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Differential Localization of A-Raf Regulates MST2-mediated Apoptosis During Epithelial Differentiation.

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Running title
Spatial regulation of A-Raf by KSR2 controls apoptosis.

Keywords:
A-Raf, apoptosis, differentiation, HNSCC, breast cancer, KSR2
**Abbreviations:**
Abstract

A-Raf belongs to the family of oncogenic Raf kinases that are involved in mitogenic signaling by activating the MEK–ERK pathway. Low kinase activity of A-Raf toward MEK suggested that A-Raf might have alternative functions. We recently identified A-Raf as a potent inhibitor of the proapoptotic mammalian sterile 20-like kinase (MST2) tumor suppressor pathway in several cancer entities including head and neck, colon, and breast. Independent of kinase activity, A-Raf binds to MST2 thereby efficiently inhibiting apoptosis. Here, we show that the interaction of A-Raf with the MST2 pathway is regulated by subcellular compartmentalization. While in proliferating normal cells and tumor cells A-Raf localizes to the mitochondria, differentiated non-carcinogenic cells of head and neck epithelia express A-Raf at the plasma membrane. The constitutive or induced re-localisation of A-Raf to the plasma-membrane compromises its ability to efficiently sequester and inactivate MST2 thus rendering cells susceptible to apoptosis. Physiologically, A-Raf re-localizes to the plasma membrane upon epithelial differentiation in vivo. This re-distribution is regulated by the scaffold protein Kinase Suppressor of Ras 2 (KSR2). Downregulation of KSR2 during mammary epithelial cell differentiation or siRNA-mediated knockdown re-localizes A-Raf to the plasma membrane causing the release of MST2. Using the MCF7 cell differentiation system, we could demonstrate that overexpression of A-Raf in MCF7 cells induces differentiation. Our findings offer a new paradigm to understand how differential localization of Raf complexes affects diverse signaling functions in normal cells and carcinomas.
Introduction

A-Raf is a member of the Raf family of serine-threonine protein kinases, which comprises A-Raf, B-Raf, and Raf-1. Raf kinases are at the apex of the 3-tiered Raf/MEK/ERK (MAPK) kinase pathway regulating fundamental cellular functions, including differentiation, transformation, apoptosis, proliferation, and metabolism (1, 2). Activation of Ras GTPases at the cell membrane initiates Raf kinase activation and sequential phosphorylation and activation of the serine/threonine kinases MEK1/2 and ERK1/2 (3, 4). In comparison to Raf-1 and B-Raf, A-Raf is only weakly activated by oncogenic H-Ras and Src (5) and is a poor MEK kinase (5-8), which is due to unique non-conserved amino acid substitutions in the N-region (9). Only recently, the first somatic oncogenic mutations of A-Raf were identified in lung adenocarcinomas (10) and Langerhans cell histiocytosis (11, 12). We recently showed that A-Raf and Raf-1, independent of their kinase activity, bind the pro-apoptotic mammalian sterile 20-like kinase (MST2) thereby suppressing MST2 activation and MST2 induced apoptosis (13-17). MST2 employs several mechanisms to induce apoptosis including the transcriptional induction of PUMA (14), a BH3 domain protein that causes mitochondrial depolarization and subsequent cell death (18). While Raf-1 counteracts MST-2-mediated apoptotic signaling by suppressing the activation of MST2 only in quiescent cells or upon apoptotic stimulation (14, 15), A-Raf binds constitutively to MST2 (16). This anti-apoptotic role may explain why A-Raf levels are elevated in a variety of malignancies (16, 19-21). Both A-Raf and MST2 were shown to localize to the mitochondria in tumor cell lines as well as primary tumors (16). A-Raf was reported to associate with the mitochondrial transport system proteins hTOM and hTIM (22), but the physiological significance of this interaction is still unknown.
A-Raf mRNA expression levels were shown to be upregulated during adipocyte differentiation (23). DA-Raf1 and DA-Raf2, two alternative splice forms of A-Raf, which contain the amino-terminal Ras-binding domain, but lack the kinase domain, act as dominant-negative antagonists of activated Ras and positive regulators of myogenic differentiation (24).

