NOVEL ROLE FOR ARAF KINASE IN REGULATING MAPK SIGNALING AND CANCER

DISSERTATION

ZUR ERLANGUNG DES DOKTORGRADES

DER NATURWISSENSCHAFTEN

VORGELEGT BEIM FACHBEREICH 14

BIOCHEMIE, CHEMIE UND PHARMAZIE

DER JOHANN WOLFGANG GOETHE-UNIVERSITÄT

IN FRANKFURT AM MAIN

VON

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(aus Berlin)

FRANKFURT AM MAIN

2015

(D30)

vom Fachbereich Biochemie, Chemie und Pharmazie
der Johann Wolfgang Goethe-Universität als Dissertation angenommen

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Datum der Disputation:

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Publications

Mooz, J., Oberoi-Khanuja, T. K., Harms, G. S., Wang, W., Jaiswal, B. S., Seshagiri, S., Tikkanen, R., Rajalingam, K. (2014) Dimerization of the kinase ARAF promotes MAPK pathway activation and cell migration. *Sci Signal, 7*(337)

Camargo Lde, L., Babelova, A., Mieth, A., Weigert, A., **Mooz, J.**, Rajalingam, K., Brandes, R. P. (2013). Endo-PDI is required for TNFalpha-induced angiogenesis. *Free Radic Biol Med, 65*, 1398-1407

Amaddii, M., Meister, M., Banning, A., Tomasovic, A., **Mooz, J.**, Rajalingam, K., & Tikkanen, R. (2012). Flotillin-1/reggie-2 protein plays dual role in activation of receptor-tyrosine kinase/mitogen-activated protein kinase signaling. *J Biol Chem, 287*(10), 7265-7278

Oberoi, T. K., Dogan, T., Hocking, J. C., Scholz, R. P., **Mooz, J.**, Anderson, C. L., Rajalingam, K. (2012). IAPs regulate the plasticity of cell migration by directly targeting Rac1 for degradation. *EMBO J, 31*(1), 14-28.

The PhD thesis presented here was conducted at the Institute for Biochemistry II, Goethe University of Frankfurt Main under the supervision of Prof. Dr. Krishnaraj Rajalingam.
Major parts of this work are published in the following paper:

Mooz, J., Oberoi-Khanuja, T. K., Harms, G. S., Wang, W., Jaiswal, B. S., Seshagiri, S., Tikkanen, R., Rajalingam, K. (2014) Dimerization of the kinase ARAF promotes MAPK pathway activation and cell migration. *Sci Signal, 7*(337)

Contributions to conferences and workshops

Mooz J. (2014) Unexpected role for ARAF kinase in cell invasion and tumourigenesis. Selected for a Talk at Science Day, Mainz Research School of Translational Biomedicine (TransMed), University of Mainz, Germany

Mooz, J., Oberoi-Khanuja, T. K., Wang, W., Jaiswal, B. S., Seshagiri, S., Tikkanen, R., Rajalingam, K. (2014) Dimerization of ARAF promotes MAPK activation and cell migration. Poster presentation at TARGETING THE KINOME III- International Symposium on molecular signal transduction in cancer, University Hospital of Basel and the Friedrich Miescher Institute for Biomedical Research, Basel, Switzerland

Mooz, J., Wang, W., Jaiswal, B. S., Seshagiri, S., Tikkanen, R., Rajalingam, K. (2014) ARAF dimerization mediates MAPK activation and cell migration. Poster presentation at Cancercon- Second International Conference on Cancer Biology, IIT Madras, Chennai, India

Mooz, J., Wang, W., Tikkanen, R., Jaiswal, B. S., Seshagiri, S., Rajalingam, K. (2013) Dimerization of ARAF promotes MAPK activation and cell migration. Poster presentation at FEBS ADVANCED COURSES on Molecular Mechanism of Signal Transduction and Cancer, International Summer School, Spetses, Greece

Abbreviations

ATP	Adenosine triphosphate
Akt	Acutely transforming retrovirus AKT8 in rodent
ALK	Anaplastic lymphoma kinase
APS	Ammonium persulfate
Bcl-2	B-cell lymphoma 2
Bl/6	Black 6 mice
BSA	Bovine serum albumin
CAAX	Cysteine; Aliphatic Amino acid, any amino acid (X)
°C	degree Celsius
cDNA	copy DNA
CDS	coding DNA sequence
cm	centimeter
CMV	Cucumber mosaic virus
CO_2	Carbon dioxide
C-terminal	Carboxy-terminal
CR	Conserved region
CRD	Cysteine rich domain
ddH_2O	Double distilled water
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleoside triphosphate
DTT	Dithiothreitol
DUSP6	Dual specificity phosphatase 6
EDTA	Ethylenediamine tetra-acetic acid
ECM	Extracellular matrix
ECL	Enhanced chemiluminescence
E.coli	*Escherichia coli*
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EML4	Echinoderm microtubule-associated protein-like 4
ERK1/2	Extracellular-regulated kinase 1/2
ETS	E26 transformation-specific transcription factor
FAK	Focal adhesion kinase
FCS	Fetal calf serum
FGFR1	Fibroblast growth factor receptor 1
Fig.	Figure
FRS2α	Fibroblast growth factor receptor substrate 2α
fw	forward
Gab1	GRB2-associated-binding protein 1
GAP	GTPase activating protein
GPB	GST pull-down buffer
GDP	Guanosine diphosphate
GEF	Guanine nucleotide exchange factor
GPCR	G-protein-coupled receptor

Grb2	Growth factor receptor-bound protein 2
GSH	Glutathione
GST	Glutathione-S-transferase
GTP	Guanosine triphosphate
h	hours
HDM	High density microsomes
HEPES	2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid
HER2	human epidermal growth factor receptor 2
HVR	Hypervariable region
iCCA	Intrahepatic cholangiocarcinoma
IFS	infectious units
IgG	Immunoglobulin G
IH-region	Isoform-specific hinge region
IP	Immunoprecipitation
JNK	c-Jun N-terminal kinases
kb	Kilobase pairs
kDa	Kilodalton
KO	knockout
KSR	Kinase suppressor of Ras
LB	Luria-Bertani medium
MAPK	Mitogen-activated protein kinase
MAP2K	MAPK kinase
MAP3K	MAPK kinase kinase
MDCK	Madin-Darby canine kidney
MEK1/2	MAPK/ERK kinase 1/2
MP 1	MEK partner 1
M2PK	Pyruvate kinase isoenzyme type M2
mg/ µg	milligram/ microgram
min	minute
ml/ µl	milliliter/ microliter
m/ µmol	milli/ micromol
MMP	Matrix metalloproteinase
MOI	Multiplicity of infection
mRNA	Messenger RNA
MS	Mass spectrometry
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium
MW	Molecular weight
NGF	Nerve growth factor
no.	number
NP-40	Nonidet P40
N-region	Negative-charge regulatory region
NRK	Normal rat kidney
NSCLC	non-small-cell lung cancer
N-terminal	Amino-terminal
PA	Phosphatidic acid
PAK	p21-activated protein kinase
PARP-1	Poly-[ADP-ribose]-polymerase 1
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction

PDGF	Platelet-derived growth factor
PI	Propidium Iodide
PI3K	Phosphatidylinositol 3-kinase
PKC	Protein kinase C
PMSF	Phenylmethylsulfonylfluorid
RAF	Rapidly accelerated fibrosarcoma
RalGDS	Ral guanine nucleotide dissociation stimulator
Ras	Rat sarcoma
RBD	Ras binding domain
rev	reverse
RFFL	ring finger and FYVE-like domain containing E3 ubiquitin protein ligase
RIPA	radioimmunoprecipitation assay
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute medium
RSK	Ribosomal S6 kinase
RTK	Receptor tyrosine kinase
SDS	Sodium dodecyl sulphate
SDS-PAGE	SDS-Polyacrylamide gel electrophoresis
SHP2	Src Homology 2
shRNA	small hairpin ribonucleic acid
siRNA	small interfering RNA
Sos1	Son-of-sevenless 1
TEMED	N,N,N,N- Tetramethylethylendiamine
TIM	translocase of the inner membrane
TK	Tyrosine kinase
Tm	Melting temperature
TOM	translocase of the inner membrane
TRC no.	The RNAi Consortium number
Tris	Tris-hydroxymethyl-aminomethane
UTR	untranslated region
VEGFR	Vascular endothelial growth factor receptor
VSVG	vesicular stomatitis virus G protein
Wt	wild type
v/v	volume per volume

Gene names are generally written in *italics* whereas protein names are written in Roman type (both in capital letters)

Summary

The RAF family of kinases constitutes the members A, B and CRAF. They mediate RAS signaling by linking it to the MEK/ERK transduction module, which regulates cellular processes such as cell proliferation, migration, survival and cell death. As the RAS/RAF/MEK/ERK (MAPK) pathway is found to be activated in human cancers, the RAF kinases have been exploited as valuable therapeutic targets and RAF inhibitors show promising results in the clinic, esp. with tumors harboring an activating *BRAFV600E* mutation. However, RAF inhibitors paradoxically accelerate metastasis in *RAS* mutant and BRAF wildtype tumors. They also become ineffective over time in *BRAFV600E* tumors because of reactivation of downstream mitogen-activated protein kinase (MAPK) signaling by promoting RAF dimerization. Aims of the present work were 1) to investigate the role of ARAF kinase in the paradoxical activation of the enzymatic cascade by RAF inhibitors downstream of mutated RAS and 2) to study the consequences of the loss of ARAF function on signal transduction *in vitro* and *in vivo* (nude mice). We have engineered several cell lines that would allow the study of basal and RAF inhibitor induced effects on MAPK activation, tumor cell migration and invasion.

In summary, we were able to show that the RAF isoform ARAF has an obligatory role in promoting MAPK activity and tumor cell invasion in a cell type-dependent manner. In these cell types, ARAF depletion prevented the activation of MAPK kinase 1 (MEK1) and extracellular signal-regulated kinase 1 and 2 (ERK1/2) and led to a significant decrease of protrusions growing out of tumor cell spheroids in a three-dimensional (3D) culture that were otherwise induced by BRAFV600E-specific or BRAF/CRAF inhibitors (GDC-0879 and sorafenib, respectively). RAF inhibitors stimulated homodimerization of ARAF and heteromerization of BRAF with CRAF and the scaffolding protein KSR1. However, induced oligomerization was not sufficient to activate MAPK signaling if ARAF was depleted. By employing full-length recombinant kinases, we were able to show for the first time that the three RAF isoforms competed for the binding to MEK1. In cell culture models, the overexpression of dimer-deficient ARAF mutants impaired the interaction between ARAF and endogenous MEK1 and thus prevented the subsequent phosphorylation of MEK1 and ERK1/2. Our findings reveal a new role for ARAF in directly activating the MAPK cascade through homodimerization and thereby promoting tumor cell invasion, suggesting the

conserved RAF-dimer interface as a target for RAS- and RAF-mediated cancer therapy. Collectively, we provide evidence for the dual role ARAF plays in controlling MAPK signaling and cancer as loss of ARAF promoted strong lung metastasis formation in nude mice. Preliminary data describing the underlying mechanisms behind ARAF-regulated metastases have been presented and discussed.

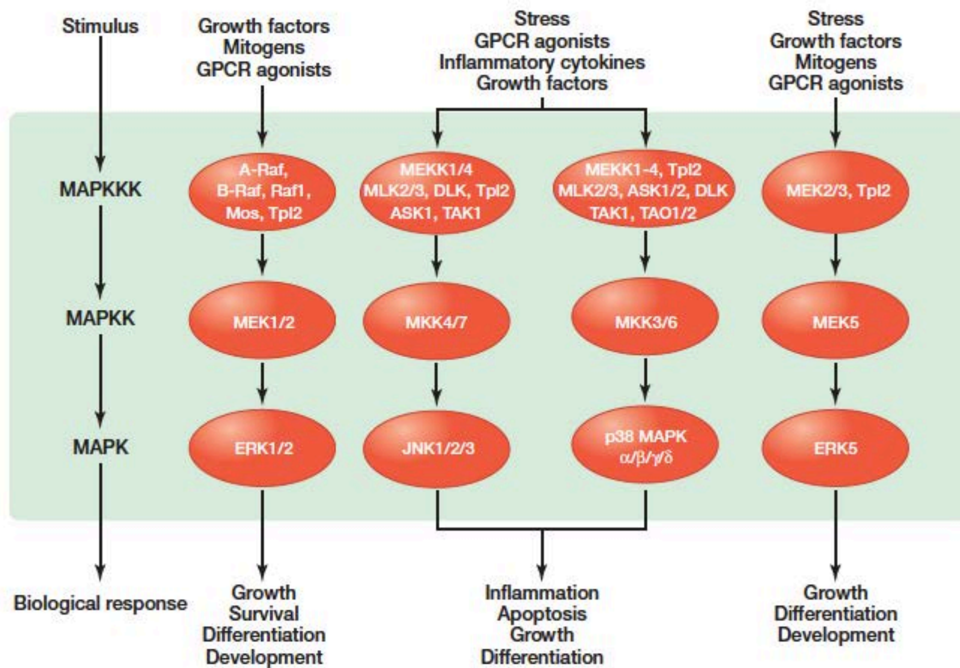
1. Introduction

1.1 The classical MAP kinase cascade

Mitogen-activated protein kinases (MAPKs) are serine/threonine-specific protein kinases that respond to extracellular stimuli (mitogens) and mediate diverse biological functions such as cell growth, survival and differentiation predominantly through the regulation of transcription, metabolism and cytoskeletal rearrangements (Wellbrock, Karasarides, & Marais, 2004). A unique feature of all classical MAPKs is that they themselves are activated by addition of phosphate groups to both their tyrosine and threonine amino acids (dual phosphorylation) in their kinase domain in response to several stimuli. The MAPK signaling pathways play an important role in relaying signals from the cell surface to the nucleus whereby each kinase in this sequence phosphorylates and thereby activates the next member of the cascade. The MAPK activation module typically consists of three protein kinases: a MAPK kinase kinase (MAP3K) that is activated by extracellular stimuli and activates a MAPK kinase (MAP2K) through phosphorylation on its serine and threonine residues. Once activated, the MAPKK phosphorylates its downstream substrate MAPK, thus constituting a three-tier kinase cascade, which represents one of the most ancient three-component module that is conserved from yeast to humans (Widmann, Gibson, Jarpe, & Johnson, 1999). In mammals, fourteen MAPKs have been categorized into seven groups. Conventional MAPKs comprise the extracellular signal-regulated kinases 1/2 (ERK1/2), c-Jun amino (N)-terminal kinases (JNKs), p38 isoforms and ERK5 (Pearson et al., 2001). Atypical MAP kinases have uncommon characteristics and include ERK3, ERK4, Nemo-like kinase NLK and ERK7/8 (Coulombe & Meloche, 2007). Of the three classical MAPK families, the ERK1/2 (Extracellular signal-Regulated Kinase) will be of special interest in this work. JNK/SAPK (C-Jun N-terminal Kinase/ Stress-Activated Protein Kinase) and p38 will be discussed in appropriate sections.

The Insulin/Mitogen-regulated ERK pathway was essentially the first mammalian MAPK pathway ever to be identified. It is regulated by the monomeric GTPase Ras, which at the plasma membrane recruits MAP3Ks of the RAF family to activate ERK1/2 via phosphorylation of dual-specificity kinases MEK1 and MEK2 (Kyriakis & Avruch, 2001; Pearson et al., 2001). The classic ERK1/2 module responds mostly to growth factors and

mitogens whereby upstream signaling is regulated through cell surface receptors, such as receptor tyrosine kinases, G-protein-coupled receptors, integrins as well as the aforementioned small GTPase Ras (Morrison, 2012) (Fig. 1).

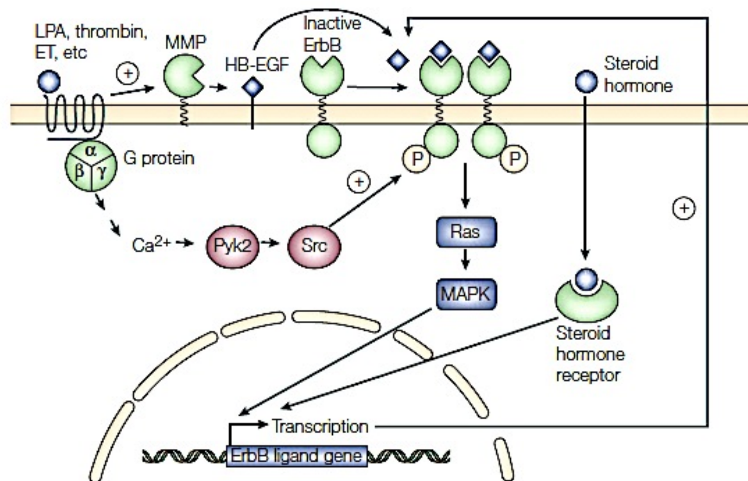


(From Morrison et al. 2012)

Figure 1: **Conventional MAPKs containing three sequentially activated protein kinases (MAP3K, MAP2K, MAPK) and their downstream implications in cells.**

One major example for a receptor tyrosine kinase is the epidermal growth factor receptor (EGFR) that is involved in the growth of epithelial cells and growth advancement in tumors of epithelial origin (Yarden & Sliwkowski, 2001). Stimulation of the EGFR pathways has been shown to promote tumor cell motility, adhesion and metastasis (Engebraaten, Bjerkvig, Pedersen, & Laerum, 1993; Shibata et al., 1996; Wells, 1999). Transmembrane signaling of the EGF receptor depends on the intrinsic tyrosine kinase activity of the receptor molecule. Phosphorylated tyrosine residues serve as a docking platform for adaptor proteins and GEFs, which in turn activate intracellular signaling pathways. The EGF family of receptor tyrosine kinases comprises four members ERBB1, ERBB2, ERBB3 and ERBB4 (Burden & Yarden, 1997). These structurally related receptors and their ligands are implicated in cell-cell interactions and in organogenesis. In the epithelium, ERBB- localization is important for their activation and biological functions as they coordinate epithelial homeostasis or the pathology of carcinomas respectively (Borg et al., 2000). Compared to the other ERBBs, ERBB2 (also known as HER2) is a more potent oncoprotein and has been shown to be

overexpressed in a variety of tumors (Olayioye, Neve, Lane, & Hynes, 2000; Ross & Fletcher, 1998). An amplification of a mutant form of the *ERBB2* gene, which encodes a variant that makes its tyrosine kinase constitutively active, is the cause of many cancers of epithelial origin (Yarden & Sliwkowski, 2001). Somatic mutations of the ERBB2 kinase domain have been detected in gastric, colorectal, and breast carcinomas (Lee et al., 2006). The Ras-activated MAPK pathway is only one of many ERBB target proteins and networks (Fig. 2).



(From Yarden and Sliwkowski 2001)

Figure 2: Crosstalk between the ERBB network and other kinase signalling pathways

1.2 MAPK signaling in cell survival and oncogenesis

Apart from cell proliferation, MAPK pathway also controls cell survival, migration and differentiation. Deviation from the strict control of MAPK signaling pathways is associated with the development of many human diseases including Alzheimer's disease (AD), Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS) and various types of cancers (Kim & Choi, 2010). Aberrant activation of the JNK or p38 signaling pathways are implicated in the mediation of neuronal apoptosis in AD, PD, and ALS, while the ERK signaling pathway is a key determinant of several steps in tumorigenesis including cancer cell proliferation, migration and invasion. The RAS-ERK pathway has long been associated with human cancers as oncogenic mutations in *RAS* occur in ~15% of cancers (Davies et al., 2002) specifically in 90% of pancreatic carcinomas, 50% colon cancers, 30% lung cancer and nearly 30% of myeloid leukemias (Bos, 1989). ERK1/2 is found to be hyper activated in ~30% of cancers (Allen, Sebolt-Leopold, & Meyer, 2003). ERK1/2- mediated transcriptional

regulation of various genes by phosphorylation contributes to cell survival and oncogenesis. Members of the ternary complex factor (TCF) subfamily of the ETS-domain transcription factors are among the first to be activated upon mitogenic and stress stimuli. TCF family of transcription factors induce immediate early genes (IEGs) such as c-Fos and c-Myc, which in turn induce late response genes that promote cell survival, cell division and cell motility (Dhillon, Hagan, Rath, & Kolch, 2007; Murphy & Blenis, 2006). c-Myc itself is a multifunctional transcription factor, which contributes to the tight regulation of gene expression and thus plays a critical role in oncogenic transformation (Morrish, Neretti, Sedivy, & Hockenbery, 2008). Proteins that are phosphorylated by ERK1/2 include myosin light chain kinase, calpain, focal adhesion kinase, and paxillin, all of which are found to be engaged in the promotion of cancer cell migration (Kim & Choi, 2010). Moreover, the ERK1/2 pathway induces expression of matrix metalloproteinases (MMP) thereby enabling the degradation of extracellular matrix proteins and consequent tumor invasion. In these lines, activated MEK1/2 has been found to protect cancer cells from anoikis, or detachment-induced apoptosis, which is a prerequisite for the formation of metastatic tumors (Voisin et al., 2008).

The RAF proteins have long been considered as important targets primarily as RAS effector proteins before they were discovered to have oncogenic activity (Moelling, Heimann, Beimling, Rapp, & Sander, 1984). Around a decade ago BRAF somatic point mutations were identified in a variety of human cancers (Davies et al., 2002), which will be discussed in more detail below. Furthermore, dysregulation of the Ras-MAPK signaling pathway has been identified as a principal cause of a class of genetic diseases, Ras-MAPK syndromes- now termed “RASopathies”, which include Noonan, LEOPARD, Costello, and cardio-facio-cutaneous syndromes as well as neurofibromatosis type I (Aoki, Niihori, Narumi, Kure, & Matsubara, 2008). Noonan syndrome (NS) is a relatively common (1 in 1,000 to 2,500 live births) autosomal dominant disorder (Tartaglia, Gelb, & Zenker, 2011) and although genetically heterogeneous, all known cases are caused by germ line mutations in conserved components of the canonical RAS-RAF-MEK-ERK pathway (Wu et al., 2012). While in half of NS cases, mutations in the protein tyrosine phosphatase SHP2 account for the disease phenotype (Tartaglia et al., 2001) other known NS genes include *SOS1* (~10%) (A. E. Roberts et al., 2007), *CRAF* (3 to 5%) (Razzaque et al., 2007), *KRAS* (<2%) and *NRAS* (Cirstea et al., 2010; Schubbert et al., 2006).

1.2.1 Ras- GTPase

RAS proteins belong to the family of small GTPases that control a variety of signaling cascades and depending on the cellular context, they mediate cell growth, cell shape and migration, endocytosis, cell cycle progression and survival among others. *RAS* oncogenes have been originally discovered as retroviral oncogenes from the genome of Harvey and Kirsten rat sarcoma viruses some 50 years ago (Harvey, 1964; Kirsten & Mayer, 1967). A lot of attention has been drawn to them with the identification of constitutively activating *RAS* mutations in human tumors ever since. The human genome comprises of three different *RAS* genes, named *Ha(rvey)-*, *K-(irsten)-* and *N(euroblastoma)-RAS.* The RAS superfamily of GTP-binding proteins regulate signal transduction across membranes where they assemble transient signaling complexes that relay information further via multilayered signaling networks (Rajalingam, Schreck, Rapp, & Albert, 2007). Figure 3 summarizes the diverse consequences of RAS activation upon stimulation of the mitogenic cascade (Kinbara, Goldfinger, Hansen, Chou, & Ginsberg, 2003; Wellbrock et al., 2004).

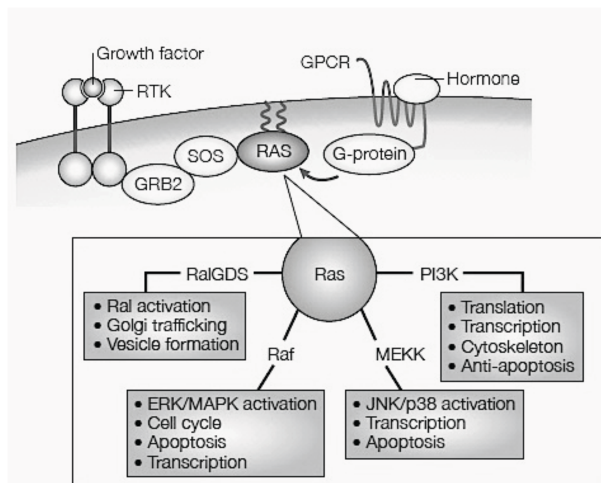
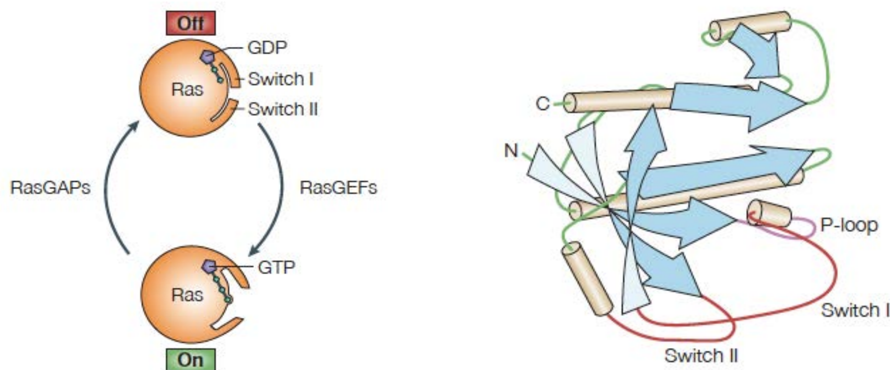


Figure 3: Ras activation and Ras effectors The binding of growth factors to their cell-surface receptor tyrosine kinases (RTKs) signals through adaptors such as growth-factor-receptor bound-2 (GRB2) and exchange factors such as Son-of-sevenless (SOS) to activate RAS. Likewise, hormone binding to G-Protein-coupled receptors (GPCRs) activates RAS through heterotrimeric G-proteins.
Once activated, RAS facilitates signal transduction via its various downstream effectors such as RAF kinases, PI3Ks and GEFs for RalGDS or MEKKs.

(Adapted from Wellbrock and Kinbara et al. 2003)

The RAS isoforms are highly homologous with their catalytic G-domain being almost identical (residues 1–165) consisting of the guanine nucleotide binding site and the effector binding site. Analysis of the approximately 50 crystal structures of HRAS and the x-ray structures of K- and NRAS confirmed the notable similarity of these proteins (Gorfe, Grant, & McCammon, 2008). Therefore it is likely that the functional differences between the RAS isoforms originate from the C-terminal hypervariable region, which comprises the last 23 of 24 amino acids. This region is responsible for membrane anchoring of Ras as well as for its intracellular trafficking. RAS proteins are guanosine-nucleotide-binding proteins and

alternate between a GTP-bound “on” conformation and GDP- bound “off” state to regulate their activity (Bourne, Sanders, & McCormick, 1991). As the cycling between these two states is intrinsically very slow, so called guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAP) aid to accelerate RAS activation and deactivation respectively (Gideon et al., 1992). The GAPs increase the GTP hydrolysis of RAS (“switch-off”), which is recovered by the dissociation of GDP through GEF action catalyzing its replacement with GTP (“switch-on”) (Fig. 4 left). Upon binding, three short segments (switch region I and II and the P-loop) that border the nucleotide-binding site, undergo dramatic structural changes. While the P-loop coordinates nucleotide binding by defining the effector specificity towards a given GTPase, the switch regions I and II make up a mobile binding surface for effector molecules in a GTP-dependent manner (Fig. 4 right).



(From Corbett et al. 2001)

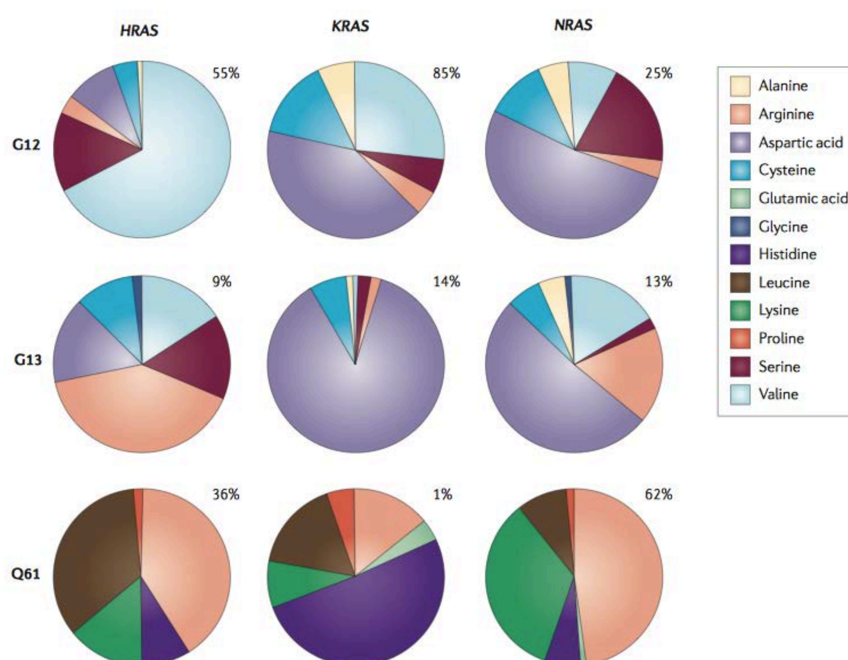
Figure 4: Structural changes in a Ras molecule upon nucleotide binding focussing the G-domain of Ras.

RAS effectors have an increased affinity for GTP bound RAS and are usually characterized by the presence of a Ras binding domain (RBD). One of the best-described RAS effector proteins is the RAF kinase, which will be the focus of this work.

In order to become biologically functional, RAS proteins need to undergo posttranslational modifications that lead to membrane anchoring and subsequent signal transduction. The initial set of modifications is directed by the carboxy-terminal CAAX motif, which is common to all RAS proteins. It is the cysteine of the CAAX box that becomes farnesylated to ensure membrane-association of the RAS proteins. Farnesylinhibitors (FTI) have thus been used to prevent proper Ras function, which is abnormally activated in cancer. Due to additional prenylation of K- and NRAS in the presence of FTI they are considered rather ineffective as this resulted in a persistent membrane localization of KRAS and NRAS and consequent upregulation of downstream signaling (Fiordalisi et al., 2003).

In human clinical trials, monotherapy with FTIs showed limited anti-tumor activity in hematopoietic cancers, and generally no or very little activity in solid tumors. The last 3 amino acids of the CAAX box (–AAX) are subjected to proteolytic processing. In particular NRAS and HRAS, and to a certain extent KRAS 4A, additionally undergo a palmitoylation/ depalmitoylation cycle by which these proteins shuttle from the ER/Golgi to the plasma membrane and back (Goodwin et al., 2005). The final CAAX processing step is carboxyl methylation that has been speculated to be required for the binding with interacting proteins. Since methylation is a reversible reaction, it has been suggested that this could represent another level of regulating RAS activity or subcellular localization.

The activation of *RAS* genes is frequently observed in human cancer. Point mutation at either position G12, G13 or Q61 in the *RAS* gene has been shown responsible for the conversion of a proto-oncogene to an oncogene (Barbacid, 1987). These oncogenic mutants of *RAS* display a whole spectrum of amino acid exchanges (Fig. 5) and the extent to which specific mutations affect the biological behavior of RAS remains to be established (Pylayeva-Gupta, Grabocka, & Bar-Sagi, 2011). These substitutions prevent the intrinsic and GAP catalyzed hydrolysis of GTP, thereby generating permanently active RAS molecules with severe consequences for the cell.



(From Pylayeva-Gupta et al. 2011)

Figure 5: Frequency of mutations at G12, G13 and Q61 in RAS isoforms. The frequency of mutational substitution at G12, G13 or Q61 for a particular amino acid has been represented using pie charts. Percentages indicate the frequency of a given residue mutated within a particular RAS isoform.

In particular, mutations in the *KRAS* gene are involved in the pathogenesis of a variety of human tumors. *KRAS* mutations are most frequently detected in colorectal tumors, lung carcinomas (mostly NSCLC) and in pancreatic carcinomas and have been shown to influence both tumor progression as well as drug resistance (Pylayeva-Gupta et al., 2011). *HRAS* mutations are associated with tumors of the skin and of the head and neck while *NRAS* mutations are common in melanomas and haematopoietic malignancies. Figure 6 summarizes the incidences of gain-of-function mutation in *RAS* genes identified in a variety of human cancers. The spectrum of RAS mutations varies with respect to organ site and allele frequency possibly due to differences in tissue specific RAS expression (Miller & Miller, 2011). Also the distribution of mutant alleles within each cancer type is disparate, pointing to non-redundant functions of the *RAS* alleles in tumorigenesis. It is further suggested that subtle differences in the (lack of) activation of down stream effectors by different *RAS* mutant alleles might account for the subsequent tumor phenotype.

There is a lot of *in vitro* and *in vivo* evidence for differential effects of *RAS* mutant alleles (Miller & Miller, 2011). In three independent studies, Miller and colleagues demonstrated that distinct *RAS* mutations are associated with different tumor stages (Gressani et al., 1999; Jennings-Gee et al., 2006; Leone-Kabler, Wessner, McEntee, D'Agostino, & Miller, 1997). Treatment of pregnant mice with a potent chemical carcinogen *in utero* resulted in a high incidence of lung tumors in the offspring half a year after birth. Mice harboring a V12, R12, D12, or D13 mutant Ki-*RAS* gene were shown to contain late stage neoplasms in contrast to mice harboring the C12 or wildtype allele. The latter exhibited mostly benign adenomas and hyperplasias. Interestingly, the mutant Ki-*RAS* alleles associated with progression to later stage tumors were the same ones associated with a trend for poorer patient outcome in a clinical study of human lung cancer (Keohavong et al., 1996). In colorectal cancer, *KRAS*G12V mutations have been associated with a worse prognosis than *KRAS*G12D mutations, underlining the possibility that particular amino acid substitutions might dictate specific transforming characteristics of oncogenic *RAS* alleles (Andreyev, Norman, Cunningham, Oates, & Clarke, 1998). In support of this idea, HRASG12V exhibits weaker GTPase activity and stronger binding to GTP than HRASG12D (Al-Mulla, Milner-White, Going, & Birnie, 1999) and additionally has been shown to be more potent in cell culture-based transformation assays. Not only initiation of tumor formation but also tumor progression is suggested to be dictated by different *RAS* variant alleles as certain *RAS* mutant alleles seem to convey a greater growth advantage than other alleles. However, these results

are still inconsistent and contradictive. A deeper understanding about the link between sequence variations and functional alterations of oncogenic forms of RAS deserves further studies.

Tissue	*KRAS**	*HRAS**	*NRAS**
Pancreas	52.6% (8758)	0% (2225)	0.48% (2063)
Large Intestine	34.6% (52724)	0.6% (1731)	4.02% (7770)
Small Intestine	22.6% (664)	0% (55)	0.71% (140)
Peritoneum	29.0% (172)	0% (13)	0% (10)
Biliary tract	24.1% (2590)	0% (349)	2.58% (543)
Lung	16.25% (27485)	0.5% (3903)	0.65% (11895)
Skin	2.23% (3493)	11.45% (4201)	15.64% (10083)
Endometrium	14.54% (3151)	0.54% (931)	2.29% (960)
Ovary	11.06% (5562)	0.08% (1253)	0.75% (1329)
Cervix	6.56% (869)	5.94% (387)	0.78% (258)
Stomach	6.15% (4455)	1.37% (1019)	1.06% (851)
Gastrointestinal tract	5.26% (1046)	0% (0)	0% (476)
Genital tract	5.15% (97)	1.52 (66)	1.14% (88)
Prostate	5.06% (1857)	3.08% (1104)	0.79% (1134)
Urinary tract	4.41% (1836)	9.33% (2796)	1.21% (1567)
Soft tissue	4.33% (1938)	3.89% (1029)	3.55% (957)
Testis	3.85% (441)	3.97% (126)	2.38% (336)
Haematopoietic and lymphoid	3.82% (12077)	0.2% (6839)	7.95% (15367)
Salivary gland	2.5% (400)	8.77% (399)	0.73% (274)
Liver	2.35% (1831)	0.13% (1496)	0.52% (1537)
Bone	2.25% (439)	1.55% (387)	2.27% (528)
Upper aerodigestive tract	1.98% (3376)	6.21% (2240)	1.6% (1871)
Breast	1.63% (4053)	0.33% (2742)	0.72% (2374)
Eye	1.57% (257)	0% (179)	2.65% (339)
Thymus	1.53% (261)	2.17% (47)	0% (49)
Thyroid	1.52% (7588)	3.67% (6049)	6.68% (7115)
Meninges	0% (173)	0% (138)	7.26% (179)

Figure 6: Frequency of RAS mutations in human cancer (in per cent). Data from the COSMIC database numbers in parentheses indicate total unique samples sequenced.

Clearly, the discovery of clinically relevant *RAS* mutations has only started and will be of great importance in tumor therapy. The lessons learned from more than 40 years of RAS-research is that the oncogenic potential of RAS is context-dependent, whereby the subcellular, cellular and tissue environments of oncogenic RAS signaling determines its functional output (Pylayeva-Gupta et al., 2011). Thus far, *RAS* is the most commonly mutated gene in human cancers as it regulates complex signal transduction modules involved in proliferation, cell survival and drug resistance among others (Fernandez-Medarde & Santos, 2011). A recent study on lung adenocarcinoma reveals the diverse nature of interconnected signaling networks in human cancers (Vandal, Geiling, & Dankort, 2014). Activating mutations in various components of different signaling cascades were not only found in RAS but included the following oncogenes: EGFR (39%), ALK fusions (notably with EML4) (4%), ERBB2 (3%) and BRAF (3%). Interestingly, oncogenic Ras mutations and mutations in other components of Ras/MAPK signaling pathways appear to be mutually exclusive events in most tumors, indicating that deregulation of Ras-dependent signaling is the essential requirement for tumorigenesis.

