoform at the mitochondrial level identified C-RAF at this organelle in HEK 293 cells (Wang et al., 1996). There, the authors showed that a stably expressed GFP-tagged C-RAF BxB localized to mitochondria upon intracellular overexpression of the anti-apoptotic protein Bcl-2. These observations prompted other studies that confirmed the localization of the kinase C-RAF at mitochondria in cancer and normal tissues (Salomoni et al., 1998; Majewski et al., 1999; Peruzzi et al., 2001; Alavi et al., 2003; Cornelis et al., 2005). However, the mitochondrial recruitment of C-RAF, the underlying mechanism and the functional role there are still not entirely resolved. Whereas some reports suggest that phosphorylation of one residue on C-RAF, Ser-338, via the p21-activated kinase (PAK), might be a prerequisite for the localization to mitochondria and conferring a

protective effect to apoptosis (Jin et al., 2005), others suggest this phosphorylation as an activation marker (Mason et al., 1999). C-RAF could recognize some structural determinants that promote the recruitment to mitochondria, among them an interaction of C-RAF with lipids (Hekman et al., 2002) as well as with proteins (Wang et al. 1996). There are already RAF-binding partners established on the mitochondrial level, like prohibitin (Rajalingam et al., 2005), the voltage dependent anion channel (VDAC) (LeMellay et al., 2002), members of the small Ras GTPases (Bivona et al., 2006; Wolfman et al., 2006) or molecules involved in signaling processes like Grb10 (Nantel et al., 1999).

There are also indications that B-RAF might be present at the level of mitochondria (Wiese et al., 2001; Götz et al., 2005). In the case of A-RAF a controversial point arose from the observation that A-RAF might be found inside this organelle via interacting with the transport machinery of mitochondria (Yuryev et al., 2000). However, the latter observations could not be confirmed to date.

1.6. General aspects on the BH3-only protein BAD

An initial report identified the pro-apoptotic protein BAD as a binding partner of Bcl-2 and Bcl-xL in 1995 (Yang et al., 1995). The work of Wang et al. (1996) established then a connection between the anti-apoptotic properties of C-RAF and the targeting of BAD. There, the authors observed that BAD was phosphorylated after introducing a mitochondrial targeted, activated C-RAF, suggesting BAD as a mitochondrial substrate. Since these initial findings BAD gained more importance and a plethora of work addressed the function and the regulation of this molecule.

Structure: Unfortunately, there is no crystallographic structural data for the full length BAD available. The only crystallographic structural analysis to date reveals the molecular binding mechanism of BAD´s BH3-domain to Bcl-XL (Petros et al., 2000). A structural analysis using

spectroscopic methods suggests that BAD is an intrinsically unstructured protein in the absence of binding partners (Hinds et al., 2007). However, the BAD sequence shares some common motifs. Beside the α-helical BH3-domain, the amino-terminus of BAD contains two PEST-sequences (Yang et al., 1995), suggestive of a high protein turnover (Rogers et al., 1986). The carboxy-terminus buries determinants important for the binding to certain types of negatively charged lipids (Hekman et al., 2006).

Function: The main function of BAD is reported in the regulation of apoptosis, where it can bind to and block the anti-apoptotic proteins Bcl-2, Bcl-xL and Bcl-w (Chen et al., 2005; Certo et al., 2006 and Fig.5). However, BAD seems not to be a strong inducer of apoptosis, nor is it a strong inducer of cytochrome c release *in vitro* (Kuwana et al., 2005). Additionally, knockout experiments in mice showed no dramatic developmental defects (Ranger et al., 2003). However, these animals developed B-cell lymphomas in higher age.
Beside the function in apoptosis, BAD plays also a regulatory role in other crucial cellular processes, like in the glucose metabolism or in autophagy (Danial NN et al., 2003; Maiuri et al. 2007).

Regulation: One particularity of BAD is its regulation via phosphorylation in response to growth factors at mainly three residues (Serine 112, 136, 155 of the murine molecule; Serine 75, 99, 118 in human). The phosphorylation of BAD results in the inactivation of its pro-apoptotic function. A knock-in approach in mice using a non-phosphorylatable mutant of BAD demonstrated the importance of these phosphorylations (Datta SR et al., 2002). The obtained animals showed no severe developmental defects. However, whereas wildtype cells could be rescued from apoptosis induction by re-applying survival factors, the mutant cells did not respond to treatment with survival factors. Clearly, this proves that the survival factor mediated phosphorylation of BAD is indeed important for apoptosis regulation.

The phosphorylation of BAD directly regulates the association with other proteins as well as its subcellular localization. In the dephosphorylated state BAD binds to Bcl-2 or Bcl-xL via its BH3 domain and localizes to mitochondria, representing the active state of BAD. Phosphorylation of Ser155, a residue located within the BH3 domain, disrupts the association of BAD to its anti-apoptotic counterparts (Virdee et al. 2000; Tan et al. 2000). The phosphorylation of Ser112 and Ser136 creates binding motives for proteins of the 14-3-3 family (Zha et al., 1996; Yaffe et al., 1997). In this state BAD is detached from mitochondria and mainly sequestered in the cytosol (Datta et al., 2000). Therefore, binding to either Bcl-2/Bcl-XL or to 14-3-3 proteins seems to be a crucial event for BAD´s intracellular localization.

Multiple signaling pathways target BAD in response to survival factors. Reported kinases with a role in BAD phosphorylation are PKA, PKB/AKT, PAK1, RSK, Abl, PIM Kinase and the RAF-pathway (Harada et al., 1999; del Peso et al., 1997; Datta et al., 1997; Schurmann et al., 2000; Eisenmann et al., 2003; Neshat et al., 2000; Yan et al., 2003; Wang et al., 1996). Whereas there is a wide consensus on the kinases that target the residues Ser136 (PKB) and Ser155 (PKA), there are multiple kinases reported to target Ser112 (Schurmann et al., 2000; Eisenmann et al., 2003; Neshat et al., 2000; Yan et al., 2003). This multiple targeting of Ser112 might be due to the different cell systems as well as the different stimuli that were used to achieve the phosphorylation. But still, this indicates that BAD phosphorylation on its different residues is an important process on which several survival signaling pathways converge (She et al. 2005). On the other hand this complex interplay of signaling pathways renders the identification of the directly responsible BAD kinase difficult.
In calcium-mediated apoptosis the first phosphatase responsible for BAD dephosphorylation was identified. Calcineurin (or PP2B) seems to be able to dephosphorylate all phospho-sites in the BAD molecule (Wang HG et al., 1999). Beside calcineurin, there are also reports

indicating PP1 and PP2A as phophatases for BAD (Danial et al., 2003; Chiang et al., 2003). Furthermore, BAD can also reach its pro-apoptotic function by other means including alternative splicing of the murine form (Ranger et al., 2003), the phosphorylation of another site (S128) (Konishi Y et al., 2002) or cleavage through caspase-3 and -7 (Seo et al., 2004).

1.6.1. BAD as a substrate for the RAF-MEK-ERK cascade

The RAF-cascade exhibits at least in part its anti-apoptotic function through the phosphorylation of BAD (Wang et al., 1996). The purified C-RAF is able to phosphorylate BAD *in vitro*, and intracellular overexpression of C-RAF also leads to a robust phosphorylation of BAD. Further indications for the relevance of RAF-kinases targeting BAD were obtained from experiments showing the phosphorylation of BAD upon addition of growth factors that mainly act on the RAF-pathway (Scheid et al. 1999). However, the direct role of RAF in BAD phosphorylation is debated, since other reports indicated a role of RAF´s downstream effector kinases like MEK and kinases of the RSK family (She et al., 2002; Eisenmann et al., 2003; She et al., 2005; Panka et al., 2006).

The difficulties in clearly identifying the contribution of RAF kinases in BAD phosphorylation can be attributed to the following points: i) Kinases of the RAF family act in a highly interconnected network, rendering the analysis of the contribution in BAD phosphorylation difficult; ii) the RAF proteins act as transforming oncogenes, thereby interfering with multiple pathways that might be directly or indirectly involved in BAD regulation. Clearly, there is evidence that sustained changes in the C-RAF protein level and activity status have an effect on BAD phosphorylation. In conditions of C-RAF overexpression BAD becomes hyperphosphorylated (Wang et al. 1996), whereas BAD is hypophosphorylated when C-RAF protein levels are reduced (Kebache et al. 2007).

Although it is rather clear that the RAF-pathway is able to target BAD, we are still far from a global understanding how this process is regulated. Neither do we have substantial kinetic

data on the phosphorylation process, nor do we know the consequences of the RAF pathway on the BAD protein.

1.7. Aim of the work

RAF kinases are an important module in signaling events initiated by growth factors and other extracellular signaling molecules. In this signaling, RAF kinases are recruited to cellular membranes, but the literature contains only multiple fragmentary reports on the ability of the RAF isoforms to interact with those membranes. Furthermore, no direct comparison between all the RAF isoforms has been performed yet.

This work aims at better understanding the localization abilities of the different RAF isoforms, particularly to mitochondria, how this process is regulated and what the cellular consequences are on this level.

Are there differencies in the binding abilities of RAF kinases to mitochondria? If yes, what accounts for these differencies? What is the nature of the mitochondrial interaction with RAF and how is this process regulated? What are the consequences of C-RAF on the cellular level?

Since the BH3-only protein BAD constitutes one target protein for the RAF pathway on the level of mitochondria, this work also aims at better understanding the regulation of BAD by RAF-kinases. Regarding the impact of the RAF-MEK-ERK cascade targeting the protein BAD, the following questions are addressed:

How relevant is the RAF-pathway for BAD regulation under acute activation in cultured cells? Which residues of BAD are targeted? What is the nature of the complex that phosphorylates BAD? What are the molecular consequences of the RAF-mediated phosphorylation for the BAD protein?

Materials and Methods

2.1. Materials

2.1.1. Instruments

Hardware	*Manufacturer*
Bacterial incubator	Heraeus B 6200
Bacterial shaker	New Brunswick Scientific innova 4330
Cell culture hood	Heraeus Instrument
Cell culture incubator	Heraeus Instrument
Confocal microscope	TCS SP2 (Leica)
Developing machine	AGFA
DNA Sequencer	ABI PRISM 373, ABI
Electron microscope	Zeiss EM900
Electrophoresis power supply	Bio-Rad
Electrophoresis unit	Bio-Rad Mini-Protean II
Mega centrifuge	J-6B, Beckman; Megafuge 1.0 R, Heraeus; RC 5B plus, Sorval
Mini centrifuge (refrigerated)	Eppendorf
Phase contrast microscope	Leica
pH meter	WTW pH525
Plastics	Sarstedt
Rotor Gene 2000 cycler	Corbett Research
Spectrophotometer	Ultraspec 3000, Pharmacia Biotech
Thermomixer	Eppendorf
Ultracentrifuge	Beckman Optima L-80 XP
Video microscope	Axiovert 200 microscope (Carl Zeiss)
Vortex	Bender Hohbein AG
Water bath	Kötterman

2.1.2. Chemical reagents

1 kb DNA ladder	Fermentas
^{35}S Redivue-Cys-Met mix	GE Healthcare
ATP, ADP	SIGMA
Acrylamide (30%)/Bisacrylamide (0.8%), 37,5:1	Roth
Agarose, ultra pure	Roth
Ammoniumchloride (NH_4Cl)	AppliChem
Ammonium peroxydisulfate (APS)	SIGMA
Ampicillin	SIGMA
Bacto-Agar	DIFCO
Bacto-tryprone	Roth
Bovine serum albumin (BSA) fraction V	SIGMA
Bromphenolblue	SIGMA
β-Glycerophosphate	SIGMA
β-Mercaptoethanol	SIGMA
Calciumchloride ($CaCl_2$)	AppliChem
Chloroform	AppliChem
4',6-Diamidino-2-phenylindol (DAPI)	SIGMA
Deoxycholate (DOC)	SIGMA
Dimethylsulfoxide (DMSO)	AppliChem
Dithiothreitol (DTT)	Roth
dNTPs	Roth

EDTA	SIGMA
EGTA	SIGMA
En3hance	Perkin Elmer
Ethanol	AppliChem
Ethidiumbromide	SIGMA
Glutathion-sepharose	SIGMA
Glycerol	AppliChem
Glycine	AppliChem
HCl	Roth
HEPES	AppliChem
Imidazole	AppliChem
Iodoacetamide	SIGMA
Isopropanol	AppliChem
KH_2PO_4	SIGMA
KOAc	SIGMA
Magnesiumchloride	AppliChem
Mannitol	SIGMA
MOPS	SIGMA
Mowiol	Calbiochem
Natrium Chloride	AppliChem
NP-40	SIGMA
Paraformaldehyde	SIGMA
PBS	Fluka
PMSF	Fluka
Ponceau S	SIGMA
Potassiumchloride (KCl)	SIGMA
protease inhibitor cocktail	SIGMA
Protein ladder	Fermentas
PROTRAN nitrocellulaose membrane	Schleicher & Schüll
Orthovanadat	SIGMA
SDS	Fluka
Sodiumhydroxide (NaOH)	Roth
Sodium-pyrophosphate	SIGMA
Succinate	SIGMA
Sucrose	AppliChem
Talon-beads	Clontech
TEMED	SIGMA
Tris-(hydroxymethyl)-aminomethane (Tris)	AppliChem
Triton-X100	SIGMA
Tween20	SIGMA
Urea	SIGMA
Whatman 3MM Paper	Schleicher & Schüll
X-ray film	Super RX Fuji
Yeast extract	DIFCO

2.1.3. Cell culture compounds

4-hydroxytamoxifene (4-OHT)	SIGMA
BAY43-9006	Bayer
BI-D1870	gift from N. Kraut (Boehringer-Ingelheim)
Cycloheximide	SIGMA
DMEM	Gibco
EGF	CellSystems Biotechnologie GmbH
FCS	PAN
H89	gift from Virchow Center
Imatinib	gift from A. Ullrich
Lipofectamine2000	Invitrogen

L-Glutamine	Gibco
MG132	SIGMA
Mitotracker Red CMX-ROS	Molecular Probes
Penicillin/Streptomycin	Gibco
phorbol 12-myristate 13-acetate (PMA)	Calbiochem
Rapamycin	SIGMA
Trypsin-EDTA	PAN
U0126	Promega
Valinomycine	SIGMA

2.1.4. Enzymes

Pfu polymerase	Stratagene
Proteinase K	SIGMA
Restriction Endonucleases	NEB
T4 Ligase	Fermentas
Trypsin	SIGMA
Trypsin inhibitor (Soybean)	SIGMA

2.1.5. Kits

ECL SuperSignal West Pico kit	Pierce
BCA-protein assay kit	Pierce
DyNAmo HS SYBR Green qPCR Kit	Finnzymes
First Strand cDNA Synthesis Kit	Fermentas
peqGOLD Trifast RNA preparation kit	peqlab GmbH
T7 TNT Quick coupled Transcription Translation system	Promega
QIAGEN Plasmid Kit (Maxi and Mini)	Qiagen
QIAquick Gel Extraction kit	Qiagen
QuickChange™ Site directed mutagenesis kit	Stratagene

2.1.6. Antibodies

rabbit α BAD	Cell Signaling
mouse α BAD pS112	Cell Signaling
rabbit α BAD pS136	Cell Signaling
rabbit α BAD pS155	Cell Signaling
rabbit α C-RAF (C20)	Santa Cruz
mouse α C-RAF	BD Bioscience
rabbit α B-RAF (C19)	Santa Cruz
rabbit α A-RAF (C20)	Santa Cruz
mouse α Cytochrome c	BD Bioscience

mouse α MEK	Transduction Lab
mouse α VDAC	Calbiochem
rabbit α TOM20	Santa Cruz
rabbit α ERK1/2	Santa Cruz
rabbit α ERK2	Santa Cruz
rabbit α pERK1/2	Santa Cruz
rabbit α PKB	Cell Signaling
rabbit α RSK	Santa Cruz
rabbit α ER	Santa Cruz
rabbit α Bcl-xL	Transduction Lab
rabbit α BIM	SIGMA
rabbit α Bak	Upstate
mouse α Tubulin	SIGMA
rabbit α Histon H3	Upstate
mouse α E-cadherin	SIGMA
rabbit α Grp98	gift from HC Drexler
Fluorescent secondary antibodies (coupled to Cy2 or Cy3)	Molecular Probes

2.1.7. Constructs

DsRED Mito	Clontech
pBABE puro-C-RAF BxB-ER	MSZ
pcDNA3-A-RAF wt	MSZ
pcDNA3-B-RAF wt	MSZ
pcDNA3-C-RAF wt	MSZ
pcDNA3-M-RAF	MSZ
pcDNA3-HA C-RAF BxB	MSZ
pGEM-Rieske	J. Rassow (Uni Bochum)
pEBG-murine BAD wt	Cell Signaling
pEGFP-human BAD wt	MSZ
pEGFP-human BAD ΔBH3 (ΔC62)	MSZ
6xHis Ubiquitin	R. Kopito (Stanford, CA)
6xHis Ubiquitin K48R	R. Kopito (Stanford, CA)

pcDNA3-B-RAF V600E (constitutively active B-RAF):
obtained by *in vitro* mutagenesis using pcDNA3-B-RAF wt as template. Primers:
V600E for: GGTCTAGCTACAGAGAAATCTCGATGG
V600E rev: CCATCGAGATTTCTCTGTAGCTAGACC

pcDNA3-C-RAF R89L (abrogates binding to RAS):
in vitro mutagenesis using pcDNA3-C-RAF wt as template; Primers:
R89L for: GCACTCAAGGTGCTGGGCCTGCAACCAGAG
R89L rev: CTCTGGTTGCAGGCCCAGCACCTTGAGTGC

pcDNA3-C-RAF DDED (constitutively active full length C-RAF):
two rounds of *in vitro* mutagenesis using pcDNA3-C-RAF wt as template; Primers:
338, 341 DD for: CGTGGACAGAGAGATGACAGCTATGATTGGGAAATAGAAGCC
338, 341 DD rev: GGCTTCTATTTCCCAATCATAGCTGTCATCTCTCTGTCCACG
491E, 494D for:
GGAGATTTTGGTTTGGCAGAAGTAAAGGACCGCTGGAGTGGTTCTCAGC
491E, 494D rev:
GCTGAGAACCACTCCAGCGGTCCTTTACTTCTGCCAAACCAAAATCTCC

pcDNA3-C-RAF DDED ΔN (constitutively active full length C-RAF without mitochondrial binding determinant):
obtained by PCR amplification with pcDNA3-C-RAF wt as template and cloning into pcDNA3 via the BamHI-XhoI sites using the primers:
for: AAAAAAAGGATCCAATGACCCAGGAGAAAAACAAAATTAGGCC,
rev: AAAAAAACTCGAGCTAGAAGACAGGCAGCCTCGGGG

pcDNA3-C-RAF S338A:
in vitro mutagenesis using pcDNA3-C-RAF wt as template; Primers:
for: CCTCGTGGACAGAGAGATGCAAGCTATTATTGGGAAATAGAAGC
rev: GCTTCTATTTCCCAATAATAGCTTGCATCTCTCTGTCCACGAGG

pcDNA3-C-RAF N-ter (1-323):
introducing a premature stop-codon into pcDNA3-C-RAF wt through *in vitro* mutagenesis;
Primer: for: GCGGGCACCAGTATCTTGAACCCAGGAGAAAAACAAAATTAGGC

rev: GGCCTAATTTTGTTTTTCTCCTGGGTTCAAGATACTGGTGCCCGC

pcDNA3-C-RAF C-ter (323-648):
obtained by PCR amplification with pcDNA3-C-RAF wt as template and cloning into pcDNA3 via the BamHI-XhoI sites using the primers:
for: AAAAAAAGGATCCAATGACCCAGGAGAAAAACAAAATTAGGCC,
rev: AAAAAAACTCGAGCTAGAAGACAGGCAGCCTCGGGG