MAPK signalling is regulated and organized by a group of molecular scaffolds, coordinating the efficient interaction of MAPK components, forming functional subcomplexes and signalling nodes thereby regulating signal intensity and specificity (2, 25). In addition, scaffolds are crucial for the spatial regulation of signaling events by targeting their clients to different localizations thereby increasing the variety of signals regulated by the cascade (26, 27). Among the best-studied MAPK scaffolds are the kinase suppressors of Ras proteins (KSR1 and KSR2), which were initially identified as a suppressor of an activated Ras phenotype in D. melanogaster (28) and C. elegans (29-31). KSR1 and KSR2 bind to Raf, MEK, and ERK thereby controlling their phosphorylation and activation (32, 33). In C. elegans KSR1/2 have overlapping but also iso-type specific interactions and functions (34). In mammalian cells KSR1 is crucial for oncogenic Ras signalling by binding to Raf-1 and B-Raf (35-37), whereas KSR2 recruits A-Raf rather than Raf-1 or B-Raf in response to TNFalpha (38). In addition to MAPK components, KSR2 was shown to interact with AMPK thereby regulating energy intake and fatty acid oxidation (39, 40). In humans mutations of KSR2 are associated with obesity and insulin resistance (41). In addition, KSR2 regulates genes controlling adipocyte differentiation in white adipose tissue (39) and is crucial for 1,25-dihydroxyvitamin D3-mediated monocytic differentiation of myeloid leukemia HL60 cells (42).
Non-keratinized stratified squamous epithelium lines the external surface of tissues such as oral cavity, esophagus, rectum and cervix, forming a protective barrier. During epithelial differentiation, epithelial progenitor cells of the highly proliferative basal membrane give rise to various cell types as they move upwards to build the multilayered epithelium. Commitment and terminal differentiation not only involves regulation of specific genes and epigenetic control, but also induction of the apoptotic machinery for controlled caspase cleavage, enculeation, and internucleosomal DNA cleavage (43-45).

We could show recently, that normal epithelia of the head and neck, colon, and liver show decreased levels of A-Raf in comparison to corresponding tumours (16, 21) (46) and that this feature is regulated via A-Raf splice form selection involving the splice factors hnRNP A2 and H. While in tumours, splicing is shifted towards enhanced expression of the full-length A-Raf protein that sequesters MST2, normal epithelia, where hnRNP H and hnRNP A2 are low, express the alternatively spliced, truncated isoform A-Raf\textsubscript{short}, which fails to control MST2. In addition, A-Raf\textsubscript{short} functions as a dominant negative mutant due to its ability to bind to activated Ras activation and suppress MAPK activation. Thus, alternative A-Raf splicing acts as a safeguard against oncogenic transformation in normal epithelia.

Here, we report that A-Raf undergoes spatial regulation upon epithelial differentiation, re-localizing from the cytoplasm to the plasma membrane. We show that A-Raf cytoplasmatic localization is crucial for sequestration and inhibition of MST2 controlling the MST2 apoptotic pathway. We show that the MAPK scaffold protein KSR2 is crucial for the integrity of the A-Raf/MST2 complex and regulation of differentiation. We
furthermore find that perturbation of either A-Raf localization or KSR2 expression causes apoptosis via MST2.