1.2.2 RAF kinases

The serine/threonine-specific protein kinase RAF is a key modulator of the classical MAP kinase cascade (mitogenic cascade) and constitutes the isoforms A, B -and CRAF. The name 'Raf' derives from the ability of the retrovirus (clone 3611-MSV) to induce 'rapidly growing fibrosarcomas' in mice. The transduced oncogene was called v-raf, while its cellular homologue was named *CRAF* (Rapp et al., 1983), which was the first out of three RAF isoforms to be discovered. It was the first oncogene kinase reported to possess serine/threonine rather than tyrosine kinase activity (Moelling, Heimann, Beimling, Rapp, & Sander, 1984). The finding that it was coexisting with the Myc oncogene in retroviruses revolutionized the concept of cellular signaling as it was proposed that upon a growth factor signal entering the cell, there is a tyrosine to serine phosphorylation switch. It further provided the mechanistic basis for a nuclear and cytoplasmic collaboration of oncogenes via phosphorylation of transcription factor class oncogenes such as Myc (T. M. Roberts et al., 1988). Homologues of *CRAF* were found in *Drosophila melanogaster* (*D-Raf*) and *Caenorhabditis elegans* (*lin-45*), and two related genes - *ARAF* and *BRAF* - were found in vertebrates (Huleihel et al., 1986; Ikawa et al., 1988; Marais & Marshall, 1996). This implies that the evolvement of RAF kinases was a prerequisite for multicellularity. Plant genomes

contain a RAF-like kinase that is deprived of an RBD, the major characteristic of all RAF isoforms. The gain of an RBD enabled RAF to become the primary messenger of RAS-mediated signals from receptor tyrosine kinases to the MEK-ERK pathway in animals (Rajalingam et al., 2007) (Fig. 7). With the emergence of the vertebrates, three RAF kinases are introduced, whereby BRAF is most likely to be the original RAF kinase as it is more closely related to all other eukaryotic RAF homologues than either A- or CRAF. Having three RAF enzymes with widely differing basal and inducible activities might significantly improve fine-tuning of the mitogenic cascade (Garnett, Rana, Paterson, Barford, & Marais, 2005). The transition of BRAF to C- and ARAF probably required a reduction of the extraordinary high basal activity of BRAF.

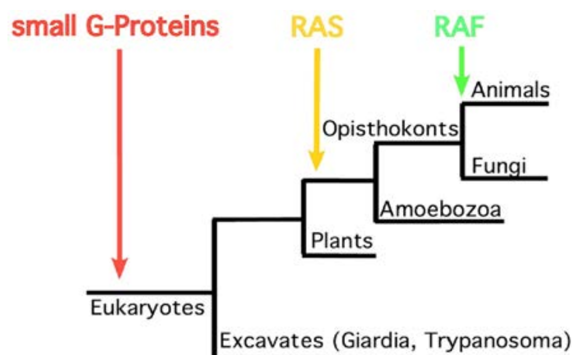


Figure 7: Major steps in the evolution of RAS and RAF signaling (left to right): Compartmentalization of eukaryotic cells into membrane-enclosed organelles goes hand in hand with gene duplication and functional diversification of small G-proteins. RAS appears at indicated point in evolution estimated by phylogenetic analysis. Introduction of RAF kinases at the level of multicellularity in animals link RAS and MAPK signaling.
(From Rajalingam et al. 2007)

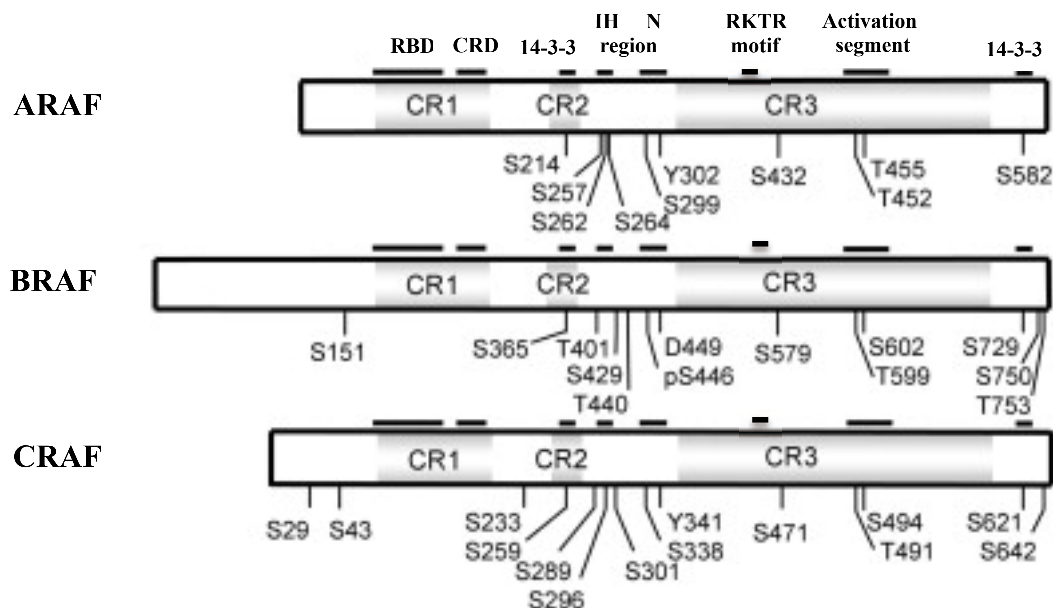
Although all mammalian RAF isoforms share considerable sequence similarity, they exhibit common and unique roles in controlling normal and pathophysiology, which needs further intensive investigations. CRAF at the mitochondrial membrane can bind Bcl-2 family proteins, thereby conferring an anti-apoptotic signal by promoting the phosphorylation of the Bcl-2 family member Bad (Salomoni et al., 1998; H. G. Wang, Rapp, & Reed, 1996). BRAF has been shown to inhibit cytochrome c- mediated apoptosis by preventing the activation of caspases (Erhardt, Schremser, & Cooper, 1999). ARAF at least has been predicted to interact with the apoptosis regulator Bcl-2 (www.compbio.dundee.ac.uk). Its role in mitochondrial function and regulation of cell survival however is not well understood.
Genetic studies in mice have shown that the RAF proteins carry out non-redundant functions during mammalian development. *ARAF –/–* mice are viable until birth, but die within 3 weeks after due to neurological and gastrointestinal defects (Pritchard, Bolin, Slattery, Murray, & McMahon, 1996). Mouse embryos that are *BRAF –/–* or *CRAF –/–* die *in utero* between 10.5 and 12.5 days postcoitum. While the *BRAF* knock out embryo displays growth

retardation, and vascular and neuronal defects, the *CRAF –/–* embryos die of massive liver apoptosis and poor development of the placenta and the haematopoietic organs (Mikula et al., 2001; Wojnowski et al., 1998; Wojnowski et al., 1997). As for the latter observation, CRAF is required to restrain apoptosis during embryogenesis. The lack of compensation between the RAF isoforms in mice point to distinct RAF functions and thus is not the consequence of a differential expression pattern. Data obtained from studies with Mouse Embryonic Fibroblasts (MEFs) regarding the role of different RAF isoforms in MAPK activation have to be carefully interpreted, as they do not necessarily recapitulate MAPK signaling in the whole organism (Galabova-Kovacs et al., 2006). Therefore, a more detailed examination of the individual roles of the three Raf isoforms in specific tissues will be required to gain a better understanding of the complex signaling function of each RAF isoform. Mammalian RAF isoforms differ in their basal and growth factor-induced activity. While BRAF displays a high intrinsic kinase activity it is weakly responsive to oncogenic RAS and as opposed to CRAF not at all stimulated by activated SRC (Marais, Light, Paterson, Mason, & Marshall, 1997). CRAF on the other hand possesses low activity in non-stimulated cells, but is readily activated by oncogenic RAS and SRC (Marais et al., 1998). While ARAF has also been shown to be activated by RAS and SRC, its activity only reaches ~20% of that for CRAF under these conditions and is altogether lower compared with BRAF (Marais et al., 1997). Regarding cell proliferation, expression of active RAF isoforms (BRAF or CRAF) have pro-proliferative effects, inducing unrestrained proliferation and transformation, and even at low conditional gene knock-in levels of the *V600E BRAF* mutant allele, transgenic mice display hyper-proliferative disorders (Mercer et al., 2005). This single point mutation in BRAF is enough to render its kinase activity much higher than it occurs in normal cells, making this RAF isoform an important player in cancer progression. This is underlined by the fact that in several cancer types, like melanoma and colorectal cancer, the single amino acid exchange in BRAF (V600E) is one of the most commonly found mutations. Interestingly, a corresponding mutation in ARAF or CRAF was not found in human cancers (Dhomen & Marais, 2007). CRAF and ARAF mutations are rare, as they cannot be activated by a single mutation but require two mutations for oncogenic activation. This is due to the composition and structure of their kinase domain, which accounts for a tighter regulation of kinase activity in these RAF isoforms (Emuss, Garnett, Mason, & Marais, 2005; Fransen et al., 2004). RAF proteins are subject to complex regulation, which is represented by the numerous phosphorylation sites distributed throughout the proteins. Some sites are conserved in all three isoforms

suggesting common regulatory mechanisms, whereas others are not, implying that these proteins can be independently regulated (Wellbrock et al., 2004).

1.2.2.1 Structure of RAF proteins

RAF kinases comprise of three conserved regions (CR1, CR2 and CR3) and can roughly be divided into the N-terminal regulatory domain and the C-terminal kinase domain. The initial process of RAF activation needs the interaction of active GTP-bound RAS with the RBD and CRD of CR1, which results in subsequent recruitment of RAF to the plasma membrane for further activation (Morrison, Kaplan, Rapp, & Roberts, 1988). CR2 is rich in serine/threonines and is therefore believed to influences RAF-localization and activation through phosphorylation and various protein-protein interactions (Guan et al., 2000; B. H. Zhang & Guan, 2000). It further contains a 14-3-3 binding site, that when phosphorylated is inhibitory, ensuring correct regulation of kinase activity (Light, Paterson, & Marais, 2002). Deletions of the N-terminal regulatory domains (CR1 and CR2) occur in several activated forms of *RAF* genes and were found in certain neoplastic human cells, which suggest that these domains negatively regulate RAF (Fukui, Yamamoto, Kawai, Maruo, & Toyoshima, 1985). CR3 is the catalytic kinase domain of RAF, which is located near the C-terminus. It is also subject to regulation by phosphorylation. A stimulatory 14-3-3-binding site occurs after the kinase domain. Figure 8 gives a simplistic overview of the organization of the three RAF enzymes with important residues and motifs indicated in a comparative manner.



(Adapted from Roskoski et al. 2010)

Figure 8: Schematic depiction of important domains in RAF protein kinases

The RAF isozymes display different intrinsic enzymatic activities with BRAF exhibiting a high basal activity compared to CRAF and ARAF, due to a motif called the N-region (negative charge region) that contains conserved serines and tyrosines. While in ARAF and CRAF, phosphorylation of these tyrosine residues are needed for proper kinase function, the N-region of BRAF is constantly negatively charged. Thus the constitutive phosphorylation of one of the serines and the substitution of the tyrosines to aspartic acid renders the basal kinase activity of BRAF rather high (Roskoski, 2010). The high degree of sequence similarity between the RAF isoforms suggests that all RAF proteins adopt a similar conformation in the inactive state. Reconstruction model of RAF kinase domains, suggest that the tight binding between the N-region and the well conserved catalytic domain acts inhibitory with regard to the kinase activity of RAF proteins, whereas release of this interaction favors the active form of the kinase (Baljuls, Mueller, Drexler, Hekman, & Rapp, 2007). Interestingly, the N-region of BRAF reveals only 60% identity compared with the N-region of ARAF and CRAF, implying a differential induction of basal as well as inducible kinase activity for this RAF isoform, favoring the active form of the BRAF monomer (Baljuls et al., 2011). Hence, the inhibitory interaction of the N-terminal regulatory half of RAF with its own C-terminal kinase domain needs to be displaced to ensure proper activation. This is facilitated through phosphorylation/ dephosphorylation events and/or interactions with other regulatory factors upon pathway stimulation (Daum, Eisenmann-Tappe, Fries, Troppmair, & Rapp, 1994). Abolishment of binding between the N-region and the catalytic domain of RAF leads to the reorganization of the complex formation between 14-3-3 proteins and RAF and subsequently preferential formation of heterodimers between C- and BRAF (Baljuls et al., 2011), a decisive step in RAF activation.

The RAF protein kinase domain is comprised of the N and C lobe characteristic for all protein kinases. The small N-lobe displays a mostly antiparallel β-sheet structure and anchors and orients ATP through a glycine-rich ATP-phosphate-binding loop, called the P-loop (Roskoski, 2010). It contains a regulatory αC helix, a short polypeptide segment, which rotates between active and inactive conformations, making or breaking parts of the active site. The αC helices of RAF subunits are for example involved in dimer formation, an important process in the activation of many kinases (Hatzivassiliou et al., 2010; Rajakulendran, Sahmi, Lefrancois, Sicheri, & Therrien, 2009). The RKTR- motif within the N- lobe plays thereby a major role as substitution of each of the basic residues within the RKTR motif resulted in inhibited kinase activity of RAF isoforms. Selective replacement of

the basic residues within this motif resulted in different phenotypes for ARAF and CRAF versus BRAF, indicating a multifunctional role for this regulatory segment (Baljuls et al., 2011).

The large C-lobe is mainly α-helical and responsible for substrate (MEK1/2) binding. Figure 9 (left) shows a ribbon diagram illustrating the structure of human BRAF, in which the ATP-competitive RAF- inhibitor sorafenib occupies the catalytic site of the kinase domain. Sorafenib stabilizes the inactive conformation of the BRAF kinase by associating the P- loop with the activation segment (AS). Before the kinase domain can become active, thus catalyzing protein phosphorylation, it needs to adjust its activation segment in the N-lobe, the so-called DFG (Asp/Phe/Gly) motif. In the inactive conformation (DFG Asp-out), the phenylalanine side chain Phe 595 occupies the ATP-binding pocket, and the aspartate side (Asp594) chain faces away from the active site (Seeliger et al., 2009). In the active conformation (DFG Asp-in), the phenylalanine side chain is rotated out of the ATP-binding pocket, enabling the aspartate side chain to face into the ATP-binding pocket to coordinate $Mg_{2+,}$ which in turn mediates the β -and γ phosphates of ATP. The multiple interactions within the BRAF kinase domain are depicted below including catalytically important core residues, secondary structures as well as motifs that are involved in the regulation of catalytic activity (Fig. 9, right).

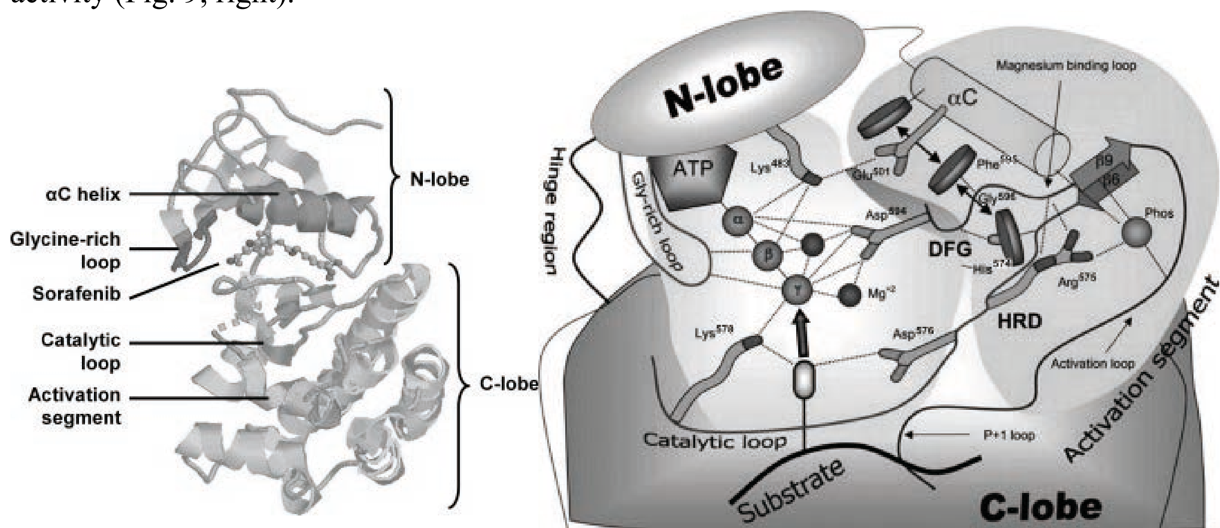


Figure 9: BRAF structure (ribbon diagram) and close up of BRAF kinase domain (depiction), *see text for details,* ***(Adapted from Roskoski et al. 2010)***

A gatekeeper residue in many protein kinases separates the adenine-binding site from an adjacent hydrophobic pocket, thereby controlling kinase sensitivity to a wide range of structurally unrelated compounds. In the human kinome, a large amino acid residue like

threonine restricts access to a pre-existing cavity within the ATP binding pocket (Liu, Shah, Yang, Witucki, & Shokat, 1998). It is readily targeted by diverse classes of small molecule inhibitors that can access this natural pocket. Mutation of the gatekeeper residue threonine to a larger one like methionine can prevent the binding of kinase inhibitory drugs, thereby conferring resistance to drugs in the clinic.

	ARAF	**BRAF**	**CRAF**
RBD	19-91	155-227	56-131
CRD	98-144	234-280	138-184
CR1	14-154	150-290	51-194
CR2	209-224	360-375	254-269
CR3, protein kinase domain	310-570	451-717	349-609
N-region	295-304	442-451	334-343
Glycine-rich loop	316-324	463-471	355-363
RKT motif	356-366	503-513	395-405
14-3-3 binding sites	S214, S582	S365, S729	S259, S261
Gate keeper residue	T382	T529	T421
HRD	427-429	574-576	466-468
DFG	447-449	594-595	486-488
AS phosphorylation sites	T452, T455	T599, S602	T491, S494
End of AS	AAE, 474-476	APE, 621-623	APE, 523-525
MEK binding site	S432	S579	471
No. of residues	604	766	648
Molecular weight (kDa)	67.5	84.4	73.0

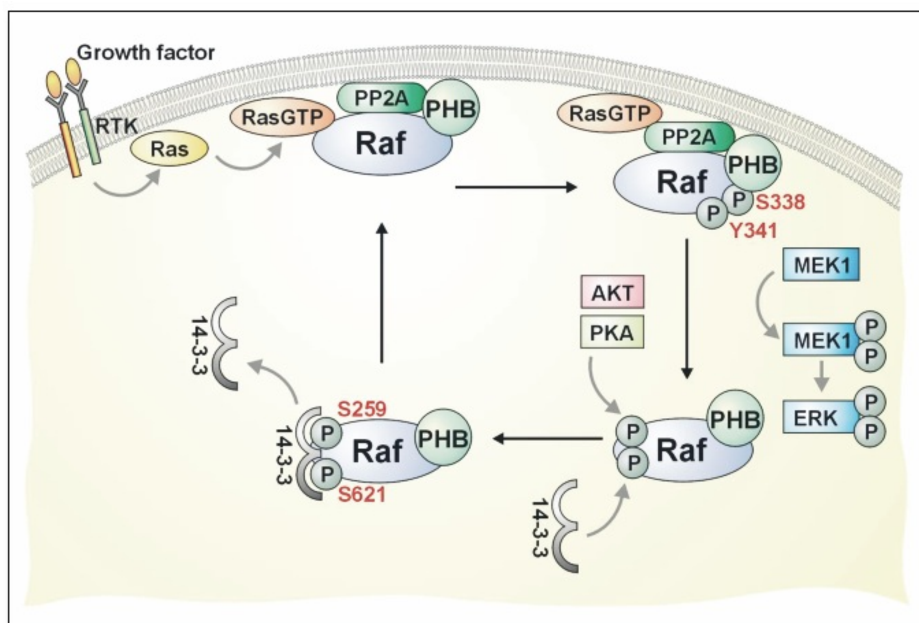
Figure 10: Important residues in human RAF kinases

The activation segment of nearly all protein kinases begins with DFG and ends with APE (Ala/Pro/Glu). However, the activation segment of ARAF ends with AAE (Ala/Ala/Glu) (Roskoski, 2010). Figure 10 summarizes most of the functionally important RAF kinase residues and motifs featured in this work.

Kinases usually contain one or more phosphorylation sites within their activation segment that are either phosphorylated by members of the same or other protein kinase families. An important example in this work will be the phosphorylation of serine residues within the activation segment of MEK1/2 catalyzed by the RAF kinases.

1.2.2.3 Regulation of RAF activity by phosphorylation

The molecular mechanism, which regulates RAF activity, is highly complex and tightly regulated. A lot of structural modifications have to happen that would allow phosphorylation/dephosphorylation events to take place (Dhillon, Meikle, Yazici, Eulitz, & Kolch, 2002). Once RAF associates with plasma membrane lipids, the assembly of a so-called RAF signalosome is facilitated through the interaction with different adaptor and scaffold proteins leading to full activation of RAF (Fig. 11). Such scaffold proteins mediate the activation of MAPK signaling pathways consisting of specific kinase components. KSR1 and MP1 function as scaffold proteins for the ERK signaling pathway.



(From Rajalingam et al. 2005)

Figure 11: The activation cycle of CRAF in short: when Ras is activated, CRAF bound to PHB is recruited to the plasma membrane enabling 14-3-3 displacement from the internal binding site (S259), and access to phosphatases (PP2A). The subsequent dephosphorylation of the internal 14-3-3 binding site initiates the activation of RAF kinase, which in turn leads to a complex set of phosphorylation events mediated by p21-Activated Kinase 1 (PAK1) at serine 338 (S338) and tyrosine 341(Y341) catalyzed by SRC family kinases resulting in full activation of the membrane bound CRAF followed by MEK1 and ERK activation.

CRAF is typically phosphorylated at serine 259 by protein kinase A (PKA) or PKB/AKT, ensuring inactivation through 14-3-3 binding to this residue. A fraction of RAF molecules (raft microdomains) however is thought to exist as membrane-prebound, which is targeted to the plasma membrane primarily upon cholesterol and ceramides stimulation (Hekman et al., 2002). The binding of the RAF catalytic domain to phosphatidic acid (PA) has also been

shown to enable RAF association with the membrane. The PA binding segment of CRAF is located between residues 389 and 423 that are highly conserved between all mammalian RAF isoforms (Rizzo, Shome, Watkins, & Romero, 2000), suggesting that RAF association with the plasma membrane lipids represents the initial step in RAF activation. Our lab could previously show that membrane targeting and activation of CRAF by RAS needs prohibitin (PHB), which recruits CRAF from the plane of the plasma membrane to special caveolin- and cholesterol-rich patches called caveolae (Rajalingam et al., 2005).

In the mechanism of RAS-induced CRAF activation, homo as well as heteromerization with BRAF is required to achieve full catalytic activity of the kinase (Z. Luo et al., 1996; Z. J. Luo, Zhang, Rapp, & Avruch, 1995). Weber et al. demonstrated that 14-3-3 binding to serine 621 (C-terminus) was important for heterodimerization, while the internal N-terminal 14-3-3 binding site of RAF (S259) was dispensible, even in the presence of active RAS (Weber, Slupsky, Kalmes, & Rapp, 2001). These data suggest that RAS induces C/BRAF complex formation through the exposure of C-terminal binding sites ensuring complete kinase activity and substrate phosphorylation. Dimerization and oligomerization will be dicussed in greater detail in the subsequent sections. Substitution of all four activating phosphorylation sites S338, Y341, T491 and S494, to acidic residues results in full CRAF kinase activity. Phosphorylation of S471, which occurs in the catalytic loop, was shown to be required for CRAF activity as this site is engaged in the interaction of CRAF with its protein substrates (Zhu et al., 2005). Generally it is agreed upon that MEK1 and MEK2 are substrates for all three kinases since the RAF enzymes have restricted substrate specificity. Serine residues 29, 43, 289, 296, 301, and 642 are ERK-catalyzed phosphorylation sites associated with feedback inhibition (Dougherty et al., 2005).

Regarding regulation of **BRAF** activity by phosphorylation, there are similarities but also essential differences compared to CRAF. While phosphorylation sites for 14-3-3 are homologues to CRAF, the N-region-mediated regulation is quite different due to two aspartic acids in BRAF (D448 and D449) leading to constitutive phosphorylation of Ser446 (Mason et al., 1999). Thus, due to the accumulated negative charge at the N-region BRAF kinase exhibits unusual high basal kinase activity and explains why BRAF can be fully induced by RAS alone, but A- and CRAF also require SRC-mediated phosphorylations for full activation (Wellbrock et al., 2004). BRAF kinase is activated by RAS induced phosphorylation of Thr599 and Ser602 in the catalytic cleft. Mutation of these residues to alanine resulted in a loss of BRAF activity induced by EGF and activated RAS, as well as by phorbol esters and

muscarinic G protein-coupled receptors (B. H. Zhang & Guan, 2000). Whereas mutation of these sites to phosphomimetic residues resulted in constitutive activity independently of activated RAS. These residues are located where the P loop and the activation segment come closer in the tertiary structure. Oncogenic mutations in BRAF tend to cluster around the P loop and the N-terminal side of the activation segment (Haling et al., 2014). These mutations disrupt the inactive state to favor the active state. The *V600E* mutation in BRAF is the most frequently occurring mutation in human malignant melanomas and to a lower frequency in other cancers. It mimics the T599 phosphorylation by RAS with the exception that now BRAF rests constitutively active in a RAS independent manner (Davies et al., 2002). The phosphorylation of serine 579, which occurs in the catalytic loop, is essential for BRAF kinase activity and is most likely related to the importance of this residue in binding its substrates MEK1/2 (Zhu et al., 2005). ERK-catalyzed phosphorylation sites are reported to be Ser151, Thr401, Ser750 and Thr753 all these sites and are involved in feedback inhibition (Ritt, Monson, Specht, & Morrison, 2010). Till today the annotated COSMIC database (Catalogue of Somatic Mutations in Cancer) was listing 40105 unique samples with BRAF mutations in a total of 205811 unique samples incorporating curated mutation data from 3288 publications (cancer.sanger.ac.uk/cosmic). The tissues represented with the highest mutation frequencies being skin (41.4 % of samples mutated), thyroid (41.5%), large intestine (12.5%), eye (10%) and bone (9.6%). The type of mutation was in 99% of cases a missense substitution.

While a lot of reports focused on BRAF and CRAF activation and their implication in tumorigenesis, relatively little is known about **ARAF** regulation in health and disease. Baljuls et al. characterized novel phosphorylation sites in the regulation of ARAF by substitution of regulatory serines and threonines. In particular, S432 was shown to participate in MEK1/2 binding and indispensable for ARAF signaling. They further identified a novel regulatory domain in ARAF (referred to as IH-segment) positioned between amino acids 248 and 267, which contains seven putative phosphorylation sites (Baljuls et al., 2008). Upon phosphorylation, the ARAF fragment including residues between S246 and E277 revealed a “switch of charge” at the molecular surface of the IH-region. It was suggested that successive high accumulation of negative charges disturbs protein and membrane interaction, which subsequently resulted in the depletion of ARAF from the membranes due to electrostatic destabilization. Three of the phosphorylation sites in the IH-region (S257, S262 and S264) were shown to stimulate ARAF in a positive manner *in vitro*.

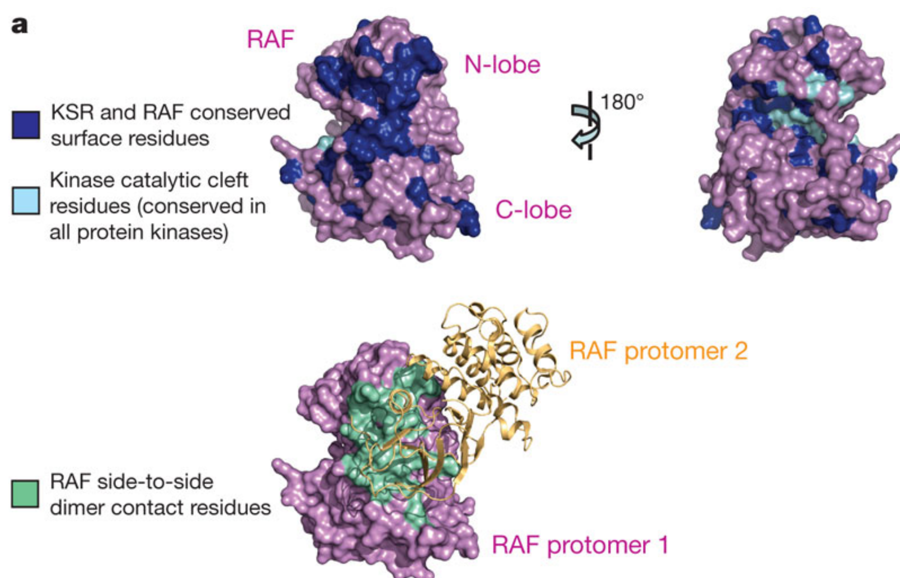
Other phosphorylated peptides obtained in that mass spectrometry screen included peptides corresponding to ARAF-14-3-3 binding sites (S214 and S582), which the authors have shown to be important for ARAF kinase activity by employing kinase assays. In samples from two patients (carcinoma and malignant melanoma), the COSMIC database listed a substitution – missense mutation at serine 214 to phenylalanine, one of the two putative 14-3-3 biding site in ARAF, confirming experimental data in patient materials. Interestingly, a recent study has identified a role for ARAF mutation S214C as an oncogenic driver in lung carcinoma, demonstrating the ability of ARAF S214C to transform immortalized human airway epithelial cells (Imielinski et al., 2014). According to Baljuls et al., the tyrosine at position 296 in ARAF favors a spatial orientation of the N-region segment, ensuing a tighter contact to the catalytic domain, whereas a glutamine residue at this position in CRAF abrogates this interaction. Thus it is suggested that the non-conserved tyrosine 296 in ARAF is a major determinant of the low activating potency of this RAF isoform (Baljuls et al., 2007). The close proximity of the regulatory N-region to the RKTR motif in all three RAF isoforms may influence phosphorylation of serine 338 in CRAF and serine 299 in ARAF and hence activation of these kinases. The RKTR motif has been previously described as a part of the phosphatidic acid binding region and recently also as a part of the RAF dimerization surface (Andresen, Rizzo, Shome, & Romero, 2002; Ghosh, Strum, Sciorra, Daniel, & Bell, 1996; Hatzivassiliou et al., 2010; Rajakulendran et al., 2009). Examination of the catalytic activity and subcellular distribution of ARAF mutants where single RKTR residues (R359, K360, and R362) were substituted for alanine revealed that the three substitutions proved to be inhibitory upon activation with HRASV12/Lck or EGF (Baljuls et al., 2011). Finally, the authors concluded that the reduced kinase activity of the RKTR mutants might result from an impaired dimer formation, as a crystal structure of a CRAF homodimer revealed that R398, K399, and R401 were part of the dimerization interface, attributing a novel function to the RKTR motif. In conclusion, the RKTR motif is described as a focus-point of multiple regulatory mechanisms, which is masked by the position of the N-region in a full-length RAF monomer and requires intramolecular rearrangements to exert its intrinsic functions in dimerization and PA binding (Baljuls et al., 2011). A recent study identified a somatic single nucleotide variant in ARAF that is predicted to result in a substitution of leucine for phenylalanine at amino acid position 351 (F351L) (Nelson et al., 2014). This is of special interest as this residue too is located in close proximity to the RKTR-motif but its mutant variant displayed a highly active MAP3K *in vitro* and was capable of transforming mouse

embryo fibroblasts. For the detection of somatic mutations in the *ARAF* gene, Lee et al. analyzed 60 human cancer cell lines along with 300 primary human cancer tissues (J. W. Lee et al., 2005). The MOLT-4 leukemia cell line was found to harbor an *ARAF* gene mutation, which resulted in an amino acid substitution (A451T) at the activation segment in the kinase domain of ARAF. Taken together, more regulatory residues in ARAF will be identified in the future accompanied by extended whole exome sequencing of patient derived material to reveal novel mutations in ARAF. In fact, a recent study by Sia et al. revealed novel mutations in the oncogene ARAF discovered by massive parallel sequencing technology that allows the characterization of cellular transcriptomes and genomes at single-base resolution, including the detection of somatic gene mutations (Sia et al., 2015). The authors had profiled a cohort of 122 iCCA cases, constituting the second most common primary liver malignant tumor after hepatocellular carcinoma. In 11% of samples tested, they detected novel ARAF mutations, which included two nonsense and nine missense mutations with the latter predicted to be damaging. Additionally, four mutations (A541V, G322S, S469F and W472) were identified to be bona fide somatic mutations. Among the ARAF mutations, N217I and G322S lead to activation of the MAPK pathway and N217I exhibited oncogenic potential *in vitro*. These findings among others show that the oncogene *ARAF* together with its two brothers in arms *BRAF* and *CRAF* represent potential therapeutic targets, that warrants further clinical evaluation. Novel mechanisms that contribute to hyperactivation or deregulation of RAF-mediated signaling pathways in human cancer are continued to be described. These are (i) Activating mutations of upstream RAF regulators, (ii) Overexpression of non mutated RAF proteins due to transcriptional upregulation or due to mutations leading to a net increase of RAF copy numbers and (iii) Mutational activation by either point mutations or chromosomal rearrangements (Schreck & Rapp, 2006).

1.2.2.3 Regulation of RAF activity through RAF dimerization

Besides RAF regulation through phosphorylation, various protein-protein interactions have been shown to be essential for RAF regulation including RAF homo- and heterodimerization (Z. Luo et al., 1996; Rushworth, Hindley, O'Neill, & Kolch, 2006). Oligomerization is a critical event in the ERK1/2 pathway as it allows signal transduction to downstream effectors (Wimmer & Baccarini, 2010). As a scaffold protein, Kinase suppressor of Ras (KSR) enhances the duration and amplitude of MAPK signaling through assembly of activating

complexes involving BRAF, MEK and ERK (Kolch, 2005; Therrien et al., 1995) via conserved interfaces (Rajakulendran et al., 2009). Like many other kinases, RAF kinases are activated by oligomerization and structural studies in recent years reveal a special mode of side-to-side dimer formation of active CRAF or BRAF kinase domains through their N-terminal lobes (Hatzivassiliou et al., 2010; Rajakulendran et al., 2009) (Fig. 12). More than 10 years ago, Weber et al. suggested a cooperative mechanism between B and CRAF in activating RAF (Weber et al., 2001). They found that active RAS induced heterodimerization of CRAF and BRAF via constitutive association of their C-termini. In kinase assays using recombinant kinase-dead MEK1 (K97M) as a substrate, they for the first time detected an increased activity with C/BRAF heterodimers.



(From Rajakulendran et al. 2009)

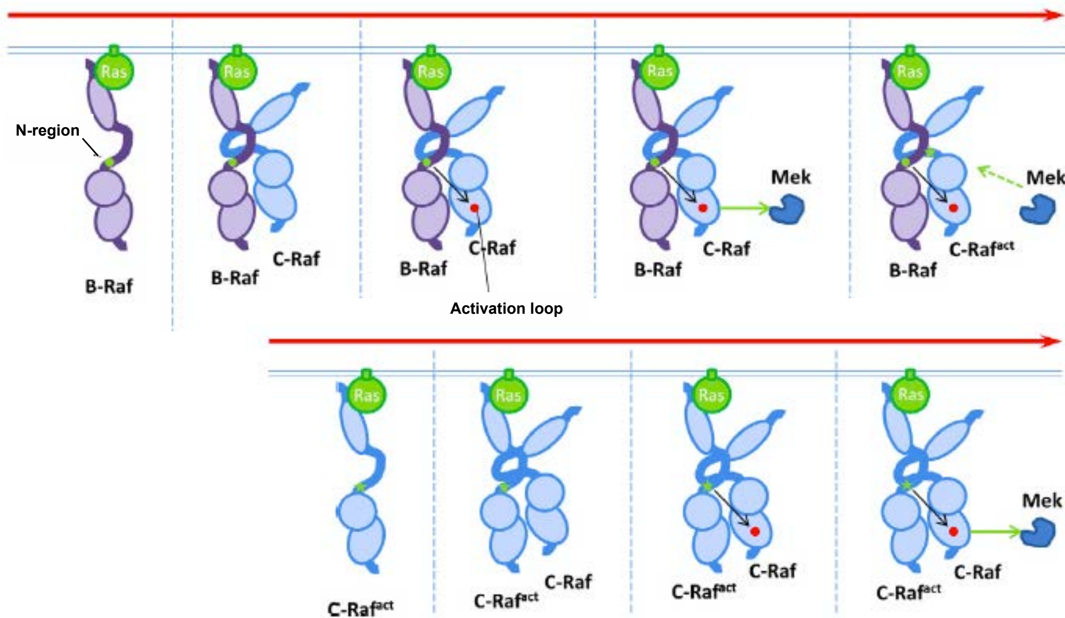
Figure 12: KSR and RAF share a conserved side-to-side dimer interface. Projection of highly conserved residues across both KSR and RAF orthologues onto the crystal structure of the BRAF kinase domain (top); common side-to-side dimer contact surfaces visualized originally in crystal structures of BRAF (bottom).

Rushworth et al. could show that isolated CRAF/BRAF heterodimers possessed a highly increased kinase activity compared to the respective homodimers or monomers (Rushworth et al., 2006). Interestingly, heterodimers between wild-type CRAF and BRAF mutants with low or no kinase activity still displayed elevated kinase activity, as did heterodimers between wild-type BRAF and kinase-dead CRAF. In contrast, heterodimers containing both kinase-dead variants of CRAF or BRAF were completely inactive, suggesting that the kinase activity of the heterodimer specifically originates from RAF and that either kinase-competent RAF isoform is sufficient to confer high catalytic activity to the heterodimer. For the first time

they demonstrated an endogenous interaction between BRAF and CRAF to form heterodimers. The basal level of association was enhanced by mitogen stimulation. The kinetics of heterodimer formation and disassembly thereby depend on the stimulus used and the cell-type, suggesting that the increase in heterodimerization is part of the activation process of RAF proteins (Rushworth et al., 2006). Heteromerization seems to be of physiological importance as it enhances the differentiation of PC12 cells (Weber et al., 2001). Increased heterodimerization ability was also shown to be the common pathogenic mechanism for Noonan Syndrome-associated *CRAF* mutations (Wu et al., 2012).