The chimeric C-RAF / B-RAF constructs were prepared after PCR amplification and cloning of the C-RAF fragment into the EcoRI-NotI sites, and subsequently of B-RAF into NotI-XbaI sites of pcDNA3. The chimeras C-RAF(AA 1-50)-B-RAF(AA 150-766), C-RAF(AA 1-200)-B-RAF(AA 300-766), C-RAF(AA 1-300)-B-RAF(AA 400-766), resulting in the swapping of each part of BRAF into the equivalent domain of C-RAF, were constructed through PCR amplification of the different fragments using the following primers:

pcDNA3-C1-50 B150-766:
C1 for: AAAAAAGAATTCCACCATGGAGCACATACAGGGAGC
C50 rev: AAAAAAGCGGCCGCATCTGTGAGTTTGCCATCATC
B150 for: AAAAAAGCGGCCGCCTCACCACAAAAACCTATCGTTAG
B 766 rev: AAAAAATCTAGATCAGTGGACAGGAAACGCACC

pcDNA3-C1-200 B300-766:
in addition to C1 for and B766 rev
C200 rev: AAAAAAGCGGCCGCAGTGGAATTTGGAAACAATAAGAG
B300 for: AAAAAAGCGGCCGCCCCACAGGAAGAGGCGTCCTTAGC

pcDNA3-C1-300 B400-766:
in addition to C1 for and B766 rev
C300 rev: AAAAAAGCGGCCGCCAGATTGTTGGGGCTACTGG
B400 for: AAAAAAGCGGCCGCCACCCCCCCCTGCCTCATTACCTGGC

pcDNA3-B1-430 C323-648:
prepared by cloning PCR-amplified fragments of B-RAF (AA 1-430) together with C-RAF (AA 323-648), respectively, into the BamHI-NotI and NotI-XbaI restrictions sites of pcDNA3
B1 for: AAAAAGGATCCACCATGGCGGCGCTGAGCGGTGGCGGTGGTGGC
B430 rev: AAAAAGCGGCCGCTGAAGATGACTTCCTTTCTCGCTGAGGTCC
C323 for: AAAAAGCGGCCGCAACCCAGGAGAAAAACAAAATTAGGCC
C648 rev: AAAAAATCTAGACTAGAAGACAGGCAGCCTCGGGG

pBABE puro-C-RAF BxB K375W-ER (kinase dead version of C-RAF BxB):
in vitro mutagenesis using pBABE puro-C-RAF BxB-ER as template; Primers:
for: GGCACGGAGATGTTGCAGTATGGATCCTAAAGGTTGTCGACCCAACC
rev: GGTTGGGTCGACAACCTTTAGGATCCATACTGCAACATCTCCGTGCC

pEGFP-human BAD S75A and pEGFP- human BAD ΔBH3 S75A:
in vitro mutagenesis using pEGFP-human BAD wt as template; Primers:
for: CGGAGTCGCCACAGCGCCTACCCCGCGGGGACG
rev: CGTCCCCGCGGGGTAGGCGCTGTGGCGACTCCG

pcDNA3-myc-DN14-3-3ζ (dominant negative version of 14-3-3):
obtained by PCR amplification and cloning into pcDNA3 via the BamHI-XhoI sites and inserting a myc-tag at the N-terminus using the primers:
for: AAAAAAGGATCCACCATGGAACAGAAACTTATTTCTGAAGAAGATCTTTA
CCGTTACTTGGCTGAGGTTGCCGCT
rev: AAAAACTCGAGTTAATTTTCCCCTCCTTCTCCTGC

2.1.8. Cell lines

All cell lines used in this study were cultured in Dulbeccos Modified Eagle Medium (DMEM) with 10% FCS , 2 mM L-glutamine, and antibiotics (penicillin-streptomycin). All works using cell culture methods were perfomed under a sterile bench. Cell lines used were the following: NIH3T3 mouse fibroblasts; Human embryonic kidney 293 cells (HEK 293); the human derived cancer cell lines HeLa (cervix cancer), HT29 (colon cancer), PC12 (pheochromocytoma); Mouse embryonic fibroblasts (MEF) immortalized with the SV40 large T-antigen (clones K2: C-RAF -/- and K9: C-RAF +/+) were obtained from C. Pritchard (University of Leicester, UK) (Huser et al., 2001). NIH3T3 C-RAF BxB-ER is a clonal derivative of the NIH3T3 murine fibroblast cell line that expresses at stable, low level a fusion between C-RAF BxB and the hormone-binding domain of the estrogen receptor (Kerkhoff, Rapp, 1997). Kinase activity of C-RAF BxB can be induced by addition of 4-hydroxytamoxifene (4-OHT). Mice of the FVB strain used in these studies were kept and anaesthesized according to protocols approved by the animal care and use committee at University of Würzburg.

2.1.9. Solutions and buffers

Bacterial medium:

LB (Luria-Bertani) medium

10g/L Bacto-tryptone

10g/L NaCl

5g/L yeast extract

Adjust pH to 7.5 with NaOH

For plates, add 15 g Bacto-agar per liter

SOB-medium:

2%Bacto-tryptone

0,5% yeast extract

10mM NaCl

2,5mM KCl

10mM $MgCl_2$

10mM $MgSO_4$

ph 6,7 – 7,0

TB-buffer:

10mM PIPES

55mM $MnCl_2$

15mM $CaCl_2$

250mM KCl

adjust first pH to 6,7 and put subsequently $MnCl_2$.

Sterile filtrate the solution.

5xLämmli sample buffer:

31 mM Tris-HCl, pH6.8

1% SDS

5 % Glycerin

2.5 % β-Mercaptoethanol

0.05 % Bromphenolblue

50xTAE buffer (pH8,5):

2M Tris base

2M glacial acetic acid

100mM EDTA

10xSDS-PAGE running buffer:

		final concentration for 1x buffer:
60,6g	Tris	25mM
376g	Glycine	250mM
20g	SDS	0,1%

pH of 10x is ≈ 8,8

1x buffer has pH 8,3

SDS-PAGE stacking-gel buffer:

250mM Tris-HCl

0,2% SDS

pH 6,8

SDS-PAGE separating-gel buffer:

750mM Tris-HCl

0,2% SDS

pH 8,8

1xBlotting buffer:		**10xTBST:**	
25mM	Tris	500mM	Tris-HCl, pH 7,4
192mM	Glycine	1,5M	NaCl
15%	Methanol	0,05%	Tween20

2.2. Methods

2.2.1. Competent bacteria

2.2.1.1. Preparation of competent bacteria ($CaCl_2$-method)

A single bacterial colony was picked of an overnight incubated LB-Agar plate and 250ml SOB-medium were inoculated. Bacteria were grown at 18°C (165rpm) for about 2 days, until OD600 = 0,6. Then chilled bacteria were pelleted in a cooled Beckman centrifuge (J-6B) at 3000rpm for 10min and the pellet was resuspended in 80ml chilled TB-buffer. After 10min on ice, bacteria were pelleted again and resuspended in 20ml TB-buffer. 1,4ml DMSO were added and bacteria were incubated for 10min on ice. The suspension was aliquoted, frozen in liquid nitrogen and stored at -80°C.

2.2.1.2. Transformation of competent bacteria

Competent bacteria were thawed on ice and desired plasmid-DNA (~0,5-1µg) was added to 50µl of bacteria and mixed carefully. The microfuge tube was incubated on ice for 10min and bacteria were subsequently heatshocked at 42°C for 1min. The bacterial suspension was immediately put back on ice for 2min and 500µl of antibiotic-free LB-medium was added. The bacteria were incubated on 37°C, 600rpm on a thermomixer for 1hr. Thereafter, 100µl of the suspension were plated on an antibiotic-containing LB-plate. Only bacteria that have taken up the plasmid-DNA, which contains the antibiotic-resistence casette (mainly ampicillin or kanamycine), can grow on the agar plates. A single colony can be expanded in LB-medium and used for DNA-preparation.

2.2.2. DNA-methods

2.2.2.1. DNA-isolation from competent bacteria

To isolate DNA from transformed bacteria we used Quiagen Plasmid Kits (Maxi or Mini) according to the manufacturers protocols. Briefly, an alkaline lysis of bacteria is followed by the binding of the plasmid-DNA to an anion-exchange resin. RNA, proteins and other

impurities are removed in salt washes and the purified DNA is eluted with high salt conditions. The salt is removed by isopropanol precipitation and DNA is dissolved in a convenient volume.

2.2.2.2. Electrophoresis on agarose gel:

Double stranded DNA fragments and plasmids with a size between 0.5 kb and 10 kb can be separated according to their size on an agarose gel. Agarose is added to 1xTAE buffer to obtain a final concentration between 0,8-2%. The suspension is boiled in the microwave until the agarose is completely solubilized. Allow the agarose to cool down, then add ethidiumbromide up to a concentration of 0,5µg/ml and pour into the gel apparatus. DNA gel loading buffer was added to the DNA sample and applied on the gel. We electrophoresed in 1xTAE buffer at 100-140 Volts. The DNA can be visualised under UV-light through the incorporation of ethidiumbromide in the DNA.

2.2.2.3. Cloning of expression vectors

To clone a DNA-plasmid of choice the DNA-fragment can be either cut out of a preexisting vector and ligated into the chosen plasmid or the desired specific fragment is amplified by PCR (polymerase chain reaction) from copyDNA (cDNA) as template. PCR is based on the use of thermostabile DNA-polymerase which elongates DNA on a single strand by the use of small oligonucleotides (primer) as priming sites. The primer pairs bind specifically to the flanking regions of the DNA to be amplified Annealing of the primers depends on the content of GC and AT nucleotides within the sequence and has to be adjusted for every primer pair. Additionally, primers are designed to contain sequences that can be cleaved by endonucleases ensuring the subsequent ligation into respective plasmid sites. PCR is performed as a repeated cycle of DNA-denaturation (95°C), primer annealing (temperature depends on the primers used, usually 52°C-58°C) and DNA-elongation (68°C-72°C). The time for elongation is estimated by the length of the amplifying fragment and the polymerase used (1kb DNA in 1-2min). Theoratically, the DNA-fragment is doubled in every cycle resulting in an exponetial amplification. The following reaction was used as standard:

Template (1:100 dil. of 1µg/µl)	2µl
Primer 20 uM (100pmol/µl)	1µl each
dNTP's 10mM each	1µl
Buffer 10x	5µl
Pfu-Polymerase	1µl
Water	Up to 50µl

Cycles:

95°C	5 min	
95°C	1 min	
56°C	1 min	
72°C	4 min	➔ 30x

The resulting PCR product mixture was separated on an agarose gel and the expected fragment was excised. The DNA was extracted from the gel slice using the QIAquick Gel Extraction kit according to the provider´s protocol. The obtained DNA fragment and the recipient plamid-DNA were digested with the respective restriction endonucleases that cleave the cloning sites of both DNA´s.

Reaction:

2µg of plasmid DNA or 5µl of extracted DNA-fragment solution

5µl endonuclease buffer 10x (in case of double digestion preferred buffer for both enzymes)

100µg/ml BSA

2µl restriction endonuclease(s)

fill with H_2O to final volume of 50µl

The mix is then incubated for 2hrs on 37°C in a thermomixer with 300rpm.

The digestion was stopped with addition of DNA-loading buffer and the reaction mix was run on an agarose gel with subsequent extraction. The purified DNA-fragment and plasmid-DNA were ligated using different ratios of insert- and vector-DNA.

Reaction:

2µl T4 ligase buffer 10x

1µl T4 DNA ligase

insert	vector
0µl	3µl
3µl	3µl
6µl	3µl

fill with H_2O to final volume of 20µl

Reaction was incubated in a thermomixer at 19°C, 300rpm for 2hrs.

The complete ligation mixes were then transformed in competent bacteria and the DNA from resulting colonies was analyzed by DNA-sequencing.

To obtain point mutations we used the QuickChange™ Site directed mutagenesis kit according to the provider`s protocol.

2.2.3. RNA-method: RealTime-PCR

mRNAs were prepared using peqGOLD Trifast RNA preparation kit. Samples were subjected to DNAse treatment before preparation of cDNAs using random primers (First Strand cDNA Synthesis Kit). Gene expression was quantified following SYBR green incorporation (DyNAmo HS SYBR Green qPCR Kit) in a cycler Rotor Gene 2000. Target genes were run in triplicate, and control reactions without template were included. Real-time PCR was performed with the following primer pairs, designed in an intron-spanning manner to avoid interference with genomic DNA; BAD: GGACTTATCAGCCGAAGCAG; GCTCAAACTCTGGGATCTGG. HPRT: CACAGGACTAGAACACCT; GCTGGTGAAAAGGACCTCT. PCR conditions were optimized to ensure the specificity of the amplification, with 40 amplification cycles (95°C for 30sec, 58°C for 30sec, 72°C for 30sec). BAD expression was analyzed in relation to the house-keeping gene HPRT.

2.2.4. Protein methods

2.2.4.1. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Proteins can be seperated on the basis of their mass by electrophoresis in a polyacrylamide gel under denaturing conditions. 50-100µg of protein from a precleared lysate were denatured in Lämmli sample buffer by boiling for 5min at 99°C. The sample buffer contained β-mercaptoethanol to reduce disulfide bonds and the anionic detergent SDS, which disrupts nearly all noncovalent interactions of native proteins and gives them a highly negative charge. Polyacrylamide gels were prepared by pouring gel solutions between two glass plates and letting it polymerize. Gels were composed of two layers: a 6-15% seperating gel (pH 8,8), which separates proteins by their size, and a 4% stacking gel (pH 6,8) on top, that ensures the simultaneous entry of proteins into the seperating gel.

	Separating gel		**Stacking gel**
separating gel buffer	5ml	stacking gel buffer	2,5ml
H_2O bidest.	0 – 2,9ml	H_2O bidest.	1,8ml
Acrylamide	2,0 – 5,0ml	Acrylamide	650µl
TEMED	3µl	TEMED	8µl
10% APS	100µl	10% APS	37,5µl

The gel was placed in gel chamber, 1xSDS running buffer was added and the samples were loaded. The gel was run at constant 100V until the migration front reached the gel bottom. The negatively charged SDS-protein complexes migrate to the direction of the anode. The smaller the protein the faster it migrates through the gel, thereby creating the separation.

2.2.4.2. Immunoblotting

After the separation of samples by SDS-PAGE, proteins were transferred by electroblotting to nitrocellulose membranes. Therefore, the gel was assembled in direct contact with the nitrocellulose membrane flanked by whatman papers, and this sandwich was placed in a buffer filled blotting chamber. Electroblotting was done at 400mA for 60min to transfer proteins from the gel to the membrane. To ensure the quality of the transfer the nitrocellulose can be stained with Ponceau S. The nitrocelluose was then blocked in TBST containing 5% milk for 1hr at room temperature. After rinsing, the primary antibody solution was applied for an overnight incubation at 4°C to ensure the specific binding to the antigen on the nitrocellulose. Usually, antibodies were used in a dilution of 1:1000 in TBST containing 1% BSA and 0,05% azide. Then, the membrane was rinsed three times for 10min in TBST and incubated for 30min at room temperature with the respective peroxidase-coupled secondary antibody solution (1:5000 in TBST). After three further rinsing steps the membrane was subjected to the standard enhanced chemiluminesence reaction using an ECL detection kit. It is based on the peroxidase catalyzed oxidation of luminol leading to the emission of light detectable by x-ray films. Thus, the peroxidase coupled secondary antibody that bound to the primary antibody detects the protein of interest.

2.2.4.3. Immunoblot stripping

Nitrocellulose membranes can be reprobed with alternative antibodies when the prior primary antibodies are removed, referred to as stripping. We used an acidic buffer to interrupt the antibody-antigen interaction. The nitrocellulose was incubated two times for 15min in 100mM Glycine-HCl (pH2,8) and 0,1% SDS. After three washing steps the membrane can be reprobed according to the above protocol.

2.2.4.4. Immunoprecipitation

To analyze the association of proteins an immunoprecipitation was performed. For that $1{,}5x10^6$ NIH3T3 C-RAF BxB-ER cells were seeded in a 10cm dish and immunoprecipitation was done the next day under different conditions. Cells were lysed in the following buffer:
25mM Hepes NaOH pH7.4
100mM NaCl
5mM EDTA
1% NP40
50mM β-glycerophosphate

1mM orthovanadate
2mM Na-pyrophosphate and a protease inhibitors cocktail
Each dish was lysed in 500µl and cell lysates were incubated for 10min on ice with subsequent clearing at 16000xg for 15min. 500µg of lysate was adjusted to 1ml volume and 2µg of a polyclonal antibody was added. The lysates were incubated overnight at 4°C on a rotary mixer. The next day 10µl of equilibrated proteinA beads were added to each condition and incubated for 1hr at 4°C on the rotary. The beads were collected by centrifugation (800xg for 3min) and washed four times in buffer. Finally, dried beads were eluted in 20µl Lämmli sample buffer by boiling for 5min at 99°C. Precipitated proteins were analyzed by SDS-PAGE and immunoblotting.

2.2.4.5. Rabbit Reticulocyte Lysates

To obtain radiolabelled proteins Rabbit Reticulocyte Lysates were synthesized using the T7 TNT Quick coupled Transcription-Translation system, according to the manufacturer's instructions. Briefly, 40µl of reticulocyte lysate was mixed with 1µg of DNA, 2µl of ^{35}S labelled Cys-Met-mix (Redivue-Cys-Met mix) and filled with water to a final volume of 50µl. The reaction was performed for 1h at 30°C at 300rpm on a thermomixer. Radiolabelled proteins were loaded for SDS-PAGE and radioactivity was detected with the autoradiography enhancer En3hance with x-ray films.

2.2.5. Cell culture

2.2.5.1. Transfections

EXGEN (for HeLa): adjusted protocol for one well of a six-well plate ($1x10^5$ cells); for a 10cm-dish the amounts to be used were considered to be multiplied by four.
Two seperate mixtures: 1) 2µg DNA + 48µl of a 150mM sterile NaCl solution
2) 40µl of 150mM NaCl + 10µl EXGEN. 1) and 2) were mixed on a vortex and the solution was incubated for 7min at room temperature. Transfection mix was applied on cells for 3hrs in fresh complete DMEM. Then, cells were rinsed once with medium and left for expression.

Lipofectamine2000 (for NIH3T3): adjusted protocol for one well of a six-well plate; for a 10cm-dish the amounts to be used were considered to be multiplied by four.
$2,5x10^5$ cells were seeded and the transfection was performed the next day. Two seperate mixtures were made: 1) 100µl of DMEM (no antibiotics, with L-glutamine) + 1µg of DNA
2) 100µl of DMEM (no antibiotics, with L-glutamine) + 2,5µl of Lipofectamine2000

Both solutions were incubated for 5min at room temperature, then mixed and subsequently incubated for further 30min at room temperature. 800µl of DMEM (no antibiotics, with L-glutamine) were added and the mixture was applied on 1xPBS rinsed cells for 6hrs. Thereafter, the transfection mix was replaced with complete DMEM.

Ca^{2+}-phosphate-transfection (for HEK293):
$4x10^5$ cells were seeded in a 10cm dish and transfection was performed the next day. Transfection mixture was the following: 340µl H_2O + 20µg DNA + 40µl 2,5M $CaCl_2$; then apply dropwise 400µl of 2xBES and incubate mix for 15-20min at room temperature. Apply mix on cells, where fresh complete medium was put, and leave over night. Next day cells were rinsed once and left for expression in complete DMEM.

Electroporation:
A confluent 10cm dish of HUVEC´s was trypsinized and from 8ml suspension 500µl were used for electroporation in a Bio-Rad Gene PulserII. 10µg of DS Red Mito DNA was applied to cell suspension in a Bio-Rad electroporation cuvette. Cells were pulsed with 300V and 450 mFarrad and then plated in one well of a six-well plate. After attachment cells were rinsed once to remove cell debris.