**Results**

*A-Raf localizes to the plasma membrane during epithelial differentiation.*

We recently demonstrated that A-Raf strongly interacts with MST2 and inhibits MST2 mediated apoptosis (16, 21). Both A-Raf and MST2 were localized at the mitochondria and cytoplasm in tumour cell lines as well as primary tumours (16). First, we confirmed our previously published data by immunohistochemistry showing a cytoplasmic expression of A-Raf in primary head and neck squamous cell carcinomas (Fig. 1A). However, in non-malignant stratified, non-keratinized squamous epithelia of the same tissue origin (Fig. 1B, S1A), A-Raf was differentially localized. While in proliferating normal cells of the basal cell layer A-Raf was cytosolic, it moved to the plasma membrane during differentiation (Fig. 1B). Interestingly, this re-localization coincides with the physiological nuclear pyknosis that takes place in the stratum spinosum. This differentiation associated pyknosis involves activation of caspase-14, which like MST2 (15) can be activated by Fas and TNFα signaling (47). These data suggest, that A-Raf changes localization in normal cells upon epithelial differentiation suggesting that A-Raf has different functions depending on its localization.

**Plasma membrane-localized A-Raf loses its ability to inhibit MST2-mediated apoptosis.**

We next determined whether A-Raf localization was important for inhibition of MST2-mediated apoptosis. We stably expressed two versions of A-Raf, wild type (wt) A-Raf which is localized in the cytoplasm, and A-Raf-CAAX which constitutively localises
to the plasma membrane due to fusion with the CAAX motif from KRas4b (48) (49) (Fig. 2A, B, C). MST2 readily bound to wt A-Raf, but poorly to A-Raf-CAAX (Fig. 2D). As expected from our previous studies (14, 15), the interaction of Raf-1 with MST2 was disrupted by growth factors, while the interaction between wt A-Raf and MST2 was largely resistant (Fig. 2D). These data indicate that A-Raf localization controls MST2 binding.

A-Raf is overexpressed in a variety of malignancies, including colon (16) (21), head and neck (21), pancreatic ductal carcinomas (20), and astrocytic tumors (19) suggesting that tumor cells maintain high-level expression of A-Raf to prevent apoptosis by antagonizing the proapoptotic kinase MST2. We therefore examined, whether overexpression of plasma membrane based A-Raf preserved this function, by activating MST2 using etoposide (16, 50). While overexpression of wt A-Raf inhibited MST2 activation (Fig. 2E) and reduced apoptosis induced by etoposide (Fig. 2F), A-Raf-CAAX could neither prevent activation of MST2 nor etoposide-triggered apoptosis (Fig. 2E, F). In addition, expression of neither wild type nor membrane targeted A-Raf influenced proliferation (Fig. S2A), ERK activation (Fig. 2E, S2B, S2C, S2D), or oncogenic transformation (Fig. S2E), indicating that the localization specific regulation by A-Raf selectively affects the MST2 but not the ERK pathway.

A dynamic recruitment of Raf proteins from the cytoplasm to the plasma membrane is well described upon activation of Ras (51-54). We therefore examined, whether dynamic changes of A-Raf localization impact on MST2 binding. For this purpose, we used the rapalogue (AP21967)-inducible heterodimerization system, which permits the efficient and selective crosslinking of FRB and FKBP-tagged proteins (55, 56). We co-expressed a flag-tagged FRB A-Raf fusion (FRB-A-Raf-flag) with a tandem FKBP
domain construct (Myr.-2xFKBP-HA) that was targeted to the plasma membrane by an N-terminal myristoylation tag (Fig. 3A,B). While FRB-A-Raf-flag localized to the cytoplasm in HeLa cells, addition of AP21967 led to membrane recruitment of A-Raf due to heterodimerization with Myr.-2xFKBP-HA (Fig. 3C, S3). The heterodimerization and membrane recruitment of A-Raf occurs over a wide concentration range and is stable for at least 8 hours allowing co-immunoprecipitation of the FRB-A-Raf-flag and Myr.-2xFKBP-HA proteins (Fig. S3). In contrast, MST2 localisation does not change upon AP21967-induced re-localisation of A-Raf (Fig. 3C). Dynamic membrane recruitment of A-Raf leads to the disruption of the A-Raf-MST2 complex (Fig. 3D). We next asked whether dynamic A-Raf relocalisation was important for inhibition of MST2-mediated apoptosis (Fig. 3E). Cytoplasmic A-Raf fully counteracts etoposide-induced MST2 activation and apoptosis as shown by cleavage of Caspase 3 and PARP, and the activation of PUMA expression. In contrast, when A-Raf was re-localised to the membrane by AP21967 treatment, it failed to inhibit MST2 activation and downstream apoptosis. Again, localisation of A-Raf did not impact on bulk ERK activation. Taken together, these results suggest that A-Raf cytoplasmic localisation is crucial for MST2 binding, inhibition, and regulation of MST2-mediated apoptosis.