Garnett et al. investigated the mechanism of CRAF activation by BRAF. A detailed analysis revealed that the phosphorylation of the N-region of CRAF was not essential, but AS phosphorylation and 14-3-3 binding to the C terminus of C-RAF are indispensable. Importantly, they show that wild-type BRAF can activate CRAF and only upon RAS-induction a BRAF/ CRAF complex is formed. However, mutant forms of BRAF bind to CRAF constitutively (Garnett et al., 2005). In summary, they suggested that BRAF activates CRAF (but CRAF does not activate BRAF) through 14-3-3 mediated heterooligomerization and transphosphorylation, implying that this pathway signals both in cancer as well as in normal cells. Structural studies on BRAF activation by Wan et al. relate AS phosphorylation to the consequential alignment of key residues within the kinase domain for catalytic activity (Wan et al., 2004). BRAF binding may stimulate CRAF activation through direct or indirectly inducing AS phosphorylation on CRAF, with BRAF acting as an inducer of successive conformational changes in CRAF (Garnett et al., 2005). In their recent work, Hu et al. elucidated the mechanism by which BRAF is able to function as an allosteric activator in the context of RAF heterodimers, independent of its kinase activity (Hu et al., 2013). The presence of negative charges in the N-region of BRAF plays thereby a pivotal role as summarized in Figure 13. CRAF mutants devoid of kinase activity cannot function as activators. Thus, kinase-sufficient CRAF is indispensable for signaling by impaired-activity BRAF mutants. These mutants activate CRAF up to 20-fold more efficiently than activated RAS. Oncogenic *RAS* was shown to cooperate with kinase-dead BRAF in inducing melanomas in mice. Tumor cells from conditional kinase-dead *BRAF* mice displayed constitutively binding of kinase-dead BRAF to CRAF (Heidorn et al., 2010). Hence, kinase-deficient BRAF dimerizes with CRAF in the presence of activated RAS, thus activating the MAPK cascade in a CRAF dependent manner (Heidorn et al., 2010; Rebocho & Marais, 2013). In conclusion, most of the publications attributed the increase in RAF kinase activity

to 14-3-3 mediated dimerization of BRAF with CRAF, triggered by activated RAS (Garnett et al., 2005; Rushworth et al., 2006; Weber et al., 2001).



(*From Cseh et al. 2014)*

Figure 13: Model of RAF transactivation and signal amplification: upon RAS activation BRAF is recruited to the plasma membrane allowing RAF monomers to bind and dimerize. The constitutively phosphorylated Serine446 in the N-region of BRAF (green dot) induces a conformational change that allows the cis-phosphorylation of the receiver kinase (here CRAF) leading to the phosphorylation of MEK. Mek in turn induces the phosphorylation of serine 338 in the N-region of CRAF converting it to a transactivator (upper panel). CRAF as a transactivator dissociates from BRAF and dimerizes with and transactivate further RAF molecules (signal amplification, lower panel).

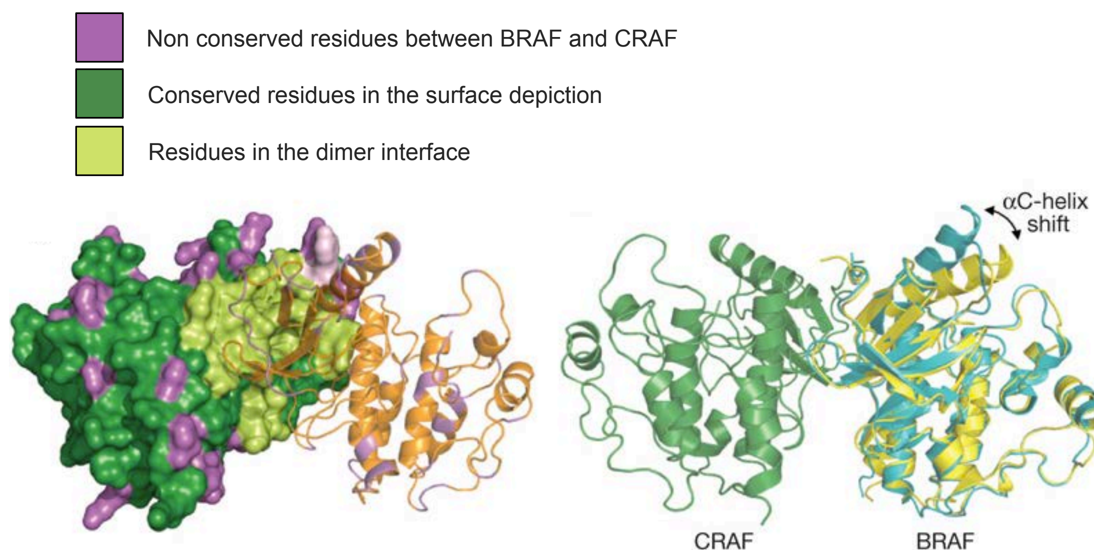
RAF-RAF as well as RAF-KSR dimerization depends on the dimer interface, a region located in the kinase domain (RKTR-motif). It comprises a cluster of basic residues that are highly engaged in dimer formation. Mutation of the critical arginine residues to histidine ($BRAF^{R509H}$, $CRAF^{R401H}$ and KSR^{R615H}) disrupts dimer formation and prevented the EGF-induced activation of BRAF and CRAF (Freeman, Ritt, & Morrison, 2013). Substitutions in the dimer interface not only altered the dimerization potential of these proteins but also severely affected the transforming ability of all but the high catalytic activity BRAF mutants. While high activity BRAF mutants such as V600E-BRAF are able to dimerize, further activation events are not required due to their already elevated enzymatic activity. Further studies from the Morrison lab show that targeting the RAF-dimer interface with short peptides could perturb RAS-MAPK signaling in a panel of tumor cell lines (Freeman et al., 2013). In RAS mutated cells, treatment with cell-permeable peptides targeting the RAF dimer interface (DIF) led to a decrease in cell viabilty. However, as high activity BRAF mutants did

not require dimerization for function, peptide inhibition had no effect on V600E-BRAF-mediated MEK phosphorylation but could prevent MEK activation mediated by lower activity RAF mutants. Taken together, with the introduction of secondary mutations in the RAF dimer interface the authors suggested a new approach in altering progression and treatment of disease states with abberant RAS/RAF signaling. Finally, inhibitors that disrupt RAF dimerization can suppress RAF-dependent signaling and have proven to be beneficial in the treatment of diseases that require dimerization for RAF function.

1.2.2.4 Paradoxical RAF-activation: Inhibitors that activate

Unrestrained signaling through the ERK1/2 pathway caused by activating mutations in RTKs, RAS or RAF has been linked to several human cancers as mentioned above. One member of the RAF family, BRAF, was propelled into the limelight, as it is the most frequently mutated oncogene in the RAF kinase superfamily. Its kinase activating *V600E* mutation that led to constitutive MEK-ERK signaling in cells (Gray-Schopfer, Wellbrock, & Marais, 2007), was also shown to be responsible for melanoma induction in mice (Dankort et al., 2009; Dhomen et al., 2009). Moreover, it is found to be present in more than 60% of human melanomas (Davies et al., 2002; Wan et al., 2004). A tremendous effort has been undertaken to interfere with the aberrant signaling of oncogenic RAF signaling. Studies with RNA interference have demonstrated that depleting oncogenic BRAF in cancer cells reduces ERK activity, inhibits proliferation, and induces apoptosis (Hingorani, Jacobetz, Robertson, Herlyn, & Tuveson, 2003; Karasarides et al., 2004). Importantly, V600E-BRAF inhibition has been shown to block melanoma cell proliferation by apoptosis induction *in vitro* and growth arrest of melanoma xenograft *in vivo* (Gray-Schopfer et al., 2007). These data confirm V600E-BRAF as a founder mutation in melanoma and a driver of melanomagenesis. Hence, this mutation soon became a therapeutic target in melanoma treatment and drugs to target the affected pathway have been developed. The first to be tested clinically were the multi-kinase inhibitor sorafenib and the MEK inhibitor PD184352 (CI1040). Although initial *in vitro* results were promising, both inhibitors failed to produce objective responses in patients because of inefficiency or undesirable toxicity (Halilovic & Solit, 2008). Recently, more potent and selective BRAF inhibitors have been described and shown to block MEK and ERK phosphorylation/ activation in cell lines and xenografts that harbor mutant BRAFV600E (Hatzivassiliou et al., 2010; Heidorn et al., 2010; Poulikakos, Zhang, Bollag, Shokat, & Rosen, 2010). For example, the triarylimidazole SB590885 was shown to be a

potent and extremely selective inhibitor of BRAF kinase (Takle et al., 2006). So was the difluorophenylsulfonamine PLX4720, which displayed excellent selectivity for BRAF *in vitro* and preferentially inhibit BRAF mutant cancer cell proliferation (Tsai et al., 2008). BRAF-selective drugs have soon after entered the clinics and are showing excellent responses in patients with BRAF mutant melanoma (K. T. Flaherty et al., 2010). In a phase I clinical trial, the BRAF- inhibitor vemurafenib (then known as PLX4032) showed a partial to complete response (tumor regression) in 11 out of 16 patients with advanced melanoma (carrying a *V600E mutation*). However, despite the dramatic regressions and increased survival seen in the Phase I trials, all the patients eventually suffered relapses. Moreover, although ATP-competitive RAF kinase inhibitors have shown outstanding activity in tumor models with oncogenic RAF mutations, their potencies in BRAF wild-type (BRAF-WT) and KRAS mutant tumor models are considerably different. Hatzivassiliou and others demonstrated in a series of publications that in KRAS mutant and RAS/RAF wild-type tumors, the selective and chemically unrelated RAF inhibitors GDC-0879 and PLX4720 activated the RAF-MEK-ERK pathway in a RAS-dependent manner, by enhancing tumor growth in some xenograft models (Hatzivassiliou et al., 2010; Heidorn et al., 2010; Poulikakos et al., 2010). The authors demonstrated by histopathological examination that mice treated with GDC-0879 developed hyperkeratosis and acanthosis of the epidermis, as well as inflammation in the dermis. Intriguingly, around 15% of patients treated with BRAF-selective drugs were shown to develop squamous cell carcinoma (SCC) caused most probably by underlying RAS mutations in premalignant skin cells (K. T. Flaherty & McArthur, 2010; Schwartz et al., 2009). Inhibitor binding triggered the main modes of wild-type RAF activation such as dimerization, membrane localization and RAS-GTP interaction (Hatzivassiliou et al., 2010). This paradoxical activation by RAF inhibitors is mediated directly through the inhibitors' effects on the RAF kinase domain (Fig. 14). Heidorn and colleagues previously presented the RAF inhibitor paradigm when they found BRAF was not active and not required for MEK/ERK activation in RAS mutant cells (Heidorn et al., 2010). By contrast, BRAF inhibitors (including the rather unspecific multikinase inhibitor drug sorafenib) hyperactivated CRAF and MEK in these cells. So at low concentrations pan-RAF inhibitors stimulated MAPK pathway presumably by formation of RAF complexes, with one kinase bound to the drug, and so inactive whereas the other is not drug bound and thus, can activate downstream signaling.

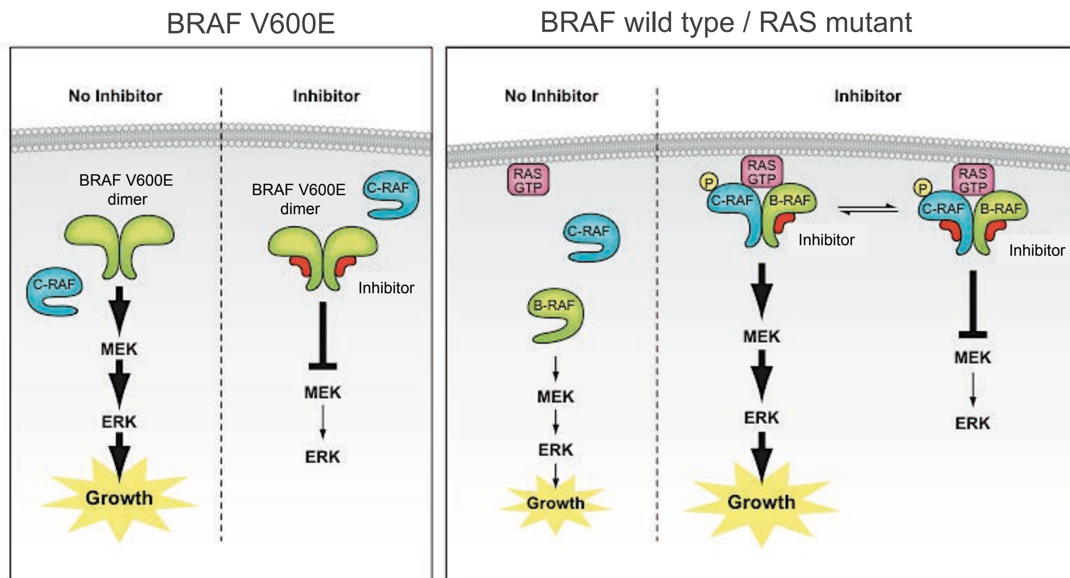


(Adapted from Hatzivassiliou et al. 2010)

Figure 14: Dimer formation upon GDC-0879 (RAF inhibitor) treatment. Crystal structure of the CRAF kinase domain complexed to a close analogue of GDC-0879 (left). The CRAF–inhibitor complex adopts a dimer conformation in the asymmetric unit as was previously shown for BRAF (Rajakulendran, T. 2009). Heterodimer model of BRAF–CRAF reveals that, in contrast to the conformation induced by GDC-0879 when bound to BRAF, PLX4720 induces a shift in the αC-helix in BRAF bringing it closer to the dimer interface (right). BRAF–CRAF heterodimer model derived from CRAF–GDC-0879 analogue (green) and BRAF–GDC-0879 analogue (yellow) or BRAF–PLX4720 (cyan).

Drugs like sorafenib induce paradoxical activation of CRAF as it inhibits BRAF and drives CRAF activation. The ability of primed CRAF to signal downstream to MEK depends on inhibitor concentration, inhibitor potency against CRAF and inhibitor off-rate (Fig. 15). Taken together, chemical or genetic inhibition of BRAF in the presence of oncogenic or growth-factor activated RAS induces BRAF binding to CRAF, leading to CRAF hyperactivation and subsequently elevated MEK and ERK signaling. Since BRAF can tolerate many different point mutations within its kinase domain, it is suggested that single agents are likely to fail in inhibiting all mutants, thereby providing a mechanism for clinical resistance (Garnett & Marais, 2004). BRAF mutant tumors could become resistant to BRAF selective drugs by different strategies i) the acquirement of a mutation in RAS or an upstream component that activates RAS, ii) the selection of a population of cells harboring pre-existing mutations in RAS (Heidorn et al., 2010). The acquisition (or selection for cells with preexisting mutations) of a CRAF mutation such as a gatekeeper mutant represents an alternative mechanism to acquire resistance in patients with RAS mutant tumors treated with pan RAF inhibitors. Clearly, BRAF is a versatile oncogene and tumor cells will acquire resistance to drug treatment via activating mutations in parallel signaling pathways or

overexpression of wild type RAF isoforms, which ultimately will result in the inability of RAF inhibitors to suppress ERK signaling (Poulikakos et al., 2010).



***(Adapted from Hatzivassiliou et al.* 2010)**

Figure 15: Transactivation of RAF by ATP-competitive inhibitors: Inhibitor binding to one protomer within a RAF dimer results in elimination of the catalytic activity of inhibitor- bound RAF and transactivation of the other. Transactivation of RAF homo- and heterodimers is therefore responsible for induction of MEK/ERK phosphorylation by RAF inhibitors in cells with wild-type BRAF/ active RAS. Inhibitors of RAF activate ERK signaling at low concentrations, but inhibit at higher concentrations in wild type BRAF/ active RAS cells.

A combinatorial use of RAF and MEK inhibitors might provide the best responses in the clinics and prevent emergence of resistance. The first combination of RAF and MEK inhibitors (dabrafenib plus trametinib) was approved for clinical use by the FDA for the treatment of metastatic BRAF- driven melanoma in the beginning of last year (K. T. Flaherty et al., 2012). It is expected to delay or better circumvent acquired resistance owed to pathway reactivation and has already been shown to prevent melanoma metastasis in a preclinical model (Sanchez-Laorden et al., 2014). Clearly a lot more functional studies including patient derived material, mouse models as well as statistical drug evaluation are warranted for an effective disease management.

1.2.3 MEK

The RAF kinases have restricted substrate specificity and catalyze the phosphorylation and activation of MEK1 and MEK2. MEK is a transferase, also classified as a dual specificity MAP2K because of its capability to transfer a phosphoryl group from ATP to both tyrosine

and threonine residues on the substrate. MEK1 and MEK2 share 79% amino acid identity and are equally competent to phosphorylate ERK substrates (Dhanasekaran & Premkumar Reddy, 1998). They form homo- and heterodimers both *in vivo* and *in vitro* (Catalanotti et al., 2009; Ohren et al., 2004). In contrast to RAF heterodimers, MEK1/2 heterodimers are stable and exist independent of growth factor stimulation. Knock-out studies showed that while disruption of MEK1 is embryonic lethal, MEK2-/- mice are viable, fertile and have no evident abnormalities (Belanger et al., 2003; Giroux et al., 1999). This observation suggests that while MEK2 homodimers cannot substitute for loss of MEK1 homodimers, the latter could compensate for the loss of heterodimers or MEK2 homodimers (Cseh, Doma, & Baccarini, 2014). MEK1 ablation leads to prolonged MEK2 phosphorylation and ERK activation due to the lack of negative feedback inhibition, which translated into upregulated MEK/ ERK signaling in mouse fibroblasts, embryos, epidermis, and brain (Catalanotti et al., 2009). MEK proteins are composed of a catalytic kinase domain (290 residues), which is flanked by a negative regulatory N-terminal domain (70 residues), and a shorter C-terminal MAP kinase binding domain required for ERK binding and activation (30 residues) (Fig. 16, left).

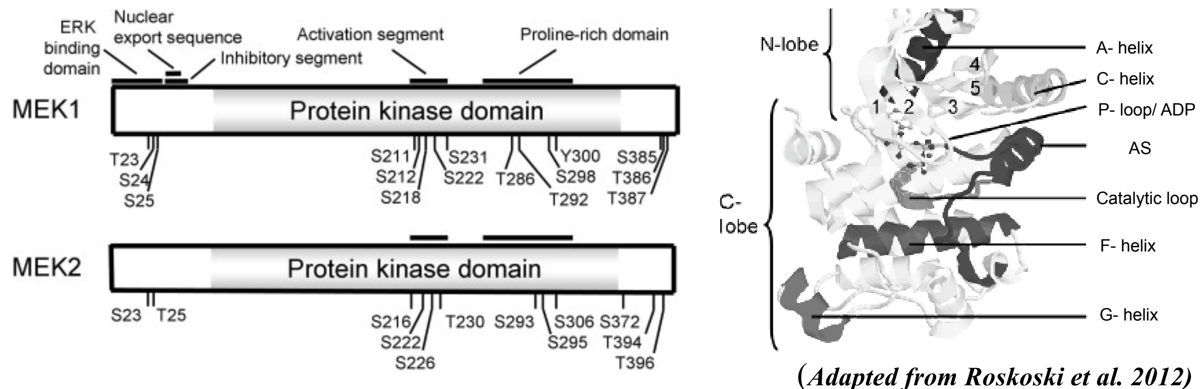


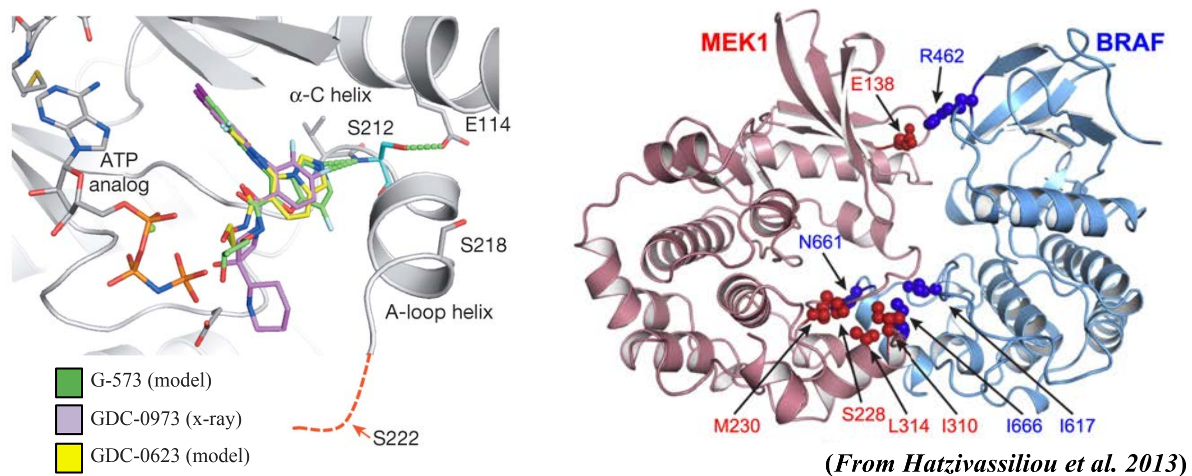
Figure 16: MEK structure comprising MEK1 and MEK2: overview of important domains and residues in MEK1/2 (left). Ribbon diagram of human MEK1 bound to MgADP (right).

Deletion of the regulatory domain results in constitutively activated MEK and ERK (Shaul & Seger, 2007). MEK1/2, like all protein kinases, have a small N- terminal lobe and large C-terminal lobe. The small loop contains an important and conserved αC-helix that occurs in active or inactive orientations, making or breaking part of the active site (Roskoski, 2012). The P-loop coordinates nucleotide binding with the help of the glycine-rich loop, which positions the γ-phosphate of ATP for catalysis thus comprising the most flexible part of the N- lobe. The majority of the binding site for ADP is on the β- sheets of MEK (numbers 1-5 in

Fig. 16, right). Most of the catalytic residues, which are engaged in the phosphoryl transfer from ATP to the ERK substrates, are part of the large C-terminal lobe. The AS of the large lobe exhibits active and inactive orientations, similar to RAF activation (DFG- Asp "in/out"). The activity of MEKs is regulated by the proline-rich insert in the catalytic core that is not present in any other known MEK family members, containing phosphorylation sites for proline-directed and other protein kinases. It needs to be phosphorylated to ensure efficient MAPK signaling in mammalian cells (Dang, Frost, & Cobb, 1998). RAFs transmit the signal downstream by phosphorylating MEK1 (Ser218/ Ser222) and MEK2 (Ser222/Ser226) in their activation segment in a complex process that involves a KSR scaffold and in some instances its catalytic activity to ensure MEK1 phosphorylation and activation by C-RAF (Hu et al., 2013). Uniquely, MEK 1 displays an inhibitory phosphorylation site in its AS (S212), which decreases its kinase activity and at the same time is not affected by other AS-phosphorylation such as S218 or S222 (Gopalbhai et al., 2003).

Substrate activation by MEK1/2 is a multistep process. First they mediate the phosphorylation of Tyr204/187 of ERK1/2 in the ERK activation segment before tyrosine-phosphorylated ERK dissociates from MEK to then re-associate with the same or another active MEK, which then catalyzes threonine- phosphorylation (Thr202/185) in the AS two residues upstream from the ERK1/2 phosphotyrosine (Ferrell & Bhatt, 1997). Phosphorylation of both activation segment residues is required for proper ERK- activation as phosphorylation of only a single ERK- residue failed to activate the enzyme (Anderson, Maller, Tonks, & Sturgill, 1990). Active ERK catalyzes a feedback inhibitory phosphorylation of MEK1 at T292 that serves to downregulate the pathway. Additionally, the action of the protein S/T-phosphatase PP2A leads to rapid down-regulation of MEK signaling through the removal of phosphate groups from S218 and S222 (Sontag et al., 1993). In contrast to ERK1/2, MEK1 stays still fully activated when dephosphorylated at only one of the two serine residues. The kinetics in the MEK1 activation cycle involves numerous phosphorylation and dephosphorylation events. Inactivation of MEK1 has many biological consequences, one of which is preventing the de-regulated cell proliferation in cancer cells. Since MEK is a common downstream effector of wild-type and mutant RAF, MEK inhibitors have the potential to target all tumors whose survival is dependent on MAPK signaling. Interestingly, MEK1 itself is mutated in 5% of 1275 samples tested from malignant melanoma patients (Cosmic database). Mutations in MEK1 either occur exclusively or together with BRAF and NRAS respectively. As activating KRAS and BRAF mutations

trigger tumorigenesis through constitutive MAPK signaling, multiple allosteric MEK inhibitors are employed in clinical trials (Hatzivassiliou et al., 2013). GDC-0623 is currently in phase I clinical trials displaying superior efficacy in KRAS driven tumors while GDC-0973 shows superior efficacy in BRAF mutant tumors and went already to phase III clinical trials. A recent study by the Shiva Malek lab employed these improved (less toxic, more specific) MEK inhibitors in BRAF-mutant versus KRAS-mutant tumors at clinically relevant doses (Hatzivassiliou et al., 2013). The potency of MEK inhibitors thereby correlated with high inhibitor binding affinity against dual phosphorylated MEK (S218/ S222, MEK sites phosphorylated by RAF) compared to un-phosphorylated MEK (Fig. 17).



(*From Hatzivassiliou et al. 2013*)

Figure 17: MEK- inhibitor interactions control individual effects on RAF-MEK complex formation. The inhibitor interaction with the S212 backbone of MEK restricts the αC-helix movement and prevents MEK phosphorylation by RAF (on S218/222) (left). Structure of the BRAF-MEK complex displaying critical residues, that when mutated reduce or disrupt complex formation of MEK1 and BRAF (right).

Hence, inhibitors like GDC-0973 proved to be very efficient in BRAF-V600E mutated tumors, as the basal activity of MEK is rather high under these settings. They are also unaffected by the ERK catalyzed phosphorylation of negative regulatory residues in CRAF, that inhibits both CRAF kinase activity and its interaction with RAS. As these inhibitors interfere with this negative feedback resulting in the reactivation of CRAF, they can more effectively down regulate the ERK pathway in these cells. However in KRAS transformed cells where concentration of phosphorylated MEK is lower due to reduced ERK signaling they are rather ineffective. MEK inhibitors like GDC-0623 that prevent phosphorylation by RAF, stabilize the RAF-MEK complex and thus leading to greater efficacy in KRAS mutant tumors (Lito et al., 2014). By generating a kind of dominant-negative inhibitor of RAF, the authors could prove that as a result, stabilization of RAF-MEK complexes had negative consequences for RAF heterodimer- formation, and consequently RAS- induced RAF

activation (Hatzivassiliou et al., 2013). Taken together, there always has to be a careful examination of the patient´s genetic background and treatment history. The only MEK inhibitor that has been approved by the FDA as a single agent for treatment of unresectable or metastatic melanoma harboring BRAF-V600E/K is Trametinib, which has been shown to reduce RAF binding to MEK thereby inhibiting the proliferation of both RAS and BRAF mutant cell lines and xenografts (Lito et al., 2014). It more over led to promising results in a transgenic model of RAS-driven epidermal tumorigenesis, where it decreased both RAS and RAS/ RAF induced tumor formation (Doma et al., 2013).

1.2.4 ERK

While the RAF and MEK family of proteins have narrow substrate specificity, the extracellular signal-regulated kinases ERK1 and ERK2 (ERK1/2) phosphorylate multiple target proteins thereby controlling a variety of cellular processes (Fig. 18). The final cellular outcome depends on the intensity and duration of ERK signaling, pathway regulation by negative feedback loops and cross talks with other signaling pathways (Deschenes-Simard, Kottakis, Meloche, & Ferbeyre, 2014).

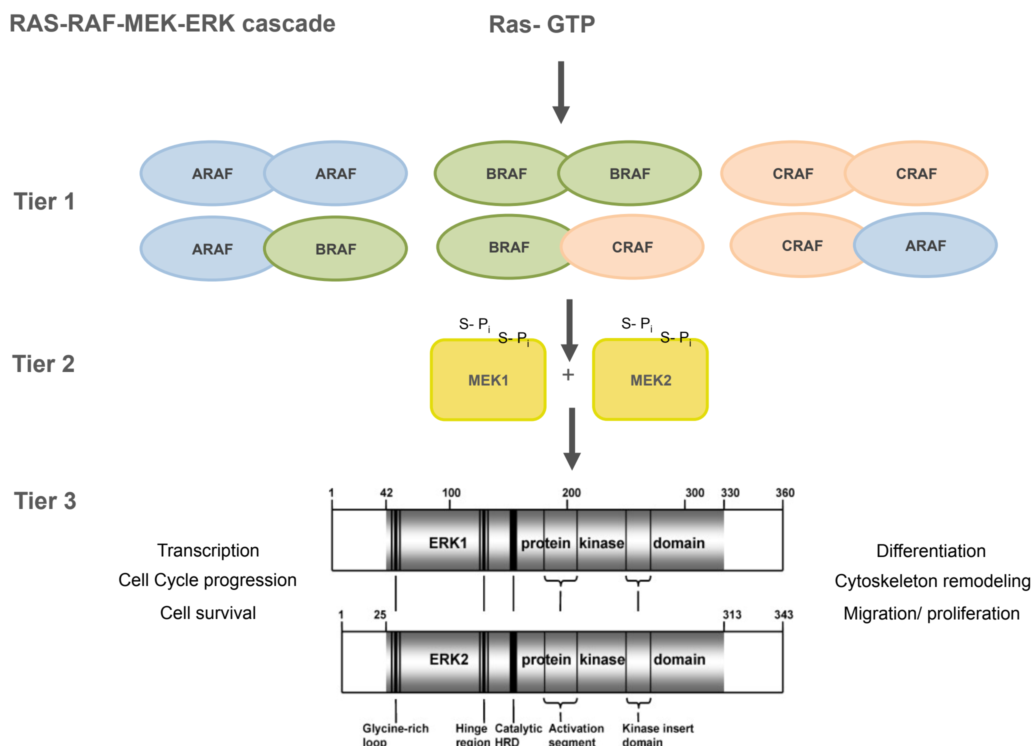
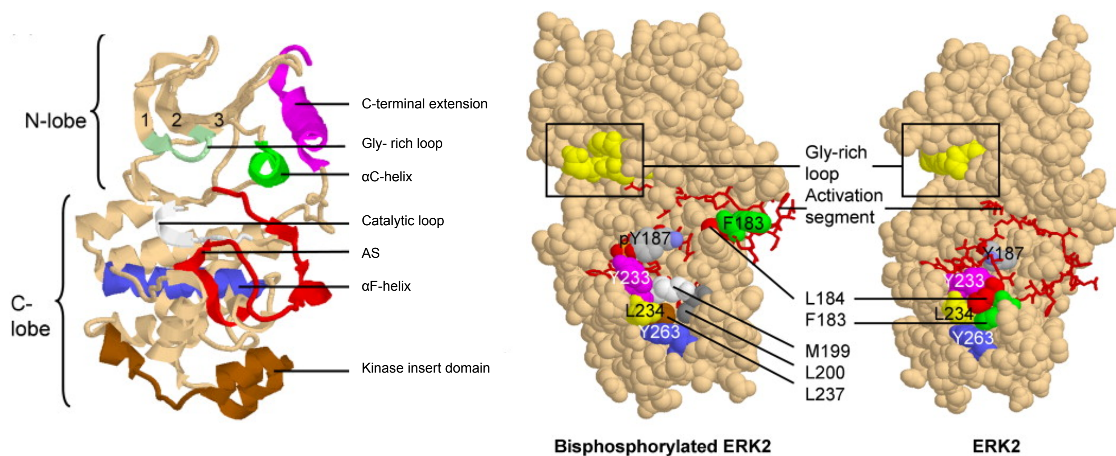


Figure 18: The components of the three- tier MAPK signaling pathway with the focus on important domains and residues within the extracellular signal-regulated kinases ERK1 and ERK2, responsible for in the indicated cellular functions.

Hence, ERK activation is a major determinant of cell fate in health and disease. Human ERK1 and ERK2 possess 84% sequence homology and share many functions.

ERK1/2, like all protein kinases, have a small amino-terminal and large carboxyterminal lobe consisting of several conserved α-helices and β-strands. The small N- lobe contains the important and conserved αC-helix that occurs in active or inactive orientations and the glycine- rich loop, which like in other kinases positions the γ-phosphate of ATP for catalysis. The large lobe characteristically binds the peptide/protein substrates with the help of the activation segment, which regulates catalytic efficiency (Kornev & Taylor, 2010) and typically begins with the DFG- motif analogous to upstream kinases (active conformation: "DFG-aspartate in"; inactive "DFG-aspartate out"). The middle of the activation segment is known as the activation loop (activation lip in ERK1/2) and displays the greatest diversity in terms of length and sequence (Roskoski, 2012). It contains residues that need to be phosphorylated to convert inactive ERK1/2 to the active form (Fig. 19). These two residues are part of the Thr-Xxx-Tyr sequence, a motif that is shared by all MAP kinases in their activation segment (Katz, Amit, & Yarden, 2007). In resting cells, ERK is kept in the cytoplasm via its association with MEK, the microtubules network or phosphatases. Phosphorylation is catalyzed by MEK1/2 and occurs as described before (1.2.3 MEK).



(*Adapted from Roskoski et al.* **2012)**

Figure 19: Model of human ERK2 ribbon structure (left, inactive enzyme "DFG-aspartate out") and a spacefilling model of the substrate recruitment sites in active (bisphosphorylated) and inactive ERK2 (unphosphorylated). Important F-site recruitment residues like M199, L200, and L237 are only observed in bisphosphorylated ERK2, but are buried in unphosphorylated ERK2, thus not expected to make contact with the F-docking site of substrates. Residues close to ERK2 phosphorylation sites catalyzed by MEK1/2 (L184, F183 and Y187) indicate the immense change in position upon activation.

Serine/threonine phosphatases (PPs), such as PP2A have been shown to inactivate bisphosphorylated ERK2 and are generally believed to regulate several steps in the MAPK pathway as many proteins in the overall cascade contain phosphate groups on their serine/threonine residues. DUSP6 is an ERK2 specific MAPK phosphatase and is one of the enzymes that terminates ERK2 signaling (Roskoski, 2012). The combination of kinases and phosphatases make the overall ERK activation process reversible, thus playing a key role in regulating the magnitude and duration of kinase activation and also the nature of the physiological responses. Signal shutdown after stimulation works via multiple feedback loops. These include inhibitory phosphorylation of the upstream kinases such as CRAF (S29, S289, S296, S301, and S642) and MEK (T292) (Dougherty et al., 2005; Shin et al., 2009).
Once activated, the ERKs phosphorylate a broad spectrum of substrates, distributed throughout the major subcellular compartments, including the nucleus and the cytoplasm (Casar, Pinto, & Crespo, 2008). The nuclear import and export of ERK1/2 are complex processes, and although intensively studied, results are controversially discussed. Taken together, it is generally agreed upon that ERK2 lacking activation loop phosphorylation, enters the nucleus by an energy-independent mechanism facilitated by direct interaction with nucleoporins at the nuclear pore (Whitehurst et al., 2002) while phosphorylated ERK2 can shuttle in and out by an energy and carrier-independent mechanism. ERK2 can be prevented from entry into the nucleus by cytoplasmic proteins either through cytoplasm anchorage or inhibition of its interaction with nucleoporins (Roskoski, 2012). Unique N- and C-terminal extension, provide signaling specificity as ERK1/2 catalyze hundreds of cytoplasmic and nuclear substrates including regulatory molecules and transcription factors. Transcription factors such as Elk or c-Fos participate in the immediate early gene response. ERK1/2 are proline-directed kinases that preferentially catalyze the phosphorylation of substrates containing a Pro-X-Ser/Thr-Pro sequence. These substrates usually also possess a D-docking or a F-docking site, which are conserved in multiple ERK1/2 interacting proteins such as in the aforementioned transcription factors. Also inactivating dual specificity protein phosphatases DUSP1/4 and the scaffold protein KSR as well as the upstream activator CRAF contain an ERK docking site (Jacobs, Glossip, Xing, Muslin, & Kornfeld, 1999). Hence, the respective ERK recruitment sites that bind corresponding modular-docking sequences in their substrates represent extremely attractive target sites for non-ATP competitive inhibitors as they lie outside the active site cleft of the kinase. The majority of available MAP kinase inhibitors target the extremely high conserved ATP binding site, thereby intrinsically

providing the basis for cross reactivity and undesirable toxicities which might ultimately limit drug potential. While all MAPKs are thought to possess a D-recruitment site (DRS), the F-recruitment site (FRS) appears to be a feature common only to ERK1/2 and p38 MAPKα, although ERK5 and other MAPKs still have to be evaluated according to a study published by Lee et al. (S. Lee et al., 2011). Non-ATP competitive inhibitors targeting sites such as the MAPK recruiting sites, might be able to modulate MAPK activities by blocking binding to upstream activating kinases, scaffold proteins or downstream substrates (Schnieders, Kaoud, Yan, Dalby, & Ren, 2012). Schnieders et al. also conceive a specific ERK2 inhibition therapeutically promising as studies in knockout mice have shown ERK2 largely compensating for the absence of ERK1 despite sequence similarities between ERK1/2 (Pages et al., 1999; Yao et al., 2003). Hence, quite some effort (mostly structure- and ligand-based computational approach) has been put into the development of small molecule inhibitors specific for ERK2. Some compounds were found to be able to inhibit the phosphorylation of protein substrates such as Elk-1 by ERK2, though they were not inhibiting the phosphorylation of ERK2. The development of protein-protein interaction inhibitors is at an early developmental stage and clearly further work is necessary to identify potential molecules and mechanisms by which aberrant ERK1/2 signaling can be suppressed. Different strategies could therefore be explored- either blocking the DRS or FRS in ERK1/2. According to Roskoski et al., the idenficiation of FRS protein-protein interaction inhibitors will be a greater challenge since the FRS is only accessible in active bisphosphorylated ERK1/2 (Roskoski, 2012). In contrast to the DRS of ERK1/2, which is accessible both in the active and inactive state. Nonetheless, it is mostly activated ERK1/2 that drives proliferation and invasion in cancer cells, thus blocking the F- recruitment site of ERK provides a more effective cancer therapeutic target. Taken together, the ERKs execute their vast cellular functions through a large number of downstream molecules (Fig. 20) some of which might be of potential use as exploitable drug targets.