2.2.5.2. Immunofluorescent labelling

Immunofluorescence labelling was performed according to a conventional protocol. Briefly, cells were fixed for 15min in 3,7% paraformaldehyd and quenched subsequently in 50mM NH_4Cl^+ in PBS for at least 5 min. The cells were then permeabilized in PBS buffer containing 0,1% TX100 and 0,5% BSA. Cells grown on glass coverslips, were then incubated for 30min in the primary antibody solution (antibody dilution 1:200 in PBS + 0,5% BSA) and for further 30min in corresponding fluorescent secondary antibody solution. Cells were then stained with 4',6-Diamidino-2-phenylindol (DAPI) for 5min and the coverslips were mounted using mowiol. NIH3T3 cells were transfected with vectors encoding untagged versions of each RAF isoform according to standard procedures (Lipofectamine2000). The following day, we performed a step of pre-permeabilization: cells were treated with digitonin (50µg/ml) in a buffer containing 10mM KCl, 1.5mM $MgCl_2$, 1mM EGTA, 1mM EDTA, 250mM sucrose, 20mM Hepes-KOH pH7.4, with protease inhibitors, for 4min on ice. The cells were subsequently fixed and processed for immunofluorescence according to the conventional protocol. Immunofluorescence pictures were acquired on a confocal microscope TCS SP2

(Leica) equipped with a HCX PL APO 63X NA 1.40 objective (Leica) under oil immersion. Following their acquisition, the images were combined and merged using the Photoshop software (Adobe).

2.2.5.3. Videomicroscopy

HUVECs were electroporated with the mitochondrial fluorescent marker DsRed-mito. Cells were filmed for 2hrs, in constant conditions of 5% CO_2 and 37°C, and observed by UV-lamp using an Axiovert 200 microscope equipped with shutter-controlled illumination (Carl Zeiss) and a cooled digital CCD camera (Roper Scientific) using a 40x lens. Images were processed using MetaMorph 2.0 image analysis software (Universal Imaging) and the QuickTime pro 7 software (Apple).

2.2.5.4. Electron microscopy

Cells grown on coverslips were fixed for 45min with 2.5% glutaraldehyde (50mM cacodylate pH 7.2, 50mM KCl, 2.5mM $MgCl_2$) at room temperature, fixed for 2 hours at 4°C with 2% OsO_4 buffered with 50mM cacodylate (pH 7.2), washed with H_2O and incubated overnight at 4°C with 0.5% uranyl acetate. The cells were dehydrated, embedded in Epon812 and ultrathin sectioned. The sections were analyzed with a Zeiss EM900.

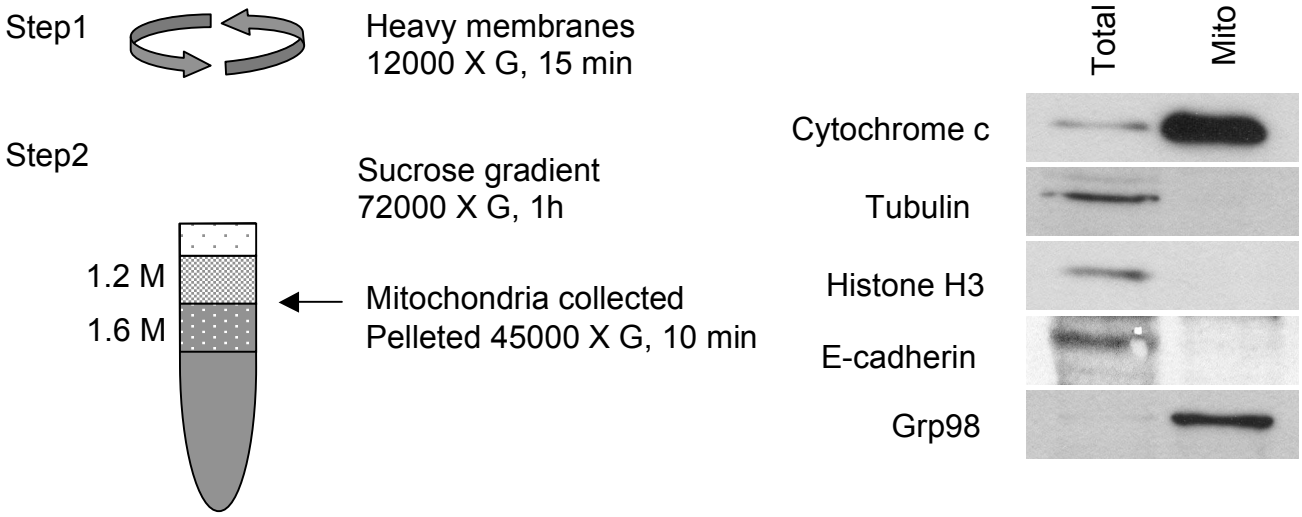
2.2.6. Experimental assays and treatments

2.2.6.1. Mitochondrial purification

Mitochondria were purified from different sources according to a protocol derived from Scaffidi et al. (1998). Cells were lysed in a mitochondrial isolation buffer containing 220mM mannitol, 70mM sucrose, 1mM EGTA, 2mg/ml BSA, 20mM Hepes-KOH pH7.4, with a protease inhibitor cocktail. Homogeneization was performed with 40 strokes in a tight-fitting Potter, and a post-nuclear supernatant was obtained after 5min of centrifugation at 1200xg. Heavy membranes were pelleted with an additional centrifugation at 12000xg for 15min. After resuspension in 1ml of MSM buffer (10mM KH_2PO_4, 300mM mannitol, 0.1% BSA pH 7.2), the membranes were applied on a sucrose gradient. Mitochondria were collected in the interphase between a lower layer consisting of 1.6M sucrose, 10mM KH_2PO_4, 0.1% BSA pH7.5, and a upper layer consisting of 1.2M sucrose, 10mM KH_2PO_4, 0.1% BSA pH7.5. The gradient was centrifuged at 72000xg in a swinging bucket rotor for 1hr. Mitochondria were reisolated by centrifugation at 45000xg for 10min, and resuspended in a storage buffer containing 250mM sucrose, 1mM EGTA, 5mM succinate, 1mM ATP, 0,08mM ADP, 2mM

KH_2PO_4, 10mM MOPS-KOH pH7.2. The protein concentration of this preparation was determined with a BCA kit.

The analysis of different markers revealed that this preparation is devoid of plasma membrane (E-cadherin), nuclear (histone H3), and cytoskeletal markers (tubulin) but contained membranes from the endoplasmic reticulum (Grp98). The mitochondria retained their functionality (polarity and ability to import intrinsic preproteins) after their isolation (data not shown).



2.2.6.2. *In vitro* assay for mitochondrial binding and import

For each mitochondrial binding reaction, we mixed 50µg of mitochondrial proteins with 10µl reticulocyte lysate in a binding buffer consisting of 55mM mannitol, 18mM sucrose, 80mM KOAc, 8mM $MgCl_2$, 1mM DTT, 20mM Hepes-KOH pH7.4, 0.5mg/ml BSA. Unless stated otherwise, the samples were incubated for 15min on ice. After a brief centrifugation, the mitochondrial pellets were rinsed, reisolated, and resuspended in sample buffer. Following separation on SDS-PAGE, radioactivity was detected with the autoradiography enhancer En3hance with x-ray films. The results were quantified using the software ImageJ (NIH) and expressed as % of input bound.

To test their ability to import RAF kinases, mitochondria were incubated with RAF kinases in the same buffer with 5mM succinate, 1mM ATP, and 0,08mM ADP. The import reactions were performed at 30°C for 20min. Following the reisolation of mitochondria, proteinase K was applied on ice for 10min (10µg/ml final concentration). Proteolysis was stopped with

addition of 1mM PMSF, and the samples were analyzed by autoradiography as previously described.

2.2.6.3. Mitochondrial treatments

Trypsinization: in order to remove proteins from the surface of mitochondria, preparations of this purified organelles were incubated with 20µg/ml trypsin for 15min on ice in storage buffer. Proteolysis was stopped with the addition of 500µg/ml soybean tryptic inhibitor, and mitochondria were reisolated and used for binding assays. EDTA treatment: mitochondria were pretreated for 5min with 20mM EDTA on ice, and later incubated with lysates, again in the presence of EDTA.

2.2.6.4. Anthrax Lethal Toxin (LT) purification and use on cells

The two components of LT, protective antigen (PA) and lethal factor (LF) were produced and purified separately as recombinant proteins in *E. coli*. Both proteins were obtained in an 6xHis-tagged form and purified by immobilized-metal affinity chromatography (IMAC) (Chelating Fast Flow, Amersham Biosciences). PA was further purified on a mono-Q column (Amersham Biosciences). Following their purification, the proteins were concentrated and dialyzed in 25mM Tris-HCl pH7.4, 250mM NaCl. PA and LF were simultaneously applied on cells, at final concentrations of 3µg/ml and 1µg/ml respectively, in normal culture medium. Under these conditions, we found that LT provides a complete and sustained inhibition of the MEK activity in NIH3T3 cells, while no apoptosis could be detected after up to 24hrs of treatment.

2.2.6.5. Analysis of BAD phosphorylation in NIH3T3 C-RAF BxB-ER cells

To probe the phosphorylation of each residue of BAD, $1x10^6$ cells were seeded in a 10cm dish and the next day transiently transfected with the pEBG-BAD construct, encoding a GST-tagged form of murine BAD. Transfections were performed using Lipofectamine2000. After overnight expression, cells were initially serum starved for 2hrs and C-RAF kinase activity was induced with 1µM 4-OHT. Cells were lysed in 500µl of a buffer containing 20mM Hepes-NaOH pH7.4, 150mM NaCl, 1mM EDTA, 1% NP40, 1mM orthovanadate, 50mM β-glycerophosphate, with a cocktail of protease inhibitors. The cleared lysates were used for immobilization of GST-BAD to equilibrated glutathione beads (20µl). After 1-2hrs of incubation at 4°C on a rotary, GST-beads were collected by centrifugation (800xg for 3min) and washed three times in buffer. Dried beads were eluted in Lämmli sample buffer by

boiling for 5min at 99°C. BAD phosphorylation was analyzed by immunoblotting with phospho-specific antibodies.

2.2.6.6. Analysis of BAD ubiquitylation

$4x10^6$ HEK293 cells were seeded in a 10cm-dish and co-transfected the next day with expression vectors encoding GFP-BAD, the active C-RAF BXB mutant, and a 6xHis-tagged ubiquitin using a calcium phosphate precipitation protocol. After overnight expression, cells were rinsed once in PBS and lyzed in 1ml of a buffer containing 8M Urea, 20mM Tris-HCl pH 8, 150mM NaCl, 10mM imidazole, 0.1% Triton X-100, 0.5% deoxycholate, 0.1% SDS and 20mM iodoacetamide at room temperature. His-ubiquitin conjugates were purified on a cobalt resin (Talon-beads) for 2hrs. Beads were washed three times in buffer and ubiquitylated proteins were detected by immunoblot.

2.2.6.7. Statistics

Statistical significance was determined with the one-tailed, paired Student's t test.

Results

3.1. C-RAF specifically interacts with mitochondria

3.1.1 RAF kinases exhibit distinct localizations on cellular membrane organelles

3.1.1.1. Immunofluorescence and cell fractionation

The RAF signaling cascade is subject of complex organization. One important parameter is the localization of RAF kinases to cellular membranes, but no direct comparison between all RAF isoforms has been performed yet. In order to analyze simultanously the membrane localization of all RAF-isoforms, we devised a strategy to solve the following technical obstacles: i) RAF kinases are expressed at low endogenous levels and the isoforms exhibit non-overlapping, tissue specific expression patterns, ii) under normal growth conditions RAF kinases tend to heterodimerize (Weber et al., 2001; Garnett et al., 2005; Rushworth et al., 2006), rendering the analysis of the individual isoforms difficult, iii) RAF kinases are predominantly present as a cytoplasmic pool. To compare directly the membrane localization of the RAF isoforms we overexpressed them in HeLa cells and applied conditions of serum starvation to prevent heterodimerization. We included then, immediately before fixation of the coverslips, a step of mild pre-permeabilization in order to eliminate the cytoplasmic pool of the kinases. These experiments revealed a striking specificity for different intracellular membranes for each RAF isoform (Fig. 7). Clearly, C-RAF was the only isoform that was detected at the level of the mitochondria (Fig. 7A-C), and to a lesser extent at the nuclear envelope. Neither A-RAF (Fig. 7D-F), B-RAF (Fig. 7G-I) nor KSR (Kinase Suppressor of Ras, a structurally closely related molecule to the RAF kinases) (data not shown), could be colocalized with mitochondria. While A-RAF fluorescence highlighted intracellular tubules and vesicles that were not mitochondrial, and KSR was present on the Golgi apparatus (data not shown), B-RAF did not associate with intracellular membranes of permeabilized cells.

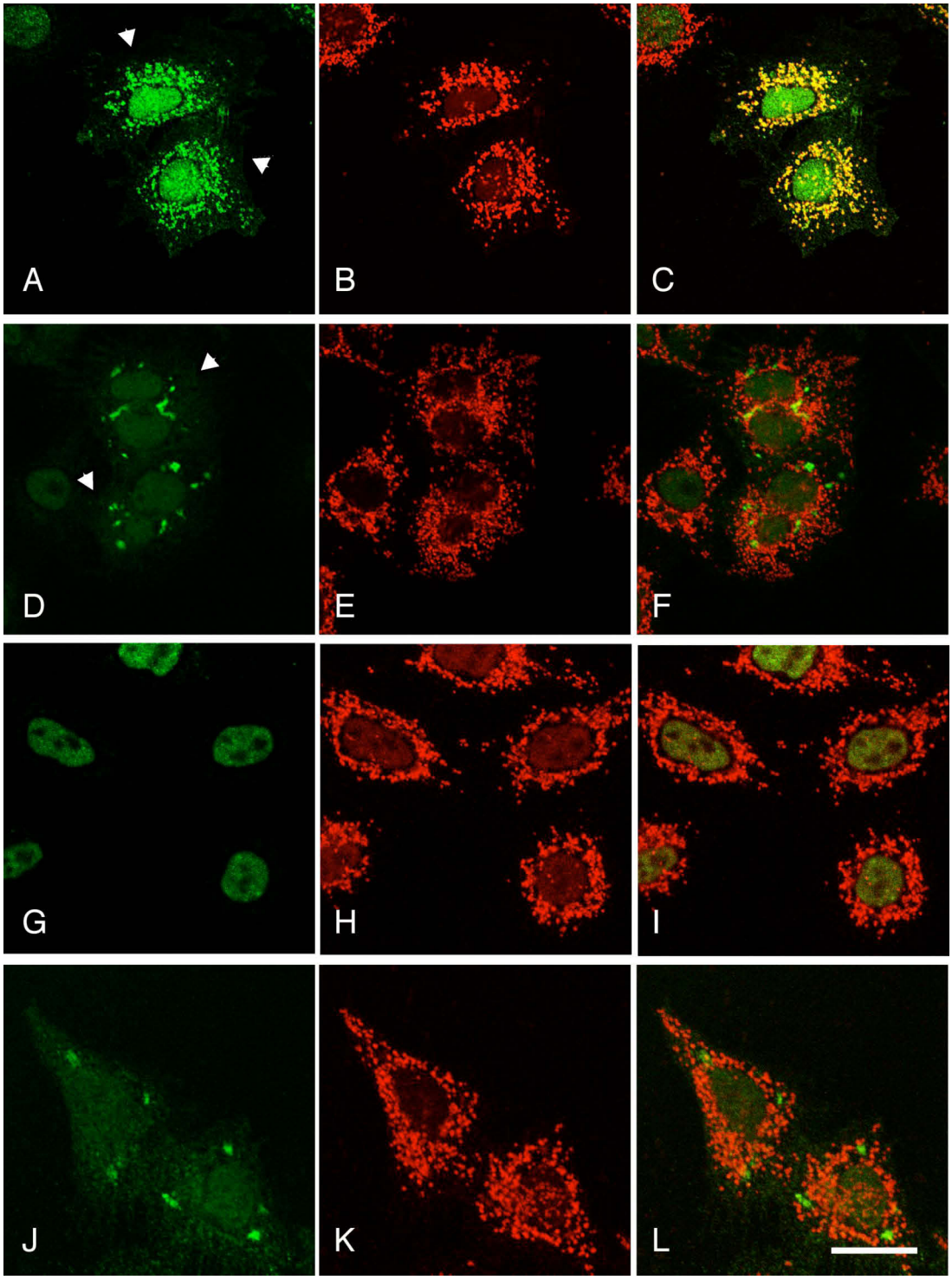


Figure 7: RAF kinases exhibit distinct distributions on intracellular membranes of eukaryotic cells, and only C-RAF is present at the mitochondrial level.
HeLa cells overexpressing C-RAF (A-C), A-RAF (D-F), or B-RAF (G-I) were serum-starved for 18h, permeabilized with digitonin, fixed, and processed for the immunolocalization of each isoform of RAF (green fluorescence) and mitochondria (cytochrome c, in red) with relevant antibodies. Transfected cells are indicated with white arrows. Cells that were transfected with B-RAF could not be identified after the digitonin extraction step (panel G), indicating that B-RAF was not present on intracellular membrane organelles. In panels J to L, HeLa cells were cotransfected with C-RAF and A-RAF and processed as previously. A-RAF distribution is shown in panel J (green), while C-RAF is shown in K (red). The overlay presented in L, indicates the absence of overlap between the membrane domains decorated by these two kinases. Note that the fragmented appearance of mitochondria is a consequence of the pre-permeabilization protocol used here. (bar: 20µm)

To obtain direct evidence that members of the RAF kinase's family exhibit distinct subcellular localization, we coexpressed C-RAF and A-RAF, and analyzed simultaneously the localization of both kinases using again the pre-permeabilization procedure (Fig. 7J-L): we noticed a complete lack of overlap between A-RAF and C-RAF, thereby confirming at the single cell level that these kinases target different intracellular membranes.
Immunoblots of purified mitochondria further confirmed that, at endogenous expression levels, C-RAF is the only isoform present in significant amounts on mitochondria of serum-starved cells (Fig. 8A). Collectively, these findings revealed the existence of distinct membrane localizations of the RAF kinases and the preferential localization of C-RAF to mitochondria.

3.1.1.2. C-RAF efficiently binds to purified mitochondria *in vitro*

To gain more insight into the interaction of RAF kinases with mitochondria, we decided to analyze this interaction *in vitro*. RAF kinases were produced and radiolabelled with ^{35}S-Cys / Met by *in vitro* transcription/translation in reticulocyte lysates (as initially reported by Stancato et al., 1993), and mixed with purified mitochondria obtained from human embryonic kidney 293 cells. Autoradiographic analysis revealed that the RAF isoforms exhibit different abilities to associate with mitochondria (Fig. 8B): while C-RAF associated with mitochondria in a fast and efficient fashion, the two other isoforms did not exhibit a significant binding and behaved in fact like the kinase MEK, included as a negative control in this assay (Fig. 8B). Under the conditions of this assay, a 15 min incubation time allowed maximal binding. The binding efficiency of C-RAF, measured as a percentage of the input bound at this time point, was more than 2 fold higher than A-RAF, and more than 5 fold higher than B-RAF (Fig. 8C). Preferential mitochondrial binding of C-RAF was not only detected with mitochondria obtained from transformed cells in culture. We extended our analysis to mitochondria obtained from a normal tissue, in this case from mouse liver (Fig. 8D). Again, we noticed a

clear difference between C-RAF and the two other isoforms: C-RAF bound almost 5 fold more efficiently to mitochondria than B-RAF and A-RAF (Fig. 8D).