A-Raf relocalizes at the plasma membrane during mammary differentiation.

We next investigated the mechanism underlying re-localisation of A-Raf and regulation of MST2-mediated apoptosis during differentiation processes in an in vitro differentiation system. MCF-7 cells, derived from breast adenocarcinoma, can undergo phenotypic changes including lipid accumulation, which can be quantified as a measure of differentiation (Fig. 4A)(57-62). We therefore stably overexpressed A-Raf fused to EGFP in MCF7 cells and induced differentiation by serum starvation and
concomitant stimulation with Herregulin (57, 62) using EGF as control. While EGFP alone localizes ubiquitously in the cell and independently of the stimulus (Fig. 4B upper panel), EGFP-A-Raf localizes to the cytoplasm under growing conditions (10%FCS) (Fig. S4A), or in cells treated with EGF (Fig. 4B). Stimulation with Herregulin induces an accumulation of EGFP-A-Raf at the plasma membrane (Fig. 4B, C) at regions reminiscent of tight junctions. Again, stable overexpression of EGFP-A-Raf inhibits activation of MST2 (Fig. 4D), but has no impact on bulk ERK activation (Fig. 4D), cell proliferation (Fig. S4B), AKT and ERK signaling dynamics except that ERK activation at 5 minutes is dampened (Fig. S4C).

To confirm the imaging data and quantify the subcellular redistribution of endogenous A-Raf upon differentiation of MCF7 cells, cytosolic and membrane fractions were isolated. While in control and EGF-treated cells, EGFP-A-Raf-EGFP and MST2 were found in the cytoplasmic fraction, stimulation with Herregulin increased A-Raf in the membrane fraction (Fig. 5A). Endogenous A-Raf and MST2 showed the same changes in distribution (Fig. 5B), corroborating the re-distribution of endogenous A-Raf to the membrane during differentiation. The re-localisation of A-Raf during Herregulin-mediated differentiation of MCF7 cells resulted in activation of MST2 (Fig. 4C, 5C) and activation of Caspase 8 (Fig. 5C), which could be reduced by A-Raf overexpression (Fig. 5C).

Taken together, these data suggest that A-Raf relocalises to the plasma membrane during Herregulin-induced differentiation of MCF7 cells, resulting in activation of MST2 and Caspases.

* A-Raf enhances Herregulin-mediated differentiation of MCF7 cells.
In an independent study, we identified A-Raf among other factors in a proteomic screen designed to probe for changes in protein expression in Epithelial to Mesenchymal Transition (EMT) of MDCK cells (63). We could demonstrate that inhibiting the MST2 pathway by overexpression of A-Raf promotes HGF-induced EMT.

Based on these data, we hypothesized that A-Raf function might be instrumental for the differentiation process. Therefore, we used the MCF7 cells, which stably overexpress EGFP-A-Raf or EGFP alone and quantified differentiation in response to Heregulin. While no difference in lipid accumulation could be detected between control cells and cells expressing EGFP, A-Raf overexpression increased differentiation as measured by lipid droplet accumulation by 33% (Fig. 6A). Decreasing A-Raf expression by transfection with specific siRNA modestly, but significantly, reduced differentiation (Fig. 6B) indicating that the effect is indeed mediated by A-Raf. In order to corroborate that the measured effects are not due to clonal selection of the stable cell line overexpressing EGFP-fused A-Raf, we repeated the experiments with a different plasmid set (pcDNA3-based) and transient transfection. Transient overexpression of wild type A-Raf or a kinase-dead version of A-Raf (K336M) increased Heregulin-mediated differentiation by 18% and 29%, respectively (Fig. 6C). These data suggest that A-Raf promotes differentiation independently of A-Raf kinase activity suggesting that this effect is not mediated through the ERK pathway but through inhibition of MST2, which does not require A-Raf kinase activity.