	Protein	Phosphorylation sites	notes
Transcription factors	AML1	Ser249, Ser266	Phosphorylation of AML1
	Elk1	Ser324, Thr336, Thr353, Thr363, Thr368, Ser 383, S389, Thr417, Ser422	Phosphorylation of this Ets tf enhances its activity, which is mainly the transcription of c-Fos
	c-Fos	Thr325, Thr331, ser374	Phosphorylation stabilizes the c-Fos protein, required for its maximal transactivation; sensor for ERKs´signal duration
	HIF1α		Phosphorylation enhances the transcriptional activity of hypoxia induced factor 1α (HIF1α)
	c-Jun	Ser63, Ser73, Ser243	Phosphorylation of Ser63/73 induces transcriptional activity of c-Jun. The phosphorylation of Ser243 may participate in its downregulation
	c-Myc	Ser62	Not clear whether this activatory phosphorylation occurs *in vivo*
	p53	Thr73, Thr55	Phosphorylation of Thr55 is necessary for doxorubicin-induced p53 activation and cell death
	Smad1	Ser187, Ser195, Ser206, Ser214	Phosphorylation inhibits nuclear accumulation of Smad1 and its TGFβ- induced transcriptional activity
	Smad2/3	Thr220, Ser245, Ser250, Ser255	Phosphorylation of Smad2/3 inhibits TGFβ- induced transcription
	Smad4	Thr276	Phosphorylation of Smad4 accelerates the rate of its nuclear accumulation and therefore facilitates its TGFβ- induced transcriptional activity
	STAT1/3	Ser727(mouse)	Phosphorylation of the signal transducers and activators of transcription (Stats) inhibits their tyrosine phosphorylation and thereby their transcriptional activity
	STAT5α	Ser780	Phosphorylation of STAT5α prevents its nuclear translocation and its transcriptional activity
Kinases and phosphatases	ERK1/2	Tyr185 (ERK2)	Role of autophosphorylation not clear. Can be followed by a slow Thr183 phosphorylation and minor activation
	FAK1	Ser910	Phosphorylation may inhibit the interaction of the focal adhesion Tyr kinase 1(FAK1) with paxillin and thereby inhibits its downstream signaling
	Lck	Ser259	Phosphorylation of this T-cell Src family protein Tyr kinase regulates the specificity of its SH2 domain
	MEK1/2	Thr292, Ser386, Thr286 (MEK1)	Phosphorylation of Thr292 inhibits the phosphorylation of Ser298 by PAK and thereby reduces association with ERK. Phosphorylation of Ser386 can facilitate the binding of MEK1 to Grb10, thereby increasing the rate of ERK activation
	MKP1/2	Ser359, Ser364 (mouse)	Phosphorylation of the MAPK phosphatase-e (MKP1, DUSP1) reduces its rate of degradation
	MKP3	Ser159, Ser197, Ser331	Phosphorylation of this ERK specific MKP3 (DUSP6) seems to lead to its enhanced degradation
	MSK1/2	Ser360, Thr518	Phosphorylation of the mitogen and stress activated protein kinase 1/2 (MSK1/2) induces its activation. Can be catalyzed by p38
	PAK1	Thr212	Phosphorylation may provide a negative feedback signal to control ERK activation
	CRAF	Ser29, Ser289, Ser296, Ser301, Ser642	Hyperphosphorylation of these sites inhibits Ras interaction with CRAF, thereby desensitizes CRAF to additional stimuli
	BRAF	Ser750, Thr753	Phosphorylation inhibits its activity, and thereby serves as a negative feedback mechanism for ERK signaling
	RSK1-4	Thr359, Ser363, Thr573 (RSK1)	Phosphorylation of the p90 ribosomal S6 kinase 1 (RSK1) leads to its activation and propagates ERK- mediated signals
	p70 S6kinase	Multiple S/P sites	Role of Phosphorylation not clear, may lead to stabilization
Signaling proteins	EGFR	Thr669	Phosphorylation might be involved in its downregulation
	Gab1	Thr312, Ser381, Ser454, Thr476, Ser581, Ser597	Phosphorylation of the Grb2- associated binder 1 (Gab1) may block insulin signaling at the level of PI3K
	Gab2	Ser623	Phosphorylation reduces its association with the phosphatase SHP-2 and decreases STAT5 activation
	Grb10	Ser150, Ser476	Phosphorylation of the adaptor molecule provides a negative feedback inhibitory step to insulin-induced signaling
	KSR1	Thr260, Thr274	Phosphorylation does not seem to affect its ability to facilitate Ras signaling but may regulate its catalytic activity
	Sos1	Ser1137, Ser1167, Ser1178, Ser1193, Ser1197	Phosphorylation of its nucleotide exchange factor prevents its association with Grb2, thereby provides a negative feedback mechanism for groth factor and GPCR signaling
Apoptotic proteins	Bad	Ser112 (mouse)	Phosphorylationof the Bcl2-antagonist of cell death (Bad) is required for the dissociation from Bcl-x(L), and thereby inhibits the proapoptotic activity of Bad
	Bim- EL	Ser69, Ser109, Thr110	Phosphorylation of the Bcl2-interacting mediator of cell death EL (Bim-EL) promotes its degradation, thereby its proapoptotic function
	Caspase 9	Thr125	Phosphorylation inhibits its activity on caspase 3, and thereby reduces its proapoptotic effect
	EDD		Role of the phosphorylation of this ubiquitin ligase E3 is not clear but may lead to its induction of its activity
	MCL1	Thr163	Phosphorylation of this antiapoptotic member of the BCL2 family stabilizes it and thereby enhances its activity

(***Adapted from Yoon et al.* 2006**)

Figure 20: List of selected ERK1/2 –substrates. Table contains ERK substrates, phosphorylation sites as well as the various cellular functions controlled by them.

1.3 Epilogue

When the first report on the *ARAF* gene was published a quarter of a century ago, nothing much was known about the role of this isoform. The gene belonged to a small group of proto-oncogenes that were located on the X-chromosome, situated in a 70 kb spanning gene cluster together with genes for the neuron- specific phosphoprotein synapsin (SYN1), the tissue inhibitor of metaloproteinases (TIMP), and the serum glycoprotein properdin (PFC) (Derry & Barnard, 1992; Huebner et al., 1986). Homologues *RAF* genes had been identified in multicellular eukaryotes as divergent as higher plants and man (Rapp et al., 1988) and due to high primary sequence conservation with *CRAF*, analogous biochemical and biological properties were assumed (Beck, Huleihel, Gunnell, Bonner, & Rapp, 1987; Huleihel et al., 1986). These included similar oncogenic activation through N-terminal deletion demonstrating the negative autoregulatory function of this region. An oncogenic *CRAF* version fused to the *ARAF*- promotor region was able to transform NIH3T3 cells, suggesting a role for *ARAF* in human pathology as was previously speculated when incorporation of 5' truncated mouse *ARAF* cDNA into a retrovirus genome established its potential transforming ability (Huleihel et al., 1986). While the 2.6 kb *ARAF* mRNA encoding for the 606- amino-acid protein is expressed in most of the murine tissues, its expression levels vary significantly (Baljuls, A. 2008). Interestingly, the highest levels are found in urogenital tissues (kidney, testis and ovary) whereas the lowest levels are present in neuronal tissue (Storm, Cleveland, & Rapp, 1990), raising the question why Bl/6 *ARAF* knockout mice display severe neurological defects, resulting in death 1-3 weeks after birth (Pritchard et al., 1996). On the other hand, the detected high level of ARAF in the steroid hormone responsive urogenital tissues, seem to correlate with the predicted control mechanism of *ARAF* promoter activity, which has been shown to depend on steroid hormone stimulation (J. E. Lee, Beck, Wojnowski, & Rapp, 1996). Interestingly, in humans, the highest *ARAF* expression has been found in skeletal muscle and bone marrow as well as the overall hematopoietic systems, whereas it was hardly detected in the central nervous system (proteinatlas.org). Meanwhile, transcripts of the proto- oncogene *ARAF* have been found in bovine chronic lymphocytic leukeamia (CCL) cells at the peak of their proliferative activity in primary culture (Kalvelyte & Pabrezaite, 1998). Also a couple of human hematopoietic cell lines such as U937 and Jurkat cells displayed ARAF- RNA levels indicative of *ARAF* gene expression (Baljuls, A. 2008; McCubrey et al., 1998). Figure 21 (attached at the end of this section) shows a detailed

list of cell lines with medium/high level of *ARAF* transcript detected. There are two ARAF splicing variants referred to as DA-RAF1 and DA-RAF2 that were generated by alternative splicing of *ARAF* pre-mRNA (Yokoyama et al., 2007). Both of them are ubiquitously expressed in a variety of mouse tissues and display a wider tissue distribution than ARAF with highest expression levels in the brain and heart. Interestingly, DA-RAF1, which contains the RAF- RBD but lacks the kinase domain, has been shown to work as a dominant-negative antagonist of the Ras-ERK pathway (Yokoyama et al., 2007). The localization as well as the activation of the ARAF protein at membranes is overall analogous to CRAF, although the implications of the mitochondrial membrane association are still controversially discussed. Reports had shown that ARAF associates with mitochondria by binding the mitochondrial inner and outer membrane import receptor proteins hTIM and hTOM (Yuryev, Ono, Goff, Macaluso, & Wennogle, 2000). The association of ARAF with the plasma membrane on the other hand is somewhat distinct from other RAF family members. As previously described, a common RAF feature upon growth factor stimulation is their relocalization to the membrane for full RAF kinase activity. The requirement for Ras- GTP binding (X. F. Zhang et al., 1993) and the interaction with membrane lipids like phosphatidic acid (Rizzo et al., 2000) or phosphoinositides (Johnson, James, Chamberlain, & Anderson, 2005) thereby applies to all RAFs. ARAF has additionally been shown to localize to the PM through EGFR and PDGFR association (Mahon, Hawrysh, Chagpar, Johnson, & Anderson, 2005), thus providing a Ras-GTP-independent membrane recruitment mechanism for this RAF isoform. Other evidence for ARAF activation independent of RAS is its regulation by G protein-coupled receptors via Gα12 (Gan et al., 2013) through its C-terminal sequence that is distinct from other RAFs. The lysophosphatidic acid (LPA)- Gα12 pathway explicitly exploits ARAF to activate the E3 ubiquitin ligase RFFL via MEK and ERK, resulting in persistent PKC activation, which ensures sustained migration of fibroblasts and tumor cells.

Taken together, a lot of mechanistic insights on the MAPK signaling module has been provided ever since the first publications entered the stage more than 30 years ago. With the advancement in reseach techniques like the application of Next Generation Sequencing (NGS) technology to human diseases and the elucidation of the crystal structure of BRAF protein, the complex nature of the signaling networks and the consequences in disease etiology have become evident. Despite these insights, there is a growing need to decipher mechanisms by which the different RAF isozymes fulfill their distinct -possibly yet unknown- tasks. Both quantitative and qualitative mass spectrometry based-analysis of

patient derived material will further assist in identifying novel regulators of RAF signaling in health and disease. Much of the study on kinases is restricted to the kinase domains and thus employing full- length kinases for further studies remains as a challenge partially because of the difficulties in purifying these enzymes. Last but not least, ARAF has slowly started to emerge from the shadow of its more famous siblings B- and CRAF. This work will further help to determine the role of ARAF in human cancers and thereby provide a new perspective on this understudied member of the prominent RAF kinase family.

RNA expression in FPKM (score 0-100)	Cell lines	Cell line summary
	Myeloid cell lines	
44	HMC-1	Mast cell leukemia
43	NB-4	Acute promyelocytic leukemia
40	HL-60	Acute promyelocytic leukemia
39	U-937	Monocytic lymphoma
33	K-562	Chronic myeloid leukemia
23	THP-1	Acute monocytic leukemia
17	HEL	Erythroleukemia
	Lymphoid cell lines	
41	U-266/70	Multiple myeloma
35	HDLM-2	Hodgkin lymphoma
34	RPMI-8226	Multiple myeloma
32	U-266/84	Multiple myeloma
30	Karpas-707	multiple myeloma
26	MOLT-4	Acute lymphomphoblastic leukemia
25	REH	Pre-B cell leukemia
17	Daudi	Human Burkitt lymphoma
13	U-698	B-cell lymphoma
	brain cell lines	
29	U-87 MG	Glioblastoma strocytoma
23	U-251 MG	Glioblastoma
21	U-138 MG	Glioblastoma
17	SH-SY5Y	Metastatic neuroblastoma
	lung cell lines	
44	A549	Lung carcinoma
23	SCLC-21H	Small cell lung carcinoma
	abdominal cell line	
36	CACO-2	Colon adenocarcinoma
33	CAPAN-2	Pancreas adenocarcinoma
20	Hep G2	Hepatocellular carcinoma
	breast, female reproductive system	
52	HeLa	Cervical epithelial adenocarcinoma
52	MCF7	Metastatic breast adenocarcinoma
30	SiHA	Cervical squamous carcinoma
28	AN3-CA	Endometrial adenocarcinoma
23	SK-BR-3	Metastatic breast adenocarcinoma
22	EFO-21	Ovarian cystadenocarcinoma
	urinary, male reproductive system	
60	PC-3	Prostrate adenocarcinoma
32	RT4	Urinary bladder transitional cell carcinoma
26	NTERA-2	Embryonal carcinoma
	skin cell lines	
38	HaCaT	Keratinocyte
27	A-431	Epidermoid carcinoma
18	WM-115	Malignant melanoma
14	SK-MEL-30	Metastatic malignent melanoma
	sarcoma cell lines	
44	U-2197	Malignant fibrous histiocytoma
29	U-2 OS	Osteosarcoma
26	RH-30	Metastatic rhabdomyosarcoma
	miscellaneous cell lines	
47	HEK 293	Embryonal kidney
23	BEWO	Metastatic choriocarcinoma
21	TIME	Telomerase-immortalized microvascular endothelial cells

Figure 21

1.4 Aim of the project

Most cancers result from the dysregulation of multiple signaling pathways. Over the past decades, the RAS-RAF-MEK-ERK signaling pathway has been identified as a key regulator of cell progression and survival by transducing multiple signals from the plasma membrane to the nucleus. Cancer cells need to exploit these survival strategies in order to grow, expand and spread within the host. As pre-existing and acquired mutations in these cells determine their oncogenic potential, it is beyond question that a detailed knowledge about underlying causes are warranted for tumor therapy. The *BRAF* gene, an effector of RAS oncogene, is somatically mutated in a number of human cancers, raising the possibility that other RAF family members such as A, - and CRAF are likely to be mutated in human cancers. Intriguingly, MEK as downstream substrates of the three RAF isoforms have only one main substrate ERK, which is unusual for protein kinases that are often relatively promiscuous. Hence, the use of phosphorylation of ERK is used to identify small molecules capable of inhibiting signaling of the RAS/RAF/MEK/ERK pathway. While inhibiting BRAF or CRAF respectively has shown tremendous success in the clinics, cancer would not be malicious if it did not find ways to circumvent this blockade by reactivating MEK/ ERK through different means. Under certain conditons, RAF inhibitors aid to tumorigenicity by enhancing MAPK signaling rather than preventing it. Given all available information on MAPK signaling in normal and malignant cells, it becomes apparent that ARAF, being the isoform with the lowest kinase activity, has been generally overlooked in these scenarios. The main focus of this study was therefore to investigate the possible role of ARAF kinase in the activation of both basal and RAF inhibitor- driven ERK1/2 activation and tumor cell invasion. The generation of various ARAF mutants through substitution of regulatory residues served as the main tool to examine kinase activity, phosphorylation patterns and paradoxical behavior of the mutants towards RAF inhibitors. Physiological consequences were studied *in vitro* in cell lines as well as *in vivo* by employing tumour xenografts in nude mice.

2. Materials and methods

2.1 Molecular biology methods

2.1.1 Vectors, cDNAs and constructs

Vectors:

pGEX-4T1 (Ritva Tikkanen)

pcDNA3.1/*myc*-HisB (Ulf Rapp/ Invitrogen)

pMCEF (Richard Marais)

pDONR223-ARAF (addgene, #23725), pLenti4TO/V5-DEST (Invitrogen)

pRK5 Flag (Genentech)

for constitutive luciferase expression (amsbio, #LVP326):

cDNAs:

pcDNA3.1/*myc*-HisB –ARAF(WT)

pcDNA3.1/*myc*-HisB –ARAF(R362H)

pMCEF-hBRAF (wt)

pMCEF-BRAF (V600E)

pRK5 Flag- KRAS (G12D)

pRK5 Flag- KRAS (G13D)

pRK5 Flag- NRAS (Q61L)

pRK5 Flag- HRAS (G12V)

pLenti4TO/V5-DEST (EV)

pLenti4TO/V5-DEST-ARAF (WT)

pLenti4TO/V5-DEST-ARAF (R362H)

pLenti4TO/V5-DEST-ARAF (R52L)

pLenti4TO/V5-DEST-ARAFY (301D/Y302D)

cDNAs used for lentivirus production (viral plasmids):

HDM VSV-G

HDM Hgpm2

HDM tat 1b

RC CMV-Rev

Oligonucleotides/ mutagenesis primers:

ARAF R362H_ fw 5´-GTGCTCAGGAAGACGCACCATGTCAACATCTTG-3´

ARAF R362H_ rev 5´-CAAGATGTTGACATGGTGCGTCTTCCTGAGCAC-3´

ARAF R52L_fw 5´-GGCCCTGAAGGTGCTGGGTCTAAATCAGG-3´

ARAF R52L_rev 5´-CCTGATTTAGACCCAGCACCTTCAGGGCC-3´

ARAF Y301D/Y302D_fw 5´-CGGGACTCAGGCGATGACTGGGAGGTACC-3´

ARAF Y301D/Y302D_rev 5´-GGTACCTCCCAGTCATCGCCTGAGTCCCG-3´

Oligonucleotides/ sequencing primers:

ARAF G163_fw 5´-GGAGGCTCCAGACAGCATGAGGCTCCCTCG-3´

ARAF E304_fw 5´- GAGGTACCACCCAGTGAGGTGCAGCTGCTG-3´

ARAF G416_rev 5´- GCCCTGGGCAGTCTGCCGGGCCACGTCGAT-3´

2.1.2 Site directed mutagenesis and plasmid generation

The various point mutations in ARAF (R362H, R52L, Y301D/Y302) were generated with the Site-Directed Mutagenesis Kit (Stratagene #200518) following maufacturer´s instructions. Depending on the type of mutation desired, the number of PCR cycles used in the cycling parameters varied between 16 and 18. The contents and timings for mutant strand synthesis- PCR are listed as followed, but were modified according to vector and insert length:

	Final concentration (50µl reaction volume)
10x Pfu buffer	1x
dNTPs 2mM	0.2 mM
forward primer 10 pM	1 pM
reverse primer 10pM	1 pM
Template DNA	25 ng
Pfu polymerase 2.5 U/µl	2.5
ddH2O	add to final volume

	Temperature	Time	
1. Initial denaturation	95°C	1 min	
2. Denaturation	95°C	45 s	18x
3. Annealing	58°C	1 min	
4. Extension	68°C	7 min	
5. Final extension	68°C	10 min	
6. Cool down	4°C	unlimited	

Annealing temperature was determined according to the melting temperature of primers (Tm-5°C) and extension time was calculated depending on the length of amplified DNA fragment (2 min per kb of DNA). After endonuclease *Dpn* I – treatment to digest parental DNA template and to select for mutation-containing synthesized DNA, the PCR product was heatshock transformed by chemocompetent *E. coli* bacterial cells as followed: Incubation of DNA with E.coli for 30 min on ice, followed by heat shock at 42 °C for 90 s before 500 µl of antibiotic-free LB-medium

(Applichem, #A4425) was added. After incubation at 37 °C for 45 min, the transformed bacteria were selected on antibiotic containing LB-agar plates. The selection marker of the pDONR223 vector is spectinomycin (Sigma), while pLenti4TO/V5-DEST contains a resistance gene against Ampicillin (Applichem, #A0839). Single colonies were then expanded in appropriate antibiotic containing LB- medium at 37 °C for >16 h and used for DNA preparation, which was achieved by use of the GeneJET- DNA purification kit (Thermo Scientific) following manufacturer's instructions. The fidelity of mutagenesis was confirmed by DNA sequencing (Eurofins MWG Operon). The pLenti4⁄V5-DEST™ Gateway® Vector system (Invitrogen) was used for lentiviral-based expression of a target gene in dividing and non-dividing mammalian cells as will be described in appropriate section.

2.2 Cell biology methods

2.2.1 Cell lines

293T (HEK), Human Embryonic Kidney	DMSZ
A549, human lung carcinoma	gift from S. Horwitz
MiaPaCa2, human pancreatic carcinoma	ATCC
MDA-MB-468, human breast adenocarcinoma	ATCC
HCT-116, human colorectal carcinoma	Genentech
SW-48, human colon adenocarcinoma	Horizon discovery

To ensure assay accuracy, cells were always counted prior to experiments described in the sections below. Cells were counted by use of TC 20™ automated cell counter (BioRad #145-0101) whereby counts have been obtained for suspension cells grown as adherent cells at concentrations up to $1x10^7$ cells/ ml. 10µl of cell suspension in the presence of trypan blue (Sigma, #T8154) have been used for determining cell viability and count.

In order to measure cell number from cells grown in suspension for 3 days, cells were collected, centrifuged and subsequently analyzed by Fluorescence Activated Cell Sorting (FACS). Therefore, lysates were dissolved in PBS (Applichem, #A0964) containing Propidium iodide (Sigma-Aldrich, #P4170) at a concentration of 10 µg/ml and incubated for 15 min in the dark. PI is generally used as an DNA intercalating

fluorescent dye to identify dead or dying cells. PI fluorescence (emitted wavelength 617 nm, captured with Fl2 channel) was determined with a FACSCANTO II flow cytometer (BD) instrument. Cells with high PI intensity were gated and the overall cell numbers were monitored. The debris was excluded from the measurement by applying gating in the FL2-FSC dot-plot.

2.2.2 Production of lentiviruses

shRNAs directed against human ARAF (NM_001654.1), human BRAF (NM_004333.2), or human CRAF (NM_002880.2) were obtained from Sigma. Cells were infected by lentiviral particles and subsequently selected for resistance to puromycin (2.5 µg/ml) until a stable knockdown culture was achieved. For complementation assays, the ARAF gene was reintroduced into shARAF (3´UTR) background by lentiviral infection [and selection with zeocin (200 mg/ml)]. Lentivirus particles were produced in human embryonic kidney (HEK) 293T cells by transfecting cells with 1.2µg of pLenti4TO/V5-DEST-ARAF and various mutants together with viral plasmids (0.2µg each), using GeneJuice transfection reagent (Merck Millipore, #70967). After two days, the virus-containing medium was sterile-filtered, and cells were infected with lentiviral particles in the presence of polybrene (10 mg/ml; Merck Millipore). Cells were then double-selected for resistance to zeocin 200 µg/ml (Invivogen, #ANT-ZN-1) and 2.5 µg/ml puromycin (Roth, #0240.3).

The lentiviral particles with various shRNAs used for stable knockdown in A549 cells were: hARAF shRNA (TRC no. 0000000567, 3´UTR region, CCGGCCAGCCAATCAATGTTCGTCTCTCGAGAGACGAACATTGATTGGCT GGTTTTT), hBRAF shRNA (TRC no. 0000006292, CDS region, CCGGCAGCAGTTACAAGCCTTCAAACTCGAGTTTGAAGGCTTGTAACTGC TGTTTTT), and hCRAF shRNA (TRC no. 0000001067, CDS region CCGGCAAGCAAAGAACAGTGGTCAACTCGAGTTGACCACTGTTCTTTGCT TGTTTTT).

Inducible shRNA-containing HCT-116 cells were generated by Jaiswal, B. S as described previously (Jaiswal et al., 2009).

Oligonucleotides used were:
luciferase shRNA (sense: 5´-GATCCCCCTTACGCTGAGTACTTCGATTCAA GAGATCGAAGTACTCAGCGTAAGTTTTTGGAAA-3´),
hARAFshRNA (sense:5´-GATCCCCCAGCTGAGGTGATCCGTATTTCAAGA GAATACGGATCACCTCAGCTGTTTTTGGAAA-3´),
hBRAF shRNA (sense: 5´-GATCCCCAGAATTGGATCTGGATCATTTCAAGA GAATGATCCAGATCCAATTCTTTTTTGGAAA-3´),
and hCRAF shRNA (sense: 5´-GATCCCCGACATGAAATCCAACAATATTCA AGAGATATTGTTGGATTTCATGTCTTTTTGGAAA-3´).
Pre-made lentiviral particles, expressing Luciferase (amsbio, #LVP326 Blasticidin resistance 10 µg/ml) were used to transduce A549 control and ARAF depleted cells for subsequent bioluminescence *in vivo* studies. In general 1.8 x 10^5 IFU/ ml were used for lentiviral transduction following the protocol mentioned before (2.2.2).

2.2.3 Transfection of siRNAs

To silence ARAF, BRAF, or CRAF translation by siRNA interference, about 75,000 cells per well were seeded on 12-well plates at least 20 h before transfection. siRNAs directed against the RAF isoform–encoding mRNAs or a scrambled control siRNA as a negative control was transfected into cultures using Lipofectamine RNAiMAX (Invitrogen, #13778-150) at a final concentration of 60 nM. For co-transfection experiments, the reverse transfection protocol was used in accordance with the manufacturer’s instructions. Unless otherwise stated, cells were lysed 48 h after transfection to test knockdown efficiency in SDS- page.

The siRNAs used in this study were purchased from Qiagen;

siControl (sense): 5´-UUCUCCGAACGUGUCACGU-3´ (#1027310);
siARAF#1 (sense): 5´-GACUCAAGGGACGAAA-3´ (#SI00287686);
siARAF#2 (sense): 5´-GGGAUGGCAUGAGUGUCUA (#S100287693);
siBRAF (sense): 5´-CAUAUAGAGGCCCUAUUGG-3´ (#SI00299488);
siCRAF (sense): 5´-GGAUGUUGAUGGUAGUACA-3´(custom-made).

2.2.4 Cell culture and transfection

A549 and HCT-116 cells were cultured in RPMI 1640 medium supplemented with 10% FCS and 0.2% penicillin (100 U/ml) /streptomycin (100 mg/ml) (all GibcoBRL). HEK293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS and 0.2% penicillin. MiaPaCa2 was cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS and 0.2% penicillin, complemented with 1mM nonessential amino acids (Gibco, #11140), 1mM sodium pyruvate (PAA #S11-003), and 2.5% horse serum (Invitrogene, #16050-122). MDA-MB-468 cells were cultured in DMEM/Ham's F12 (1:1, v/v), 2mM L-glutamine (Gibco, #25030) and 10% FCS. Knockdown in HCT-116 cells was induced with doxycycline hyclate (Sigma, #D9891) for 3 days. Isogenic SW-48 cell lines containing heterozygous knock-ins of individual RAS-activating mutations were cultured in RPMI 1640 medium supplemented with 10% FCS and 0.2% penicillin (100 U/ml) /streptomycin (100 mg/ml). G418 (Sigma, A1720-1G) was added to the culture of KRAS-mutant cell lines (at 0.4 mg/ml) and H/NRAS-mutant cell lines (at 0.8 mg/ml). No antibiotic was added in the case of wild-type/ parental cells.

All cells were grown at 37°C at 5 % CO_2 containing air. After reaching confluency, the cells were passaged at regular intervals. Unless otherwise stated, A549 cells were transiently transfected with various plasmids, using polyethylenimine/ PEI (Polysciences Inc., #23966) at a concentration of 10 mM. Where indicated, cells were treated with DMSO (Applichem #A3672), GDC-0879 (Selleckchem, # S1104), UO126 (Calbiochem, # 662005), and sorafenib p-toluenesulfonate salt (LC Laboratories, #S-8502) in the presence of serum for the times and at the concentrations (ranging from 0.1 to 10 mM) indicated in the figures. Different transfection reagents and protocols (see manufacturer´s protocol) were used best suited for the specific cell line, whereby usually 1 µg of DNA was transfected into adherent cells for simple overexpression experiments.

2.3 Biochemical methods

2.3.1 Antibodies

Antibodies used in this study were generated against human antigens. This included phosphorylated ERK1/2 at Thr202/Tyr204 (# 9101L), total ERK1/2 (p44/42 MAPK;

#9102L) and PARP (#9542) rabbit polyclonal antibodies, BRAF (55C6; #9433S), phosphorylated CRAF at Ser338 (56A6; #9427S) and phospho-MEK1/2 at Ser217/221 (41G9; #9154S) rabbit monoclonal as well as phospho-Akt at Ser473 (#4051S), which is a mouse monoclonal antibody- all from Cell Signaling Technology. Total CRAF (#610151), a mouse monoclonal antibody was purchased from BD Transduction Labs. ARAF (sc-408), BRAF (sc-166), and CRAF (sc-133) rabbit polyclonal antibody, c-Myc (9E10; sc-40) mouse monoclonal antibody, normal mouse IgG (sc-3877), normal rabbit IgG (sc-3888) were all obtained from Santa Cruz Biotechnology. Tubulin (T9026) and Flag (M2, F3165) are mouse monoclonal antibodies from Sigma; V5 (#R960-25) mouse monoclonal IgG2a antibody from Invitrogen; RAS (#3233-1), MEK1 (N-term) (#1518-1); KSR-1 (#04-1160) rabbit monoclonal from Millipore; M2-PK (#S-1) mouse monoclonal from Schebo Biotech and NaK-ATPase (#MA3-928) mouse monoclonal antibody from Thermo Scientific. Conditions and dilutions for the usage of primary and secondary antibody were followed according to producer´s datasheets.

2.3.2 SDS-PAGE and Western blotting

By electrophoresis proteins were separated on the basis of mass in a polyacrylamide gel* under denaturing conditions disrupting nearly all non-covalent interactions (SDS-page). For that, cells were lysed in 5x laemmli-buffer (SDS- loading buffer) and boiled at 100°C for 5 min before loading onto polyacrylamide gels. After separation by length of the polypeptide, the proteins were transferred to nitrocellulose membranes (Whatman Protran BA83 #10401396). For immunoblot analysis, membranes were blocked with 5% low-fat milk (Carl Roth, #T145.2) in PBS for 1 h at room temperature and then incubated with indicated primary antibodies (according to manufacturers' datasheet). Antigen-antibody complexes were detected by horseradish peroxidase-coupled secondary antibodies followed by enhanced chemiluminescence (Amersham Biosciences, Millipore, #RPN2209), visualized on X-ray films (Agfa Cronex, #ECOAA). Quantification of Western blots was performed by densitometry using ImageJ software (NIH).

*The polyacrylamide gels used in this work were composed of two layers: a 6–15% separating gel (pH 8.8) that separates the proteins according to size and a lower

percentage (5%) stacking gel (pH 6.8) that insures simultaneous protein entry into the separating gel at the same height.

2.3.3 Immunoprecipitation

To immunoprecipitate endogenous proteins, two million A549 cells were seeded on 10 cm dishes and after 48 h treated with indicated small molecular inhibitors. After 4 to 6 h incubation, the cells were lysed in 500 µl RIPA buffer for 30 min on ice. Lysates were cleared by centrifugation for 15 min at 14,000 rpm and 50µl was used for total lysate control (TLC). Endogenous ARAF, BRAF, CRAF, MEK1, or V5 were then immunoprecipitated from the remaining supernatant with a target antibody overnight. Antigen-antibody complexes were precipitated by agarose-coupled protein A/G beads (Roche, #11-134-515-001 and 11-243-233-001). Beads were washed with RIPA buffer, and bound proteins used for subsequent experiments (kinase assay or SDS page). For immunoprecipitation of co-expressed proteins in A549 cells, 250 000 cells were transfected with various plasmids using Turbofect® transfection reagent (Thermo Scientific, Merck Millipore, #70967) in 6-well plates. Cells were lysed in RIPA buffer at 48 h after transfection, and proteins were pulled down as mentioned above. A total protein amount of 1 mg was used for endogenous pull-down experiments. Control experiments were performed with IgG isotype antibodies.

2.3.4 RAF kinase assay

For the kinase assay, V5-tagged ARAF was immunoprecipitated from reconstituted shARAF A549 cells treated with RAF inhibitors (either sorafenib or GDC-0879) using the V5 antibody and agarose-coupled protein A/G beads, according to the immunoprecipitation methods described here. Beads were washed three times with RIPA lysis buffer, and all remaining buffer was removed using an insulin syringe. A reaction mix of 4 µl of 10x kinase buffer, 2 µl of 20x Mg-ATP (Enzo Lifesciences), 1µg of GST-MEK1 and up to 40 µl distilled water was added to the beads. The reaction was incubated at 30°C for 30 min and then stopped by adding 8 µl of 5x Laemmli. The entire reaction mix was loaded on SDS-PAGE gel for immunoblot analysis.

2.3.4 RAF Competition Assays

Human MEK1-GST was purified as previously described (Amaddii et al., 2012). Recombinant, purified ARAF, BRAF, and CRAF were purchased from Origene or Abnova. For competition assays, 2 µg of MEK1-GST was bound to glutathione (GSH) sepharose beads (GE Healthcare) and incubated with 200 ng recombinant ARAF or BRAF for 2 h. After washing, 200 ng of the competing RAF was added and incubated for further 3.5 h. After SDS-PAGE and Western blotting, MEK1-bound RAF was detected with RAF antibodies. For the saturation assay, 2 µg GSH-bound MEK1-GST was incubated with an increasing amounts of a specific recombinant RAF which was detected as above. In addition, competition experiments were also performed with RAF isoforms produced in cell free systems. ARAF, BRAF, CRAF, and MEK1 proteins were transcribed and translated using TNT® Coupled Rabbit Reticulocyte Lysate System (Promega, #4611) in vitro using pCDNA3-RAF constructs in accordance with the manufacturer's protocol. To assess the competition from other RAF proteins with BRAF for binding MEK1, BRAF and MEK1 were initially incubated together for 1 h before the addition of ARAF and CRAF proteins. Finally, beads were washed thrice with RIPA buffer, and bound proteins were dissolved in Laemmli loading buffer for SDS-PAGE and Western blotting.

2.3.6 GST pull-down

GSH-agarose beads (GE Healthcare, #17-0756-05) were washed and equilibrated in GST pull-down buffer (GPB). For each condition, 50 µl of beads was resuspended in 300 µl of GPB and incubated on a rotator for 2 h at 4°C with 1 µg of GST or GST-tagged protein. The beads were then washed three times with GPB and incubated on a rotator for 1 h at 4°C with BSA-GPB solution (100 mg/ml). After being washed thrice, the beads were finally incubated on a rotator for 2 h at 4°C with protein lysates from ARAF-knockdown cells that were reconstituted with wild-type or mutant ARAF (RIPA buffer). The final washing was performed with RIPA buffer. Buffer was removed using an insulin syringe, and samples were then prepared for SDS-PAGE by addition of Laemmli buffer.

2.3.7 Subcellular fractination

A total of 250,000 A549 cells were seeded into 6 well plates before treatment with GDC-0879. After an incubation time of 6 h, subcellular fractions were then prepared

using the proteome extraction kit (Calbiochem, #539790) according to manufacturer's instructions. RAF isoforms were immunoprecipitated from cytosolic (fraction 1) and membrane fractions (fractions 2 to 4) as described for immunoprecipitation and analyzed for proteins of interest by immunoblot analysis.

2.3.8 Phospho Kinase array

In order to estimate relative levels of protein phosphorylation, the human Phospho-Kinase Array Kit (R&D Systems# ARY003B) was used, allowing for parallel detection of 43 kinase phosphorylation sites. Therefore, control and ARAF depleted A549 cells (2.5 x 10^6) have been seeded into ultra low attachment surface plates (COSTAR, #3471) to maintain cells in a suspended, unattached state before they were collected after two hours and further processed according to assay instructions. Briefly, the assay employs phospho-specific antibodies spotted in duplicates on nitrocellulose membranes. Cleared cell lysates were mixed with biotinylated detection antibodies and then incubated with the array membrane overnight. To capture spots corresponding to the amount of phosphorylated protein bound, streptavidin-HRP and chemiluminescent detection reagents were applied for signal detection. The analysis of spot pixel density was done with ImageJ software. The assay contained the following Kinase antibodies (sensitive to indicated phosphorylation sites):

EGF R	Y1086	Tyrosine-protein kinase receptor
PDGF Rβ	Y751	
p38α	T180/Y182 DYC8691B DYC869B	MAPK
ERK1/2	T202/Y204, T185/Y187	
JNK 1/2/3	T183/Y185, T221/Y223	
MSK1/2	S376/S360	mitogen- and stress- activated kinase, nuclear, downstream of MAPKs
RSK1/2/3	S380/S386/S377	Ribosomal S6 kinase, serine/threonine kinase, downstream MAPK substrate
Akt 1/2/3	S473 and T308	kinases activated by phosphoinositide 3-kinase (PI3K)
Src	Y419	Src family, cytoplasmic tyrosine kinase
Lyn	Y397	
Lck	Y394	
Fyn	Y420	
Yes	Y426	
Fgr	Y412	
Hck	Y411	
FAK	Y397	non-receptor protein tyrosine kinase, integrin-enriched focal adhesion sites
PLC-γ1	Y783	phospholipase Cγ1, integrin signaling
PYK2	Y402	non-receptor tyrosine kinase, focal adhesion kinase family
GSK-3α/β	S21/S9	Glycogen synthase kinase 3, β -catenin/Wnt pathway
β-Catenin	-	
p53	S392 and S15 and S46	tumor surpressor
p27	T198	tumor suppressor

AMPKα1	T183	AMP-activated protein kinase, cellular energy homeostasis
AMPKα2	T172	
mTOR	S2448	(mammalian) target of Rapamycin serine/threonine protein kinase engaged in protein synthesis etc.
p70 S6 Kinase	T389 and T421/S424	serine/threonine protein kinase, target of mTOR
PRAS40	T246	Proline-Rich Akt Substrate, involved in mTOR signaling
CREB	S133	cAMP response element- binding protein, transcription factor
c-Jun	S63	transcription factor, JNK substrate
HSP27	S78/S82	heatshock proteins
HSP60	-	
STAT2	Y689	signal transducer and activator of transcription, transcription factors
STAT3	Y705 and S727	
STAT5a	Y694	
STAT5b	Y699	
STAT5a/b	Y694/Y699	
STAT6	Y641	
eNOS	S1177	endothelial nitric oxide synthase, MAPK target among others
Chk-2	T68	checkpoint kinase, serine/threonine protein kinase, cell cycle control
WNK1	T60	serine/threonine protein kinases, ion transport regulation, Akt substrate

2.4 Phenotypical studies

2.4.1 Cell Proliferation assay (MTT)

To measure the proliferation rates of cells, the Cell Proliferation Kit I (MTT) (Roche, 11465007001) was used following assay guidelines. Equal number of control and ARAF-depleted A549 cells (30,000 cells) were seeded on 96-well plates. After an overnight incubation, cells were treated with MTT, and 4 h later a solubilization solution was added, reducing the yellow MTT dye to its insoluble formazan, which has a purple color. The absorbance of the samples was measured the next day marking the start of proliferation (Plate T-0). The proliferation of A549 control versus ARAF-knockdown cells treated with indicated concentrations of GDC-0879 was assessed by spectrophotometry 72 h after treatment with inhibitor (Plate T-72). The values were normalized to T-0 absorbance readings.