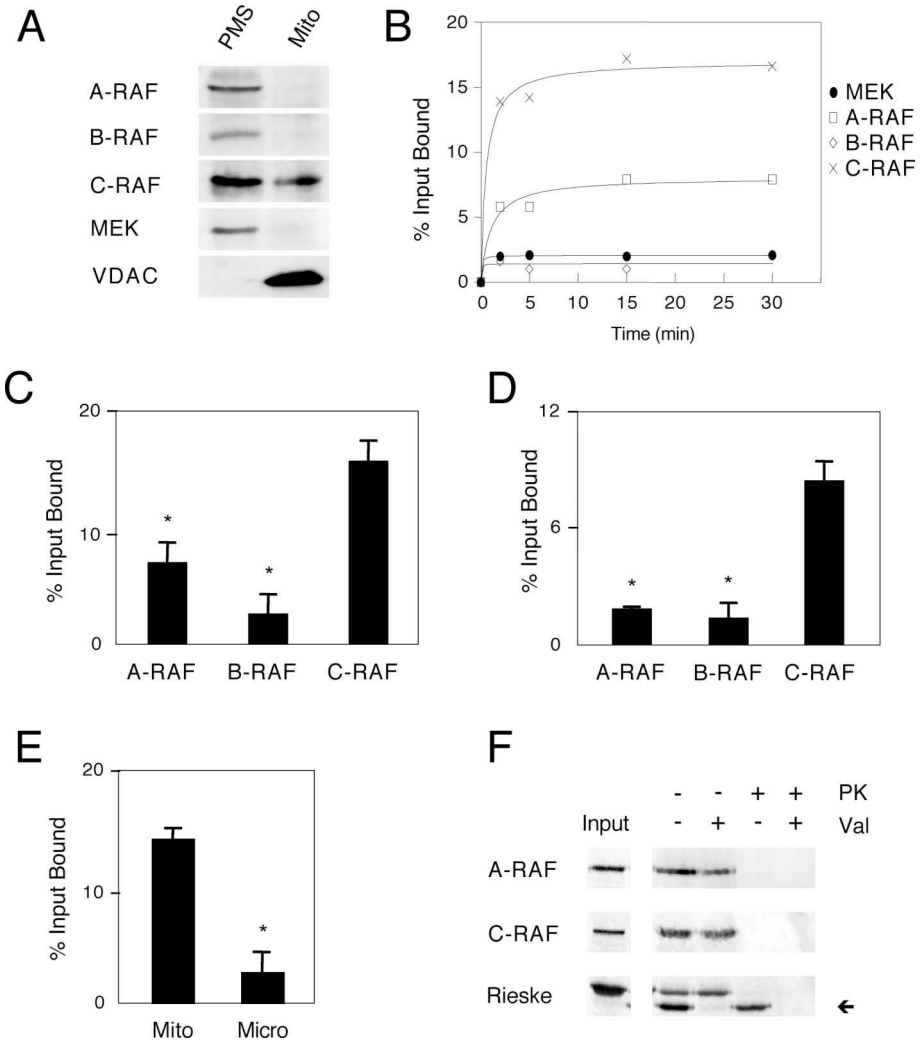


Figure 8: C-RAF efficiently interacts with purified mitochondria.
A: C-RAF is the only isoform detectable in mitochondria prepared from 293 cells. Equal amounts (50µg) of purified mitochondria (Mito) and post-mitochondrial supernatant (PMS) were compared by immunoblot for their content in the different isoforms of the RAF kinase family. The Voltage Dependent-Anion Chanel (VDAC) is used here as a mitochondrial marker. B: Time course analysis of RAF kinases binding to mitochondria: A-RAF, B-RAF, C-RAF and MEK produced as reticulocyte lysates were incubated with mitochondria purified from HEK293 cells, and the amount of radioactively-labelled RAF that bound this organelle was analyzed over the indicated time points (2 to 30min). The results are expressed as % of input bound. C. A statistical analysis based on 3 independent experiments (mean + SD). The binding of the different isoforms of RAF were compared at 15 min (* for $p<0,01$ compared to C-RAF). D. Binding of RAF kinases to purified mouse liver mitochondria: Mitochondria obtained from mouse liver were used as described previously to analyze their ability to interact with RAF kinases (* $p<0,05$ compared to C-RAF). E: Lack of C-RAF binding to purified microsomal membranes. Reticulocyte lysates containing C-RAF were incubated with purified mitochondria or with microsomes (Micro), under the same conditions. Results are expressed as % of input bound, based on two independent experiments (mean + SD, * $p<0,02$ compared to mitochondria) F. RAF kinases are not imported in vitro inside purified mitochondria. The iron-sulfur protein Rieske was used as a positive control for mitochondrial import: the appearance of a lower band (arrow) indicates that the signal sequence of Rieske has been processed. This band is proteinase K (PK)-resistant, and does not appear when mitochondrial potential is abolished with valinomycine (1µM). In conditions where Rieske protein was imported, we found that the RAF kinases remained accessible to PK, indicating that they were associated with the cytosolic side of mitochondria.

We verified the specificity of our binding assay by replacing mitochondria with purified microsomal membranes (Fig. 8E), and detected no binding of C-RAF to microsomes, suggesting that the experiments presented indeed reflected C-RAF-specific interaction with mitochondria.

Since the possibility that one of the RAF kinase isoforms (A-RAF) might be partially present inside mitochondria had been proposed by Yuryev et al. (2000), we decided to test if RAF kinases would be imported into this organelle. We tested this possibility by performing proteolytic treatments of mitochondria that had been incubated with RAF kinases and we observed that none of the RAF kinases demonstrated resistance to externally added protease upon incubation with mitochondria (Fig. 8F). Therefore, RAF kinases were not imported inside mitochondria *in vitro*. At this point of the work, we concluded that C-RAF exhibits a specific capacity to bind to the surface of mitochondria. These results strongly confirmed our initial observation that RAF kinases have different tropism for membrane organelles and encouraged us to use further our acellular system to learn more about C-RAF interaction with mitochondria.

To examine the possibility that the activation of these kinases, for example obtained through the introduction of an oncogenic mutation, might interfere with their recruitment on mitochondria, we compared C-RAF and B-RAF wild-type with their oncogenic versions C-RAF DDED (mutated on the residues 338, 341, 491, 494) (Chong et al., 2001) and B-RAF V600E. We found that these mutations did not alter the mitochondrial binding properties of either C-RAF or B-RAF (Fig. 9A). We also focused our attention on the role of Ser338, a residue whose phosphorylation had previously been proposed to constitute a mitochondrial targeting signal (Alavi et al., 2003; Jin et al., 2005). Neither a non-phosphorylatable mutant at this position (C-RAF S338A) (Fig. 9B) nor a phosphomimetic mutant (C-RAF S338, 339D) changed the binding properties of C-RAF to mitochondria (data not shown). These findings

indicated that the phosphorylation of Ser 338 is neither necessary nor sufficient for the mitochondrial targeting of C-RAF. We concluded that the conformational changes that accompany the activation of RAF kinases do not change their affinity for mitochondria.

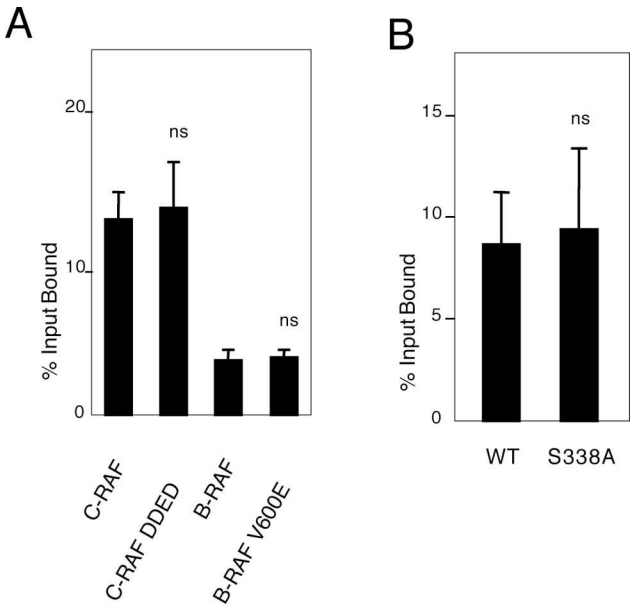


Figure 9: The introduction of oncogenic mutations and the phosphorylation of C-RAF on the residue Ser338 do not change the interaction of C-RAF with mitochondria.

A: B-RAF and C-RAF wild-type were compared with the oncogenic mutants C-RAF DDED (in which phosphomimetic mutations were introduced on the residues 338, 341, 491, 494) and B-RAF V600E. The mitochondrial binding was determined as described previously. The results presented here are based on two independent experiments (ns: non significant compared to wild-type B-RAF and C-RAF). B. Role of Serine 338 phosphorylation. C-RAF S338A was compared to C-RAF wild-type for its ability to bind mitochondria *in vitro*.

To address directly the possibility that C-RAF affinity for mitochondria might be an intrinsic property of this isoform, we decided to identify the sequence determinants that would account for it. We constructed a set of vectors allowing the expression of different parts of C-RAF and B-RAF, as well as chimeras between these two kinases (Fig. 10). Each protein was synthesized and radiolabelled *in vitro* in reticulocyte lysates, and incubated with purified mitochondria as before. Interestingly, all chimeras in which the first 100 amino-terminal residues of B-RAF were substituted with equivalent residues of C-RAF showed an increased recruitment to mitochondria (Fig.10). These results indicated that the amino-terminal regulatory region of RAF kinases determines their mitochondrial affinity, a point that is of

interest considering that this region concentrates almost all sequence divergences between the RAF isoforms. To determine directly which part of the C-RAF kinase binds mitochondria, two constructs covering the carboxy-terminus of C-RAF (AA 324-648) or its amino-terminus (AA 1-323) were compared in this assay. This analysis revealed that the amino-terminal domain of C-RAF binds efficiently to mitochondria, a finding that suggested that the amino-terminus directly confers its mitochondrial interaction properties to the entire C-RAF kinase.

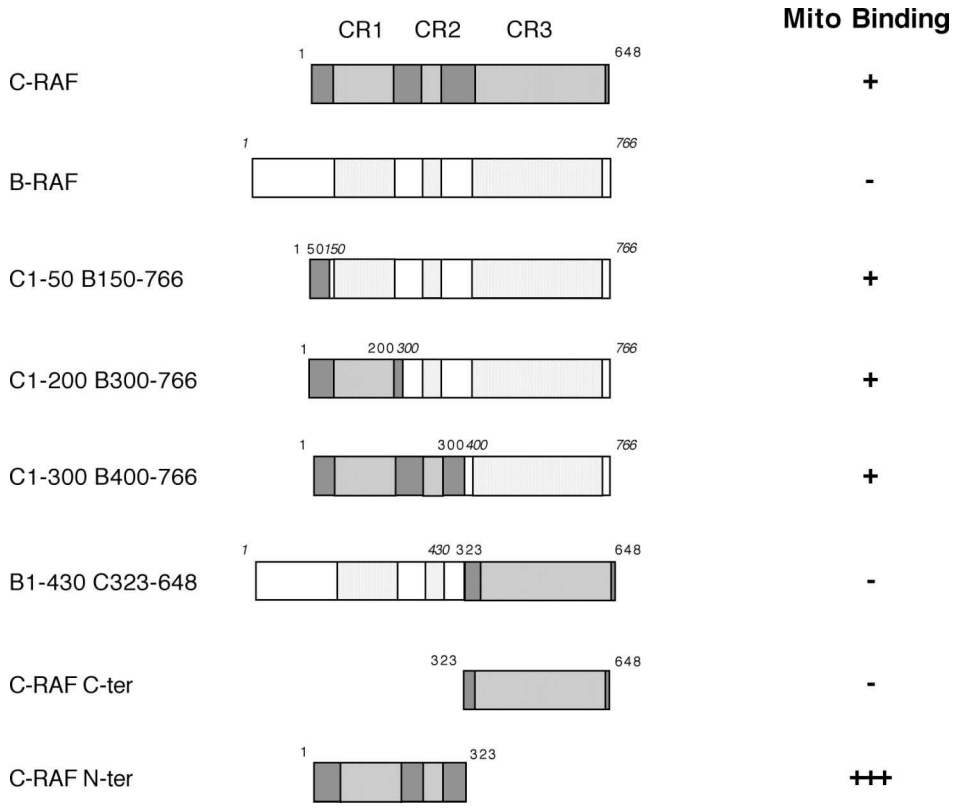


Figure 10: Identification of the region determining C-RAF ability to bind mitochondria.

A set of chimeric proteins between C-RAF and B-RAF were constructed, as well as different fragments of C-RAF. Each protein was produced as reticulocyte lysate and used for mitochondrial binding. Amino-acid positions are given for C-RAF (normal characters) and B- RAF (italics). The conserved regions of each RAF kinase are indicated as CR1 to CR3. Each protein was tested at least in two independent experiments. The results are summarized here: - indicates less than 10% of input bound, + between 10 and 25 %, +++ more than 50 %

RAF kinases, and in particular C-RAF, can interact *in vitro* both with proteins and also with lipid mixtures (Hekman et al., 2002). To determine which of these molecules C-RAF would recognize, we treated purified mitochondria with trypsin: this procedure, which removed the

proteins of the mitochondrial outer membrane (BCL-XL) but left intact intrinsic mitochondrial proteins (the matrix protein MnSOD), almost completely abolished the recruitment of C-RAF (Fig. 11A). Therefore, we concluded that C-RAF interacts with proteins of the mitochondrial surface. Small GTPases of the RAS family constitute a prime candidate as binding partners for RAF kinases at this level. To test their contribution to the recruitment of C-RAF, we used the following approaches: i) we tested the effect of Mg^{2+} chelation by EDTA, a procedure that triggers the transition of small GTPases to an inactive, nucleotide-free form (Fig. 11B), ii) we compared the binding of C-RAF WT with the mutant R89L (Fig. 11C), a mutation that abrogates the binding of C-RAF to activated RAS (Bondeva et al., 2002). Clearly, neither of these two procedures abrogated the binding of C-RAF to mitochondria. We confirmed this observation in cell culture by showing that the mutant C-RAF R89L localizes with the same efficiency to mitochondria like C-RAF WT (data not shown). We concluded that, under the conditions of our assay, protein binding partners other than RAS GTPases account for most of the binding of C-RAF to mitochondria.

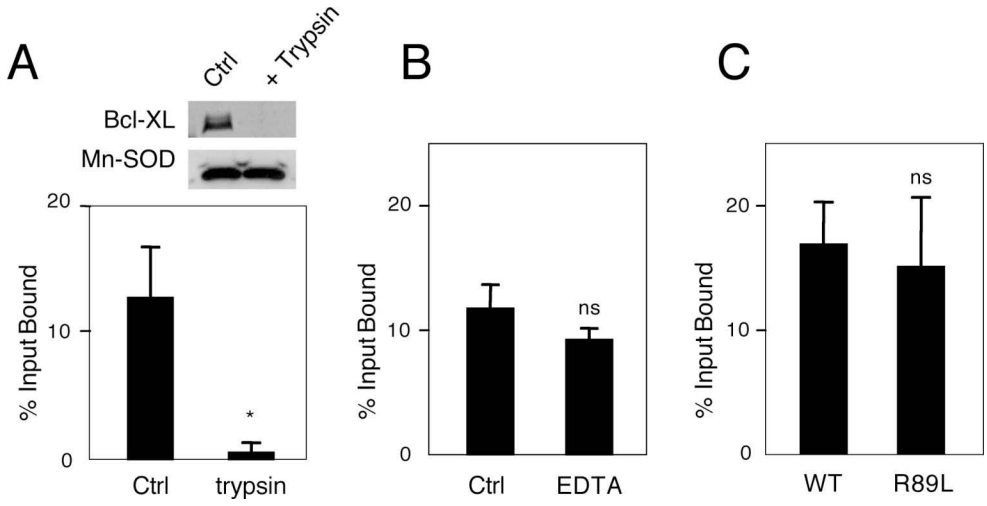


Figure 11: Role of mitochondrial surface proteins and of the small GTPases of the RAS family as interaction partners for C-RAF.

A: Mitochondrial surface proteins are required for C-RAF binding. Purified mitochondria were treated with trypsin. To monitor the selective removal of mitochondrial surface proteins by this protocol, mitochondria were analyzed by immunoblot for their content of proteins of the mitochondrial surface (BCL-XL) or matrix (MnSOD). Trypsinized mitochondria exhibit a drastically reduced ability to bind C-RAF (* $p<0,01$ compared to ctrl). B: EDTA treatment. Mitochondria were pre-treated on ice with 20 mM EDTA, and used later for a binding assay with C-RAF. „ns“ indicates the lack of significant difference compared to ctrl. C: Mitochondrial interaction properties of the mutant C-RAF R89L. A mutant C-RAF R89L, which abolishes the interaction of C-RAF with RAS GTPases, was compared to C-RAF WT for its ability to bind mitochondria *in vitro*.

3.2. Cellular effects of C-RAF at the level of mitochondria:

Active C-RAF specifically changes mitochondria's subcellular distribution in a MEK-dependent fashion

To analyze the consequences of C-RAF activation on mitochondria, we decided to use an inducible system, allowing a kinetic analysis of the consequences of C-RAF activation. We used a cell line stably expressing low levels of a fusion protein between an activated form of C-RAF (C-RAF-BXB) and the hormone-binding domain of the estrogen receptor (Kerkhoff and Rapp, 1997). This oncogenic version of C-RAF, which consists of an internal deletion of its Ras-binding domain (between AA 65 and 304), still bound mitochondria with high affinity *in vitro* and *in vivo* (data not shown). In this system, C-RAF activation could be achieved through the application of the estrogen receptor agonist 4-hydroxytamoxifene (4-OHT, 1 μM). Using this system, we observed that C-RAF activation causes an intense remodeling of this organelle, characterized by a change from long, filamentous to short, spherical mitochondria clustered around the nucleus. The activation of C-RAF was sufficient to produce this mitochondrial remodeling, because it could be observed in serum-free conditions (Fig 12A-D). Transmission electron microscopy confirmed these observations (Fig. 12E,F): while mitochondria from control cells were visible as longitudinal as well as cross-sections, we only observed spherical elements in cells in which C-RAF had been activated for 12h. In spite of this intense change in shape and distribution, the inner architecture of mitochondria remained normal (Fig. 12E,F). In these conditions, mitochondrial remodeling occurred progressively upon 4-OHT addition, and was systematically noticed after 12h of treatment (data not shown). At the same time, all mitochondrial markers that we tested remained at a stable level (data not shown). The distribution of other membrane organelles was insensitive to C-RAF activation: early endosomes, labelled with the fluorescent marker GFP-Rab5, were not redistributed (data not shown).