*The scaffold Kinase Suppressor of Ras 2 (KSR2) regulates A-Raf localization and MST2 activity.*
We next investigated the mechanism underlying the re-localization of A-Raf during differentiation. A-Raf was shown to interact with a plethora of other proteins (2, 64) including scaffold proteins regulating ERK signalling (25). Recently, it was suggested that the scaffold Kinase suppressor of Ras 2 (KSR2) mediates A-Raf signalling, while a close homolog, KSR1, would facilitate Raf-1-mediated ERK signalling (38). Based on these data, we hypothesized that KSR2 may function as a scaffold to control A-Raf localization and its ability to regulate MST2 activity. First, we confirmed that KSR2 can interact with A-Raf and MST2, while no interaction between A-Raf and KSR1 could be observed (Fig. 7A) (38). Then, we determined, if KSR2 expression is regulated during Heregulin-mediated differentiation of MCF7 cells. KSR2 expression is downregulated during differentiation, while the expression of KSR1, A-Raf and its upstream regulator hnRNPH (16, 21) is not changed, (Fig.7B). Monitoring KSR2 expression over a timecourse of differentiation, we found that KSR2 expression is lost five days post induction with Heregulin (Suppl. Fig. S5), suggesting that KSR2 loss represents a late event during MCF7 differentiation.

We next determined whether the changes in A-Raf localisation and regulation of MST2 are dependent of KSR2 (Fig. 7C). Knocking-down KSR2 by siRNA induced MST2 activation in serum starved MCF7 cells. Interestingly, knocking-down KSR2 also abolished the interactions between MST2 and A-Raf, suggesting that KSR2 is required as a scaffold for the MST2 interaction with A-Raf, or that it directs A-Raf to a subcellular compartment that enables this interaction. Importantly, KSR2 depletion causes a re-localisation of A-Raf from the cytosol to the membrane as shown by cell fractionation (Fig. 7C). In addition, siRNA-mediated knock-down of A-Raf does abolish the interaction between KSR2 and MST2 (Fig. 7D), suggesting that MST2 binds
directly to A-Raf and not KSR2 and that A-Raf seems to be crucial to keep the trimeric complex intact.

Taken together these data suggest a model where the scaffold KSR2 is required for the sequestration and inhibition of MST2 by A-Raf in tumour and proliferating cells (Fig. 7E). Loss of KSR2 during differentiation results in A-Raf re-localisation to the plasma membrane, the disruption of the anti-apoptotic A-Raf-MST2 complex and activation of the MST2 pathway.

Discussion

Spatial regulation of Raf kinases within the cellular compartments is regulated in a highly dynamic and concerted manner (2). In growth factor deprived cells, Raf-1 localises in the cytoplasm in an inactive state. Here, Raf proteins are stabilised in a closed conformation by binding to 14-3-3 dimers. Upon activation through growth factors, dephosphorylation at the cell membrane by PP2A and PP1 phosphatases releases 14-3-3 from Raf thereby enabling Ras binding and membrane recruitment (51, 53, 65-67). However, due to the exchange of an arginine for a lysine at position 22 in its RBD and a non-conserved tyrosine 296 in the N-region, A-Raf is only weakly activated by oncogenic H-Ras(9, 68). In addition, several phosphorylation sites between amino acids 248 and 267 seem to facilitate A-Raf dissociation from the plasma membrane(69). This electrostatic destabilisation together with low kinase activity towards MEK seems to direct A-Raf to other subcellular compartments. We could demonstrate recently, that A-Raf localizes to the mitochondria in tumor cell lines as well as primary tumors, where it binds the proapoptotic kinase MST2 and suppresses its activation and MST2 induced apoptosis (16, 21). Here, we demonstrate that A-Raf localisation is regulated upon epithelial differentiation. While in highly proliferating cells of the basal cell layer
of non-malignant stratified, non-keratinized squamous epithelia, A-Raf localized to the cytoplasm and mitochondria, differentiated non-carcinogenic cells of head and neck epithelia expressed A-Raf at the plasma membrane. We observe a sharp demarcation between basal cells expressing A-Raf at the cytoplasm or mitochondria and the juxtaposed suprabasal cells with A-Raf at the plasma membrane. Our results suggest a thorough relocalisation of A-Raf, which correlates with morphological transition from undifferentiated stem cells to terminal differentiation. Intriguingly, in the cell models used, this spatial regulation of A-Raf correlates with its anti-apoptotic function. While cytoplasmic and mitochondrial A-Raf keeps anti-apoptotic signaling via MST2 in check, plasma-membrane standing A-Raf is not capable of MST2 binding and inactivation.