In order to visualize increase in cell number in different assay conditions, equal amounts of A549 cells have been seeded into 6-well plates, left to proliferate for three days before staining with crystal violet (dissolved in 20% Methanol). Quantification of proliferation rates has always been performed by MTT-method described above.

2.4.2 Wound healing assay

A549 control and ARAF, BRAF, or CRAF depleted cells were seeded onto 12 well plates and scratches were made on confluent monolayers with a pipette tip. Cells were

then washed and treated with DMSO or 4 μM GDC-0879 and the extent of wound closure was assessed at 0 and 6 h. The images were acquired with a Leica microscope using a 10x objective (Live Cell Imaging System) and the percentage of wound closure was calculated from the width of the wound formed after time using IMAGEJ software tools (Oberoi et al., 2012).

2.4.3 Transwell migration assay

Control A549 cells and ARAF-knockdown cells were treated with BRAF-inhibitor (2.5 or 5 μM GDC-0879 for 6 h) in serum-free media. Cells (100,000) were then transferred into 8 μm Transwell migration chambers (Corning, #3422). 10% FCS was added to the lower chamber to serve as chemo-attractant. The cells were left to migrate for 12 h or overnight. Cells that successfully migrated and attached to the bottom of the chamber were considered for quantification (pictures taken by Leica cell culture microscope). Cells were counted from three random fields per condition from each experiment. For the sake of illustration, pictures were taken from one representative experiment in which every transwell had been stained with crystal violet as described (Dogan et al., 2008).

2.4.4 Matrigel invasion assay

Tumor cell invasion studies were performed on control and ARAF-knockdown A549 cells using Matrigel invasion chambers (BD BioCoat™ Growth Factor Reduced, #354483). Approximately 100,000 cells were seeded onto 8 μm Matrigel invasion chambers in RPMI media supplemented with 0.1% FCS and 0.5% BSA with BRAF inhibitor. 10% FCS was added to the lower chamber to serve as chemo-attractant. After 30 h incubation at 37°C, cells were washed twice with PBS and fixed with 3.7% paraformaldehyde (Applichem, #A3813) for 2 min before being permeabilized in 100% methanol for 20 min. Invading cells were stained with crystal violet (dissolved in 2% ethanol) for 15 min and the cells on the upper surface of the membrane were mechanically removed with a cotton swab. Images (three to five fields per chamber filter) were acquired using a Leica cell culture microscope, and the invasion index was calculated by determining the total area of invaded cells in the matrix with Adobe Photoshop CS5 software.

2.4.5 3D spheroid cell invasion assay

To evaluate the ability of tumour cells to invade the matrix, an organotypic 3D invasion assay was used. Control A549 cells together with ARAF-knockdown cells were cultured and used in a 3D spheroid cell invasion assay according to the manufacturer's instructions with slight modifications (Cultrex, #3500-096-K). In brief, 3000 cells per condition were suspended in a specialized spheroid formation matrix and left for 72 h at 37°C to induce the formation of spheroids. Spheroids were subsequently embedded in an invasion matrix that consisted of basement membrane components, and invasive cells were left to penetrate this barrier over a period of three to six days. Increasing amounts of the BRAFV600E-specific inhibitor GDC-0879 were added to evaluate its invasion-modulating capacity on these cells. Cell invasion was visualized by photography; images were taken every 24 h using a 10x objective on a light microscope. To measure changes in the area of the invasive structure, images were analyzed with Adobe Photoshop CS5 software. The start time (0 h) was noted at the initiation of cells projecting out of the spheroid, and the change in surface area over a period of 2 days was calculated.

2.4.6 Random motility assay

Equal numbers of shControl- or shARAF-transfected A549 cells were seeded at a low confluency on a 0.5% gelatin (Sigma, #G2500) coated tissue culture plate. Once settled, the cells were treated with DMSO or GDC-0879 (2 μM) and subjected to time-lapse imaging (37°C, 5% CO_2) on a Leica microscope using a 10x objective over 24 h. The resulting movies were used for cell tracking analysis as described previously (Oberoi et al., 2012). In short: extracted images from the movies were processed with standard routines ("plugins") on Image J (Scion Image, NIH, USA), and single cells were tracked with three different programs to ensure consistency of results: the imaging and analysis software on Meta Morph (Universal Imaging, USA), on Volocity (Improvision, USA) and with Cell Track (Sacan, Ferhatosmanoglu, & Coskun, 2008). The cell speed and distance traveled was calculated for single cells indicated by tracks in the representative image frames.

2.4.7 Colony formation assay (Soft agar)

For the anchorage-independent growth assay, control and RAF knockdown cells (A, B and CRAF respectively) were grown in soft agar. The ability of cells to form colonies in soft agar was assayed by seeding 10^4 cells in a suspension of top agar (2 ml of: 6x RPMI high glucose, conditioned media containing growth factors and nutrients, 10% FCS and 0.7% agar) above of a layer of bottom agar (2 ml of: 6x RPMI high glucose, conditioned media containing growth factors and nutrients, 10% FCS, 2% agar) in 6 cm plates. Colonies were fed weekly for 14 days by applying an additional 500 µl RPMI media, supplemented with 10% FCS to each plate. After two weeks, colonies were stained using crystal violet diluted in water (0.01%). Photographs were taken using GelDoc instrument and the number of colonies was counted under a light microscope.

2.4.8 Bioimaging of luciferase expression in mice

A549 cells were stably transfected with the firefly luciferase gene, under the control of a strong CMV promotor, which lead to constitutive luciferase expression. One million cells per mouse and condition (-/+ ARAF knock down) were suspended in cell culture medium and injected into the tail vain of 6-8 weeks old nude female mice (Janvier, NMRI-nu). The experiment was carried out twice, each time employing 2 x 6 mice per assay condition to ensure reproducibility. The mice had free access to food and water, were maintained in a climate-controlled room at a 12-h light–dark cycle. The experiments were approved by the local Ethics Committee for animal research (Darmstadt, Germany) and were conducted in collaboration with the Tegeder lab (Uni Frankfurt). *In vivo* luciferase and fluorescence analysis of tumor growth was done according to measurement routines in Tegeder´s lab 2-3 weeks post injection (Pickert, G. 2013). In short: *In vivo* luciferase imaging was made with an IVIS Lumina II imaging system that employs XENOGEN technology (Caliper LifeSciences). 100 µl d-luciferin (150 mg/ml) was injected intraperitoneally 10 minutes prior whole live animal imaging. *In vivo* near-infrared (NIR) fluorescence was analyzed on a Maestro imaging system that employs CRi's *in vivo* spectral imaging technology with the mean bioluminescence correlating with mean tumor volume. The quantification was done in the B-focus, with a close up on mice while shielding of the tail. Representative images were taken in the C-focus detecting luciferase activity in the whole organism

(tail, paws, abdominal region). Mice were kept under 1–1.5% isoflurane anesthesia during imaging.

2.5 Appendix- material

Chemicals

Acrylamide/Bis solution, 40%	Bio Rad
APS	Applichem
Albumine bovine fraction V	Sigma, Taufkirchen, Germany
Bactoagar	Bector, Dickinson
Benzamidine	Sigma, Taufkirchen, Germany
Bromphenolblue	Roth, Karlsruhe, Germany
β-mercaptoethanol	Applichem
β-Glycerophosphate	Sigma, Taufkirchen, Germany
Crystal violet	Sigma, Taufkirchen, Germany
DTT	MP biomedicals, France
Ethanol	Roth, Karlsruhe, Germany
HEPES	Roth, Karlsruhe, Germany
Hydrochloric acid (HCl)	Sigma, Taufkirchen, Germany
Low melting point agarose	Sigma, Taufkirchen, Germany
Glycerol	Roth, Karlsruhe, Germany
Glycine	Applichem
Magnesiumchloride ($MgCl_2$)	Applichem
Methanol	Sigma, Taufkirchen, Germany
NP-40	Sigma, Taufkirchen, Germany
PMSF	Sigma, Taufkirchen, Germany
Protease inhibitor cocktail	Roche, Mannheim, Germany
Proteinase K	Roth, Karlsruhe, Germany
SDS	Sigma, Taufkirchen, Germany
Sodium Chloride (NaCl)	Roth, Karlsruhe, Germany
Sodium Orthovanadate ($NaVO_3$)	Sigma, Taufkirchen, Germany
Sodium Fluoride (NaF)	Sigma, Taufkirchen, Germany
Sodium Hydroxide (NaOH)	Riedel-de-Haën,

T-EDTA, 10x	Sigma, Taufkirchen, Germany
Temed	Sigma, Taufkirchen, Germany
Tris-base	Applichem
Trypsin/EDTA	PAA Laboratories, Austria
Triton X-100	Applichem

Solution and buffers

LB (Luria-Bertani) medium

[10 g/L Bacto-tryptone, 10 g/L NaCl, 5 g/L yeast extract, pH 7.5 (NaOH)]

For plates, addition of 15 g Bacto-agar per liter

5X SDS-loading Buffer, laemmli (for SDS-PAGE)

[70 mM Tris-HCl (pH6.8), 3 % SDS, 40 % Glycerin, 5 % β-Mercaptoethanol, 0.05 % Bromphenoblue]

10X Running Buffer (for SDS-PAGE*)*

[143.75 g Glycine, 30 g Tris, 10 g SDS add 1 L dH_2O]

10X Blotting Buffer

[29 g Glycine, 58 g Tris, 18.5 ml of 20% SDS (or 3.7 g SDS) add 1 L dH_2O]

RIPA buffer (for lysis)

[50 mM Tris-HCl (pH 7.5), 250 mM NaCl, 1% Triton X-100, 1 mM $NaVO_3$, 25 mM NaF, 1.5 mM $MgCl_2$, 1 mM PMSF, β-mercaptoethanol (1:1000 dilution), protease inhibitor mixture (1:100 dilution), 10% glycerol]

10x kinase buffer

[100 mM $MgCl_2$, 250 mM β-Glycerophosphate, 250 mM HEPES pH 7.5, 50 mM Benzamidine, 5 mM DTT, 10 mM $NaVO_3$]

GST pull-down buffer (GPB)

[50 mM tris (pH 7.5), 150 mM NaCl, 1% NP-40, 1 mM DTT]

3. Results

3.1 Role of ARAF kinase in regulating MAPK activation

3.1.1 RAF inhibitors paradoxically activate MAPK signaling

To investigate the potential involvement of ARAF in driving RAF inhibitor (RAFi)–mediated paradoxical MAPK activation, the pan-RAF inhibitor sorafenib as well as the BRAFV600E-specific inhibitor GDC-0879 were employed in various cell lines with defined KRAS mutations (**Figure 3.1**). A549 lung cancer cells (KRASG12S mutation), HCT-116 colorectal carcinoma cells (KRASG13D mutation), MiaPaCa2 pancreatic cancer cells (KRASG12D mutation), and MDA-MB-468 breast carcinoma cells were screened (no reported RAS mutations) initially. As shown in **Figure 3.1 A** and **C** these inhibitors at low concentrations increased MEK1/2-ERK1/2 phosphorylation in these cell lines. To check for the possible activation of CRAF kinase, its phosporylation at Serine 338, was tested (pCRAF).

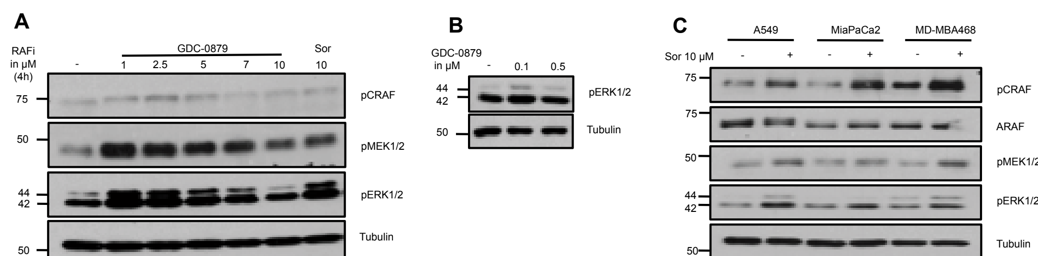


Figure 3.1 RAF inhibitors paradoxically trigger MAPK pathway activation in cells with RAS mutations. (**A**) A549 and (**B**) HCT 116 cells were treated with increasing concentrations of BRAF inhibitor GDC-0879 for 4 h. Shown are representative immunoblots to check for activation of MAPK signaling using pMEK1/2 and pERK1/2 antibodies; Tubulin served as a loading control. (**C**) Various cell lines were treated with activating concentrations of pan- RAF inhibitor sorafenib and analyzed by immunoblotting with indicated antibodies representative of RAF/ MEK/ ERK signaling.

Concentrations of 2.5 or 5 µM for GDC-0879 and 10 µM for sorafenib therefore were identified as MAPK pathway activating and thus these concentrations were used throughout the work.

3.1.2 ARAF is required for basal and RAF inhibitor-induced MAPK activation

To elucidate the role of ARAF kinase in regulating MAPK activation in detail, the abundance of RAF isoforms was decreased by employing small interfering or short

hairpin RNAs (siRNAs or shRNAs, respectively) in these cell lines. The knock down efficiency of individual RAFs was validated by western blot analysis. As shown in **Figure 3.2** loss of ARAF, but not BRAF or CRAF, reduced RAF inhibitor–driven ERK1 and ERK2 activation in a panel of KRAS mutated cell lines.

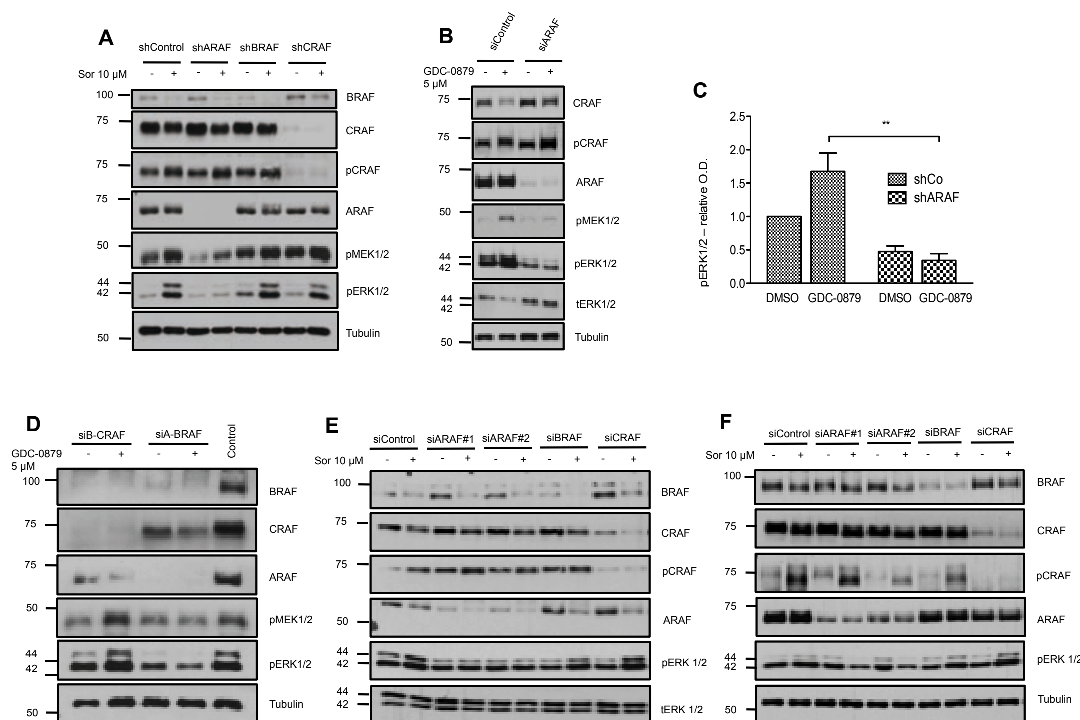


Figure 3.2 ARAF is critically required for RAF inhibitor-mediated MAPK activation. (A) A549 cells were stably transduced with lentiviruses carrying various shRNAs directed against RAF isoforms. Upon selection with puromycin, A549 cells expressing either control shRNAs or shRNAs against the three RAF isoforms were treated with sorafenib (10 µM) for 4 h. The activation of CRAF, MEK1 and ERK1/2 was analyzed by immunoblots, employing phospho-specific antibodies. p, phosphorylated protein. Total levels of ARAF, BRAF and CRAF were also monitored. Tubulin served as loading control. (**B**) A549 cells were transiently transfected with siRNAs directed against ARAF and treated with GDC-0879 (5 µM) for 4 h. Activation of MAPK signaling was assessed as mentioned before. t, total protein. (**C**) A549 cells, stably transfected with shRNA against ARAF were treated with GDC-0879 and phosphorylation of ERK 1/2 in the control and ARAF depleted cells was shown by immunoblots. Density of the bands obtained was quantified using ImageJ software. Data represent the mean optical density of the bands ± SEM from three independent experiments. **P < 0.005, one-way analysis of variance (ANOVA) with Bonferroni's multiple comparison test. (**D**) Shown are immunoblots of lysates from A549 cells transiently transfected with siRNAs against BRAF and CRAF together (siB-CRAF), or ARAF and BRAF together (siA-BRAF), treated with GDC-0879. (**E**) MiaPaCa2 and (**F**) MDA-MB-468 cells were transiently transfected with siRNAs against individual RAF isoforms and treated with sorafenib (10 µM) for 4 h. Total cell lysates were analyzed by immunoblots using indicated antibodies. siARAF#1 and siARAF#2 indicate two different siRNAs .

In A549 cells, basal as well as RAFi-induced phosphorylation levels of ERK 1/2 were significantly down regulated when ARAF expression was silenced as summarized in **Fig 3.2 C**. Double depletion of BRAF and CRAF failed to prevent RAFi–mediated

ERK1 and ERK2 activation (phosphorylation) in these cells (**Figure 3.2 D**). To exclude clonal effects multiple siRNAs and shRNAs were used (**Figure 3.2 E** and **F**). To further confirm these observations and to exclude any potential off-target effects of the siRNAs or shRNAs used, complementation experiments were performed. Cells depleted of endogenous ARAF with an shRNA against the 3´untranslated region (3UTR) were transduced with lentiviruses carrying V5 tagged ARAF-encoding complementary DNAs (cDNAs). As demonstrated in **Figure 3.3 A**, expression of ARAF cDNA in trans displayed a band around 5 kDa (size of the V5 tag) bigger than the one of endogenous ARAF (68 kDa) in an SDS-page.

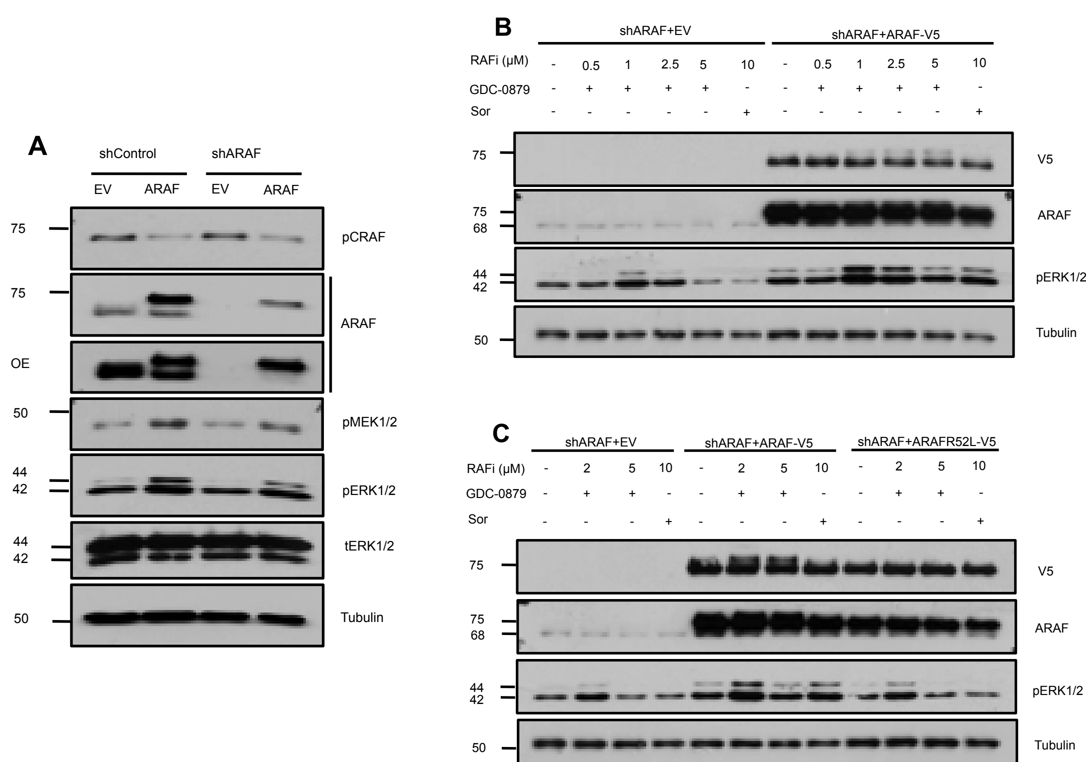


Figure 3.3 Reconstitution of ARAF expression restores basal and RAF inhibitor induced MAPK signaling. (**A**) ARAF and an empty vector control (EV) were stably transfected into control and ARAF depleted cells employing lentivirus particles as mentioned in the methods. Activation of ERK1/2 and MEK1/2 was checked in reconstituted ARAF knock down as well as in over expressed control cells by immunobloting. p, phosphorylated protein. t, total protein. OE, overexposed blot. (**B**) Western blotting in lysates from ARAF-knockdown A549 cells reconstituted with ARAF (V5 tag) or empty vector and treated with indicated concentrations of GDC-0879 or sorafenib for 4 h. (**C**) Same as in (B). A549 cells were also reconstituted with ARAF R52L (Ras binding deficient mutant), and ERK1/2 activation was monitored by Western blots.

Furthermore, reconstitution of ARAF expression restored basal MAPK signaling and reverted RAFi–mediated ERK1 and ERK2 activation in these cells, proving that the observed effects are dependent on ARAF (**Figure 3.3 B** and **C**). As the interaction with RAS is required for normal RAF activation, ARAF-knockdown cells were

reconstituted with mutant ARAF protein, where the Arginine at position 52 was replaced by Lysine impairing this interaction. Consistently, interaction with RAS was required for ARAF to activate ERK1 and ERK2 in these cells under RAF inhibitor treatment. During the course of these experiments a consistent reduction in the protein abundance of BRAF was detected upon treatment with sorafenib or GDC-0879 (**Figure 3.2**). To test whether the loss of BRAF under these conditions primed ARAF for activating MEK1 and MEK2, wildtype BRAF was overexpressed in A549 cells along with the most common mutant form of BRAF, the constitutively active state BRAFV600E. The latter markedly increased the activation of MEK1/2 and ERK1/2 in these cells (**Figure 3.4**). In contrast, transient expression of wild-type BRAF was not sufficient to trigger the MEK1/2- ERK1/2 pathway upon ARAF depletion in these cells, proving that ARAF is the prime MAP3K in these cells to activate MEK1.

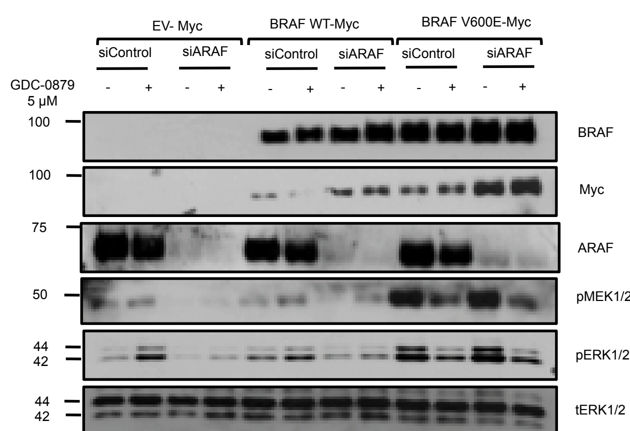


Figure 3.4 ARAF is the prime MAP3K in these cells to activate MEK1. Western blotting in lysates from A549 cells transiently transfected with siRNAs against ARAF and cotransfected with cDNAs encoding of wild-type (WT) BRAF or mutant Myc-tagged BRAFV600E. Control and ARAF-knockdown cells were treated with GDC-0879 (5 µM) for 4 h and probed for MAPK activation using specific antibodies.

3.1.3 ARAF kinase directly phosphorylates MEK1 regardless of BRAF and CRAF

To prove that ARAF functions indeed as direct MAP3K, other RAF isoforms were depleted in a variety of cell lines using different RNA interference strategies. In A549 cells, ARAF-knockdown cells complemented with wild-type ARAF fused with a V5 tag were used for these studies. To test whether ARAF directly activated MEK1, endogenous CRAF was depleted in these cells. The cells were then treated with RAF inhibitors to induce paradoxical MAPK activation, followed by ARAF immuno-

precipitation with V5 antibody and an *in vitro* kinase reaction using recombinant MEK1 protein as a substrate. As shown in **Figure 3.5 A,** loss of CRAF did not prevent the ARAF-mediated phosphorylation of MEK1 in response to RAF inhibitors, suggesting an imminent involvment of ARAF in mediating MEK1 activation.

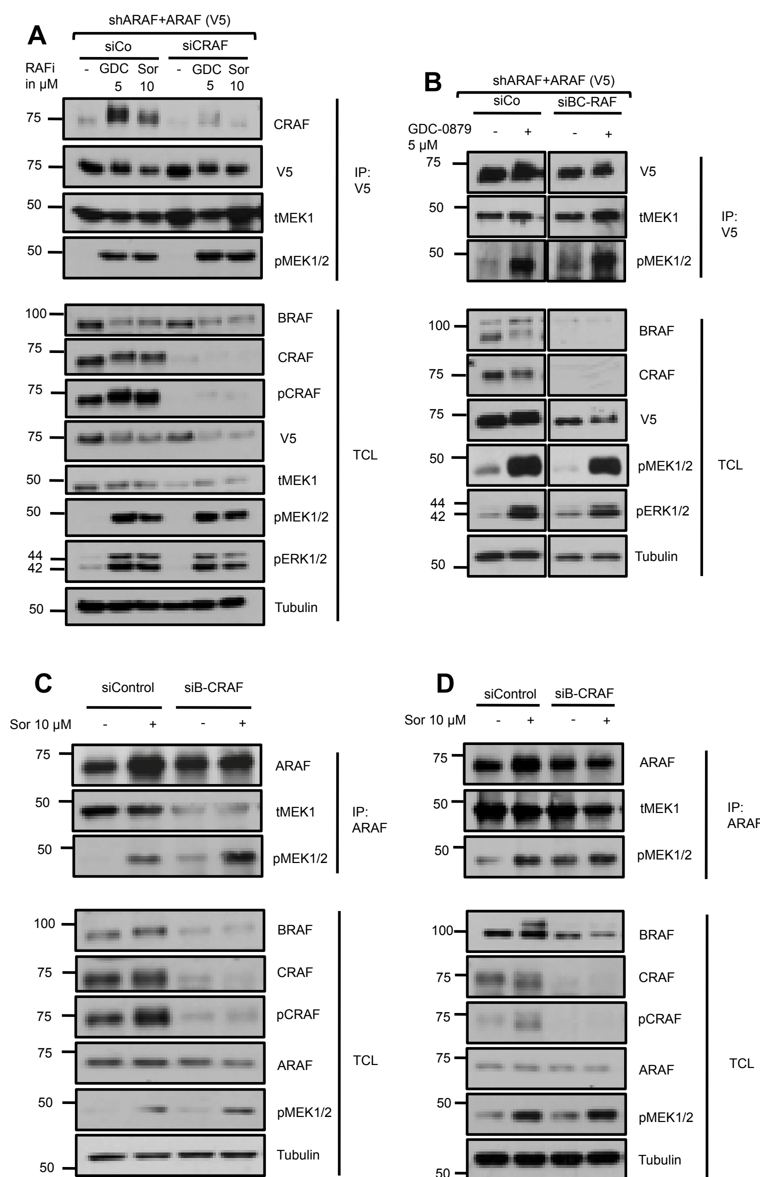


Figure 3.5 ARAF phosphorylates MEK in the absence of BRAF and CRAF in various cell lines upon RAF inhibitor treatment. (**A** and **B**) Immunoprecipitation–Western blotting for the indicated proteins in lysates from reconstituted A549 shARAF cells that were transiently transfected with control and CRAF siRNAs (A) or with control and BRAF and CRAF siRNAs together (B) and treated with GDC-0879 (5 μM) or sorafenib (10 μM) for 4 h. Reconstituted ARAF (V5) was immunoprecipitated (IP) and blotted for pMEK 1/2 and MEK1. The latter was used as a substrate in an in vitro kinase assay. t, total protein; p, phosphorylated protein; TCL, total cell lysate. (**C** and **D**) Immunoprecipitation and Western blotting in lysates from MiaPaCa2 cells (C) and MDA-MB-468 cells (D) that were transiently transfected with control and BRAF and CRAF siRNAs together and treated with sorafenib. Endogenous ARAF was immunoprecipitated and blotted for MEK1 that was used as a substrate in an in vitro kinase assay and pMEK1/2 was analyzed.

In support of these observations, RAF double-knockdown experiments were carried out using siRNA against BRAF and CRAF respectively. Depletion of both CRAF and BRAF did not prevent the activation of MEK1 in response to RAF inhibition by GDC-0879 (**Figure 3.5 B**). Similar results were obtained in other *in vitro* kinase assays conducted in MiaPaCa2 and MDA-MB 468 cells where endogenous ARAF was immunoprecipitated after simultaneous B and CRAF knockdown and RAF inhibitor treatment (**Figure 3.5 C** and **D**). In all these cell lines RAF inhibitors directly activated ARAF, which in turn phosphorylated MEK1 in the absence of BRAF and CRAF.

3.1.4 ARAF kinase is activated by RAS isoforms and their mutants

As the binding of RAF to activated Ras reorients RAF molecules and induces structural modifications that allow phosphorylation/dephosphorylation events crucial for proper RAF activity, it was tested whether the requirement for ARAF in activating MEK1 was confined to a specific RAS isoform or its mutant. To address this issue, BRAF and CRAF were depleted in isogenic SW48 knock-in colorectal carcinoma cell lines expressing an endogenous abundance of NRASQ61L, HRASG12V, KRASG12D, or KRASG12S. Loss of BRAF and CRAF mostly prevented MEK1/2-ERK1/2 activation as a consequence of RAF inhibitor treatment regardless of the RAS isoform or mutation present (**Figure 3.6 A** and **B**). In A549 cells, overexpression of NRASQ61L, HRASG12V, KRASG13D or KRASG12D largely restored basal MEK1 and MEK2 activation despite the loss of ARAF in these cells (**Figure 3.6 C** and **D**). The addition of GDC-0879 to the culture medium activated MEK1 and MEK2 only in control A549 cells transfected with empty vector, but not in the same cells transfected with various mutants of RAS isoforms. However, loss of ARAF blocked the phosphorylation of MEK1 and MEK2 completely in RAF inhibitor–treated A549 cells irrespective of RAS status (**Figure 3.6 C** and **D**). Taken together, these data indicate that ARAF is required in RAF inhibitor induced MEK1/2-ERK1/2 activation in a cell type–dependent manner and thus not confined to a particular RAS isoform or specific mutant.

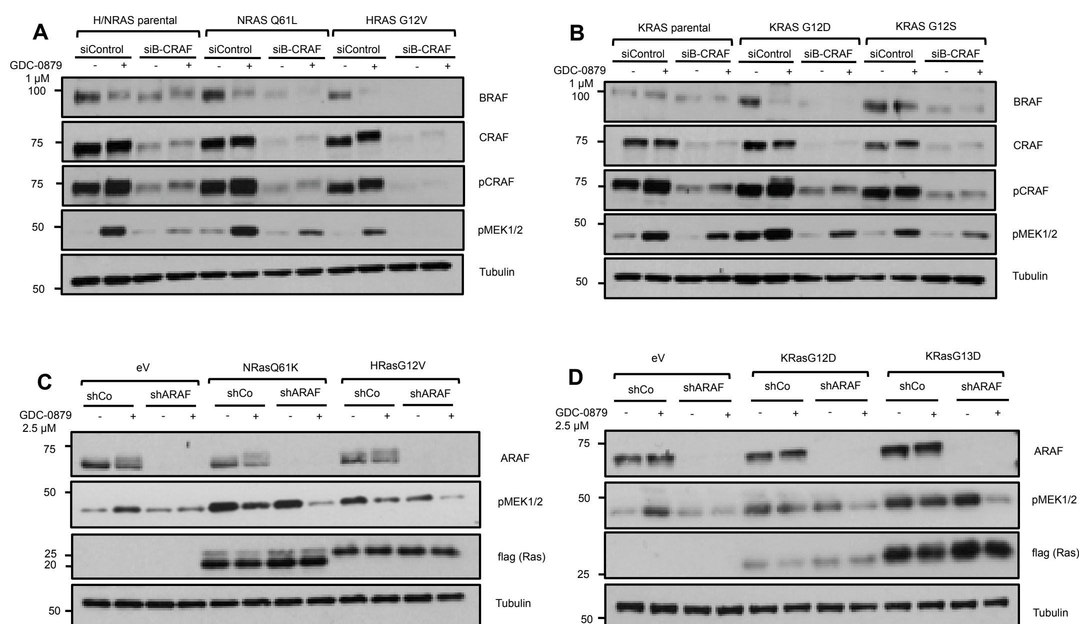


Figure 3.6 Requirement of specific RAF isoforms for MAPK activation in response to various RAS mutants. (**A**) Isogenic HRAS and NRAS wild-type and mutant SW- 48 knock-in colorectal carcinoma cells were depleted of B and CRAF together and treated with GDC-0879 (1 µM) for 4 h. Activation of MAPK signaling was assessed by immuno blotting using phosphospecific antibodies against CRAF (Ser338) and MEK 1/2 (Ser218/222) p, phosphorylated protein. (**B**) As in (A) Cells were isogenic KRAS wild-type and indicated mutant variants (**C**) Western blotting in lysates from A549 control or ARAF depleted cells that were transfected with various cDNAs of mutant NRAS (Q61L) and mutant HRAS (G12V) or (**D**) mutant KRAS (G12D and G13D) (all Flag-tagged). Cells were treated with GDC-0879 (2.5 µM) and lysates were probed for MEK 1/2 phosphorylation.

3.1.5 Loss of ARAF does not prevent complex formation of BRAF with CRAF and KSR1 upon RAF inhibitor treatment

Inhibitors, such as GDC-0879 efficiently target the constitutively active form of BRAF (V600E), whereas in RAS- mutant cell lines where BRAF is wildtype, drug treatment triggers MAPK signaling through the induction of RAF- oligomerization. To test whether this was applicable in KRAS- mutated tumor cell lines, various RAF isoforms from control or inhibitor-treated cells were immunoprecipitated from lysates and the pathway activation was monitored in the total lysate controls. As shown in **Figure 3.7 A** and **B** heteromers consisting of BRAF-CRAF and KSR1 were readily immunoprecipitated at endogenous amounts in A549 and MiaPaCa2 cells upon treatment with RAF inhibitors. Although small amounts of ARAF were detected in CRAF immunoprecipitates in A549 cells treated with GDC-0879, BRAF or CRAF protein kinases were barely present in ARAF immunoprecipitates. These results

indicate that treatment with RAF inhibitors preferentially triggered BRAF-CRAF-KSR1 complex formation.