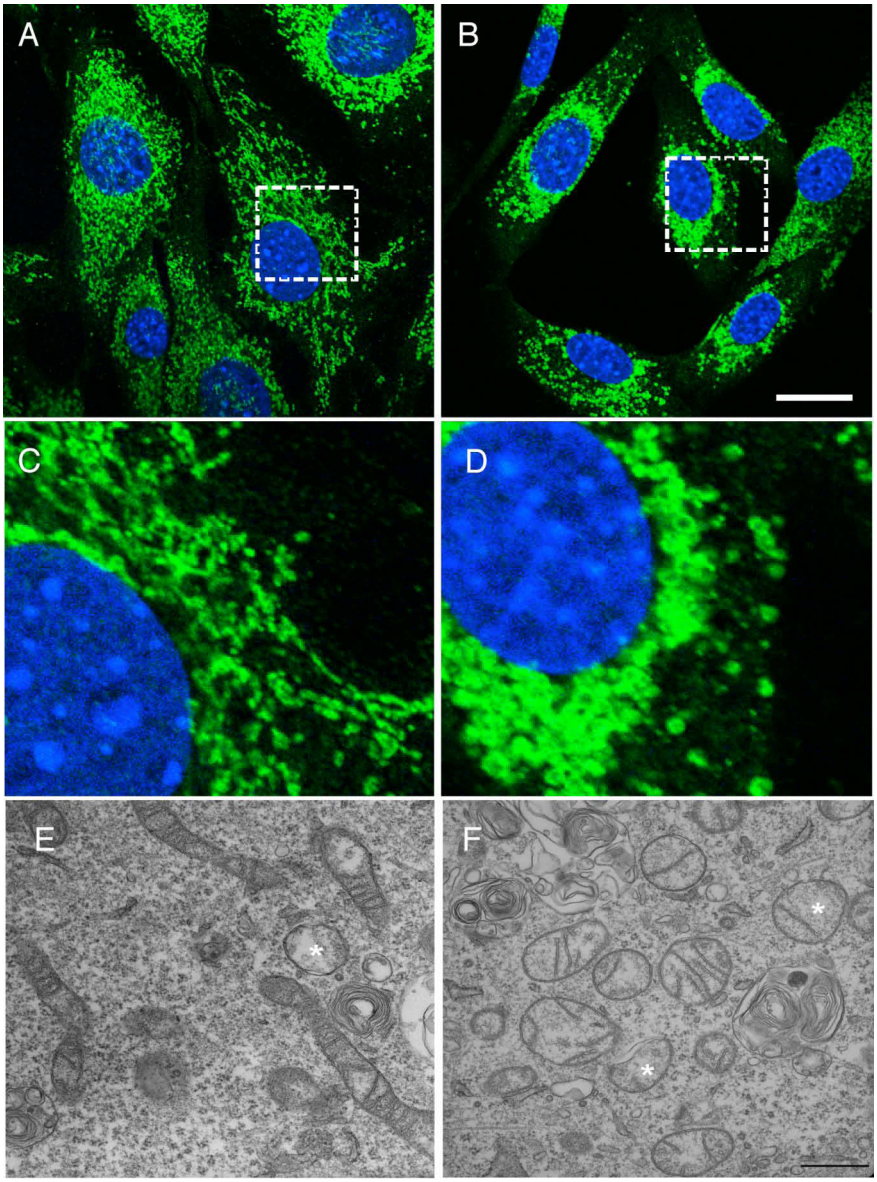


Figure 12: C-RAF activation changes mitochondrial subcellular distribution.
A-D. NIH 3T3 cells stably expressing low levels of an activatable version of C-RAF (C-RAF BXB-ER, fused with the hormone-binding domain of the estrogen receptor) were used to analyze kinetically the effects of C-RAF activation on mitochondria. Cells placed in conditions of serum starvation were either incubated with 1µM 4-OHT for 12h or a vehicle. The pictures are representative findings that were obtained upon confocal examination after staining for the mitochondrial marker Tom20 (green) and nuclei (DAPI, blue) without (panels A and C) or after C-RAF activation (panels B and D). In panels C and D, we present close- ups corresponding to the area indicated with boxes in A and B. E, F: Mitochondrial ultrastructure in control cells and after C-RAF activation. In control cells (panel E) the majority of mitochondria have an elongated shape and only few mitochondria with spherical profiles are visible (indicated here with an asterisk). After 12hrs of activation of C-RAF by addition of 4-OHT (panel F), we noticed predominantly spherical mitochondria (bar: 0,5µm).

Changes in mitochondrial morphology, such as fragmentation and perinuclear clustering, are common findings in cells that undergo apoptosis, or the loss of mitochondrial membrane potential. To rule out the contribution of a form of toxicity, we checked that mitochondria

from these cells retained cytochrome c and a normal uptake of mitotracker, a dye whose uptake is sensitive to $\Delta\Psi m$ (data not shown), suggesting that these organelles had kept their functionality. In addition, we verified that the phenotype induced by C-RAF activation was not abolished in the presence of zVAD-fmk, a broad spectrum inhibitor of caspases (data not shown). The remodeling was not dependent on transcriptional events, because actinomycin D (1μg/ml) applied at the time of C-RAF activation did not prevent its occurrence (data not shown). Collectively, these findings suggested that mitochondrial remodeling was possibly a direct consequence of C-RAF activation.

To analyze the contribution of the kinase MEK, the classical effector for the kinases of the RAF family, we used two different inhibitory approaches: the chemical inhibitor U0126, and the bacterial toxin LT from *Bacillus anthracis*. LT is a protease that cleaves MAPKK at their amino-terminus in a potent and specific fashion (Duesbery et al., 1998; Chopra et al., 2003). This cleavage reduces the affinity of MEKs for their substrates ERKs as well as their intrinsic kinase activity (Chopra et al., 2003). Compared to the other approach that can be used to inhibit the kinase MEK, LT exhibits two important advantages: i) its specificity, conferred by the enzymatic nature of its activity, and ii) LT affords a direct monitoring of the inhibition of MEK, through the visualization of an electrophoretic mobility shift consecutive to its cleavage. In our hands, both U0126 and LT resulted in a complete inhibition of the mitochondrial remodeling induced by activated C-RAF (data not shown).

To relate directly the property of C-RAF to remodel mitochondria with the ability of this kinase to interact with these organelles, we compared the distribution of mitochondria in NIH3T3 cells expressing an active version of C-RAF (C-RAF DDED) with different mutants that either abolished mitochondrial binding (C-RAF DDED ΔN, a deletion of C-RAF N-terminus), or strictly restricted its localization to mitochondria (the mutant M-RAF, a fusion

of C-RAF BXB with the mitochondrial localization determinant of the protein Tom70, previously reported in Wang et al., 1996) (Fig.13).

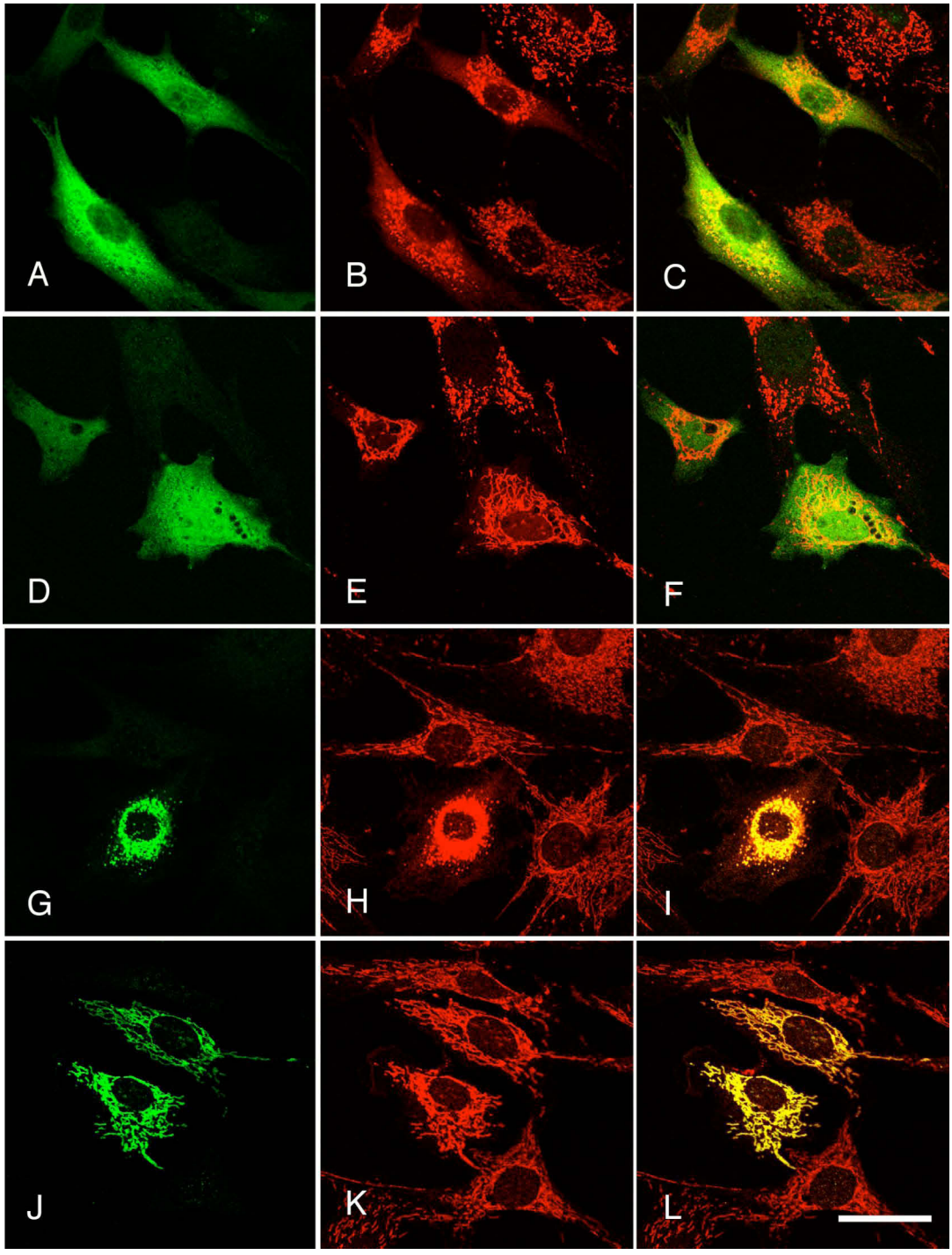


Figure 13: Mitochondrial remodeling depends on C-RAF´s mitochondrial binding properties.

NIH 3T3 cells were transfected with an active version of C-RAF (C-RAF DDED, panels A-C), the same mutant in which the mitochondrial binding region was deleted (C-RAF DDED DN panels D-F), or a mutant in which an active C-RAF is fused to the constitutive mitochondrial targeting determinant of Tom70 (M-RAF , panels G-L). In the experiments presented in the panels J-L, anthrax LT was applied immediately after transfection. After overnight expression, cells were processed for simultaneous immunolocalization of C-RAF (green) and mitochondria (cytochrome c, red) (bar: 20µm).

All constructs were expressed at high level, and turned-out to be functional kinases based on the increased levels of phospho-ERK that were measured upon their intracellular expression. While the expression of C-RAF DDED led to a clear mitochondrial remodeling consisting in the disappearance of all filamentous aspects (Fig. 13A-C), neither an activated A-RAF (A-RAF Y299D/Y300D) (data not shown) nor C-RAF DDED ΔN did exert any such effect (Fig. 13D-F), suggesting that the mitochondrial binding ability of C-RAF was required for the remodeling. This point was further confirmed with the observation of cells that expressed M-RAF: while we noticed that M-RAF was strictly localized to mitochondria (Fig. 13, panels G-L), the intracellular expression of this mutant of C-RAF produced again the phenotype that we had noticed earlier, consisting of multiple short mitochondria clustered around the nucleus, with a remarkable intensity (Fig. 13G-I). Interestingly, the mitochondrial remodeling produced by M-RAF was prevented when LT was applied at the time of the transfection (Fig. 13J-L). These findings collectively indicated that the ability of C-RAF to remodel mitochondria depends on the mitochondrial binding of C-RAF, and can be produced through a locally-restricted activation of this kinase.

To establish the relevance of our findings in a context that would not rely on the overexpression of C-RAF, we compared the distribution of mitochondria in MEF obtained from WT and C-RAF knock-out animals (Huser et al., 2001) (Fig. 14). In standard culture conditions, MEF derived from C-RAF WT and KO animals exhibit only subtle differences in mitochondrial shape and distribution. However, in the presence of phorbol 12-myristate 13-acetate (PMA, 100 nM) a strong PKC agonist that can be used to activate C-RAF (Kolch et al., 1993) (Fig. 14A), we noticed a striking difference between these cells: mitochondria from WT C-RAF cells appeared intensely fragmented and perinuclearly clustered (Fig. 14C-D), in contrast to mitochondria from C-RAF KO cells (Fig. 14E-F), that remained long and were almost not redistributed (compare Fig. 14, panels D and F). The PMA induced remodeling was systematically observed in WT cells, but not in the KO cells (Fig. 14B). We concluded

that our findings regarding C-RAF regulation of mitochondrial subcellular localization were not only observed under conditions of overexpression of this kinase, and were therefore relevant to a physiological situation.

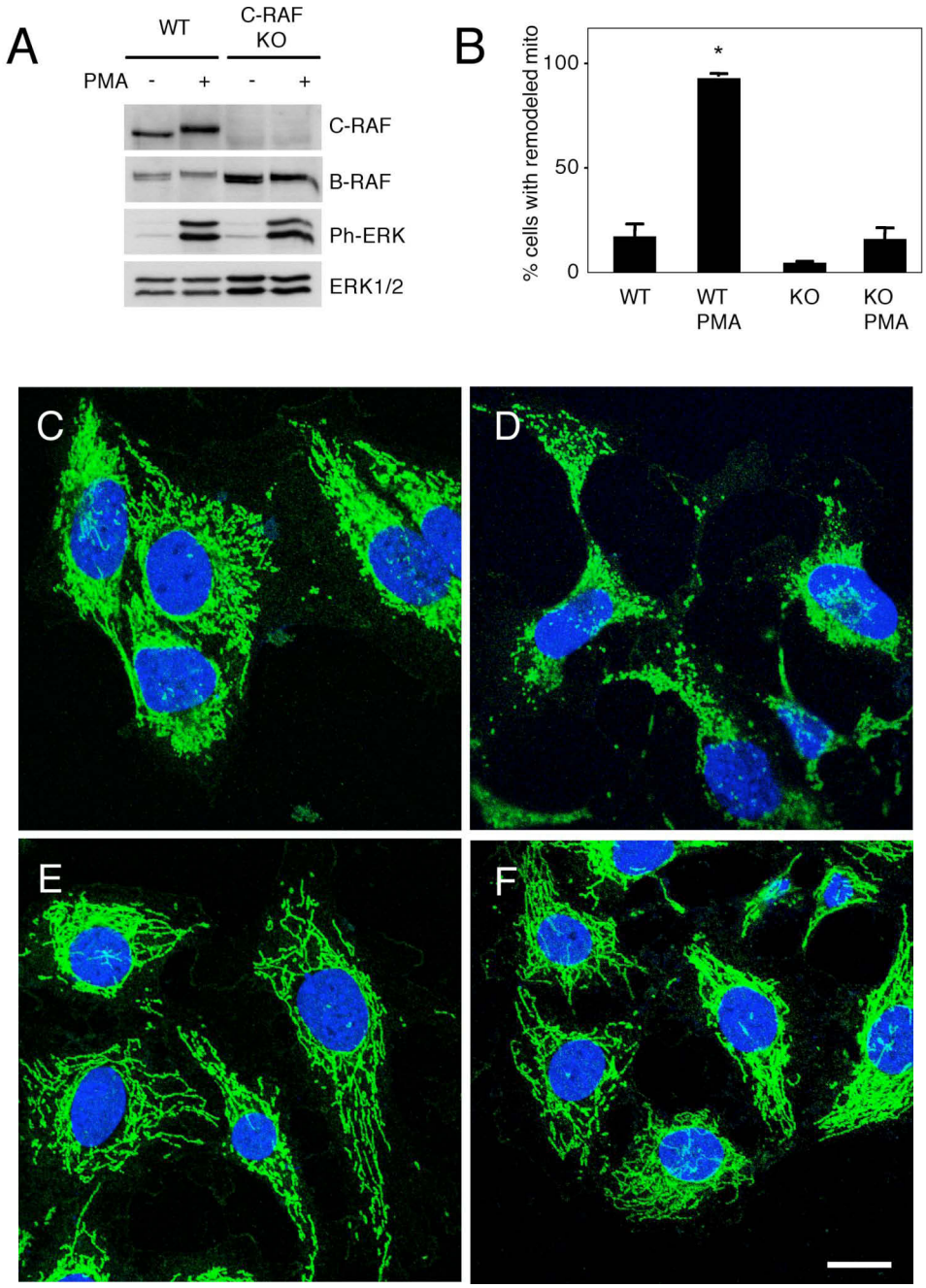


Figure 14: PMA, a potent tumor-promotor, remodels mitochondria in a C-RAF-dependent fashion. A. MEF derived from WT and C-RAF knock-out mice were grown under standard culture conditions, or stimulated for 1h with 100nM PMA and the corresponding extracts were analyzed for their content in B-RAF, C-RAF, and ERK / phospho-ERK. B-F. Effect of PMA on mitochondrial distribution of WT / C-RAF knock-out MEFs. WT and C-RAF knock-out MEF grown under standard culture conditions, or stimulated with PMA, were fixed and processed to label their mitochondria with an antibody raised against the mitochondrial marker Tom20 (green) and nuclei (DAPI, blue). A quantification of the % of cells exhibiting short spherical mitochondria is presented in panel B (results based on 2 independent counting of each time 50 cells, * $p<0,01$ compared to untreated). Representative pictures are shown for WT (C, D) and C-RAF knock-out cells (E, F), in control conditions (C, E) or after 1h of treatment with PMA (D, F) (bar: 20µm).

We went further to investigate the mitochondrial effects of C-RAF on the molecular level. The pro-apoptotic BH3-only protein BAD constitutes an important target of C-RAF at the mitochondrial level (Wang et al. 1996) and connects C-RAF with the regulation of apoptosis. Therefore, we focused our attention on the regulation of BAD by C-RAF to learn more about the mode of action of this kinase on mitochondria.

3.3. Molecular effects of C-RAF activation:

Acute C-RAF activation phosphorylates BAD in a fast and potent fashion at Ser-112

Several reports set C-RAF as a regulator of the BAD protein, but this observations were mainly achieved in the context of cancer or in conditions of sustained changes of the protein level or kinase activity of C-RAF. This leaves open the question how C-RAF regulates BAD in conditions of acute activation. To obtain a clear answer, whether C-RAF regulates BAD also in these settings, we performed our analysis in the cell line NIH3T3 C-RAF BxB-ER (Kerkhoff, Rapp 1997). We transfected these cells with a GST-tagged version of murine BAD in order to purify BAD via its tag after C-RAF activation for subsequent analysis of the respective phosphorylation sites. Using this system for a kinetic analysis of the effect of acute C-RAF activation on BAD phosphorylation, we could clearly show that BAD is quickly and potently phosphorylated on only one residue, Serine 112 of the murine form (Fig.15A,B). We could only observe a modest increase in the other phosphorylatable residues of BAD Serine 136 and Serine 155. In this analysis the initial phase of the increase in BAD phosphorylated at S112 (pS112) even paralleled the increase of phosphorylation of ERK (pERK). However, whereas the level of pERK remained stable, the level of pS112 BAD peaked at 30 min and decreased at later timepoints.

To reach the conclusion that C-RAF´s kinase activity is important in this settings, we followed two approaches. First, we blocked the RAF kinase activity with the chemical inhibitor BAY-43-9006 (Fig. 16A) and second, we analyzed BAD phosphorylation under 4-

OHT addition of cells transfected with a kinase-dead version of the C-RAF BxB-ER (K375W) fusion construct (Fig. 15C). Both strategies revealed that in case of blocked C-RAF kinase activity virtually no phosphorylation of BAD at Serine 112 occurs.