Interestingly, A-Raf expression and anti-apoptotic function is consistent with our previous reports that the splice factor hnRNP H controlling A-Raf splicing is highly expressed in basal cells but get lost during epithelial differentiation (70).

Furthermore, we could demonstrate that the scaffold protein KSR2 is crucial for the A-Raf-MST2 complex. KSR2 expression is lost during epithelial differentiation thereby releasing A-Raf to the plasma membrane leading to activation of MST2 and subsequent apoptosis. While previous reports demonstrated interaction between A-Raf and KSR2 only in response to TNFalpha (38), we observe interaction also in quiescent cells and upon stimulation with EGF. KSR2 is also known to bind AMPK mediating its stimulatory effects on glucose uptake and fatty acid oxidation (39) suggesting that A-Raf may play a direct role in the cross talk between apoptosis (MST2), energy homeostasis (AMPK), MAPK signaling and glucose metabolism by binding and inhibiting pyruvate kinase M2 (PKM2)(71, 72).
In addition to its functional role of regulating MST2-mediated apoptosis in differentiation, A-Raf expression also correlates with the extent of differentiation in the described MCF7 cell model. Here, overexpression of A-Raf independent of kinase activity increases differentiation, which is in line with an independent study from our group (63), where A-Raf overexpression inhibits the MST2/Hippo pathway thereby promoting HGF-induced EMT in MDCK cells. This is also in line with previous reports, where A-Raf expression correlated with adipocyte (23) and myogenic (24) differentiation.

While our results demonstrate a causal role and spatial regulation of A-Raf during epithelial differentiation, its target MST2 is stably localised in the cytoplasm. The MST2 tumour suppressor pathway is a master regulator of proliferation, cell death and cellular differentiation controlling normal tissue development, organ size and cancer (73-75). Activation of the MST2/Hippo pathway leads to the activation and cytoplasmic retention of the transcriptional coactivators YAP/TAZ, the subsequent formation junction formation in epithelial cells through interaction with members of the angiomotin (AMOT) family (76-78). The involvement of the Hippo/MST2 pathway in epithelial behaviour and downstream processes were recently reviewed in (79). MST2 itself and its closest isoform MST1 were shown to be crucial for 1,25-dihydroxyvitamin D₃-mediated monocytic differentiation of myeloid leukemia HL60 cells (42), control the downstream kinase YAP to control pancreatic acinar differentiation mice (80), regulate trophoblast differentiation via the transcription factor Mash2 in the placenta (81), and are required for embryonic stem cell differentiation (82). In addition, MST1/2 play a role in differentiation of hematopoietic and endothelial progenitor cells in Xenopus (83) and MST1 was identified as a crucial caspase-3 effector in myoblast differentiation (84).
For epithelial differentiation, proliferating cells of the basal cell layer withdraw from
the cell cycle, progressively differentiate towards the surface of the epithelium, flatten
and lose their nucleus, before they are finally shed from the surface. This commitment
and terminal differentiation also involves induction of the apoptotic machinery for con-
trolled caspase cleavage, enculeation, and internucleosomal DNA cleavage as demon-
strated in human keratinocyte differentiation (43-45). Relocalisation of A-Raf in our
model systems led to activation of MST2 and subsequent activation of Caspase 3,
cleavage of PARP and transcriptional activation of PUMA, suggesting a direct in-
volvement of A-Raf in epithelial differentiation.