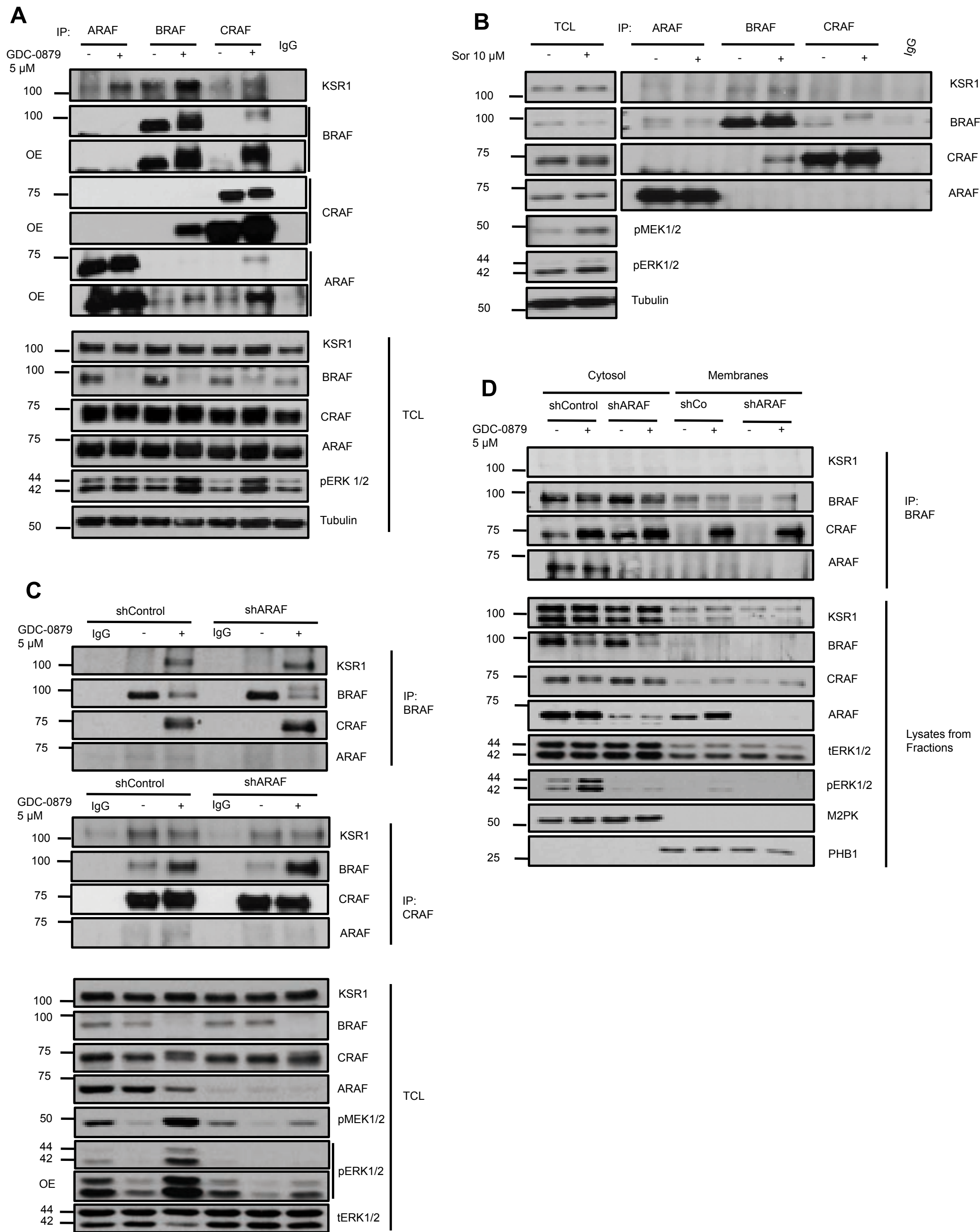


Figure 3.7 Complex formation between CRAF-BRAF-KSR1 in A549 cells upon GDC-0879 treatment. (**A** and **B**) A549 cells were treated with 5 μM GDC-0879 (A), and MiaPaCa2 cells were treated with 10 μM sorafenib (B) for 4 h, and each of the three RAF isoforms was independently immunoprecipitated (IP) from lysates and blotted for the other isoforms and KSR1. OE, overexposed blot; TCL, total cell lysate. (**C**) Control and ARAF knockdown A549 cells were treated with GDC-0879, and BRAF or CRAF was independently immunoprecipitated from lysates and blotted for other complex members. t, total protein; p, phosphorylated protein. (**D**) Same as in (C). BRAF was immunoprecipitated from different cell fractions. Marker proteins were used to validate the purity of fractions (PHB1 for membrane fraction, M2PK for cytosol fraction). Arrow marks KSR1. Blots are representative of three independent experiments.

Because ARAF was indispensable for RAF inhibitor–mediated ERK1 and ERK2 activation in these cells (Figure 3.2 A and B), the formation of BRAF-CRAF-KSR1 complexes in ARAF-deficient A549 cells was tested. Surprisingly, loss of ARAF did not prevent the formation of such complexes upon treatment with GDC-0879 in these cells as shown in **Figure 3.7 C**. Similar outcomes were also observed when the cytosol and membrane fractions were analyzed under these conditions (**Figure 3.7 D**). However, MEK1 or ERK1/2 phosphorylation representative of MAPK pathway activation was severely impaired in these cells, despite the induction of B-/CRAF/ KSR oligomerization through RAF- inhibitor application. Furthermore, loss of ARAF did not prevent CRAF translocation to the plasma membrane as it was phosphorylated at serine 338, yet BRAF and CRAF failed to signal downstream under these circumstances.

3.2 Characterization of ARAF dimerization

3.2.1 Model of the RAF dimer interface

Results so far showed that RAF inhibitors could directly activate ARAF in the absence of BRAF and CRAF, suggesting that ARAF dimers and/or oligomers are directly involved in the activation of the MEK1-ERK1/2 pathway in these cell lines. Based on published crystal structures for drug- induced BRAF dimer formation (Hatzivassiliou et al., 2010), the dimer interface of a possible ARAF-B/CRAF heteromer was modeled. ARAF was there by docked onto the BRAF structure using the BRAF homodimer as a guide. The resulting ARAF/BRAF dimer resembled BRAF homo-dimers. **Figure 3.8 A** shows a projection of highly preserved residues across RAF orthologues onto the crystal structure of the BRAF kinase domain. RAF kinases oligomerize via a specific mode of dimerization of their kinase domains in a side-to side fashion, which is virtually conserved among the RAF isoforms (Rajakulendran et al., 2009). The Argenine at position 362 of the catalytic domain in ARAF (second Argenine of the RKTR motif), as well as the corresponding amino acids in CRAF and BRAF are indicated in **Figure 3.8 B and C**. The ARAF based homology modeling on wild-type BRAF crystal structure not only showed that contact residues at the dimer interface are conserved between ARAF and B/ CRAF but also that Arginine 362 was highly engaged in the dimer interactions.

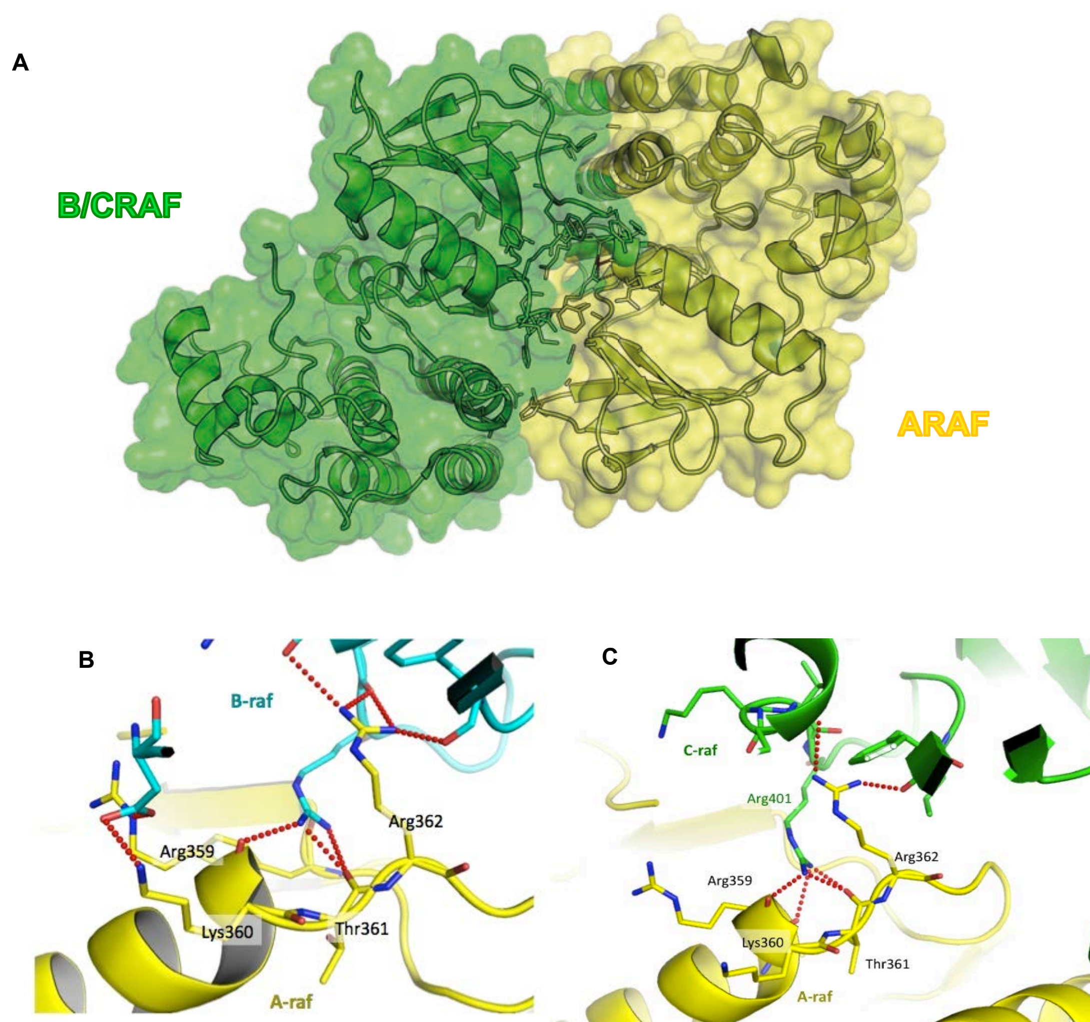


Figure 3.8 Model of the dimer interface of a possible ARAF side to side heteromer (**A**). (**B**) Shown is a model of the RAF dimer interface of [BRAF in cyan (residue R509H) or (**C**) CRAF in green (residue R401) and ARAF in yellow (residue R362H)] based on the published BRAF structures. In detail: Generation of a homology modeling of A-RAF based on WT B-RAF crystal structure. The contact residues at the dimer interface are conserved between ARAF and B/ CRAF. Arginine 362 was highly engaged in the dimer interactions. The dimer modeling was carried out by Weiru Wang as described in (Hatzivassiliou et al., 2010).

3.2.2 ARAF homodimers are required for activation of MAPK signaling

The dimerisation of RAF kinases involves a central cluster within the kinase domain and substitution of arginine to a larger histidine in the dimer interface impairs CRAF-BRAF heteromerization thereby reducing the cellular MEK phosphorylation potential of wildtype BRAF by more than half (Roring et al., 2012). To test the effects of interfering with the dimerization in ARAF, the corresponding dimer deficient ARAF point mutant (ARAFR362H) was generated using site-directed mutagenesis.

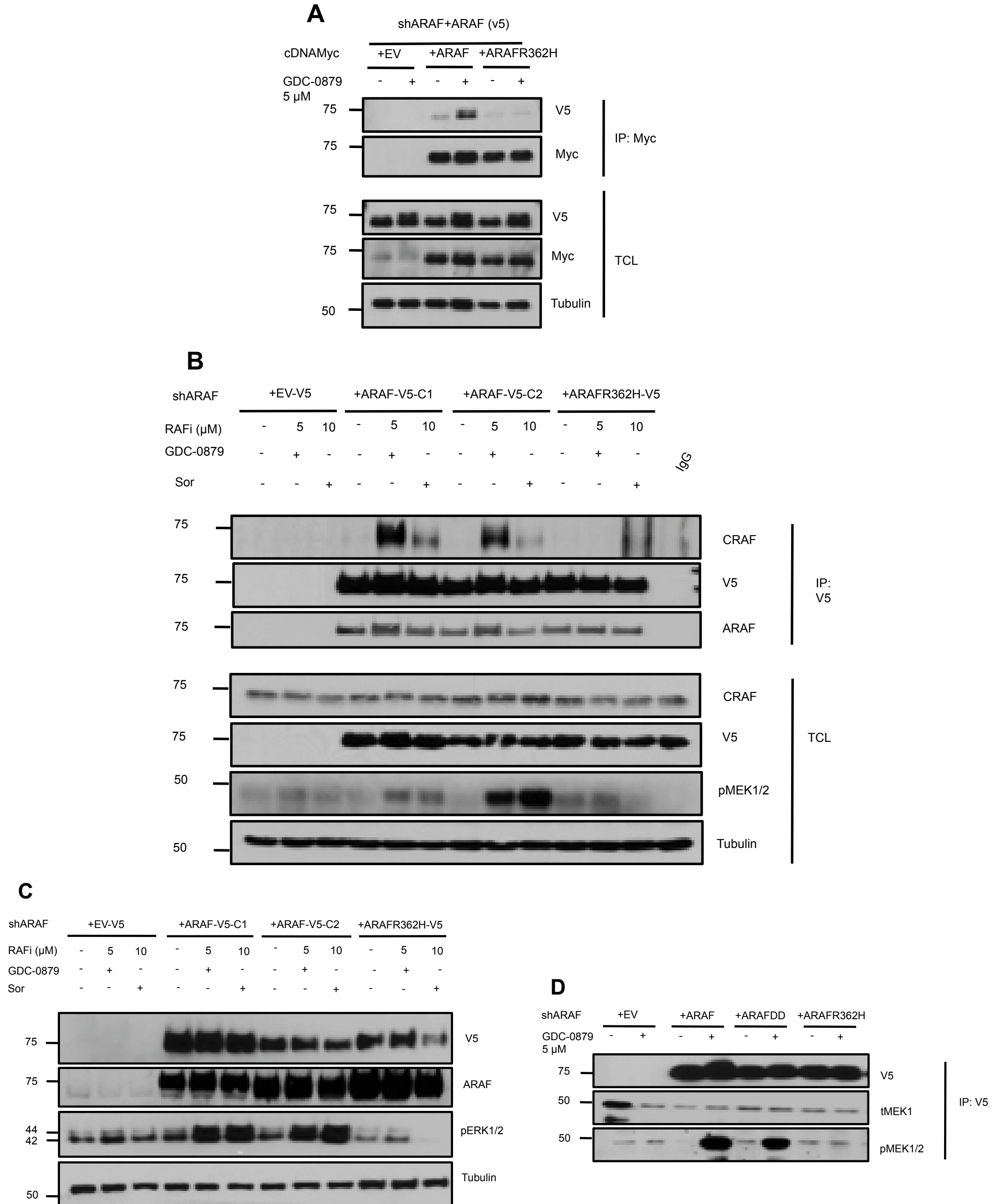


Figure 3.9 ARAF homodimers are required for activation of the MEK1/2-ERK1/2 pathway. (**A**) Reconstituted ARAF-knockdown A549 cells (ARAF-V5) were transiently transfected with the indicated plasmid DNA (Myc-tagged ARAF) and treated with GDC-0879 (5 µM) for 4 h. Overexpressed ARAF (Myc) was immunoprecipitated (IP) from lysates and blotted for ARAF (V5). TCL, total cell lysate. (**B**) Heteromerization-Western blotting for the indicated proteins in lysates from ARAF knock down cells re-constituted with wild-type and mutant ARAF R362H that were treated with GDC-0879 (5 µM) and sorafenib (10 µM). Reconstituted ARAF (V5) was immunoprecipitated (IP) and blotted for CRAF. (**C**) Western blotting for the indicated proteins in lysates from A549 ARAF-knockdown cells reconstituted with wild-type and mutant ARAFR362H (dimer deficient mutant) that were treated with sorafenib (10 µM) and GDC-0879 (5 µM) for 4 h. C1 and C2 indicate two different clones of ARAF-reconstituted cell lines. (**D**) Immunoprecipitation and Western blotting in lysates from A549 ARAF-knockdown cells reconstituted with wild-type and mutant ARAF R362H or ARAF DD (kinase active) that were treated with GDC- 0879. After 4 h, reconstituted ARAF (V5) was immunoprecipitated (IP) and blotted for MEK1 that was used as a substrate in an in vitro kinase assay and pMEK1/2. t, total protein; p, phosphorylated protein.

In order to study ARAF homomer formation, reconstituted A549 cells depleted of ARAF were transiently transfected with ARAF wild-type and ARAFR362H Myc-tagged plasmid DNA and treated with GDC-0879. Immunoprecipitation of overexpressed ARAF (Myc) showed homodimerization only in case of intact wild-type ARAF protein (**Figure 3.9 A**) but not in case of mutant ARAF protein (R362H). When dimer deficient ARAF was reconstituted in ARAF depleted A549 cells using stable lentiviral transduction, the mutation of Arg 362 to His (R362H) in ARAF prevented also heteromerization of ARAF with CRAF induced by RAF inhibitors (**Figure 3.9 B**), consistent with published data (Roring et al., 2012).
Interestingly, reconstitution of ARAF-deficient cells with the dimer deficient mutant not only prevented RAF-oligomerization but also the basal and RAF inhibitor–induced activation of MEK1/2-ERK1/2 in these cells, implying that this mutant works in a dominant negative manner (**Figure 3.9 B and C**). In order to test if the mutation of the dimer interface directly influenced the kinase activity of ARAF toward its putative substrate MEK1, kinase assays using MEK1 as a substrate were carried out. The extent of kinase activity was monitored by anti-pMEK 1/2 antibody. As expected, treatment with RAF inhibitors strongly triggered the kinase activity of wildtype ARAF and ARAFY301D/Y302D (referred in here as ARAF-DD, corresponding to CRAFY340D/Y341D), a constitutively active ARAF mutant. However, kinase activity was impaired when ARAF dimerization was compromised using ARAFR362H mutant (**Figure 3.9 D**). These data underscores the importance of ARAF homodimers in mediating MEK1/2-ERK1/2 activation in response to RAF inhibitors in A549 cells.

3.2.3 ARAF dimer interface engaged in the interaction between ARAF and MEK1

As the activation of MEK1/2-ERK1/2 was constantly impaired when RAF dimerization was affected it was further investigated whether the ARAF dimer interface contributed to the interaction between ARAF and MEK1 upon RAF inhibitor treatment at activating concentrations. In order to test this hypothesis, cell lines depleted of ARAF and reconstituted with either wildtype or the impaired dimer mutant of ARAF (R362H) were treated with RAF- inhibitor and co-precipitation of RAF family members with endogenous MEK1 was determined. Two different clones

(C1 and C2) of ARAF-deficient A549 cells reconstituted with wild-type ARAF were used for these experiments. Compared to either of the clones reconstituted with wild-type ARAF, there was relatively higher abundance of BRAF, CRAF, and KSR1 that co-precipitated with MEK1 after GDC-0879 treatment in the absence of ARAF (shARAF+EV) or in the presence of the ARAF dimer-deficient mutant (shARAF+ARAFR362H) (**Figure 3.10 A**). This data is a clear indication that ARAF possibly competes with BRAF and CRAF for the binding to MEK1, which was further tested by immunoprecipitation of ARAF from A549 cells stably transfected with control or CRAF shRNA. Compared to control cells, a higher amount of MEK1 protein readily co- precipitated together with ARAF in the CRAF depleted cells- both at steady state and upon exposure to either RAF inhibitor (**Figure 3.10 B**).

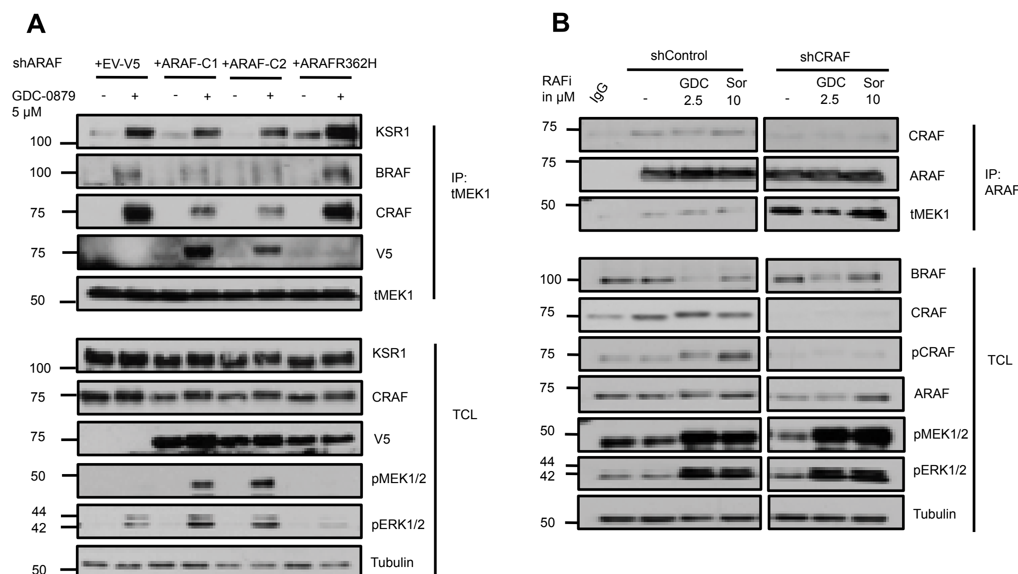


Figure 3.10 RAF isoforms compete among themselves for binding to MEK1. (**A**) Immunoprecipitation–Western blotting in lysates from A549 cells depleted for ARAF and reconstituted with ARAF or ARAF R362H and treated with GDC-0879 (5 μM) for 4 h. Endogenous MEK1 was immunoprecipitated (IP) from lysates and blotted for RAF isoforms and KSR1. t, total protein; p, phosphorylated protein; TCL, total cell lysate. **(B**) Immunoprecipitation and Western blotting to assess the activation of MEK1/2 and ERK1/2 in control A549 cells and CRAF depleted cells treated withGDC-0879 (2.5 μM) and sorafenib (10 μM) using phosphospecific antibodies. Endogenous ARAF was immunoprecipitated from lysates and blotted for CRAF and MEK1.

To demonstrate a direct competition between the RAF isoforms in binding to MEK1, full-length recombinant RAF kinases and GST-tagged MEK1 were employed. As shown in **Figure 3.11 A** increased concentrations of RAF augmented the binding of the respective RAF isoform to MEK1. In an *in vitro* solution of recombinant BRAF

and MEK1, the addition of recombinant ARAF decreased the amount of BRAF that interacted with MEK1 by more than half (**Figure 3.11 B**). Reciprocally, BRAF and CRAF did reduce the binding of ARAF to MEK1 respectively (**Figure 3.11 C**). To discern the MEK binding properties of different ARAF mutants, *in vitro* GST pull-down experiments were conducted with recombinant GST-MEK1 as a bait to capture co-expression of wildtype ARAF, kinase active ARAF or dimer deficient ARAF in reconstituted ARAF depleted cells. In accordance with the data obtained in cells (Figure 3.9), in which the dimer- deficient mutant was not able to phosphorylate MEK1- GST, the ARAF-R362H mutant failed to bind GST-MEK1, possibly due to the lack of dimerizing ability (**Figure 3.11**).

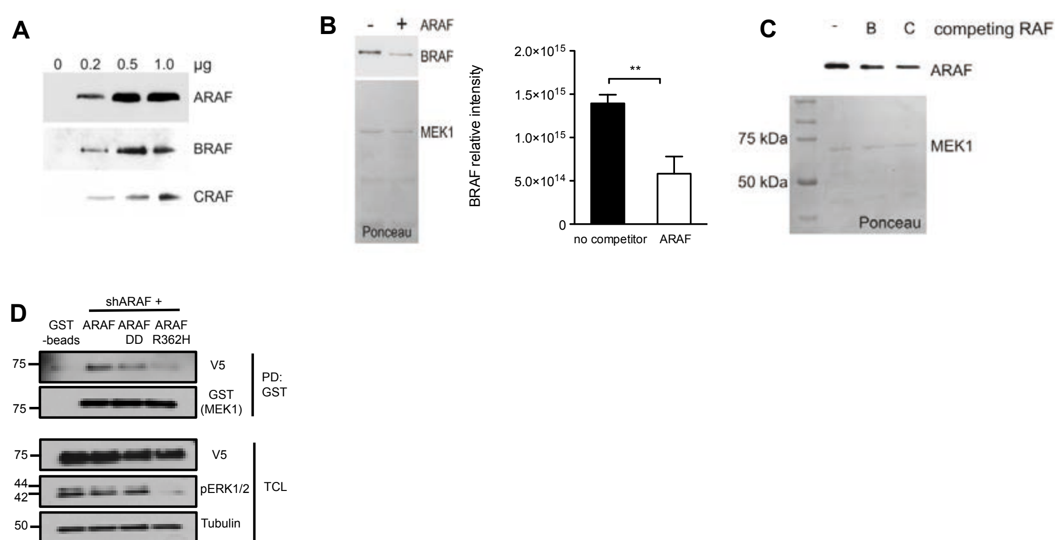


Figure 3.11 Competition between RAF isoforms for binding to MEK1. (**A**) 2 μg of MEK1-GST was bound to glutathione sepharose beads and incubated with the indicated amount of the recombinant RAF protein. RAFs were detected with specific antibodies after SDS-PAGE and Western blot. (**B**) Quantification of Western blotting for BRAF after a MEK1-competition binding assay with ARAF. Data are means ± SEM from three independent experiments. **P < 0.005, Student's t test. (**C**) 2 μg of MEK1-GST was bound to GSH beads and incubated with 200 ng of ARAF. Purified BRAF or CRAF was added and ARAF was detected with specific antibodies after SDS-PAGE and Western blot. (**D**) Western blotting from lysates of A549 ARAF-knockdown cells reconstituted with wild-type and mutant ARAF R362H or ARAF DD from which MEK1-GST was pulled down (PD) in an in vitro kinase assay and blotted for exogenous ARAF (V5). p, phosphorylated protein; TCL, total cell lysate. (A) to (C) *in vitro* competition assays carried out by our collaborator Ritva Tikkanen.

In summary, these results confirm that RAF isoforms compete among themselves for binding to their common substrate MEK1 and ARAF dimerization is required for its interaction with MEK1.

3.3 Role of ARAF kinase in regulating cell migration and invasion

3.3.1 Loss of ARAF results in defects in cell migration and motility

As shown above (Figure 3.11), RAF proteins share MEK1/2 kinases as substrates that in turn activate ERK1/2. This pathway regulates many cellular processes such as cell proliferation, migration, invasion or apoptosis. In order to find out whether the loss of MEK1/2 and ERK1/2 activation upon knockdown of ARAF had any functional relevance in cells, different biological assays associated with MAPK signaling have been carried out. At first, cell proliferation in different tumor cell lines was tested employing several RAF- inhibitors. In **Figure 3.12 A**, loss of ARAF led to a slight but reproducible decrease in the proliferation of these cells.

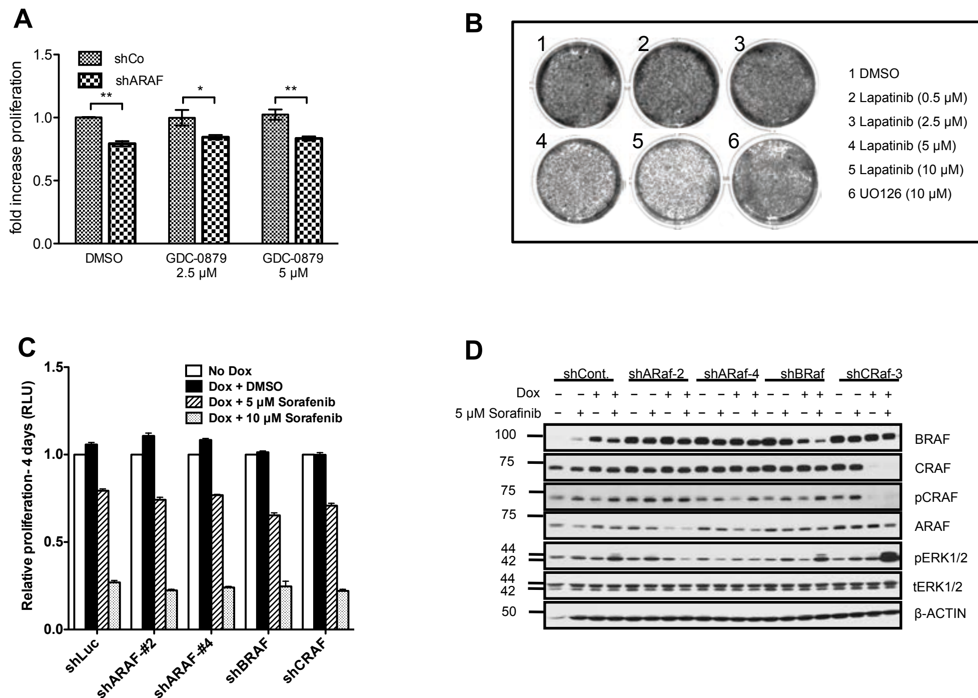


Figure 3.12 MAPK signaling but not proliferation is impaired in ARAF depleted cell lines upon RAFi-treatment (**A**) Quantification of cell proliferation in A549 control and ARAF knockdown cells that were treated with GDC-0879 (2.5 or 5 µM over 72 h) Data are mean ± SEM from three independent experiments, **P< 0.01, *P< 0.05 two way ANOVA with Bonferroni post-test. (**B**) Crystal violet staining assay for cell viability/ proliferation. A549 cells were seeded in a 6 well plate and treated with different concentrations of Her2/ERBB inhibitor lapatinib for 72 h. (**C**) Quantification of cell proliferation in MiaPaCa2 control and RAF knockdown cells upon sorafenib (5 and 10 µM) treatment. Knockdown was induced with doxycycline for four days. (**D**) Western blotting of cell lysates from (C) The activation of CRAF, MEK1 and ERK1/2 was analyzed by immunoblots, employing phospho-specific antibodies. p, phosphorylated protein. Total levels of ARAF, BRAF and CRAF were also monitored. β-Actin served as loading control. (C) and (D) experiments carried out by our collaborator Bijay S. Jaiswal

However, addition of the BRAF inhibitor GDC-0879 to A549 cultures did not trigger cell proliferation (no change in proliferative index), suggesting that these cells are not entirely dependent on the ERK1 and ERK2 pathway for their proliferation. Furthermore, treatment with UO- 126, a well-studied MEK-inhibitor did not block proliferation significantly in these cell lines (**Figure 3.12 B**). Instead, treatment with lapatinib led to a decrease in the proliferation of these cells and thus these cell lines are probably addicted to Her2/ERBB signaling as shown by Diaz et al. (Diaz et al., 2010). It is to be noted that neither of ARAF, BRAF or CRAF knockdown decreased proliferation in Miapaca2 (KRAS G12D mutation) cells (**Figure 3.12 C**). The addition of pan RAF inhibitor sorafenib decreased proliferation in all cases although lysates of the knock down cells showed paradoxical ERK1/2 activation in shBRAF and shCRAF cells respectively (**Figure 3.12 D**). These results show that neither in A549 nor other Ras mutated cell lines, the increase in pERK with inhibitors was translated to an increase in cell proliferation.

It is known that Raf kinases relay signals inducing cell migration among others (Baccarini, 2005). In that study the authors showed that CRAF was required for normal wound healing *in vivo* and for migration of keratinocytes and fibroblasts *in vitro*. Because the MAPK pathway was shown crucial for driving cell migration, the migration ability of A549 cells was tested through the use of wound healing assays. As shown in **Figure 3.13 A**, loss of ARAF exhibited reduced basal and RAF inhibitor-induced wound closure in A549 cells when compared with BRAF- or CRAF-depleted cells. When control and ARAF depleted A549 cells were grown to confluency and wounded as described in the methods, shARAF cells displayed a slower closure of the wound with and without RAFi treatment after 24 h as visualized in **Figure 3.13 B**. In the time frame of experiment, the wound closure of ARAF depleted cells was delayed by almost half compared to control cells. Mitomycin C was further employed to rule out any possible role for proliferation effects in the wound healing phenotype. As expected, presence of mitomycin c did not change the outcome in these experiments (**Figure 3.13 C**). In all the experiments carried out, lysates have been taken for immunoblotting to confirm the knockdown of RAF and MAPK activation upon RAF inhibitor treatment as exemplified in **Figure 3.13 D**. All RAF- inhibtor concentrations used led to phosphorylation of ERK1/2 and MEK 1/2 in the control but not in ARAF depleted cells.

Taken together, ARAF deficient cells were viable except for a slight reduction in their proliferation index and they displayed a reduced basal and RAFi- induced wound closure when compared with BRAF- or CRAF depleted A549 cells.

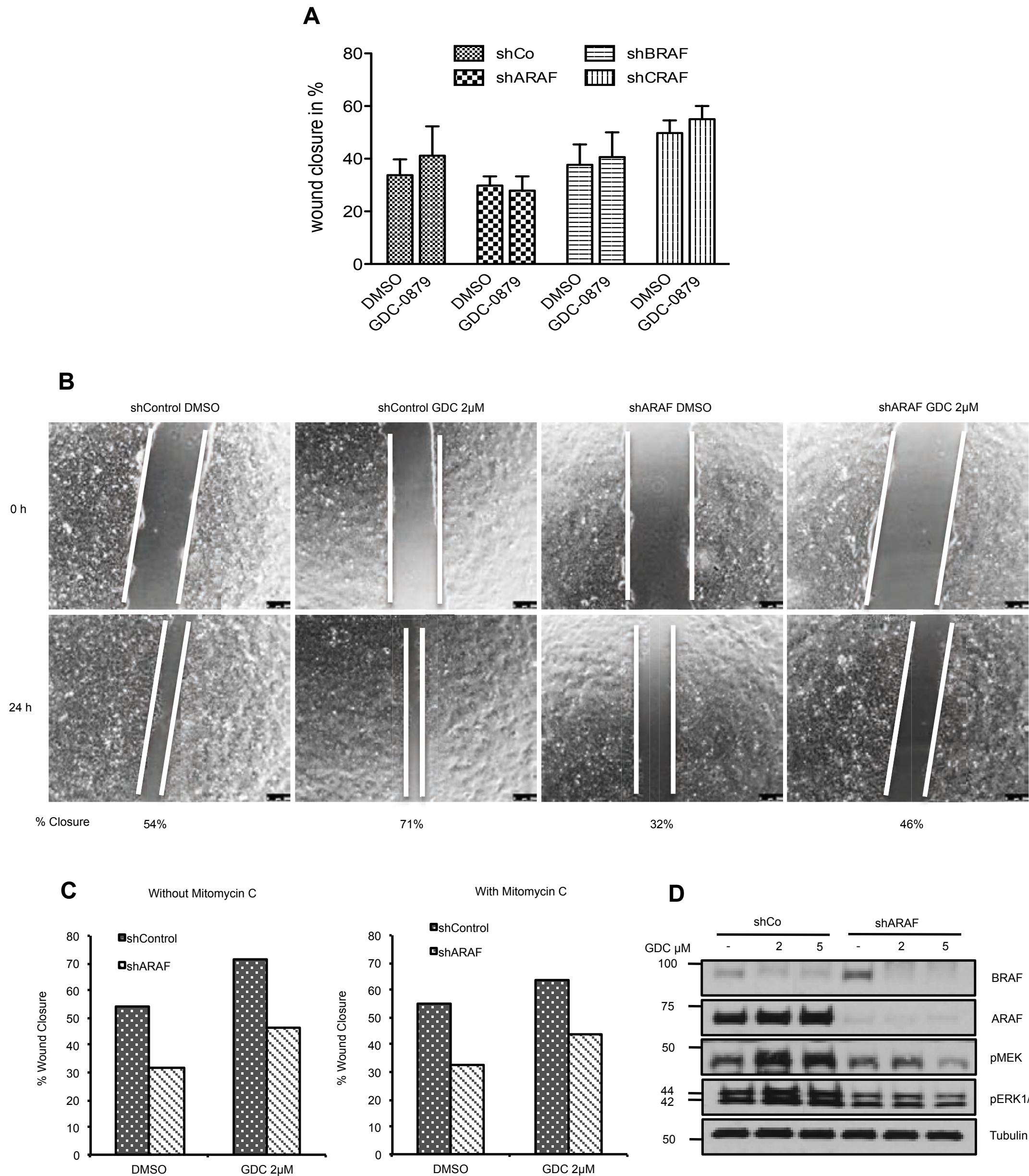


Figure 3.13 A549 cells display growth and proliferation defects upon ARAF knock down (**A**) Quantification of wound healing assays from A549 control and A,- B,- and C-RAF depleted cells that were treated with GDC-0879 (4 µM, 6 h). Data are means ± SEM from two independent experiments. (**B**) Shown are the images from 0 h and 24 h time points in a wound healing assay using confluent monolayers of control and ARAF depleted cells. The cells were scratched and treated with DMSO or GDC-0879 (2 µM) for 24 h. (**C**) Quantification of (B) as percentage of wound closure for shControls and shARAF cells untreated (left panel) and pretreated with 5µM proliferation inhibitor Mitomycin C for 2 h before addition of GDC-0879 (2 µM, right panel). (**D**) Representative Western blotting for woundhealing experiments indicative of MAPK signaling, using specific antibodies.

To confirm these observations, transwell migration experiments were performed and the migratory response of A549 cells to RAF inhibitors tested. Enhanced migration was detected and quantified by the fold increase of cells that successfully migrated through a membrane and attached to the cell culture dish stimulated by the addition of serum. As seen in **Figure 3.14** treatment with RAF inhibitors steadily enhanced cell migration in control cells. The loss of ARAF led to a reduced basal migration by more than half whereas the inhibitor-driven migration was decreased by 3-4 fold in these cells (**Figure 3.14 A to C**). Consistent with these findings, reconstitution of ARAF (V5-tagged) rescued ERK1/2 activation and directional migration in ARAF depleted cells (**Figure 3.14 D and E**).