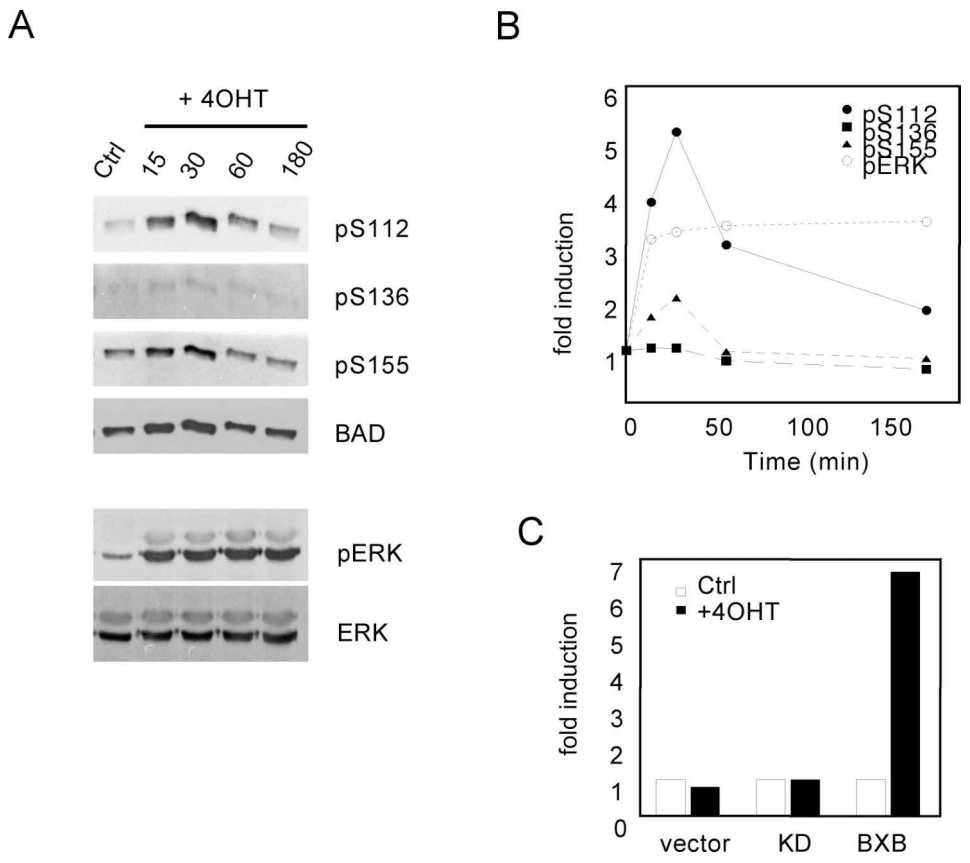


Figure 15: C-RAF activation specifically promotes BAD phosphorylation on Ser-112.

A: NIH3T3 C-RAF BXB-ER cells were used to analyze kinetically the effects of C-RAFactivation, achieved with the addition of 4-OHT (1µM). Cytosolic extracts and immobilized BAD were analyzed separately by immunoblots using relevant antibodies. B: densitometricdetermination of the experiment shown in A. C: comparative estimation of BAD phosphorylation on Ser112 in NIH 3T3 cells transfected with a vector control or a kinase deadversion of C-RAF BXB-ER upon 4-OHT addition.

In order to analyze the specificity of this phosphorylation events on BAD under physiological activation, we applied epidermal growth factor (EGF), a growth factor that mainly works through the RAS-RAF-ERK pathway (Kratchmarova et al., 2005), on NIH3T3 cells transfected with GST-BAD. We could confirm the observations of the inducible system in these settings (data not shown). The specificity of this phosphorylation events could be further attributed to the kinase activity of RAF under these conditions, since using the RAF-specific

inhibitor BAY-43-9006 we were able to reach an IC50 value below the micromolar range (0,76μM).

3.3.1. C-RAF acts indirectly in BAD phosphorylation: involvement of the downstream cascade

In order to test the direct contribution of C-RAF in this process we used the inducible system to analyze the effects of downstream kinases in the RAF pathway under acute activated conditions. For this, we blocked MEK, the downstream effector kinase of C-RAF and several other survival pathways with implications in BAD phosphorylation (PKA, mTOR, Bcr-Abl and PKB) with chemical inhibitors in the presence of 4-OHT (Fig. 16B). While none of the tested pathways participated in BAD phosphorylation under these conditions, we could only block the pS112 appearance by inhibiting the kinase MEK. This indicated that in the conditions of our assay the phosphorylation of BAD is dependent on the downstream cascade of the RAF-pathway. In addition, we extended this analysis by using a non-chemical approach, applying the bacterial factor Lethal Toxin from *Bacillus anthracis*. Applying the anthrax toxin on cells in conditions of C-RAF acute activation, we observed the same loss of BAD S112 phosphorylation as observed previously (Fig.16D).

We further decided to analyze the contribution of the RSK kinases in this assay, since these kinases are downstream effectors of the RAF cascade and they reportedly contribute in BAD phosphorylation in cancer cells (Eisenmann et al., 2003). Therefore, we included a recently developed specific inhibitor of RSK (BI-D1870) (Sapkota et al., 2007) in our assay (Fig. 16C). There again, we could completely block the phosphorylation of BAD on S112. These results indicated that even under conditions of acute C-RAF activation the downstream components of the pathway present on endogenous levels are involved, rather than C-RAF phosphorylating BAD directly. It further suggested that the kinases of the RSK family are the effectors of the RAF-pathway in the phosphorylation of BAD on S112.

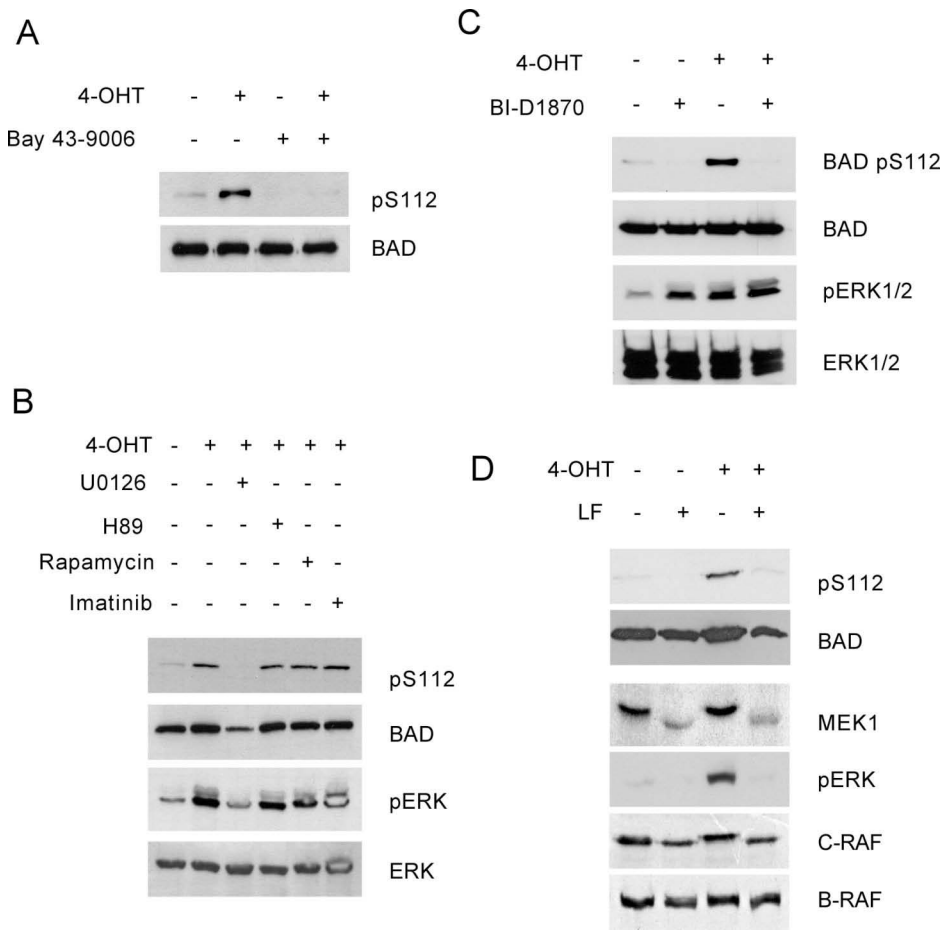


Figure 16: BAD phosphorylation on Ser112 depends on the kinases MEK and RSK.

A: NIH 3T3 C-RAF BXB-ER were treated with the inhibitor BAY43-9006, applied at 10µM and pre-incubated for 15min before 4-OHT (30min at 1µM). B: Chemical inhibitors were applied to probe the contribution of the kinases MEK (U0126, 10µM), PKA (H89, 10µM), mTOR (rapamycin, 1µM), and Abl (Imatinib, 5µM) downstream of C-RAF. Inhibitors were preincubated for 30 min, and cells were stimulated for 30 min with 4-OHT. C: The RSK inhibitor BI-D1870 blocks BAD phosphorylation downstream of C-RAF. BI-D1870 (10µM) was pre-incubated for 30 min before 4-OHT treatment. D: Lethal toxin from *Bacillus anthracis* blocks BAD phosphorylation downstream of C-RAF. LT was applied in the culture medium of the cells 3hrs before 4-OHT.

3.3.2. C-RAF activation recruits B-RAF, MEK, ERK, AKT, in association with BAD

Since RAF kinases mediate BAD phosphorylation indirectly and act in large multi-molecular complexes, our findings prompted us to reach further insight into the organization of the complex that mediates BAD phosphorylation. In a timing that correlated with the peak in pS112 appearance we observed components of the RAF-pathway in association with BAD (Fig.17). We detected C-RAF BxB-ER, MEK, ERK and even B-RAF 20 - 30 min after C-RAF activation. However, we could not detect RSK in this experiment, which could be due to a low antibody specificity or the labile association of RSK with BAD. However, we could

observe the molecular interaction of BAD with RSK in an immunoprecipitation approach on endogenous protein levels in a kinetic analysis comparable to the previous experiment (data not shown).

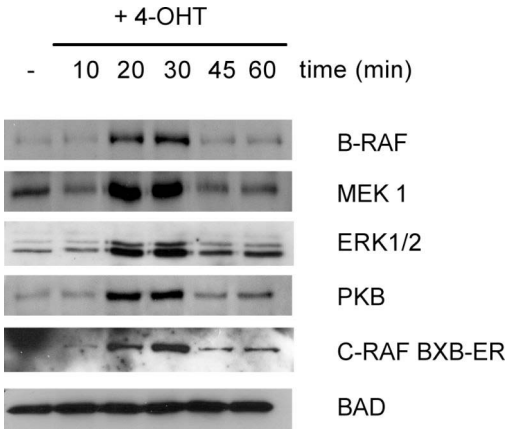


Figure 17: C-RAF activation recruits B-RAF, MEK, ERK, RSK and PKB in association with BAD.

The association of different kinases together with BAD was determined after C-RAF activation. NIH3T3 CRAF BXB-ER cells were stimulated for different time points with 4-OHT and immobilized BAD with co-purified proteins were assessed by immunoblot with relevant antibodies.

Although we cannot exclude that this association was just an association of different pools of BAD with the detected kinases, we believe that the recruitment of B-RAF to the „kinase-complex“ might be an attractive possibility in terms of heterodimer formation. This concept is supported by the observation that the main residue responsible for the heterodimer formation (Serine 621 in C-RAF) was detected with an phospho-specific antibody (6B4) raised against this residue (Hekman et al., 2004) on C-RAF BxB-ER (data not shown). Furthermore, we also observed an immunoreactivity with the 6B4 antibody when blotting for co-purified components on GST-BAD from cells that were stimulated for 30 min with 4-OHT. Interestingly, we could also detect the kinase PKB together with the components of the RAF-pathway in association with BAD at the same time scale (Fig.17). But PKB associated in an inactive state, determined by the lack of an activating phosphorylation at Serine 473 (data not shown). This correlates well with the fact, that we did not observe an increase in BAD pS136 at this time points. Therefore, C-RAF activation probably recruits B-RAF and MEK, but also

the kinase PKB in an inactive form in association with BAD at a time scale that matched the appearance of pS112.

3.4. Consequences of C-RAF activation on BAD: C-RAF increases the protein turnover of BAD

Phosphorylation of BAD is currently the main regulation process of this protein described. The consequences of these phosphorylation events can reach from changes in the molecular interaction partners to changes in the subcellular distribution. Serine 136 and Serine 112 constitute in their phosphorylated state a binding motif for proteins of the 14-3-3 family (Masters et al., 2001). But Serin 112 might also be required in regulating the interactions to other Bcl-2 proteins (Scheid et al. 1999). We decided to test both, the change in the interaction partners and the change in the subcellular localization of BAD in the context of acute C-RAF activation. To address the subcellular localization, we compared the presence of BAD on the mitochondrial level in control and 4-OHT treated cells. Phosphorylation of Serine 112 shifted the concentration of BAD from the mitochondrial level into the cytosol (data not shown).

In order to confirm BAD phosphorylation on endogenous protein level and to test the changes in the interaction with the molecular interaction partners, we performed an co-immunoprecipitation of BAD in conditions of acute C-RAF activation (Fig. 18A). We observed a reduction of the level of co-immunoprecipitated Bcl-xL, but this was difficult to interprete since, surprisingly, also the precipitated BAD protein level was reduced after C-RAF activation. Since we found only small variations in the cellular content of BAD mRNA upon 4-OHT addition quantified by RT-PCR (data not shown), our initial observation prompted us to measure BAD´s halflife in this system (Fig. 18B). While we activated C-RAF for different time length, we blocked the *de novo* protein synthesis with the drug

cycloheximide at the same time. This approach enabled us to follow the disappearance of the BAD protein over time by immunoblot analysis as result of its protein turnover.

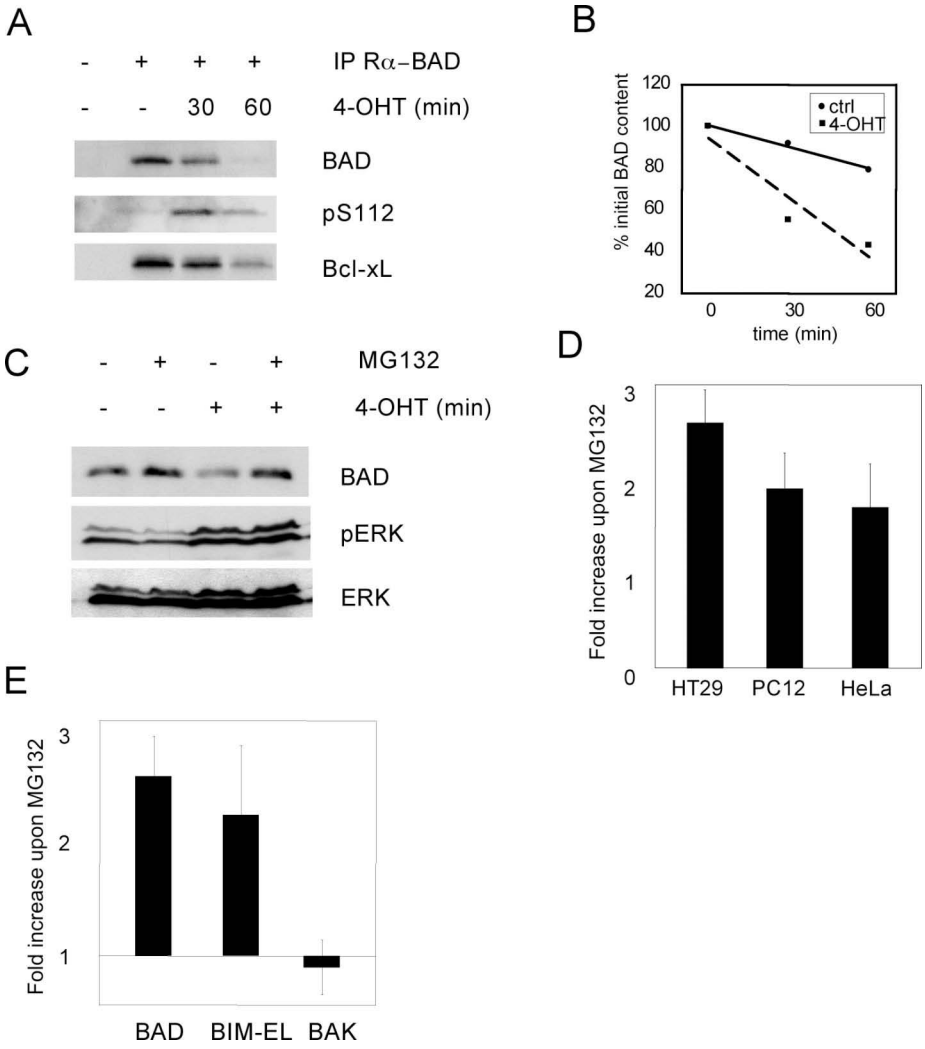


Figure 18: C-RAF activation reduces the protein level of endogenous BAD through proteasomal degradation

A: Immunoprecipitation of endogenous BAD after C-RAF activation with 4-OHT for different time points. Immunoblot analysis of precipitated proteins with indicated antibodies. B: BAD turn-over increases upon C-RAF activation. Densitometric determination of the half life of BAD protein in control conditions or under C-RAF BxB-ER stimulation with 4-OHT for indicated time points. Cells were pretreated for 30min with 25µg/ml cycloheximide. BAD protein levels were detected by immunoblot and are expressed as % initial protein content. C: BAD disappearance is reverted with the proteasome inhibitor MG132 when C-RAF is activated. NIH3T3-C-RAF BxB-ER cells were pretreated with cycloheximide. MG132 (50µM) and 4-OHT (1µM) were applied as indicated. Cell lysates were analyzed by immunoblot with relevant antibodies. D: Proteasome-dependent protein turn-over of BAD in a panel of human cancer cell lines. Extracts were prepared from HT29, PC12 and HeLa cells in control conditions or after treatment for 6hrs with 10µM MG132. Content in BAD protein was analyzed by immunoblot and results are indicated as fold increase upon MG132 treatment (average of three experiments $\pm$ s.d.). E: Comparison of the effect of MG132 on different members of the Bcl-2 family. Samples were prepared from HT29 cells as previously (average of three experiments $\pm$ s.d.).

We determined that the BAD protein on basal level has a calculated halflife of approximatively 145 minutes. However, in conditions of C-RAF activation the halflife of BAD was dramatically decreased by a factor of three-fold, resulting in a calculated halflife of approximatively 45 minutes. To get a first indication which system might account for the reduction in BAD protein, we turned our attention to the proteasome. Interestingly, we were able to block the disappearance of BAD with the proteasome inhibitor MG132, but only in conditions where C-RAF was activated (Fig. 18C), indicating an active role for C-RAF in regulating BAD protein level through proteasomal degradation.

To probe the relevance of our findings, we analyzed the role of the proteasome in the turn-over of BAD in different cancer cell lines. For this, we used the cell lines HeLa (cervix cancer), HT29 (colorectal cancer) and PC12 (pheochromocytoma cells) and treated those for 6 hours with MG132 (10μM). We detected an increase in the protein level of BAD by a factor of two to three fold (Fig. 18D). This effect was most likely specific, because MG132 did not affect cell viability nor the activity of the ERK kinases under these conditions (data not shown). Similar results were obtained using epoxomycin, another proteasome inhibitor structurally non related to MG132 (data not shown). Furthermore, MG132 induced an inrease in BAD protein level to an extent that was comparable to that measured for another proteasomal regulated BH3-only protein, BIM-EL (Luciano et al., 2003; Ley et al., 2003). In contrast, the protein level of the pro-apoptotic protein Bak remained stable under this condition (Fig. 18E). We concluded that the proteasome plays a major role in the turn-over of BAD protein when C-RAF is activated and in cancer cells. Therefore, we decided to investigate how C-RAF targets BAD for degradation.