In summary, our data and previous reports suggest that A-Raf is a crucial signalling
hub controlling cellular processes such as proliferation, apoptosis, and glucose metabo-
lism. In addition, our data suggest a potential link towards energy homeostasis control
via KSR2 and AMPK, which warrants further validation. The fine-tuning of such di-
verse signalling events is controlled by adjusting A-Raf expression levels in normal
cells and disease, alternative splice form selection, phosphorylation, and interaction
with specific proteins. Here, we describe for the first time how ARAF spatial regula-
tion through interaction with the scaffold protein KSR2 regulates MST2-mediated
apoptosis and differentiation.

Materials and Methods

Immunohistochemistry

Healthy tissue and carcinoma specimen were obtained during routine biopsy or surgery
after informed consent, snap frozen, cryopreserved, and processed to generate serial
sections (4 μm). Polyclonal goat anti-human A-Raf antibody (Santa Cruz Biotechnolo-
gy) was used for immunohistologic staining and antigen-antibody complexes were
visualized using the avidin-biotin-peroxidase method (85). Counterstaining was achieved with haematoxylin (blue).

**Cell lines**

HEK293 (human embryonic kidney 293 cells), NIH3T3 (mouse fibroblast cells), and MCF7 (human breast cancer cells) cells were cultured in standard DMEM containing 10% fetal calf serum (FCS).

**DNA constructs, siRNAs, Transfections**

For membrane targeted A-Raf (Flag-A-Raf-CAAX), human full-length A-Raf was fused N-terminal with a flag-tag and C-terminal with a CAAX motif (20 C-terminal amino acids of KRas4b) (48, 49) and inserted into Nhel/BamHI sites of pcDNA3.1(+)-hyg (Invitrogen).

For the rapalogue-induced recruitment of A-Raf to the plasma membrane we used plasmids containing the tandem FK506-binding protein domain (2xFKBP) and the mutant FRB domain (FRB) of the ARGENT Regulated Heterodimerization Kit provided by ARIAD (now: iDimerize Inducible Heterodimer System; Clontech) (55, 56). To generate the A-Raf recruitment fusion construct (FRB-A-Raf-flag), human full-length A-Raf was amplified and subcloned into SpeI/BamHI sites of pC4-RshE of the ARGENT Regulated Heterodimerization Kit (now: pHet-1, Clontech). As a plasma membrane target construct we used the plasmid pC4M-F2E of the original ARGENT Regulated Heterodimerization Kit (now: pHet-Mem1, Clontech) coding for an N-terminal myristoylated tandem FK506-binding protein domain (Myr.-2xFKBP-HA).

For Flag-A-Raf_{WT}, amplified human full-length A-Raf was fused N-terminal with a flag-tag and inserted into Nhel/BamHI sites of pcDNA3.1(+)-hyg (Invitrogen). Site-
directed mutagenesis was used to produce a kinase-dead version of A-Raf (K336M; Flag-A-Raf<sub>kd</sub>).

For EGFP-A-Raf, A-Raf was fused C-terminal to EGFP into XhoI/HindIII of pEGFP-C3 (Clontech). For V5-A-Raf, A-Raf from pDONR223-ARAF (Addgene Plasmid 23725; (86)) was fused N-terminal with a V5-tag of pcDNA6.2/V5-DEST using Gateway technology.

For transient knockdown of A-Raf expression, we used Silencer Validated siRNA 151 (Ambion, Austin, US) as described previously (21). For knockdown of KSR2 expression, ON-TARGETplus SMARTpool Human KSR2 (Dharmacon) was used.