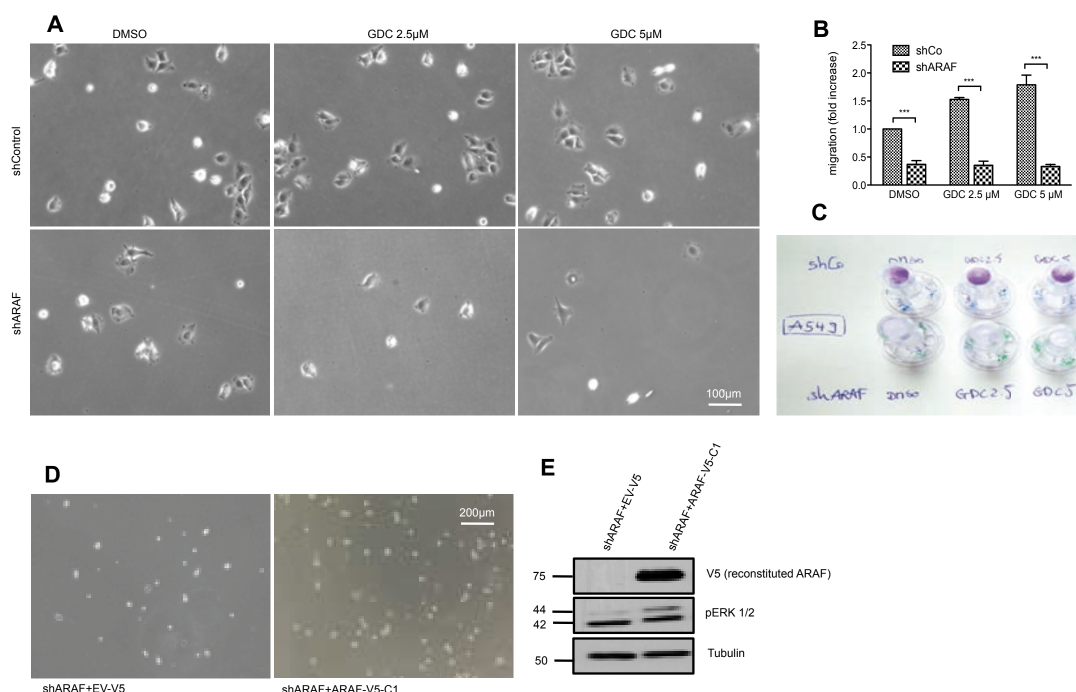


Figure 3.14 A549 cells display migration defects upon ARAF knock down (**A**) Shown are representative images of A549 cells that successfully migrated through a migration chamber and attached to the bottom of the dish upon overnight treatment with different concentration of GDC-0879 (upper panel shows control cells, lower panel ARAF knock down cells). (**B**) Quantification of migrating cells that were counted from three random fields per condition. Data are means ± SEM from three independent migration experiments. ***P < 0.001, two-way ANOVA with Bonferroni posttests. (**C**) Control and ARAF depleted cells were allowed to migrate in a transwell migration chamber and stained with crystal violet after 12 h. Image from one representative experiment is shown. (**D**) Shown are representative images of control cells and re-constituted ARAF (V5) knock down cells that successfully migrated through a migration chamber and attached to the bottom of the dish. (**E**) Representative Western blotting for migration experiments in (D) indicative of MAPK signaling, using specific antibodies.

To measure the random two-dimensional (2D) migration and potential different migratory behaviour of control versus ARAF knock down cells, time-lapse imaging

studies were carried out after seeding them onto thin gelatin matrices. Subsequent cell tracking analysis over one day revealed that loss of ARAF led to a clear reduction of basal and RAF inhibitor induced random motility of A549 cells (**Figure 3.15**). While the control cells wandered and subsequently changed their position, ARAF deficient cells often failed to displace from the point of origin within the time frame of the experiment despite producing membrane protrusions like lamellipodia and filopodia.

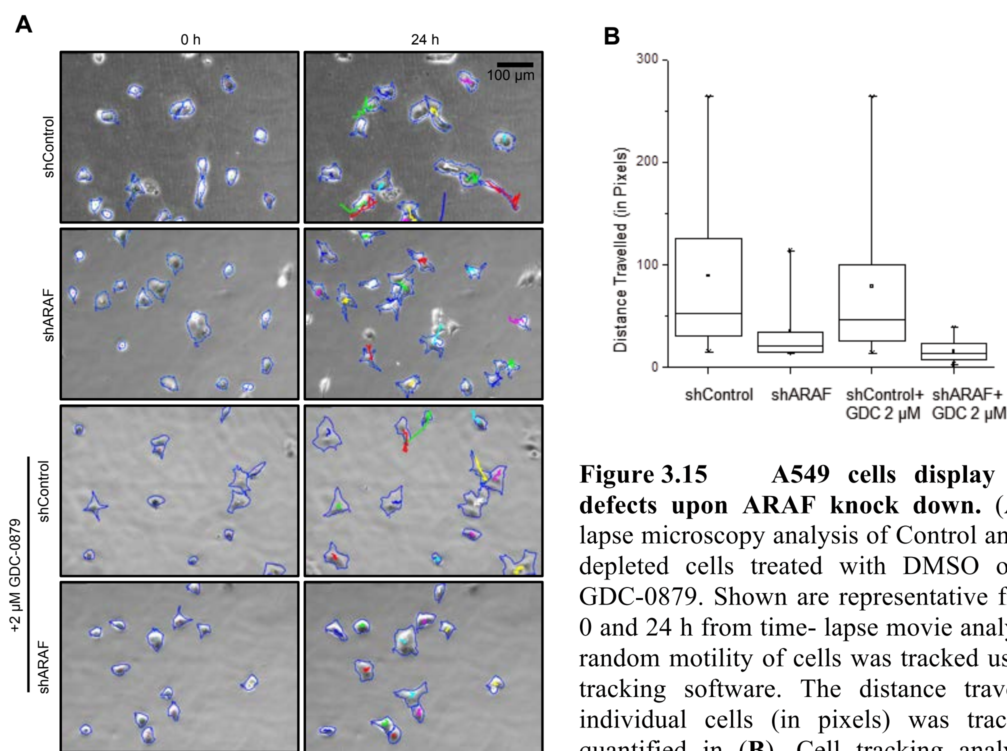


Figure 3.15 A549 cells display motility defects upon ARAF knock down. (**A**) Time lapse microscopy analysis of Control and ARAF depleted cells treated with DMSO or 2 µM GDC-0879. Shown are representative frames of 0 and 24 h from time- lapse movie analysis. The random motility of cells was tracked using cell-tracking software. The distance travelled by individual cells (in pixels) was tracked and quantified in (**B**). Cell tracking analysis was performed by Gregory S. Harms (see methods)

3.3.2 Loss of ARAF prevents tumor cell invasion

As tumor cells exhibit different strategies to migrate and invade in 2D and 3D matrices, Matrigel invasion assays were carried out to study the mechanisms by which A549 cells invade the matrix. Matrigel contained basement membrane components as collagens, laminin, and proteoglycans among others that facilitated a well- defined, yet still *in vitro* environment by which non-invasive cells were blocked from migrating through that reconstituted membrane. In control cells, treatment with the RAF inhibitor GDC-0879 strongly induced tumor cell invasion into matrigel and led to a 2-3 fold increase of the invasion index. Whereas the loss of ARAF significantly impaired the basal as well as the drug- induced tumor cell invasion into matrigel by up to 4-fold (**Figure 3.16 A and B**).

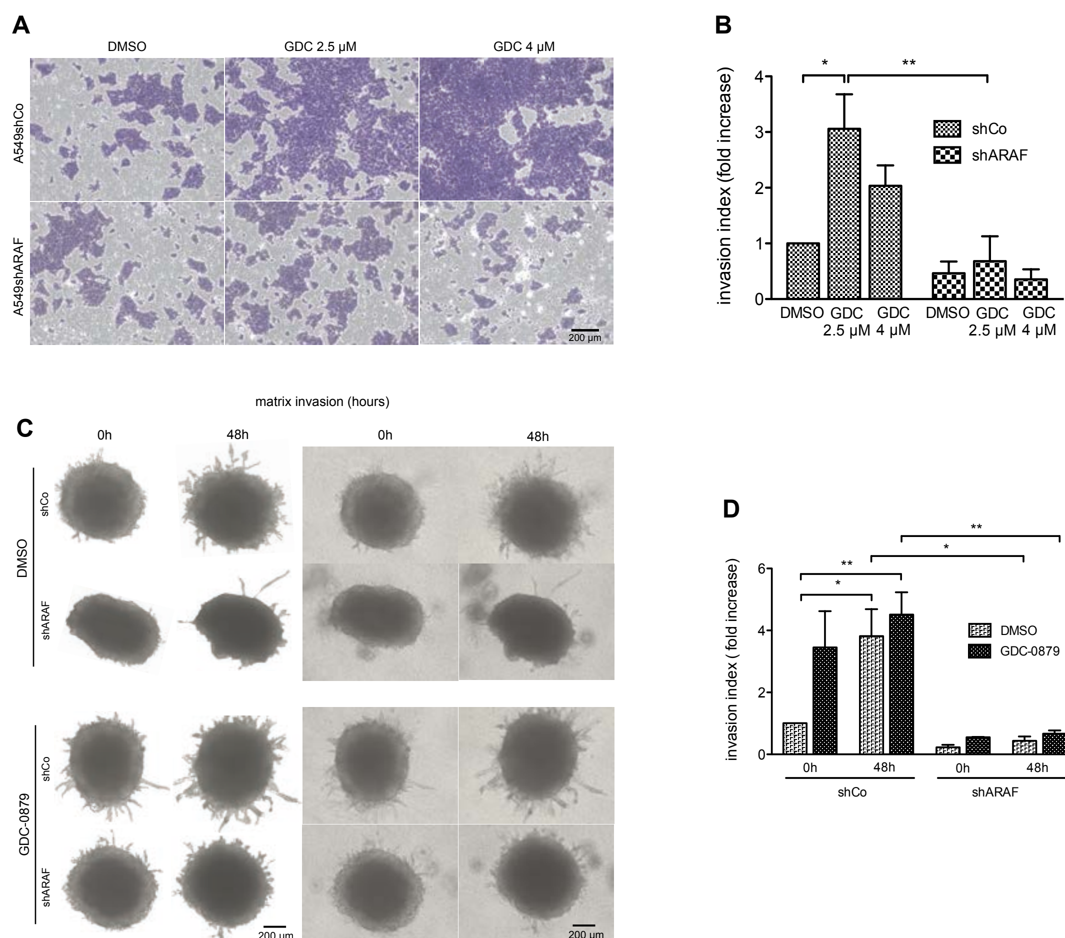


Figure 3.16 Loss of ARAF prevents both basal and RAF inhibitor–mediated tumor cell invasion of A549 cells (**A**) Representative images from Matrigel invasion assay of control and ARAF-depleted A549 cells upon GDC-0879 treatment. Scale bar, 200 µm. (**B**) Quantification of Matrigel invading control and ARAF-depleted A549 cells. Data are means ± SEM from three independent experiments. $**P < 0.005$, $*P < 0.05$, one-way ANOVA with Bonferroni posttest. (**C**) Representative images from a 3D spheroid cell invasion assay of control and ARAF-depleted A549 cells upon GDC-0879 treatment over 48 h. Scale bar, 200 µm. (**D**) Quantification of matrix invading control and ARAF-depleted A549 cells. Data are means ± SEM from three independent experiments. $**P < 0.005$, $*P < 0.05$, one-way ANOVA with Bonferroni posttest.

To further investigate the consequences that loss of ARAF displayed in tumor formation, a three-dimensional organotypic spheroid invasion assay was used as a cancer invasion model. There is an increasing number of publications using organotypic 3D spheroid assays as a tool to study the pathophysiology of *in vivo* tumors (Hirschhaeuser et al., 2010; Vinci et al., 2012), which guarantee the same test conditions and reproducibility. An important characteristic of solid tumors is their rapid expansion through secretion of the extracellular matrix in which they reside to interact with cells from their original microenvironment. To mimick tumor growth in

a 3D environment, cells are usually cultured as aggregates that subsequently grow free of foreign materials. These so formed multicellular tumor spheroids exhibit numerous physiological traits including similar *in vivo* morphology, formation of cell-cell bonds, decreased proliferation rates and increased cell survival. They also display greater chemotherapeutic resistance than the same cells grown in monolayer culture. Therefore, control and ARAF-deficient A549 cells were first tested whether they were able to form spheroids in 3D cell culture. Once the formation of spheroids was evident, a hydrogel network consisting of basement membrane proteins was added in the presence or absence of RAF inhibitors at activating concentrations. As shown in **Figure 3.16 C and D** the invasive cells formed spindle-like structures over time and intruded into the matrix. GDC-0879 thereby triggered substantial invasion at early time points up to 48 h and both, DMSO and drug treated control cells showed a 4-fold increase in the invasion index. The loss of ARAF in contrast, prevented the invasion of A549 cells from the spheroid into the matrix almost completely. Together, these results indicate a crucial role for ARAF in mediating tumor cell invasion in 3D matrices.

3.4 Role of ARAF kinase in anchorage independent growth (ANOIKIS)

3.4.1 Loss of ARAF promotes anchorage independent growth in A549 cells

Tumor cell invasion is a complex process. The expansion of tumor cells into surrounding tissues leads to proteolysis and destruction of biological barriers. It involves multiple processes such as cellular adhesion to specific glycoproteins, degradation of extracellular matrix (ECM) components by tumor-associated proteases, migration, local invasion, dissemination and angiogenesis (Bacac & Stamenkovic, 2008; Jung, Park, & Hong, 2012). Cells only grow and differentiate when in the correct context within a tissue, sensing their location through specific interactions with the ECM as well as neighbouring cells (Gilmore, 2005). Normal cells undergo apoptosis in response to inappropriate cell/ECM interactions. This process, termed Anoikis is a special form of cell death initiated by signals emanating from the lack of their attachment to a proper matrix or substratum. Tumor cells need to resist Anoikis to grow in an anchorage-independent manner in order to expand and invade the adjacent tissues, and to disseminate through the body, giving rise to metastasis (Guadamillas, Cerezo, & Del Pozo, 2011). To investigate the role of

ARAF in oncogenic transformation, soft agar colony formation assays were carried out to monitor anchorage-independent growth, which measures proliferation in a semisolid culture media after 2-3 weeks. As shown in **Figure 3.17 A** and **B**, A549 cells that were deficient in ARAF expression, bypassed the need for anchorage as five times more colonies were detected under these conditions when compared to the cells depleted of B or CRAF respectively. Control and ARAF deficient cells were therefore employed in further studies.

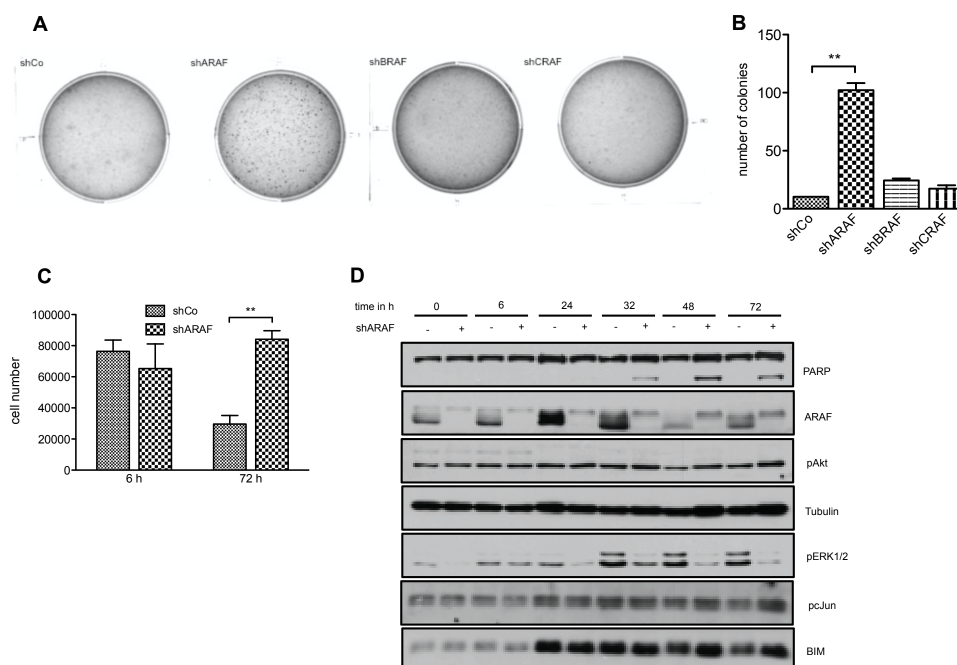


Figure 3.17 Loss of ARAF promotes anchorage independent growth in A549 cells. (**A**) Representative images from a soft agar colony formation assay of control cells and cells that were depleted of A, B, and CRAF respectively. Cells were mixed with 0.7% agarose and poured onto plates containing a 2% agarose base. Colonies were stained with Crystal violet and photographed 14 days later. The number of colonies was manually counted under a light microscope. (**B**) Quantification of colonies stained as described in (A), Data are mean with SD from three independent experiments. **P < 0.005, Student's t test. (**C**) Quantification of cell number obtained from two time points (6 and 72h) per each experiment by FACS counting. Data are means ± SEM from three independent experiments. **P < 0.001, two-way ANOVA with Bonferroni posttests. (**D**) Western blotting of control and ARAF depleted cells grown in ultra-low attachment 6 well plates. Lysates were taken at indicated time points and probed for differential MAPK pathway activation using specific antibodies.

Anoikis can be induced *in vitro* by transferring epithelial cells from standard, adhesive cell culture dishes (with a hydrophilic surface that supports cell attachment and spreading) to ultra-low cluster (ULC) plates with a covalently bound hydrogel layer that effectively inhibits cellular attachment. When control and ARAF knock down cells were shifted from adhesive to ULC dishes and grown for three days, control cells were significantly reduced in number (**Figure 3.17 C**) when compared to ARAF knock down cells. **Figure 3.17 D** shows western blotting from lysates that

were taken over the course of experiment to check for differential signaling pathway activation and apoptosis induction that could explain the change in cell number. Mitogen-activated protein kinase signaling (MAPK) modules that include extra cellular signal regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38 cascades play a significant role in communicating changes from membrane receptors to different cellular processes. Three kinase pathways in particular are important for apoptotic signaling: c-Jun N-terminal kinase (JNK), glycogen synthase kinase-3b (GSK3b), and protein kinase B (AKT) pathways. ARAF deficient and control cells have been tested for ERK, JNK and AKT activation after removing adhesion signals by growing them in suspension. In accordance with all results described so far, ERK1/2 activation was almost absent in ARAF depleted cells, unperturbed by the inappropriate ECM environment. However, it strongly increased after 32 h in the control cells. In both cell lines higher levels of phosphorylated c-Jun, indicative of JNK pathway activation, could be detected after 24 h. Accumulated evidence showed that AKT and its downstream targets constitute a major cell survival pathway (Dan et al., 2004; Ui et al., 2014). However, no differences were found in the expression of activated AKT (pAKT) in control versus ARAF depleted A549 cells. AKT-phosphorylation remained constant throughout the experiment. Bim, as an apoptotis sensor, has been reported to respond to loss of survival signals delivered by EGF (P. Wang, Gilmore, & Streuli, 2004) and increased Bim levels have been detected after loss of cell adhesion in some epithelial cells (Reginato et al., 2003). Here, the involvement of Bim in the rapid anoikis response has been tested and elevated Bim expression was detected after 24 h in both cell lines. Caspase mediated apoptotic cell death is accomplished through the cleavage of several key proteins required for cellular survival (Fischer, Janicke, & Schulze-Osthoff, 2003). PARP-1 is one of several known cellular substrates of caspases. Its cleavage by caspases is considered to be a hallmark of apoptosis (Kaufmann, Desnoyers, Ottaviano, Davidson, & Poirier, 1993). PARP-cleavage (85 kDa fragment) was detected only in shARAF cells after 32h. However, tubulin levels were similar, if not more in ARAF depleted cells over the course of experiment, pointing to a higher proliferation potential. In summary, to gain a deeper understanding of the pathways active that triggered anchorage independent growth in ARAF knock down cells, parallel determination of the relative levels of protein phosphorylation of 43 kinase substrates was undertaken as follows.

3.4.2 Differential kinase expression in ARAF depleted cells

As a broad range of cellular responses to loss of adhesion utilizes diverse signaling and apoptotic pathways, a screen was carried out to gain insights into the phosphorylation profiles of 43 kinases and their protein substrates in control vs. ARAF depleted cells grown in suspension for 4 h. In **Figure 3.18** the average signal (pixel density) of the pair of duplicate spots representing each phosphorylated kinase protein (**Figure 3.18 A**) has been quantified to determine the relative change in phosphorylated kinase proteins between control and ARAF depleted cells.

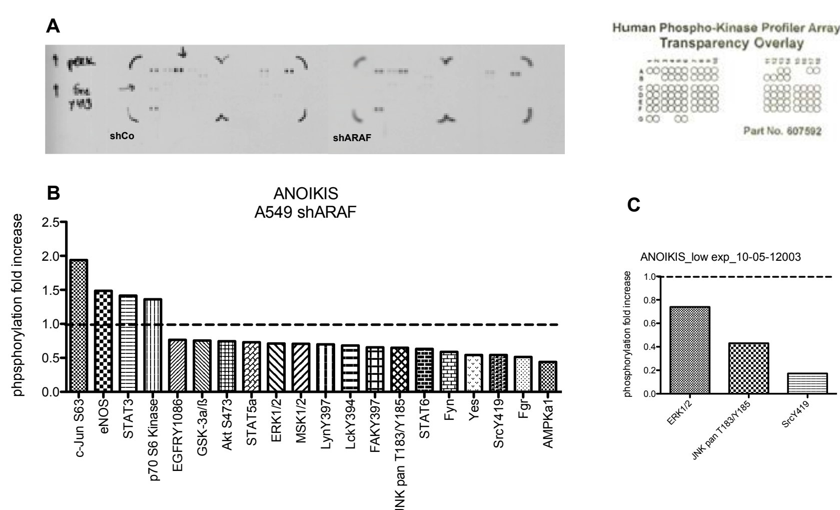


Figure 3.18 Differential phospho proteome profiling in control and ARAF depleted A549 cells grown under ultra low attachment conditions. (**A**) Lysates of control and ARAF depleted cells (shARAF) were incubated with the human phospho-kinase array as described in the methods. Shown are images of the signals produced at each spot in the membrane corresponding to the amount of phosphorylated protein (left); overlay indicative of phosphorylated Kinase substrates (right). (**B**) Graphs showing levels of protein phosphorylation of indicates kinases in ARAF depleted cells as fold increase when compared to control cells (dotted line); representative of one experiment. (**C**) Graphs showing phosphorylation levels of Src kinase and potential downstream kinases in shARAF cells relative to controls (dotted line).

Shown are phosphorylation profiles of kinases involved in cell survival that displayed the most striking differences in ARAF depleted cells when compared to control cells (**Figure 3.18 B**). Figure **3.18 C** focused on the change in phosphorylation of Src kinase that is stimulated by cues from the ECM (Integrins) and its potential downstream kinases ERK 1/2 and JNK. The phosphorylation of Tyrosine 419 in Src kinase was five times lower in shARAF cells compared to control cells. Taken together, these preliminary results point to differential activities in different MAP signaling pathways in control and ARAF deficient cells. In order to identify proteins

involved in the ARAF-mediated regulation of invasion and tumorigenesis, quantitative mass spectrometry needs to be conducted and is currently under investigation

3.4.3 Loss of ARAF promotes lung metastasis in nude mice

To address whether the loss of ARAF endowed epithelial cells with metastatic potential, cell lines were re-engineered to express luciferase, allowing non-invasive *in vivo* imaging in live mice. Each cell line was administered intravenously (tail vein) to nude mice, and outgrowth and the location of tumors was monitored. After two weeks Luciferase imaging showed that cells were viable and had accumulated in different regions in the mice (**Figure 3.19**). While the control cells mostly accumulated in the tail of mice and hardly any lung metastasis was present, the luciferase signal of ARAF deficient cells was mostly detectable in the lungs of mice in seven out of eleven of them exhibiting strong lung metastasis (**Figure 3.19 A** and **B**).

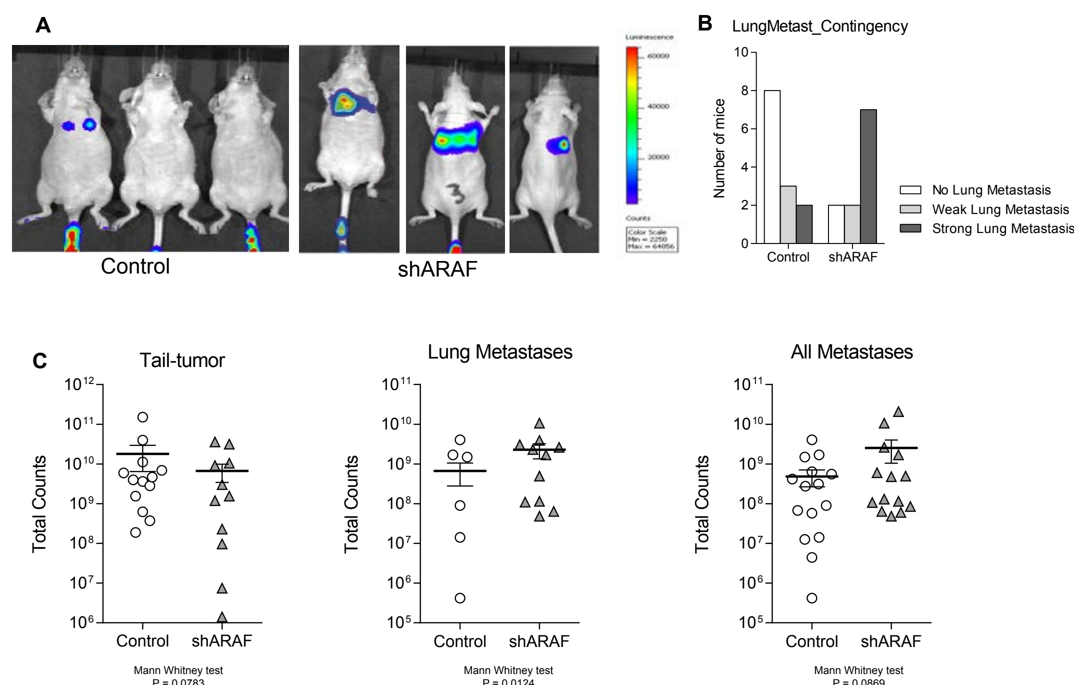


Figure 3.19 Loss of ARAF promotes lung metastasis in nude mice. (**A**) Expression of luciferase in tumor-bearing mice from recombinant lentiviruses harboring promoter–luciferase gene using bioluminescent imaging. Mice were injected with 1x 10^6 control and ARAF depleted (shARAF) A549 cells (tail vein) by A. Häußler. Luciferase activity was imaged after 14 days by Irmgard Tegeder. (**B**) Graph showing the incidences and quality of metastasis developed in indicated number of mice; Contingency analysis with Chi-Square test P=0.039 (**C**) Scatter plots showing in vivo luciferace activity (flux) of tail-tumors (left panel), lung metastases (middle panel) and all metastases (right panel) that developed after tail vein injection. Results are representative of 13 mice in the control and 11 mice in the shARAF group, statistical analysis below individual panels performed by I. Tegeder.

Interestingly, the local tail-tumor growth of shARAF luciferase expressing cells was somewhat weaker or slower than with the control cells. Furthermore, the shARAF local tumors were all distal in the tail (towards the tip) whereas normally, tail tumors are found at the base of the tail. Scatter plot analysis represented primary tumor burden and metastasis in mice of both groups evaluated by bioluminescent imaging (**Figure 3.19 C**). Significantly more ARAF depleted A549 cells exhibited metastasis in the lung, implying that ARAF may affect the extravasation of lung tumor cells compared to wild type cells (Figure 3.19 C, middle panel). The intensity differences were not all significant as the intensity compares only the tumors to metastases ratio (which was there) but does not take into account that some mice had no metastases. Therefore the contingency table in Figure 3.19 A was more relevant for data interpretation and Chi-Square test revealed significant qualitative and quantitative differences in metastatic spread in mice injected with either control or ARAF deficient A549 cells (Figure 3.19 B). In summary, the loss of ARAF contributed to the pathogenesis of lung metastasis, suggesting that ARAF might be a regulator of metastasis in lung cancer. However, the means and circumstances by which ARAF is exerting its function need to be studied in greater detail.

4. Discussion

4.1 ARAF is indispensable for basal and RAFi induced MAPK signaling

With the discovery and identification of the *RAFs* as proto oncogenes thirty years ago (Sutrave et al., 1984), an intensive research on serine/ threonine kinases had been set into motion not least because they had been shown to posses the potential to transform cells in culture and induce tumours *in vivo.* The Raf kinase family consists of three RAF isoforms that share a common structure comprising three conserved regions to function in the context of the Ras-MEK-ERK cascade that is involved in cell proliferation, migration and invasion (Leicht et al., 2007). With CRAF being found to be ubiquitously expressed in mouse tissue and further characterization as oncoprotein, it became the most intensively studied member of the RAF family (Bonner et al., 1985; Morrison et al., 1988) until BRAF somatic point mutations were identified in a variety of human cancers (Davies et al., 2002). Among the different *BRAF* mutations, the missense substitution that changes valine to glutamic acid at codon 600 (V600E) in exon 15 was found to be prevalent in 90% of melanoma tumors with BRAF mutations, rendering the protein kinase activity ten times higher than it occurs in normal cells (Davies et al., 2002). The resulting hyperactivity of the MAP kinase pathway promoted tumor development. Hence, *BRAF* genetic mutations that were identified in a large number of tumors further intensified drug development efforts (Madhunapantula & Robertson, 2008; Sharma et al., 2005). Chemical inhibitors targeting the mutated form of BRAF have shown remarkable effectiveness in the clinics in patients that have been tested positive for *BRAFV600E* mutation (Bollag et al., 2010). However, these inhibitors could drive downstream ERK1/2 signaling in cells with underlying *RAS* mutation by promoting dimerization with CRAF, contributing to resistance in tumors treated with these drugs (Hatzivassiliou et al., 2010; Heidorn et al., 2010). Moreover, patients with melanoma treated with BRAF inhibitors developed cutaneous squamous cell carcinomas (Bollag et al., 2010; K. Flaherty, 2010). CRAF, but not BRAF was shown to be essential in mediating oncogenic signaling in *KRAS*G12V-driven NSCLCs in mice (Blasco et al., 2011). While much attention has been devoted to the dimerization-mediated activation of B-

and CRAF, relatively little is known about the ARAF activation process. In this work, the role of ARAF kinase in regulating MAPK activation under basal as well as RAFi inhibitor induced conditions was elucidated in greater detail.

As A549 cells have been frequently used to study KRAS-driven NSCLCs (Diaz et al., 2010; Wada, Horinaka, Yamazaki, Katoh, & Sakai, 2014), initial RAF knock down studies have been performed on this particular $KRAS^{G12S}$ mutant- NSCLC-lung carcinoma cell line. Loss of ARAF prevented both basal and RAF inhibitor-driven MEK1-ERK1/2 activity (Fig. 3.2 A to C), whereby the multikinase inhibitor sorafenib and the more BRAF specific inhibitor GDC0879 was used to investigate the paradigm of RAF activation downstream of mutated RAS (Fig. 3.1). Surprisingly, we found that ARAF was critically required for MAPK activation as double knock down of B and CRAF still resulted in ERK phosphorylation upon inhibitor treatment (Fig. 3.2 D). To exclude off-target and clonal effects, multiple siRNAs and shRNAs were employed and the same ARAF-knock down cells were reconstituted with the wild-type ARAF to confirm these observations (Fig. 3.3). The latter restored MAP kinase signaling in these cells and treatment with increased concentrations of GDC-0879 induced the paradoxical activation of RAF that could be detected in control A549 cells. Interestingly, reexpression of ARAF, but not the RAS-binding mutant of ARAF ($ARAF^{R52L}$), rescued MAPK activation in ARAF depleted cells, suggesting that RAS-RAF interaction is indeed required for MAPK activation under these settings. RAS binding to RAF kinases is pivotal for proper RAF activity as it induces structural changes and recruitment to plasma membrane that allow subsequent (de)phosphorylation events to happen in order to be activated (Weber et al., 2000; Wittinghofer & Nassar, 1996). It was shown that RAS isoforms interact dynamically with specific microdomains of the plasma membrane whereby Ras signal output is regulated through differential interaction with RAF isozymes (Weber et al., 2001). Recent studies also revealed that not all activating RAS mutations are equal with respect to their downstream signaling and oncogenic transformation (Miller & Miller, 2011). KRAS ablation in A549 cells did not detectably suppress activation of the ERK1/2 cascade (Singh et al., 2009). We could demonstrate that the requirement for ARAF in signaling to ERK1/2 was not confined to a specific RAS isoform in A549 cells since overexpression of various RAS isoforms and their mutants blocked MEK1 activation upon inhibitor treatment in an ARAF depleted background (Fig. 3.6 C and

D). However, the effects of different *RAS* point mutations on MAPK signaling in A549 cells should be considered suggestive rather than confirmatory since they were gained through transient overexpression. In accordance with published observations (Miller & Miller, 2011) we found that the need for ARAF in RAFi- triggered MEK1/2-ERK1/2 activation was more a cell-type dependent phenomenon since in the isogenic SW48 RAS knock-in cells we employed, it was BRAF and CRAF that activated the pathway (Fig. 3.6 A and B). Nevertheless, to gain further mechanistic insights of the tissue specificity and dose related effects of *RAS* gene expression, it would be pertinent to engineer A549 cells to express various *RAS* mutants at endogenous levels to test for the requirement of ARAF in mediated MEK-ERK activation. Further, it would be interesting to test if these results can be extended to primary epithelial cells like the pulmonary alveolar cells. Interestingly, overexpression of a cancer-associated ARAF mutant (S214C/F) transforms human immortalized lung epithelial cells (Imielinski et al., 2014). Taken together, treatment with GDC-0879 or sorafenib at RAF-activating concentrations required ARAF for MAPK activation in a cell type dependent manner under these experimental conditions. This was further strengthened by the fact that overexpression of BRAF but not $BRAF^{V600E}$ failed to rescue MEK1/2-ERK1/2 activation in cells treated with ARAF siRNA, implying that ARAF functions as the MAP3K that activates MEK1 (Fig. 3.4).

4.2 ARAF is the prime MAP3K to activate MEK1

Mitogen-activated protein kinase/ERK kinase (MEK1) is a tyrosine (Y-) and S/T-dual specificity protein kinase that is positively regulated by RAF phosphorylation on Serine residues in the catalytic domain (Alessi et al., 1994). All three RAF family members have been shown to phosphorylate and activate MEK but with different biochemical potencies, though BRAF is considered as the main activator of MEK (Marais et al., 1997). To compare the activation of CRAF, ARAF, and BRAF by oncogenic *RAS*, the authors had used mammalian cell expression systems. The level of ARAF activity obtained under maximal activation conditions was only 20% of that for CRAF. In this work, we identified an obligatory role for ARAF in directly activating MEK1 in a panel of sequence-verified cell lines despite the presence of kinase competent B -and CRAF (Fig. 3.2). Apart from cell lines with RAS mutations

(A549 and MiaPaCa2), the requirement of ARAF for mediating MEK1 activation in response to RAF inhibitor treatment was also evident in MDA-MB-468 cells, which have wild-type RAS/ RAF isoforms. Double depletion of B and CRAF in all cell lines studied, did not prevent the activation of MEK1 in response to RAF inhibition by GDC-0879 (Fig. 3.5). RAF inhibitors directly activated ARAF, which in turn phosphorylated its putative target MEK1. These findings were further strengthened with kinase assays in A549 cells where ARAF was re-expressed in an ARAF depleted background. Upon RAF inhibitor treatment MEK1 was only phosphorylated in the presence of ARAF, proving that ARAF solely functions as MAP3K under these circumstances (Fig. 3.9). Further studies are clearly needed to uncover the concoction of factors that forbid the BRAF-CRAF heteromer to phosphorylate MEK1 in ARAF dependent cells.

4.3 ARAF homodimerization is required for MAPK activation

Oligomerization of RAF per se promotes RAF activation through a RAS-dependent mechanism (Z. Luo et al., 1996). Active RAS induces heterodimerization of BRAF with CRAF and isolated CRAF/BRAF heterodimers possessed a highly increased kinase activity compared to the respective homodimers or monomers (Rushworth et al., 2006; Weber et al., 2001). BRAF-specific inhibitors, such as GDC-0879 and sorafenib triggered heteromerization between RAF isoforms in RAS-mutant cell lines resulting in pathway activation through allosteric activation of CRAF kinase activity (Hatzivassiliou et al., 2010; Heidorn et al., 2010). We could demonstrate that ARAF was required for RAF inhibitor-mediated ERK1 and ERK2 activation in A549 cells and that exposing A549 cells to RAF-activating concentrations of RAF inhibitors, triggered the formation of ARAF homodimers as well as BRAF-CRAF-KSR1 oligomers (Fig. 3.7) though the latter is unable to activate MEK1 under these conditions. Also the inhibitor induced membrane localization of CRAF or the phosphorylation of CRAF at Ser338, a crucial event in the activation cycle of CRAF was not impaired in an ARAF depleted background. This also indicates that the phosphorylation of CRAF at Serine 338 may not necessarily be a maker for the activation of this kinase. Thus in these cell lines, only ARAF homomers are functional and its currently unclear why the CRAF-BRAF complex was not active in the absence of ARAF. One possibility is that loss of ARAF affects the stoichiometry

of a kinase competent RAF complex with MEK1, which in turn can influence the activation of MEK1. Indeed, we further demonstrate that RAF isoforms compete among themselves in binding to MEK1, which is further discussed below. Our studies showed that BRAF inhibitor treatment induced priming of the MAPK pathway through enhanced ARAF-ARAF dimerization, underlining ARAF´s exclusive role as MAP3K kinase in A549 cells.