3.4.1. The C-RAF cascade targets BAD for poly-ubiquitylation in a phosphorylation-dependent fashion

Proteasomal degradation is already described for other Bcl-2 family proteins, like Mcl-1 and BIM-EL, where phosphorylation creates the signal for destruction (Maurer et al., 2006; Luciano et al., 2003, Ley et al., 2003). In the case of BIM, the RAF pathway targets BIM-EL by direct phosphorylation through ERK for ubiqitin-mediated proteasomal degradation. To test this possibility for BAD we devised a new strategy. We transiently co-expressed a GFP-tagged form of human BAD together with 6xHis tagged ubiquitin in the presence or abscence of C-RAF BxB in human embryonic kidney 293 (HEK 293) cells. After 24 hrs of expression cells were lysed in an urea containing, reducing buffer and a step of immobilized metal affinity chromatography (IMAC) was performed in order to purify every ubiquitin-conjugated protein via the 6xHis tag of ubiquitin. The purified beads were then subjected for a BAD immunoblot analysis. The non-phosphorylatable mutant of BAD at Serine 112, from now on called Serine 75 to follow the human nomenclature, was included as a control. With this strategy we could show that by co-expression of BAD with the oncogenic C-RAF-BxB, BAD becomes highly ubiqitylated, visualized through a smear on the blot of purified ubiquitin (Fig. 19A). Furthermore, we could illustrate that this ubiquitylation occurs in a RAF mediated, phosphorylation-dependent fashion on Serine 75 of BAD, since the non-phosphorylatable mutant at this position did not show any ubiquitylation. In order to ensure that the ubiquitylation observed is indeed a result of poly-ubiquitin chains and not a multiple mono-ubiquitylation, we tested an ubiquitin that is deficient of forming poly-ubiquitin chains via its lysine at position 48 (K48R). We co-expressed either His-tagged wildtype ubiquitin or the K48R-mutant of ubiquitin together with BAD and C-RAF BxB and determined the poly-ubiquitylation state of BAD (Fig. 19C). Clearly, we could observe that the K48R-mutant of ubiquitin was defective in poly-ubiquitylating BAD, indicating that BAD indeed becomes poly-ubiquitylated for proteasomal degradation.

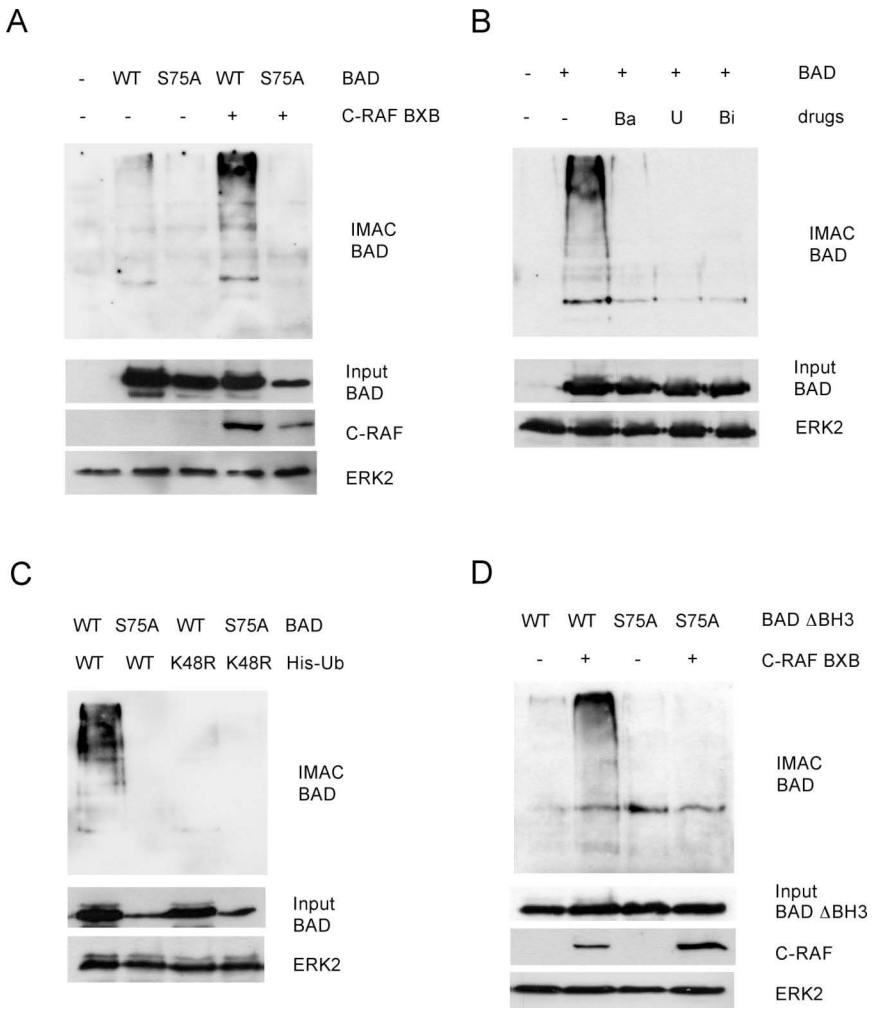


Figure 19: C-RAF activation promotes BAD poly-ubiquitylation

A: C-RAF activation promotes BAD ubiquitylation in a phosphorylation-dependent fashion. HEK293 cells were co-transfected with 6xhis ubiquitin, C-RAF BxB and GFP-BADwt or S75A mutant. Total cell lysates were subjected for immobilized metal affinity chromatography (IMAC) and purified proteins were analyzed by immunoblot with BAD antibody. Cell lysates for expression controls are shown below. B: RAF, MEK and RSK kinases activity is required for C-RAF induced ubiquitylation of BAD. Cells were incubated for 12hrs with different inhibitors against RAF (BAY43-9006 10µM; Ba), MEK (UO126 10µM; U) and RSK (BI-D1870 10µM; Bi). Cells were processed for the detection of ubiquitylated forms of BAD. C: Ubiquitylation of BAD depends on K48-linked internal ubiquitin branching. Ubiquitylation approach was performed using either 6xhis ubiquitin wt or a K48R mutant. D: BAD amino-terminus is sufficient for C-RAF induced poly-ubiquitylation. A fragment of BAD comprising aminoaacids 1-106 (BAD ΔBH3) or the corresponding phospho-deficient mutant were processed for poly-ubiquitylation analysis.

To prove the contribution of the downstream pathway of C-RAF in the ubiquitylation process of BAD, we used the same strategy but included the chemical inhibitors against RAF, MEK and RSK. We co-expressed C-RAF BxB with 6xHis-ubiquitin and GFP-BAD over night and incubated cells for 12 hours with the inhibitors BAY48-9006, U0126 and BI-D1870 (all at 10 µM) prior lysis. Using this strategy we determined no poly-ubiquitylation of BAD when

blocking the C-RAF cascade at any level (Fig. 19B). Noteworthy, the C-RAF downstream kinases were present at endogenous levels, indicating the specificity of this process. We concluded, that activation of the C-RAF pathway leads to a poly-ubiquitylation of BAD, which is dependent on the phosphorylation of Serine 75.

To get first indications of the mechanism that would lead to the poly-ubiquitylation of BAD, we tested a mutant that lacks the main structural determinant of this molecule. This construct encodes only the amino-terminal part of BAD, lacking the BH3 domain and the following carboxy-terminal part (called BAD ΔBH3). We detected that BAD ΔBH3 was still efficiently poly-ubiqitylated (Fig. 19D). This points to the fact that the interaction with other Bcl-2 family proteins and therefore also the mitochondrial localization seems not to be a crucial parameter in this process. Rather, the ubiqitylation of this BAD fragment was a direct consequence of the phosphorylation on Serine 75, since a non-phosphorylatable mutant of this construct did not reveal any ubiquitylation (Fig. 19D).

Taken together these results indicate that the activation of the C-RAF pathway phosphorylates BAD on Serine 112 (or Serine 75 in the human molecule), which creates a signal for poly-ubiquitylation targeting for proteasomal degradation. The amino-terminal part of BAD is sufficient to be ubiquitylated via K48-branching and the ubiquitylation is therefore most likely independent of the interaction with other Bcl-2 family proteins.

Discussion

In this work we could establish that the isoforms of the RAF kinases exhibit a striking specificity for intracellular membranes. While C-RAF is the only isoform with pronounced intrinsic properties to localize to mitochondria, we also determined the effects of this kinase on the cellular level in live cells, *i.e.* its capacity to change the morphology of mitochondria. Furthermore, we analyzed the effects of activated C-RAF on the molecular level on the basis of the regulation of the pro-apoptotic protein BAD.

4.1. C-RAF interaction with mitochondria and resulting cellular effects

4.1.1. Isoform-specific distribution of RAF kinases at the level of membranes

To our knowledge this work represents the first report comparing the binding properties of all three human RAF kinases to natural membranes. While analyzing the membrane binding abilities of the three different RAF-isoforms in cells, we noticed that they exhibit a striking non-overlapping localization pattern. There, C-RAF is the only isoform with high affinity for mitochondrial membranes. Until now, the RAS family of small GTPases was the only established component of the RAF-signaling cascade for which this specificity for intracellular membranes was noticed (Omerovic et al.; 2007). Therefore, RAS proteins were thougth to place the RAF-signaling cascade to certain intracellular compartments and therefore establish a compartmentalization of the cascade. But with the data presented here, we propose a scenario where not only the RAS proteins, but also the RAF isoforms would activley contribute to the compartmentalization of the signaling. This would draw a slightly more complex picture of the RAF-signaling, where the sequestration of the signaling is achieved at different levels of the cascade. Furthermore, the RAF and RAS isoforms would meet only in conditions where they share the same localization pattern. For example, there are

indications that N-Ras and K-RASb can be present at mitochondria (Bivona et al., 2006; Wolfman et al., 2006), which would point to these isoforms as primary activators for C-RAF at this level, although they are apperently not directly involved in the recruitment of C-RAF (see 8.1.2). However, the isoform-specific distribution of RAF kinases does not exclude the possibility that RAS proteins might further facilitate the sub-compartmentalization into specialized microdomains.

We demonstrated that the mitochondrial binding of C-RAF is an intrinsic property of this kinase. We identified the amino-terminal part of this kinase as the mitochondrial binding determinant. The amino-terminal part of RAF kinases was already known to bear binding abilities to partner molecules correlating with its localization (e.g. RAS) , whereas the carboxy-terminus contains the catalytic activity (Wellbrock et al. 2004). This clearly highlights the role of the RAF amino-terminus in establishing different localization patterns. Comparing the amino-acid sequences of the three RAF it is obvious that the carboxy-terminal region is of high homology throughout the isoforms, with the exception of some amino-acids being important in the regulation of the kinase activity (Baljuls et al. 2007). The amino-terminal part of RAF is, beside the conserved region 1 and 2, more divergent throughout the isoforms (Fig. 2). Especially the very amino-terminal stretch of RAF kinases represents the region with the highest sequence divergencies. Experiments using the chimeric constructs between C-RAF and B-RAF point to the importance of these stretches, since the replacement of the first 50 aminoacids of C-RAF into the corresponding region of B-RAF created clearly an increased binding affinity to mitochondria. In the same line we could decrease the mitochondrial affinity for a chimera fusing the B-RAF amino-terminal part to C-RAF (Fig. 10). A first indication for a regulatory role of the very amino-terminal stretch was suggested for B-RAF (Fischer A. 2007). There, the very amino-terminal stretch of B-RAF creates differences in the binding affinites to H-RAS compared to C-RAF. In our work we illustrated

a specialized role for the C-RAF amino-terminus in bearing the property to interact with mitochondria. How could this stretch regulate this interaction? The very amino-terminal stretch of RAF kinases might exert on the one hand motives that bind certain proteins, which define their binding properties. On the other hand this region could modulate the accesibility of the adjacent conserved domains to certain binding partners, for example through sterical changes in the protein fold. Unfortunately, there is no data available yet regarding the mitochondrial binding partner of C-RAF. Identification of this receptor will shed light on the involved membrane targeting mechanisms.

Taken together, these observations point to the importance of the amino-termini of the RAF kinases in the subcellular localization, probably by using both conserved and non-conserved sequences that might play in concert for the defined membrane binding abilities.

4.1.2. Effects of C-RAF on mitochondria at the cellular level

4.1.2.1. Effects in live cells

Here, we described for the first time the effect of activated C-RAF on mitochondria in live cells. We found that the local activation of C-RAF on mitochondria was sufficient to initiate an intense mitochondrial remodeling, creating small spherical elements clustered around the nucleus. Usually, mitochondria constitute a wide network spanning throughout a cell, but in several situations a cell changes this mitochondrial morphology (Cereghetti, Scorrano, 2005). For example, mitochondrial fragmentation is a hallmark of apoptosis progression. Nevertheless, in the experiments presented here the observed mitochondrial remodeling upon C-RAF activation was clearly not a matter of apoptosis, revealed through the retention of cytochrome c and the lack of caspase processing under these conditions (data not shown). The mitochondrial network is constantly remodeled by fission and fusion, two highly regulated processes (Chan, 2006). An indication whether C-RAF activation interferes with one of these processes was derived from the observation that Drp1 (dynamin related protein

1), an important regulator in mitochondrial fission (Jagasia et al. 2005), colocalized with mitochondria in PMA treated HeLa cells (data not shown). Usually, Drp1 is sequestered in the cytosol and gets only recruited to mitochondria when fission is initiated. This suggests that mitochondria under these conditions remodel their shape including fission where C-RAF might participate in the regulation. However, the recruitment of Drp1 could just constitute a secondary consequence of another process initiated by C-RAf activation.

The compartmentalization of the RAF cascade suggests that the individual RAF isoforms also contribute to different effects on the cellular level (Pelkmans et al., 2005). The fast centripete movement of mitochondria and the kinetics of the mitochondrial remodeling upon C-RAF activation suggest that these are active processes rather than a secondary consequence. The clustering of small units of cellular compartments in the cell center is also observed in the process of cell division to ensure the appropriate sequestration and inheritance of organelles. Components of the RAF pathway were already implied in this regulation for other organelles, like the Golgi-apparatus and endosomes (Shaul, Seger 2006; Teis et al. 2006). The effect of active C-RAF on mitochondria could establish a comparable regulation although we did not directly investigate this matter.

Intracellular transport of mitochondria and other cellular organelles uses the cytoskeleton as „railroads“ (Boldogh and Pon, 2007; Frederick and Shaw, 2007). Interestingly, C-RAF is also able to interfere with the organization of the cytoskeleton (Mavria et al., 2006; Lovric et al. 1998). The observed pronounced centripete movement of mitochondria suggets that active C-RAF creates changes in the organization of the cytoskeleton or in the interaction of mitochondria with components of the cytoskeleton. Both possibilities are likely to affect the intracellular transport mechanism of this organelle. Possibly, the localization of mitochondria could also influence the cytoskeletal dynamics. Cytoskeletal reorganization is an energy dependent coordinated assembly and disassembly of cytoskeletal fibers. Since mitochondria represent the „powerhouse“ of a cell that produces the energy required for many different

processes (Galmiche, Fueller 2007), C-RAF mediated mitochondrial remodeling might constitute an important parameter in cytoskeletal organization. Therefore, regulation of the mitochondrial distribution could locally modulate the accessible amount of energy, which could affect processes, like cell migration (Campello et al., 2006).

The mitochondrial remodeling could also influence signaling processes where this organelle is central. Beside the endoplasmic reticulum, mitochondria play an important role in the storage and buffering of Ca^{2+}. There are already indications that the mitochondrial shape and the intracellular Ca^{2+}-concentration are interconnected (Szabadkai et al., 2006). Additionally, the RAF-dependent sequestration of mitochondria away from the cell periphery towards the cell center might influence Ca^{2+} mediated signaling events, since the influx of cortical Ca^{2+} is then probably less efficiently buffered. Furthermore, mitochondria signal through the production of reactive oxygen species (ROS) and the activation of the hypoxia-like pathway (Gottlieb et al., 2005). However, the participation of C-RAF and changes in mitochondrial shape and localization in this signaling remains unclear.

The effects of RAF are especially acknowledged in the context of cell transformation. Transformed cells often show a dramatically changed metabolic state (Warburg-effect). Whereas these cells obtain their energy rather from glycolysis, mitochondria are more important in producing cellular metabolites, supporting the increased requirement of those for cancer cells (Gottlieb et al., 2005). Although many cancer cells show also changes in mitochondrial distribution (Alirol, Martinou, 2006), a participation of the mitochondrial remodeling in these metabolic changes is only speculative.

4.1.2.2 Effects on apoptosis regulation

Clearly, C-RAF negatively regulates apoptosis, which constitutes one crucial aspect in its oncogenic potential. The change in mitochondrial shape and distribution might represent an

additional parameter in modulating the apoptotic response. Changes in mitochondrial distribution are common features observed in various cancer cells (Alirol, Martinou, 2006). Considering the fact that cancer cells have a reduced susceptibility to apoptosis and many cancer types show a frequent upregulation of the RAS-RAF-ERK pathway, the mitochondrial remodeling might represent a further mode in establishing and/or maintaining cancerogenous properties. How could small mitochondrial units protect from apoptosis? Possibly, C-RAF mediated mitochondrial remodeling could limit the release of apoptogenic factors from an mitochondrial network. Separating mitochondria into small units means that a resulting unit contains less of apoptosis promoting factors compared to a normal elongated unit from a mitochondrial network. In case of limited apoptosis induction the release of such factors from a proportion of small mitochondria would set the threshold for apoptosis to a higher level compared to the situation where mitochondria constitute a wide spread network.

4.2. Molecular mode of action of C-RAF on the protein BAD

4.2.1 Mode of action of C-RAF in conditions of acute activation

Literature contains multiple reports that connect C-RAF with apoptosis regulation (Fig. 6 and text). One crucial regulatory target is the BH3-only protein BAD. However, regulation of BAD by the RAF pathway is mostly described in the context of sustained changes in the protein level or activity of RAF (Wang et al. 1996; Jin et al. 2005; She et al 2005). Here, we focused our attention on the phosphorylation of BAD in conditions of acute activation of C-RAF. Using an inducible system to obtain kinetic informations on BAD phosphorylation, we observed that the RAF pathway targets only one residue of BAD (Ser112 in the murine molecule) in a fast and potent fashion. This phosphorylation is most likely indirect, since C-RAF activation required the downstream effector MEK. Furthermore, we found that in these conditions the kinases of the RSK family are likely to be involved in BAD phosphorylation,

as already suggested in other settings (Bonni et al., 1999; Eisenmann et al. 2003, She et al. 2002).

Our results further indicate that C-RAF activation temporally assembles a large multi-molecular complex to achieve BAD phosphorylation, including in addition to BAD itself B-RAF, MEK, ERK and PKB. Unfortunately, we were not able to detect RSK in this complex, which could be due to a reduced antibody specificity for RSK or to the labile nature of the association between RSK and BAD. Nevertheless, we were able to establish a direct interaction between these two molecules in a co-immunoprecipitation approach (data not shown). Although we cannot exclude that different sub-pools of BAD associate with the detected kinases, we believe that the recruitment of a multimolecular „kinase-complex“ might be an attractive possibility. The recruitment of B-RAF after C-RAF activation is reminiscent of the described heterodimer formation between RAF kinases (Weber et al. 2001; Rushworth et al.; Garnett et al. 2005). This concept is supported by the observation that the major reported residue responsible for the heterodimer formation (Serine 621 in C-RAF) was detected with a phospho-specific antibody (6B4) (Hekman et al. 2004) on C-RAF BxB-ER as well as in complex with BAD. What would be the advantage of a heterodimer in this respect? As we observed C-RAF is the only isoform of RAF kinases that has the intrinsic potential to localize to mitochondria, the location where BAD displays it pro-apoptotic potential and needs to get inactivated. Here, C-RAF would represent the localizing component of the cascade. On the other hand B-RAF is the isoform that displays the highest basal kinase activity (Marais et al., 1997). Heterodimer formation combines the properties of both isoforms. In this context C-RAF would localize the kinase activity of B-RAF to the mitochondria, which would initiate a kinase cascade targeting BAD.