4.4 Arginine 362 is highly engaged in ARAF dimerization

Dimerization is the key step in the activation of many kinases, whereby the heterodimers have been shown to be the most active forms (Weber et al., 2001). Although RAF kinases are known to dimerize during normal and disease-associated RAF signaling, key questions regarding the mode and effects of RAF dimerization were only recently studied in greater detail (Freeman et al., 2013). These authors investigated to which extend all RAF family members dimerize with one another and whether homo- or heterodimerization was most critical. In accordance with published data (Hatzivassiliou et al., 2010; Heidorn et al., 2010; Poulikakos et al., 2010), mostly BRAF-CRAF heterodimerization was found in normal growth factor stimulated RAF signaling, with some BRAF and CRAF homodimers present. Further, RAF inhibitors promoted and stabilized RAS dependent RAF dimerization causing paradoxical ERK activation in cells that express wild-type RAF proteins as has been reported before. Analysis of the kinase domain structure of both BRAF and CRAF indicated that RAF isoforms form side-to-side dimers, with specific residues in the dimer interface being critical for dimer formation (Hatzivassiliou et al., 2010; Rajakulendran et al., 2009). Mutational analysis of the dimer interface (DIF) revealed that replacement of a critical arginine residue in the dimer interface with a bulky histidine disrupted RAF dimerization and function of wildtype RAF. However, given that ARAF showed weak dimerization and little activity change upon depletion of fellow RAF isoforms, all studies investigating RAF dimerization and downstream function were focused mainly on BRAF and CRAF. Our studies showed a clear requirement for ARAF homomers in mediating MAPK activation in A549 cells. To investigate the dimerization potential of ARAF and the events that regulate ARAF dimer formation following RAF inhibitor (GDC-0879) treatment, we focused on the highly conserved RAF dimer interface comprising a cluster of basic residues- the so called RKTR motif

(Baljuls et al., 2011). Dimerization deficiency of the BRAF kinase domain-containing the R509H mutation had pointed to an involvement of the RKTR motif in dimer formation (Baljuls et al., 2011) and subsequent MAPK signaling. As all RAF isoforms behave similarly concerning the RKTR motif-dependent dimerization, we found that mutation of Arg362 to a Histidine residue in ARAF prevented both homo- and heteromerization with CRAF (upon overexpression of ARAF) induced by sorafenib or GDC-0879 (Fig. 3.9 A and B). Drug binding to one member of a RAF oligomer leads to dimerization and transactivation of the drug-free protomer stimulating paradoxical ERK signaling (Poulikakos et al., 2010). The ARAF R362H mutant possibly works thereby in a dominant-negative manner as we could show that RAFi- induced ERK activation was severely impaired in ARAF depleted cells reconstituted with the dimer-deficient mutant. This dominant-negative effect is in full agreement with the behavior of BRAF R509H mutation (equivalent to ARAF R362H) in MEFs that were either stimulated with EGF or Tamoxifen released $ERTmRAS^{V12}$ in a study by the Brummer lab (Roring et al., 2012). They demonstrated that an intact dimer interface was indispensable for BRAF homodimerization and subsequent MAPK signaling. Sorafenib-treatment of the $ERTmRAS^{V12}$ induced BRAF -/- MEFs re-constituted with BRAF R509H, prevented thereby strong formation of ARAF containing heteromers, while CRAF interaction with BRAF was hardly impaired. Intriguingly, the analogous CRAF R401H mutation still allowed the formation of CRAF homodimers, but like its BRAF counterpart, quenched $K\text{-}Ras^{G12V}$-mediated MEK phosphorylation. The formation of RAF/MEK/ERK signalosomes clearly depends on the cell type and underlying RAF/ RAS mutational status as well as the consequential mode of (trans) activation triggered by different RAF inhibitors. The impact of the single amino acid substitution in the dimer interface of ARAF (R362H) was as such that it prevented the basal and RAF inhibitor-induced activation of MEK1/2-ERK1/2, indicating that ARAF homomerization was a prerequisite for the activation of MEK1 in the cell lines that were subject of investigation in this work. We could further confirm these observations with *in vitro* kinase assays, proving that expression of the RAF dimer mutant R362H indeed impaired kinase activity of ARAF when dimerization was compromised (Fig. 3.9 D).

4.5 ARAF dimer interface is engaged in the binding to MEK1

Rajalkulendran et al. suggested a side-side dimer formation for the activation of RAF kinases (Rajakulendran et al., 2009). We could show that interfering with the formation of ARAF homodimers prevents both basal as well as RAF inhibitor-stimulated MEK1 phosphorylation. Interestingly, the ARAF dimer interface mutant failed to bind MEK1 (Fig. 3.10 A), which could possibly be attributed to the lack of kinase activity. It would be worth investigating whether kinase activity is required for phosphorylation of ARAF at Ser432, which facilitates MEK1 binding. We expect that in accordance with Zhu and colleagues (Zhu et al., 2005) who reported that the corresponding phosphorylation sites in BRAF (Ser579) and CRAF (Ser471) were essential for kinase activity, phosphorylation at this site in ARAF (S432) might be required for binding to MEK1. This data would need to be verified with the kinase-dead mutant ARAF^{D447N} (in the DFG-motif) to show the requirement of ARAF kinase activity for MEK binding. In BRAF, mutation at the corresponding Aspartate (D594A) is inhibiting as it interferes with the phenylalanine side chain switch that usually renders the inactive conformation of the αC helix (DFG Asp-out) towards the active one (DFG Asp-in). It is worth mentioning that in an oncogenic RAS background, kinase-dead BRAF drove tumor progression through CRAF (Heidorn et al., 2010). *BRAF*^{D594A} and *KRAS*^{G12D} induced melanoma when expressed together in transgenic mice, with only the mutant BRAF priming CRAF to signal downstream to MEK. However, the settings were as such that BRAF and CRAF were considered the prime MAP3Ks. Given our findings that in A549 cells (KRAS^{G12S}), ARAF is the prime MAP3K to drive tumorigenesis, it would be indeed exciting to see the cellular outcome when employing the kinase-dead variant of ARAF in an analogous mouse model.

Although it has been shown that BRAF activation through dimerization (DFG Asp-in) was important for enzymatic activity (Rushworth et al., 2006), Haling and colleagues investigated whether RAF dimerization per se was a prerequisite for MEK1 interaction (Haling et al., 2014). Cellular studies had revealed a pre-existing quiescent BRAF-MEK complex in the cytosol that could be activated through RAF dimerization. Binding studies with truncated versions of the proteins showed that monomeric BRAF (BRAF^{R509H}) was still competent to bind MEK1 with a similar

affinity as the dimeric $BRAF^{WT}$ proving that the BRAF-MEK1 interaction was independent of BRAF dimerization. Also overexpression experiments showed that MEK co-immunoprecipitates with wild-type BRAF and monomeric $BRAF^{R509H}$. However, in our experiments with ARAF, the dimer deficient monomeric $ARAF^{R362H}$ mutant failed to bind GST-MEK1 (Fig. 3.11 D), suggesting that only the intact dimer interface assures proper interaction of ARAF with MEK1. This seems to be in stark contrast to what Haling et al. reasoned from BRAF-MEK crystal structure analysis where they propose that BRAF can adopt an active conformation in a complex with MEK1 in the absence of phosphorylation of its activation segment, hence independent of its kinase activity. Although the residues within the HRD motif in the activation segment of BRAF that are responsible for this "behavior" are conserved across the RAF family, further studies employing full-length kinases are needed to make any general conclusions. Further, as most of their structural insights were gained from work with recombinant proteins and focused exclusively on BRAF kinase dimerization and BRAF dependent phosphorylation of MEK, it would be interesting to evaluate how the different RAF isoforms come together and interact with MEK1 *in vivo*. This also raises the question whether ARAF is in complex with MEK prior to activation and /or dimerization. Further the role of mutations in RAS and RAF kinases (especially of ARAF and CRAF) in altering the stability, affinity and the stoichiometry of RAF-MEK1 complex needs to be investigated. Indeed, the stoichiometry of kinase-substrate moieties strongly determines the type of signaling complexes assembled in a spatio-temporal manner to relay MAPK activation in cells. In BRAF wild type and KRAS mutant cell lines where paradoxical RAF activation is observed, BRAF-MEK1 complexes were found to be enriched (Haling et al., 2014). BRAF inhibitor GDC-0879 stabilizes the BRAF-MEK1 complex as it favors the "active-like" orientation of the αC helix in BRAF that is preferred for binding to MEK. The authors demonstrated by *in vitro* biochemical assays that GDC-0879 promotes binding of BRAF kinase domain to MEK1 as well as in KRAS mutant cells, where the inhibitor at low concentrations, primed the pathway with elevated pMEK levels. We suspect that in the absence of ARAF, the BRAF-MEK1 heteromers that are stabilized by RAF inhibitor treatment could potentially inactivate the complex. Nevertheless, we failed to detect any increase in MEK1 phosphorylation despite a strong complex formation between BRAF and MEK1 in the absence of ARAF (Fig.

3.10 A). Expression of BRAFV600E triggered MEK1-ERK1 activation in A549 cells, despite the absence of ARAF (Fig. 3.4). This suggests that the wild type BRAF-CRAF kinase complex fails to gain an active conformation in the absence of ARAF despite being in complex with MEK1. Interestingly, we uncovered a competition among the RAF isoforms for MEK binding. Our cellular studies showed that CRAF depletion led to increased ARAF binding to MEK1 under steady state as well as inhibitor action (3.10 B). The absence of ARAF increased the binding of the remaining RAF family members to MEK1 upon GDC-0879 treatment while recombinant ARAF was able to displace BRAF bound to MEK and vice versa (Fig. 3.11). Already more than 10 years ago Mercer and colleagues had reported a significantly increased BRAF and CRAF activity towards MEK in ARAF deficient MEFs (Mercer et al., 2002). However, this work presented here for the first time suggests that a RAF competition for binding to MEK might be a general feature in MAPK signaling and activation. Further studies using full-length RAF-MEK complexes are clearly required to uncover the stoichiometry and the activity of such complexes. Future experiments employing RAF wild-type and respective dimer-deficient mutants in MEK binding studies might add additional insights on how MEK1-ERK1/2 activation is co-ordinated by a complex of RAF isoforms.
In summary, we could show that in A549 cells ARAF was required for RAFi-mediated ERK1/2 activation and it was the intact (A)RAF dimer interface that was needed for MEK binding and activation, making ARAF the obligatory MAP3K. Further, our work revealed that RAF isoforms compete directly among themselves for binding to MEK1, a major conceptual advance to our current understanding of MAPK activation.

4.6 ARAF kinase regulates cell migration and tumor cell invasion in A549 cells

The RAF/MEK/ERK pathway upon activation regulates a broad range of biological responses from cell proliferation/migration extending to transformation to the cancerous state. Different *in vivo* and *in vitro* models have been employed to study the role of MAPK signaling in cancers and metastases. Blasco and colleagues investigated the role of individual members of the RAF/MEK/ERK cascade in the onset of *KRAS* oncogene- driven non-small cell lung carcinoma (NSCLC). They

could show that CRAF, but not BRAF was specifically required for $KRAS^{G12V}$-driven NSCLC in mice (Blasco et al., 2011). However, extending these findings to human NSCLC cell lines containing different *KRAS* oncogenes, the authors could no longer generalize the CRAF requirement as proliferation rates of these cells did not vary between BRAF and CRAF knockdowns. In these lines, BRAF and CRAF both were not essential for proliferation and immortalization of primary MEFs driven by wild-type or oncogenic KRAS (G12V) signaling (Blasco et al., 2011). In contrast, Karreth et al. found CRAF to be responsible for the proliferative effects of $KRAS^{G12D}$ in primary epithelial cells (Karreth, F. A., et al. 2011). In a lung cancer model they further found an obligatory role for CRAF in tumor initiation by oncogenic KRAS G12D. Despite numerous studies focusing on RAF in tumorigenesis, little is known on the role ARAF has in tumor cell migration and invasion, partially because it exhibits weak kinase activity relative to BRAF or CRAF. Like CRAF, a concerted action of RAS activation and Src phosphorylation activates ARAF kinase (Marais et al., 1997). Also, loss of ARAF in mouse embryonic fibroblasts does not impair either ERK1/2 signaling or oncogenic transformation by RAS and SRC (Mercer et al., 2002). ARAF is activated by heterodimer formation with BRAF and CRAF upon GDC-0879 treatment, which goes together with increased kinase activity in MeWo melanoma cells that are wild-type for BRAF and KRAS (Hatzivassiliou et al., 2010). In a KRAS mutant cell line like HCT 116, dual ARAF and CRAF knock down was shown to be synergistic in decreasing inhibitor-induced phosphoMEK levels. Taken together, the authors showed that GDC-0879 increased the growth rate of KRAS mutant lung xenografts in accordance with enhanced proliferation in cell culture studies. However, in A549 cells, where RAS activation is HER2 dependent, GDC-0879 treatment did not increase proliferation of cells irrespective of (A)RAF knockdown (Fig. 3.12). The HER2 inhibitor lapatinib rather than MEK inhibitor UO-126 decreased cell proliferation, indicating that RAS was required for MEK-ERK activation by RAF inhibitors as we had proven before with the Ras binding mutant of ARAF (R52L) no longer being able to phosphorylate ERK. We could show that GDC-0879 induced migration of tumor cells rather than stimulating cell proliferation (Fig. 3.13 and 3.14). Moreover, loss of ARAF prevented both basal and RAFi induced invasive behavior in A549 (Fig. 3.16). Reconstitution of ARAF rescued ERK1/2 activation and directional migration in ARAF depleted cells, suggesting that the

kinase competent ARAF protein is needed for proper cellular function. However, studies employing different kinase mutants such as a kinase-impaired and constitutively active-kinase variant of ARAF have to be carried out to substantiate these initial findings.

Interestingly, cell-tracking analysis revealed that ARAF expression also contributes to random two-dimensional migration in A549 cells as its depletion led to a marked reduction in both basal as well as RAF inhibitor-mediated displacement of cells (Fig. 3.15). However, membrane protrusion such as lamellipodia and filopodia were not impaired despite a reduction in cell motility. In ARAF depleted cells, the assembly and disassembly of focal adhesions was disturbed as a protrusive directional migration could not be achieved. As the ERK-MAPK pathway is one of the principal signaling cascades by which cells respond to extracellular and intracellular cues it is likely that the lack of basal and RAFi- mediated ERK activation in these cells, prevented effective adhesion turnover. ERK has been shown to be involved in coordinating adhesion and actin polymerization to promote productive leading edge advancement during cell migration (Mendoza et al., 2011). Depending on the cell type and stimulus from the receptors, Ras can also activate PI(3)K (phosphatidylinositol 3-kinase), which functions with its downstream effector kinase Akt to regulate migration speed and directionality (Kolsch, Charest, & Firtel, 2008). Also the extra cellular matrix itself can induce ERK activity during adhesion through the activation of PAK (p21-activated kinase) by the small GTPases Rac and Cdc42 (Eblen, Slack, Weber, & Catling, 2002; Slack-Davis et al., 2003). Thus, the role of ARAF and its interacting partners in regulating adhesion turnover has to be studied in greater detail. Further, it would be worth investigating if the kinase activity of ARAF is required for regulating focal adhesions turnover. Along these lines it is interesting to indicate that CRAF kinase can regulate cell migration in a kinase independent manner (Ehrenreiter et al., 2005; Ehrenreiter et al., 2009).

The initial evidence we have gained from our directional migration and random cell motility experiments had prompted us to investigate further whether the loss of ARAF also compromised the cells′ ability to invade the matrix, which is one of the hallmarks of cancerous cells. In order to reproduce the complexity and pathophysiology of *in vivo* tumor tissue, we have employed three-dimensional cell culture systems to replicate to a greater extent, tissue architecture and the ECM as they significantly

influence tumor cell responses to microenvironmental signals. In addition to other cell lines, that have been tested elsewhere (Vinci et al., 2012), we could show that spheroid formation, indicative of tumor microregions or micrometastases also occured in A549 cells which was not compromised upon ARAF knock down (Fig. 3.16). However, the anti cancer drug GDC-0879 did not significantly trigger invadopodia advancing into the matrix in the absence of ARAF that was enhanced in control cells, indicative of tumour cell dissemination. Already the basal cell invasion was severely impaired upon loss of ARAF, which could not be compensated for over time. Thus, how ARAF is employed in the intrusion of the matrix and how it influences potential key players like integrins or cadherins that control matrix integrity, needs to be assessed and evaluated. Therefore, mass spectrometry analyses in the presence or absence of ARAF and RAF inhibitor, are being conducted to identify the factors that determine the role of ARAF in mediating tumour cell invasion.

4.7 Loss of ARAF promotes anchorage independent growth and lung metastasis in nude mice

Invasion of tissues by malignant tumors is facilitated by cross talk between the tumour and the microenvironment. Therefore a constant contact to the substratum to maintain the signaling from the ECM is essential for the survival of many cell types. Our *in vitro* biochemical assays in A549 cells suggested a role for ARAF in cancer cell invasion as depletion of ARAF reduced ERK1/2 signaling thereby decreasing tumor cell migration and motility. GDC-0879 stimulation of these ARAF depleted cells could neither trigger the paradigm of RAF activation downstream of mutated RAS nor the phenotypes observed in wildtype cells.

Surprisingly, depletion of A, but not B or CRAF resulted in greatly enhanced colony formation when cells were grown in soft agar (Fig. 3.17 A and B), suggesting a higher oncogenic potential for this RAF isoform. Transformation by most oncogenes is characterized by the acquisition of anchorage independence (Qiu, Abo, McCormick, & Symons, 1997) and apoptosis resistance (Reed, 1995; Thompson, 1995) both of which are critical for tumorigenesis. Our experiments indeed showed a better survival for A549 cells that lacked ARAF expression grown in an anchorage independent manner (Fig. 3.17 C), possibly by evading apoptosis due to detachment from the extra cellular matrix- a process termed anoikis. A variety of signaling pathways are

engaged in the induction of anoikis as in the resistance to this type of cell death, conferring the ability to survive without signals normally provided by contacts with the ECM (Buchheit, Weigel, & Schafer, 2014). Lack of ECM attachment has been shown to increase the level of the pro-apoptotic protein BIM (Reginato et al., 2003) and cancer cells use several molecular mechanisms to evade BIM-mediated anoikis (Reginato et al., 2005). By the activation of c-Jun-N-Terminal Kinases (JNKs), epithelial cells usually induce anoikis after the detachment from matrix and the disruption of integrin-mediated cell-matrix interactions (Frisch, Vuori, Kelaita, & Sicks, 1996). In accordance with the literature we detected in A549 cells grown under suspension conditions, increased Bim and p-cJun (JNK substrate) levels over time, indicative of anoikis induction (Fig. 3.17 D). However, ARAF depletion did not block Bim induction suggesting possible upregulation of other compensatory anti-anoikis factors in shARAF cells. ERK activation is engaged in the regulation of anoikis by mediating proteasomal degradation of BIM (Fukazawa, Noguchi, Masumi, Murakami, & Uehara, 2004) or its repression due to overexpression of oncogenic BRAF/NRAS in human melanocytes (Becker et al., 2010) and other melanoma cell lines (Boisvert-Adamo & Aplin, 2008). It is also involved in RAS-mediated anoikis suppression as KRAS activation promoted anchorage-independent growth in NRK cells, which could be reversed by the inhibition of MEK (Fukazawa & Uehara, 2000). In all cases, ERK signaling stimulated cell survival during detachment from the ECM mediating resistance to anoikis. Yet, throughout this work all cell culture experiments and differential proteome studies revealed a persistent inhibition of the ERK pathway in the absence of ARAF. However, this did not result in the induction of anoikis despite upregulation of BIM over time. On the contrary, shARAF cells tolerated the loss of attachment to the ECM to an extent that they were able to survive in suspension for more than 7 days (Fig. 3.17), ensuring that anoikis was not simply delayed. Moreover, these cells when injected into the tail vain of nude mice were able to colonize the lung, exhibiting strong lung metastasis after two to three weeks (Fig. 3.19). This is in accordance with studies showing that anokis resistance is one of the major prerequistes for tumour cells to metastasize (Simpson, Anyiwe, & Schimmer, 2008). Hence, the anoikis resistant phenotype resulting from the loss of ARAF in A549 cells must have been acquired in an ERK1/2 independent mechanism, challenging the consensus that lack of signaling through the ERK pathway contributes

to anoikis. Ras activation triggers two divergent signaling cascades that activate distinct MAPKs with different substrate specificities and transcriptional functions (Feig & Cooper, 1988). Both the ERKs and JNKs are activated by EGF and oncogenic *RAS* (Minden et al., 1994). There are reports in which ERKs were not activated in MDCK cells in response to cell-matrix adhesion (Frisch et al., 1996) but disruption of the cell-matrix interactions led to the activation of the JNK pathway instead. Further, a dominant negative mutant (JNKK-dn) significantly reduced JNK activity and these cells became resistant to anoikis when grown in suspension. Phosphorylation of JNK at T183/Y185 which marks its endogenous, active form was markedly decreased in ARAF depleted A549 cells. In these lines, a rather unexpected observation is the strong phosphorylation of the JNK substrate c-Jun at Serine 63 (Fig. 3.18). JNK had been demonstrated to be essential for a basal level of c-Jun expression and its phosphorylation at this specific residue in response to stress such as UV irradiation and oncoprotein activity (Derijard et al., 1994; Kayahara, Wang, & Tournier, 2005). Behrens and colleagues showed that oncogenic transformation by Ras required phosphorylation of c-Jun at S63 (Behrens, Jochum, Sibilia, & Wagner, 2000). Mice with mutant Jun, which was incapable of N-terminal phosphorylation, exhibited impaired skin tumor and osteosarcoma development. In mouse models of intestinal cancer, genetic abrogation of c-Jun activation attenuated cancer development and prolonged the animals' life span (Nateri, Spencer-Dene, & Behrens, 2005). The 2-fold increase in phosphorylation of c-Jun upon ARAF knock down might account for the oncogenic transformation potential of these cells. In a study using NSCLC, c-jun was found to be overexpressed in around one third of the cases in primary and metastatic lung tumors (Szabo, Riffe, Steinberg, Birrer, & Linnoila, 1996). However, our results are only preliminary and further experiments such as JNK kinase activity assays are warranted to estimate a potential role for Jun-N-Terminal Kinase and its substrate in mediating anoikis resistance under suspension conditions in A549 cells.

How the loss of ARAF kinase contributes to tumor cell survival during detachment from ECM needs to be evaluated in this context. First of all, reconstitution experiments should be carried out to demonstrate that ARAF expression itself reduces the ability of cancer cells to grow in an anchorage-independent state and impair tumor formation *in vivo* (either by xenografts or tailvain injection into nude mice). ARAF

mutants could be additionally employed to investigate the contribution of kinase activity (constitutively active DD/ kinase dead D447N); the influence of Ras binding (R52L); dimerization (R362H) or MEK binding (S432D/A) on anoikis regulation.

In epithelial cells, integrins establish a physical link between the ECM and the cytoskeleton. Furthermore they prevent the activation of the common anoikis pathway while at the same time drive the stimulation of various survival promoting pathways to support cell survival and anoikis suppression (Streuli, 2009; Vachon, 2011). Interestingly, important players in downstream signaling of integrin receptors such as focal adhesion kinase (Fak; p125Fak), the phosphatidylinositol-3 kinase (PI3-K)/Akt-1 and Src (p60Src) pathway are strongly down regulated with their representative substrates less phosphorylated in ARAF depleted cells. It is worth mentioning that all members of the Src kinase family tested showed reduced phosphorylation in their respective activation sites. Src phosphorylation at Y419 was found to be severely impaired in shARAF cells which seems rather unexpected as activity of src tyrosine kinase is suggested to be linked to cancer progression (Wheeler, Iida, & Dunn, 2009). The activation of the c-Src pathway due to genetic mutations has been observed in about 50% of tumors from colon, liver, lung and breast (Dehm & Bonham, 2004). EGFR as well as overexpression of HER2 has been shown to activate c-Src, which is correlated with a poor prognosis for breast cancer among others (Slamon et al., 1989). A549 cells, which have long been used to study KRAS-driven NSCLCs, not only carry an activating Ras-mutation but also amplifications of *EGFR* and *HER2* oncogenes, which encode members of the epidermal growth factor receptor family (Diaz et al., 2010). In this context, it would be interesting to decipher the contribution of *HER2* amplifications and the loss of ARAF to survival and invasion if it is not via Src-activation that previously has shown to be employed in the aberrant growth of tumors and metastasis formation (Ottenhoff-Kalff et al., 1992; Slamon et al., 1987). Proteome analysis showed further that ARAF depleted cells had higher levels of STAT3 phosphorylation. STAT3 is persistently active in a wide variety of human solid tumors whereby the tumor cells acquire the ability to proliferate uncontrollably and to resist apoptosis (Yu & Jove, 2004). A recent article established STAT3 as a critical player that confers anoikis resistance to melanoma cells and enhances their metastatic potential (Fofaria & Srivastava, 2014).

However, the phosphorylation profiles we gained from A549 cells grown in suspension need to be repeated, possibly also over a longer period of time with their respective adherent controls to estimate the changes in protein expression. They further have to be validated by mass spectrometry analyses (Phospho proteomics) to strengthen the results we obtained from initial phosphor-kinase array screenings. This will help us to understand how the loss of ARAF protects cells from anoikis despite the common survival pathways being down regulated in these cells. The identification of the factors that allow ARAF depleted A549 cells to evade apoptosis will gain us a better understanding of how cancer cells survive during detachment from ECM and might be valuable for developing novel chemotherapeutic strategies to eliminate ECM-detached metastatic cells.

P.s. It is worth noting that protection from anoikis has been associated with cell cycle arrest (Collins et al., 2005) but due to technical problems we did not obtain consistent data for cell cycle distribution using FACS analysis and BrdU incorporation.

Zusammenfassung

Einleitung

Der Lebenszyklus von Zellen ist durch Wachstum, Vermehrung, Differenzierung, Überleben sowie dem Sterben gekennzeichnet. Diese vielfältigen zellulären Vorgänge werden mittels so genannter Signalkaskaden sorgfältig reguliert (Wellbrock, Karasarides, & Marais, 2004). Dabei leiten die einzelnen Komponenten extrazelluläre Stimuli in das Innere der Zelle weiter, wodurch eine entsprechende Reaktion auf die erhaltenen Informationen ausgelöst wird. Einer der best erforschten Signalwege ist der durch Mitogene Aktivierte Protein (MAP) Kinaseweg. MAPK-Proteinkinasen zeichnen sich dadurch aus, dass sie durch die Zugabe von Phosphatgruppen aktiviert werden und so ihrerseits das nächste Mitglied in der Sequenz durch Anhängung einer Phosphatgruppe an entsprechende Aminosäurereste (duale Phosphorylierung), aktivieren. Die Bindung von Wachstumsfaktoren an Rezeptoren an der Zelloberfläche setzt z.B. die mehrstufige RAS/RAF/MEK/ERK Signaltransduktionkaskade im Inneren der Zelle in Gang. Die kleine GTPase RAS fungiert dabei als eine Art molekularer Schalter (Goetz, O'Neil, & Farrar, 2003). Ist dieser durch eine Mutation konstitutiv aktiviert -wie es in etwa einem Drittel aller menschlichen Tumore der Fall ist, kommt es zur Deregulierung des Zellwachstums (Bos, 1989). In vielen menschlichen Krebsarten sind zudem die nachgeschalteten Proteinkinasen hyperaktiviert, d.h. einzelne Mitglieder dieser "in Serie" geschalteten MAP-Kinasen sind konstant phosphoryliert und entgehen somit jeder Regulation (Davies et al., 2002). Die RAF-Proteine, welche durch RAS aktiviert werden, waren die ersten beschriebenen Serin/ Threoninkinasen, denen krebserregende Aktivität nachgewiesen werden konnte (Moelling, Heimann, Beimling, Rapp, & Sander, 1984). Die humane Familie der RAF-Proteine setzt sich aus A, B und CRAF zusammen, denen RAS als Aktivator und MEK als Substrat gemein ist. Während BRAF bereits durch die Bindung an RAS aktiviert wird, sind für die Aktivierung von ARAF und CRAF weitere Faktoren erforderlich (Marais et al., 1997). Hierin liegt auch die Ursache für das ungebremste Zellwachstum, welches durch die häufigste Mutation im BRAF-Protein, die BRAF-V600E-Mutation, hervorgerufen wird. Der Austausch einer

einzigen Aminosäure Valin gegen Glutamat an Position 600 des BRAF-Exons 15 (Davies et al., 2002) führt zu einer andauernden Aktivierung des MAPK-Signalweges und somit unter Umständen sogar zu aggressiven Karzinomen (meist Melanomen).
RAF-Kinasen stellen deshalb ein vielversprechendes Ziel in der Behandlung von Tumoren dar (besonders Tumore mit zugrunde liegender aktivierender *BRAFV600E* Mutation). Krebstherapien zielen darauf ab, die hyperaktivierten Kinasen zu hemmen und somit das Tumorwachstum zu unterbinden. Dabei werden unterschiedlich selektive RAF-Inhibitoren (small molecules) in der Behandlung von Patienten eingesetzt (Takle et al., 2006; Bollag et al., 2010). Spezifische BRAF-V600E-Blocker konnten das progressionsfreie Überleben, sowie das Gesamtüberleben von Patienten mit metastasiertem Melanom im Vergleich zur Chemotherapie deutlich verbessern (K. T. Flaherty et al., 2010). Patienten, deren Tumorerkrankung auf eine RAS-Mutation zurückzuführen ist, entwickelten bei gleicher Behandlung nach einiger Zeit jedoch kutane Plattenepithelkarzinomen und Keratoakanthomen aufgrund einer paradoxen Aktivierung des MAPK-Weges (Hatzivassiliou et al., 2010; Heidorn et al., 2010; Poulikakos et al., 2010). Zusammenfassend beschleunigen RAF-Inhibitoren das Wachstum von Metastasen in *RAS* mutierten Tumoren und werden mit der Zeit unwirksam in Tumoren, in denen sie zuvor effektiv (*BRAFV600E)* waren (erworbene Resistenzen).

Zielsetzung

Zielgerichtete Therapien, die spezifische Kinasen innerhalb eines Signalweges angreifen und somit die deregulierte Signalübertragung unterbrechen, sind wichtige Bestandteile onkologischer Behandlungen. Die Rolle des MAP-Kinase-Signalweg bei der Entstehung und dem Wachstum bösartiger Karzinome ist vielfältig beschrieben worden. Vor allem der Einfluß BRAFs im Zusammenspiel mit CRAF lag dabei im Fokus der (klinischen) Studien. Mit dieser Arbeit sollte die weniger intensiv untersuchte RAF-Kinase ARAF in das Zentrum der Betrachtung gerückt werden. Die Ziele der hier vorliegenden Arbeit waren dabei im Einzelnen 1) die Ermittlung des Zusammenhangs zwischen ARAF-Funktionsweise und der paradoxen Aktivierung der MAPK-Signalkaskade durch RAF-Inhibitoren in RAS mutierten Zellinien sowie 2) die

Untersuchung der Auswirkungen auf den MAPK-Signalweg bei Verlust von ARAF-Expremierung *in vitro* als auch *in vivo* (Nacktmäuse). Die dazu verwendeten Zellinien wurden so verändert, dass wichtige Prozesse der Krebsentstehung wie MAPK-Aktivierung, Tumorzellmigration und- invasion zeitgleich untersucht werden konnten.

Ergebnisse und Diskussion

Welchen Beitrag die ARAF-Kinase an der paradoxen Aktivierung des MAP-Kinase-Signalweges leistet, wurde mit Hilfe von knock- down Experimenten in verschieden *KRAS*- mutierten Zelllininen untersucht. Dabei wurde das Expressionslevel von ARAF mittels shRNA herunterreguliert und die daraus resultierenden Veränderungen auf die basale wie durch RAF-Inhibitoren hervorgerufene, Aktivierung von ERK1/2 analysiert. Die hierzu verwendeten RAF-Inhibitoren waren Sorafenib, ein Multi-Kinase-Blocker, sowie der spezifische BRAF-Inhibitor GDC-0879. Durch die Herstellung unterschiedlicher ARAF-Mutanten, bei denen wichtige regulatorische Aminosäurereste ausgetauscht wurden, war es zudem möglich, die für ARAF charakteristischen Auswirkungen auf Phosphorylierungsmuster, Dimeresierung und Komplexbildung infolge von RAF-Inhibitorbehandlung, zu bestimmen. Schließlich wurden die *in vitro* erhaltenen Resultate phenotypisch mit Hilfe von Proliferations- und Migrationstudien geprüft, um letztlich die Übertragbarkeit der *in vitro*-Ergebnisse auf die *in vivo*-Situation mittels Invasionsstudien zu beweisen.
Zusammenfassend konnte gezeigt werden, dass die Proteinkinase ARAF bei der Aktivierung des klassischen MAPK Signalwegs und der zelltyp- abhängigen Migration von Krebszellen unerlässlich ist. Die Herabsetzung der ARAF-Expression verhinderte die Phosphorylierung von MAPK-Kinase 1 (MEK1) sowie dessen Substrat ERK1/2. Darüber hinaus waren unter diesen Umständen weniger Auswüchse aus einem dreidimensionalen Tumorgewebe zu beobachten als es die Behandlung mit BRAFV600E- spezifischen oder pan-RAF-Inhibitoren (GDC-0879 oder Sorafenib) normalerweise in diesen Zellen bewirkt. Die verwendeten RAF-Inhibitoren führten zur Bildung von ARAF-Homodimeren sowie zur Oligomerisation anderer RAF-Kinasen mit dem Stützprotein KSR 1. Auch bei verminderter ARAF-Expression bildeten sich die eben beschriebenen Signal-Komplexe, welche jedoch nicht

(ausreichend) aktiv waren, um die MAP-Kaskade und die damit verbundene Migration der Tumorzellen in Gang zu setzen. Warum BRAF und/ oder CRAF-Kinasen in der Abwesenheit ARAFs nicht in der Lage waren, MEK 1 zu phosphorylieren, muss durch weiterführende Experimente geklärt werden. Dabei könnten massenspektrometrische Untersuchungen helfen, Faktoren zu identifizieren, die durch unterdrückte ARAF-Expremierung beeinflusst werden und so für entscheidende Veränderungen im komplexen MAPK-Signalosom verantwortlich sind. Zum ersten Mal konnte gezeigt werden, dass die drei rekombinanten RAF-Isoformen in einer aufgereinigten Proteinlösung miteinander um die Bindung an ihr Substrat MEK 1 konkurrierten. Zellkulturexperimente bewiesen zudem, dass ARAF-Mutanten, die nicht in der Lage waren sich zu Dimeren zusammenzulagern, auch nicht ihr zelluläres Substrat MEK 1 binden konnten und es als Folge zu keiner nachgeschalteten Phosphorylierung kam.

Unsere Ergenisse beschreiben die vielfältige Funktionsweise der ARAF-Proteinkinase wenn es um die zellinterne Signalübertragung (Aktivierung des MAPK-Signalwegs) und die sich daraus ableitende Invasierung von Tumorzellen geht. Damit stellt die vormals wenig bedeutsame Serin/ Threoninkinase ARAF eine neuartige Option in der Behandlung bösartiger Tumore dar, welche auf mutiertes RAS und/ oder RAF zurückzuführen sind. Die vorliegenden Untersuchungen weisen ARAF einerseits als Onkogen aus, andererseits führte der Expressionsverlust von ARAF bei der Injektion solcher Zellen in die Schwanzvene von Nacktmäusen zu starker Metastasenbildung in der Lunge. In diesem Zusammenhang bleibt die Frage zu klären, inwieweit die Kinaseaktivität ARAFs für die beschriebenen Ergebnisse verantwortlich ist oder es sich dabei um eine kinaseunabhängige, protektive Funktion des ARAF-Proteins handelt.

Fazit und Ausblick

Bis zum heuigen Zeitpunkt konnten wir mittels Vergleichsstudien erste aufregende Erkenntnisse über die Ursachen dieser unerwarteten ARAF- Doppelfunktion als Tumorsurpressor/ aktivator gewinnen, welche den Anfang eines neuen spannenden Kapitels in der mittlerweile schon über 40- jährigen RAF-Geschichte bilden könnten.

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internet sources (tissue and protein information):

www.cancer.sanger.ac.uk

www.proteinatlas.org

Acknowledgements

Ich möchte DANKE sagen an all die Menschen, die mir immer zur richtigen Zeit am richtigen Ort begegnet sind. Das bedeutet auch gleichzeitig DANK an jene, deren Wege sich nicht mit meinen gekreuzt haben. Ohne sie alle wäre diese Doktorarbeit wohl so nicht zustande gekommen. Es war die Neugier, die mich am Ende von Würzburg über Göttingen und Berlin nach Frankfurt gebracht hat. Alle Ideen und Talente wollten erst ausgetestet werden bevor das Abenteuer Doktorarbeit beginnen konnte. Eine intensive Lebensphase geht nun mit der Dissertation zu Ende- doch wie sooft sind die Fragen eher mehr als weniger geworden...So gibt es denn wohl immer genug zu erforschen, um dem Geheimnis des Lebens auf die Spur zu kommen, in der wissenschaftlichen Arbeit wie im wirklichen Leben ;)

„Die Glücklichen sind die Neugierigen.“ *Friedrich Nietzsche*

Thank you Krishna for letting me find and develop this exciting ARAFstory in your lab plus for all the fiftythousand ideas we came up with along the way to finally present a fine piece of research in the Science Podcast...not to mention the conference(s) in India we went to. Tamil Nadu- not only a scientific but also a visual, acoustic, olfactory, tasty and altogether historical sensation. நன்றி !

A special thanks goes to Volker Dötsch, who I appreciate for his enthusiasm in science and for discussions on this pHD project. I am also happy and grateful to have had such a good time at the ibc2, which is passionately run by its tireless director Ivan Đikić, who I also want to thank for the great people he invited for lectures. Not only the Nobel laureates have been inspiring but also every scientist with their own personal story to tell.
Dhanyavaad ! Thank you ! xièxie ! Nandri ! Kiitos ! Danke ...
... all my collaborators – what a truly successful international enterprise!

Finally, I am looking forward to seeing my dearest lab mates again who have left me before I could do so: Tanerle, Tripat, Carrie and Armelle. Bisous!

Curriculum Vitae

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Education

2001-2003 Undergraduate studies in biology at Würzburg University

2004-2007 Postgraduate studies at Würzburg University including year(s) abroad: Umeå University, Sweden (molecular biology and cancer research) Bangor University, Wales (cell/ developmental studies on marine life) fieldwork in Malaysia (biodiversity studies of invasive ant species and investigation of animal-plant mutualisms in Brunei Darussalam, Borneo)

2007 Diploma/ master thesis: "Modulation of cell survival by Prohibitins" under the supervision of Krishnaraj Rajalingam/ Ulf Rapp

2008 "Talking about science" - exploring the field of scientific journalism via radio broadcast and lectures for an interested lay audience

2009 Ph.D student at Frankfurt University since

Work experience

2008 Technical assistant/ lab manager at the Institute for Clinical Radiology and Cell Research, Würzburg

2009-2014 Preparation and support of the public lecture series „Perspectives in Molecular Medicine" at the University hospital Frankfurt Main in collaboration with Sanofi and Merck Serono (press release, podcast interviews and presentations

Eidesstattliche Erklärung

Ich erkläre hiermit, dass ich mich bisher keiner Doktorprüfung im Mathematisch-Naturwissenschaftlichen Bereich unterzogen habe.

Frankfurt am Main, den ....

Juliane Mooz

Ich erkläre hiermit, dass ich die vorgelegte Dissertation über

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