A previous report suggests BAD as a convergence point of the RAF- and the PKB-pathway (She et al. 2005). This model is further supported by the observation that BAD can associate with PKB and B-RAF on the mitochondrial level (Götz et al. 2005). Interestingly, we detected

also the kinase PKB in association with BAD upon C-RAF activation, but in an inactive state correlating with the abscence of a change in the phosphorylation of Ser136, the preferred residue of BAD for this kinase. A recent report illustrates that cells deficient in C-RAF show also a reduced phosphorylation of Ser136 in addition to Ser112 (Kebache et al. 2007). Taken together, this suggests that there is an interplay between the RAF- and the PKB-pathway at the level of BAD. Our observation that acute C-RAF activation only targets Ser112 and recruits PKB, but that the lack of C-RAF has also an impact on Ser136 (Kebache et al. 2007), suggests that C-RAF can function as a node of signaling pathways (Taniguchi et al, 2006). There, C-RAF might coordinate the assembly, recruitment and possibly the activity of other kinases resulting in the correct signaling output.

We noticed that the phosphorylation of BAD at Ser112 was of temporary nature correlating with the association of the different kinases to BAD, peaking at 20-30 min after induction of C-RAF and declining thereafter. The fact that Ser112 is probably the first residue of BAD changing its phosphorylation state was observed previously (Chiang et al. 2003). Several mechanisms could account for this decline in phosphorylation. Although we do not favour this possibilty, one explanation could be a temporary activation of C-RAF-BxB-ER. Secondly, the phosphorylation of BAD might be counteracted by a dephosphorylation process. The third possibility, which we consider the most likely, is that phosphorylation of BAD at this residue would target the protein for degradation.

4.2.2. BAD turn-over, a new consequence of the activation of RAF kinases

Little data address directly the consequences of the C-RAF mediated phosphorylation of BAD. There are clear consequences for the phosphorylation of the residues Serine 155 and Serine 136 of BAD. Serine 155 lies within the BH3 domain of the BAD molecule and the phosphorylation of this results in the dissociation of BAD from its anti-apoptotic binding partners Bcl-2 and Bcl-xL (Virdee et al.; Tan et al. 2000). Serine 136 and Serine 112 are

included in their phosphorylated state in a binding motife for proteins of the 14-3-3 family (Zha et al., 1996; Yaffe et al., 1997). But only Serine 136 seems to be the major binding site for 14-3-3 proteins (Masters et al, 2001). Serine 112 seems to be neglectable or is functioning as a gatekeeper in the association to 14-3-3 molecules. Although BAD Serine 112 is targeted by many different pathways, to date no clear role for this residue has been described. A first indication for the role of Serine 112 came from the report of Scheid et al. (1999), which indicated that the phosphorylation of this residue plays a role in the dissociation of BAD from Bcl-xL and therefore also from mitochondria. Surprisingly, we found that C-RAF activation reduces the BAD protein level (Fig. 18). This observation opened a new possibility in the regulation of BAD.

4.2.2.1. Regulation of protein stability of Bcl-2 family proteins

Different mechanisms can account for the regulation of the protein level. While the protein expression is regulated by transcriptional as well as translational mechanisms, a change in the protein stability can also account for the abundance of proteins. Protein degradation can be achieved by several means, including the lysosomal or the proteasomal turn-over as well as the cleavage by specialized enzymes, like calpains or caspases (Mizushima, 2007; Ciechanover 1998; Wu et al, 2007; Lamkanfi et al., 2007).

Some members of the Bcl-2 family, for instance PUMA, Noxa and BIM, are regulated via transcriptional mechanisms (Oda et al. 2000; Nakano et al. 2001, Dijkers et al. 2000). Moreover, many proteins of the Bcl-2 family are also subject for ubiquitin-mediated proteasomal turn-over (Table 1). Ubiquitylation is a post-translational modification achieved through a highly coordinated covalent binding of small ubiquitin molecules (Ciechanover, 1998). This modification targets proteins for different fates (Wilkinson et al., 2005), where the proteasomal degradation via the 26S proteasome probably represents the most acknowledged. The attachment of ubiquitin to target proteins requires the sequential action of

protein		reference
Bax	Proteasome inhibtion accumulates ubiquitylated Bax	Li and Dou; 2000
BIM-EL	Phosphorylation by ERK1/2 (Ser 69) targets for proteasome	Luciano et al.; 2003
	Phosphorylation by ERK1/2 (Ser 65 murine) ubiquitylates and targets for proteasome	Ley et al.; 2003
	Dissociation from Mcl-1 and Bcl-xL upon phosphorylation by ERK1/2 (S69) and targets for proteasome	Ewings et al.; 2007
Mcl-1	Phosphorylation by ERK1/2 (Thr163) targets for proteasome	Domina et al.; 2004
	Association with Noxa leads to degradation	Willis et al.; 2005
	Association with Ubiquitin E3 ligase MULE-1 leads to ubiquitylation	Zhong et al.; 2005
	Phosphorylation by GSK3b (S159) leads to ubiquitylation	Maurer et al.; 2006
	Upon phosphorylation by GSK3b (S155, 159 T169) association with βTRCP and ubiquitylation	Ding et al; 2007
BIK/NBK	Protein accumulation upon proteasome inhibitor	Zhu et al.; 2005
Bfl-1/A1 (human)	Continous proteasomal degradation Cleavage by m-calpain	Kucharczak et al.; 2005
Bfl-1/A1 (murine)	Carboxy-terminus targets for ubiquitylation; BIM binding stabilizes A1	Herold et al.; 2006
Bcl-2	Blocking dephosphorylation by PP2A (T69; S70; S87) ubiquitylates and targets for proteasome	Lin et al; 2006
BIM, BAD, PUMA	Infection with *Chlamydia spp.* targets for proteasome	Ying et al.; 2005
PUMA	Inhibition of the proteasome leads to p53 dependent upregulation of PUMA	Ding WX et al.; 2007
BID	N-terminus is ubiquitylated and releases autoinhibition	Tait et al.; 2007

Table 1: A non-exhaustive summary of reported evidences for proteasomal regulation of Bcl-2 family proteins

three emzymes. First, an ubiquitin molecule becomes activated by an E1-enzyme and subsequently gets transferred to an E2-ubiquitin-carrier enzyme. The specificity of the final ubiquitin-protein ligation is determined by the selective interaction between the large family of E3 ubiquitin-ligases and their target proteins.

To get insights into the mechanism of BAD degradation it is usefull to relate it to other Bcl-2 proteins for which similar mechanisms are reported (Table 1). The mechanism underlying the proteasomal degradation of BIM-EL and Mcl-1 are rather well described. In the case of the anti-apoptotic protein Mcl-1 even the relevant E3-ubiquitin ligase is known. MULE-1/ARF/LASU1 comprises a HECT domain with E3 ligase activity and binds directly via its BH3 domain to Mcl-1 (Zhong et al., 2005). Therefore, the protein stability of Mcl-1 appears to be regulated by the binding competition with other BH3 containing proteins and MULE-1 (Warr et al. 2005). Additionally, the protein stability of Mcl-1 is also decreased by phosphorylation through glycogen synthase kinase 3 (GSK3) (Maurer et al., 2006), possibly through the modulation of the binding to BH3 only proteins and MULE-1. But Mcl-1 phosphorylation can also create a direct binding motife for the binding of an E3-ligase resulting in its ubiquitylation (Ding Q et al., 2007).

BIM-EL constitutes another BH3-only protein, whose ubiquitin-mediated proteasomal degradation is regulated via the phosphorylation through ERK at its amino-terminus (Ley et al., 2003; Luciano et al., 2003). This phosphorylation triggers the dissociation from anti-apoptotic proteins, like Mcl-1 and Bcl-xL, resulting in proteasomal turn-over (Ewings et al. 2007). In this case the phosphorylation targets for poly-ubiquitylation through the dissociation of BIM from its partner molecules.

4.2.2.2. How does phosphorylation of BAD signal for its turn-over?

Surprisingly, the acute activation of C-RAF reduced the protein level of BAD (Fig.18). While we could detect no changes in the mRNA level of BAD after C-RAF activation (data not

shown), the reduction clearly was a result of an increased protein turn-over (Fig.18). We concluded that the protein stability of BAD represents a new aspect of its regulation. The half life determination of BAD pointed to a crucial role of activated C-RAF in modulating BAD stability. Unfortunately, it remains unclear how C-RAF contributes in this respect. Since we detemined BAD Ser112 as the main residue targeted by C-RAF, its phosphorylation appears to be an important parameter. However, Ser112 was also suggested to function as gatekeeper for the accesibility of BAD´s other phosphorylatable residues (Chiang et al., 2003). Phosphorylation of Ser112 by the RAF pathway might therefore represent only an initiating event that requires subsequent steps, like further phophorylation(s) or changes in the protein fold.

By introducing an constitutively active version of C-RAF, we further demonstrated that BAD is readily ubiquitylated and marked for proteasomal degradation in a phosphorylation-dependent fashion of Ser75 (the human homologue to Ser112). The specificity of this process is further underlined by the fact, that we could completely abrogate BAD ubiquitylation with different chemical inhibitors targeting the endogenous downstream effectors of the RAF pathway. This indicates that, although we induced BAD ubiquitylation by the overexpression of C-RAF BxB, ubiquitylation of BAD proceeds through the involvment of the downstream pathway. Furthermore, this sets the phosphorylation of BAD directly in correlation with the ubiquitylation.

Ubiquitylation of proteins can be both the modification with (multiple) single ubiquitin molecules (mono-ubiquitylation) or with ubiquitin chains (poly-ubiquitylation). Additionally, ubiquitin can be linked through different lysine residues within the molecule creating poly-ubiquitin chains. The different types of ubiquitylation reflect also the fate of the modified target-molecule (Wilkinson et al., 2005). We illustrated that BAD is a subject of poly-ubiquitylation through branching of Lys48 in the ubiquitin molecule, further supporting that BAD is indeed targeted for proteasomal degradation.

To investigate the mechanism(s) of BAD ubiquitylation, we first focused on the role of the adapter proteins of the 14-3-3 family, since these proteins bind BAD in a phosphorylation-dependent fashion. We suggest that 14-3-3 does not constitute a major regulator of BAD ubiquitylation due to the following findings: i) We did not detect an increased binding of 14-3-3 proteins to BAD after C-RAF activation (Fig. 18A). ii) The overexpression of a dominant negative 14-3-3, that competes with the substrate binding of 14-3-3 ζ, did not abrogate BAD poly-ubiquitylation under the conditions of our assay (data not shown). Insights into the possible regulations of BAD´s protein stability came from the observation that the amino-terminal part of BAD is still highly poly-ubiquitylated. This observation highlights two important points: i) The localization of BAD to mitochondria seems not to be required to establish its ubiquitylation, since the amino-terminus alone has no determinant to bind to membranes (Hekman et al., 2006). ii) Unlike BIM and Mcl-1, the interaction to other Bcl-2 family proteins is not an essential component in regulating the ubiquitylation process for BAD, since the BH3 domain represents the only binding motife for other Bcl-2 family proteins.

How could the phosphorylation of Ser112 interfere with the protein stability? Currently, the crosstalk between different types of posttranslational modifications, like phosphorylation and ubiquitylation, is an emerging field (Hunter, 2007). In this context, the phosphorylation of Ser75/112 might constitute a direct marker for degradation, *i.e.* a specialized sequence motif that is commonly called phosphodegron (Orlicky et al., 2003). This motif can be directly recognized by specific molecules, such as F-box proteins, that serve as substrate recognition subunits for specific E3 ligases (Nakayama, Nakayama, 2006). A role for such an F-box protein, the β transducin repeats containing protein (βTRCP), in mediating the degradation of Mcl-1 was recently described (Ding Q et al., 2007). Although the BAD sequence does not show a characteristic consensus motife for a βTRCP recognition, phosphorylation of Ser112 might still form a degron for other E3-ligase substrate recognition subunits.

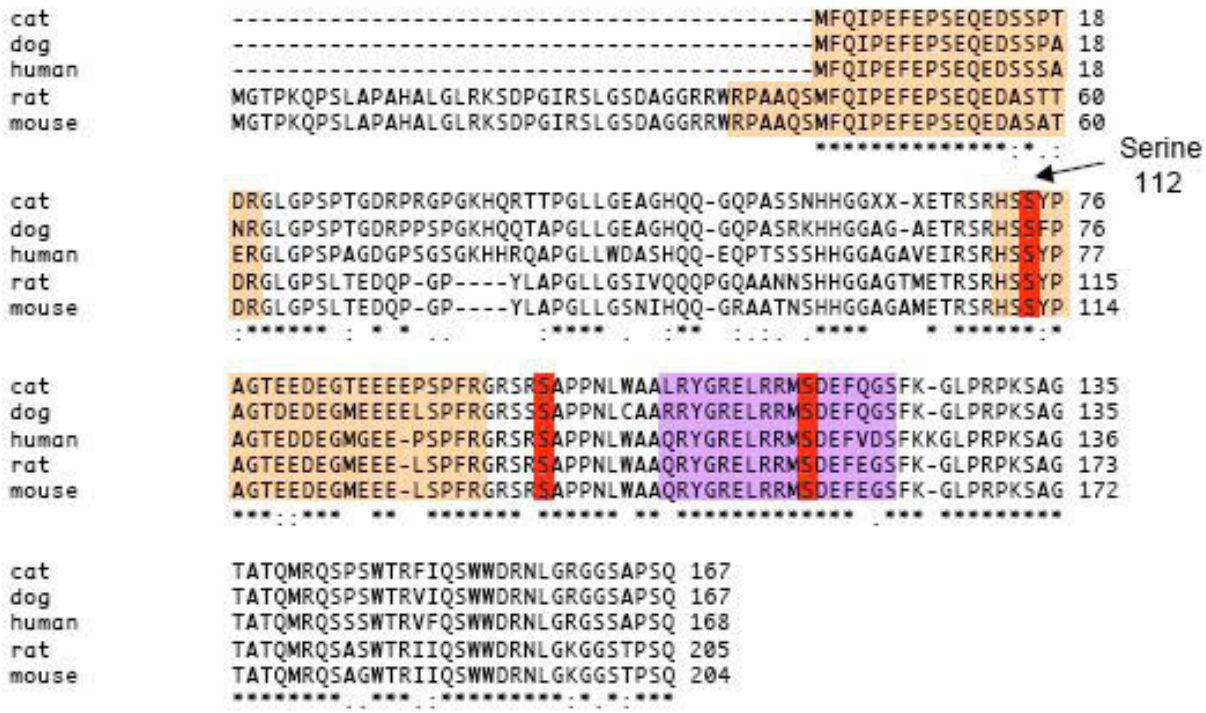


Figure 20: A sequence analysis of the determinants that are putatively involved in the regulation of BAD turn-over.

Amino-acid sequences of BAD from different species were aligned using the ClustalW algorithm (http://www.ebi.ac.uk/Tools/clustalw2/index.html). The BH3 domain is highlighted in lilac and the three phosphorylation sites (S112, S136, S155 in murine) are in red. The presence of two PEST sequences (orange) was predicted using the PESTfind algorithm (http://www.at.embnet.org/toolbox/pestfind). This analysis revealed that the phosphorylation site, targeted by C-RAF (Serine 112), lies within the second PEST sequence. In addition, the two PEST-sequences are highly conserved throughout species, compared to the surrounding amino-terminal sequences of BAD.

Phosphorylation can also serve indirectly to modulate protein stability. It can lead to exposure of degrons by inducing a conformational change, even though the phosphate does not directly contribute to the recognition of the degron. The amino-terminal part of BAD contains two PEST regions that are highly conserved between species (Fig. 20). PEST regions are sequence motives rich in the aminoacids proline (P), glutamate (G), serine (S) and threonine (T). These regions constitute a marker for proteins that undergo proteasomal degradation (Rogers et al. 1986), but are not obligatory. Since the PEST sequences are continously present, proteins are just recognized when they are marked for degradation. Interestingly, Ser75/112 is located within the second PEST sequence. Phosphorylation of such a residue might acquire a change in the protein fold, important for the following recognition and degradation, as described

previously (Garcia-Alai et al., 2006). Such a conformational change might unfold the protein structure of the PEST sequence or the entire BAD molecule.

4.2.3. Implications in therapeutic strategies

In many cancer cells the apoptotic pathway is deregulated by several mechanisms, for example through downregulation of pro-apoptotic proteins. Restoring the sensitivity to apoptosis might be one way to fight cancer. Proteasome inhibitor mediated induction of apoptosis is a rising field in cancer treatment. The proteasome inhibitor bortezomib, which is the first Federal Drug Association (FDA) approved compound of this class for the treatment of multiple myeloma, represents a promising medication (Bross et al. 2004). The accumulation of pro-apoptotic proteins might confer partially an explanation for proteasome inhibitor mediated killing of cells. By blocking the proteasome in different cancer cell lines, we detected elevated protein levels of BAD (Fig. 18). Interestingly, the analyzed cancer cell lines seemed to have acquired a constant protein turn over of BAD, since blocking the proteasome in non-transformed cells did not change its protein level unless the C-RAF pathway was activated (Fig. 18C). BIM, another pro-apoptotic protein that is regulated in its stability by the RAF-pathway, was also upregulated by blocking the proteasome in cancer cells. Probably, the higher activity of survival signaling pathways in cancer cells might produce a constant downregulation of such pro-apoptotic molecules. This suggests a mechanism for the anti-apoptotic effects of C-RAF in certain cancer types, where synergistic downregulation of BAD and BIM could increase the threshold for apoptosis induction. Such a mechanism would go in line with the phenomenon of the so called „oncogene addiction“ (Sharma, Settleman, 2007). Additionally, cancer cells that got used to low amounts of such pro-apoptotic proteins might become more sensitive to apoptotic triggers when the levels of such proteins are raised again, for instance by blocking the proteasome. The treatment with proteasome inhibitors of cancer could restore, at least partially, the sensitization to apoptosis.

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Abbreviations

cAMP	cyclic Adenosine monophosphate
CNK	Connector-enhancer of KSR
CR1, 2, 3	conserved region 1, 2, 3
CRD	cysteine rich domain
DMEM	dulbecco's modified eagle medium
DMSO	dimethyl sulfoxide
DTT	dithiothreitol
ECL	enhanced chemoluminiscence
E. coli	Escherichia coli
EDTA	ethylenediamine tetraacetic acid
EGF	epidermal growth factor
ER	estrogene receptor
ERK	extracellular signal-regulated kinase
et al.	et alii
FCS	fetal calf serum
FDA	food and drug administration
GDP	guanosine diphosphate
Grb2	growth factor receptor-bound protein 2
GST	glutathione S-transferase
GTP	guanosine triphosphate
HEK293	human embryonic kidney cells
Hepes	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IAP	Inhibitor of apoptosis protein
i.e.	id est
KSR	kinase suppressor of Ras
MAPK	mitogen-activated protein kinase
MEF	Mouse embryonic fibroblast
MEK	mitogen-activated protein kinase kinase
MEKK	MAPK/ERK activating kinase kinase
MOM	mitochondrial outer membrane
MST2	Mammalian sterile 20-like kinase

OD	Optical density
PAK	P21 activated kinase
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PC12	rat pheochromocytoma
PI3K	Phosphatidyl inositide 3-kinase
PKA	Protein kinase A
PKB (AKT)	Protein kinase B
PKC	Protein kinase C
PMA	phorbol 12-myristate 13-acetate
PMS	post mitochondrial supernatant
PMSF	Phenylmethylsulfonylflourid
PP1, 2A	Protein phosphatase1, 2A
RAF	rapidly growing fibrosarcoma
RBD	Ras binding domain
rpm	rounds per minute
Rsk	Ribosomal S6 kinase
RT	Room temperature
RTK	receptor tyrosine kinase
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
Ser	serine
SOS	son of sevenless
V	Volt
VDAC	Voltage-dependent anion channel
wt	wild type

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