Transient transfections were conducted with Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer’s instructions.

**Immunoblot analysis**

Protein lysates or immunoprecipitates were resolved by SDS PAGE (10–15%) and blotted on polyvinylidene difluoride membrane (Millipore). Protein visualization was performed using the following antibodies in combination with horseradish peroxidase–conjugated secondary antibodies and the enhanced chemiluminescence system (GE Healthcare): Polyclonal goat anti-human A-Raf antibody sc-30703 (Santa Cruz), monoclonal mouse anti-human A-Raf antibody (12C9, sc-100420)(Santa Cruz Biotechnology), polyclonal rabbit anti-human MST2 antibody (Stratagene), monoclonal rabbit anti-human MST2 (N terminus) antibody (Epitomics, Inc.), monoclonal mouse anti-flag antibody M2 (Sigma), monoclonal mouse anti-human tubulin antibody (Santa Cruz Biotechnology), monoclonal mouse anti-human alpha 1 Sodium Potassium ATPase (ATP1A1) antibody (plasma membrane marker) (Abcam), polyclonal rabbit
anti-human MEK-1/2 antibody (12-B, sc-436) (Santa Cruz Biotechnology), monoclonal rabbit anti-human phosphorylated Mst1 (pT183)/Mst2 (pT180) (Epitomics), monoclonal mouse anti-HA tag antibody 3F10 (Roche Diagnostics), polyclonal rabbit anti-human Puma antibody (Sigma), polyclonal rabbit anti-human caspase-3 antibody (Santa Cruz Biotechnology), monoclonal rabbit anti-human caspase-3 antibody (8G10; Santa Cruz Biotechnology), monoclonal mouse anti-human poly(ADP-ribose) polymerase (PARP) antibody (Becton Dickinson), polyclonal rabbit anti-human hnRNP H antibody, polyclonal rabbit anti-human mitogen-activated protein (MAP) kinase [extracellular signal-regulated kinase (ERK) 1 and ERK2] antibody (Sigma), monoclonal mouse anti-human MAP kinase activated (diphosphorylated ERK1 and ERK2) antibody (Sigma), monoclonal rabbit anti-GFP antibody (D5.1) (Cell Signaling Technology), monoclonal rabbit anti-human KSR1 antibody (Epitomics, Inc.), mouse monoclonal anti-human KSR2 antibody (K75, sc-100421) (Santa Cruz Biotechnology), polyclonal rabbit anti-human AKT antibody (Cell Signalling), polyclonal rabbit anti-human activated Akt (phosphorylated at Ser473) antibody (Cell Signalling), and monoclonal mouse anti-human Caspase 8 (1C12) (Cell Signaling Technology)

**Immunoprecipitation**

Immunoprecipitations were conducted as described previously (21) with the following immobilized antibodies: Monoclonal mouse anti-HA tag antibody 3F10 (Roche Diagnostics), monoclonal mouse anti-flag antibody M2 (Sigma), polyclonal goat anti-human MST2 antibody sc-6211 (Santa Cruz), polyclonal goat anti-human A-Raf antibody sc-30703 (Santa Cruz), monoclonal mouse anti-V5 tag antibody (Invitrogen).

**Apoptosis Assays**
Apoptosis levels were determined as described previously (16) by measuring subgenomic DNA.

**Subcellular fractionation**

Subcellular fractionation of cytoplasmatic and plasma membrane-associated proteins was conducted with Subcellular Protein Fractionation Kit for Cultured Cells (Thermo Scientific) according to the manufacturer’s instructions.

**Cell Differentiation Assay**

For differentiation of MCF7 breast carcinoma cells, we used a modified protocol of a published method (62). MCF7 cells were seeded in standard 24-well plates (0.4x10^6/well) and serum-starved (1% FCS) 24 hours prior to stimulation with either 10nM Epidermal Growth Factor (EGF) (#11376454001, Roche) or 2nM recombinant Human NRG1-beta 1/Heregulin1-beta 1 EGF Domain (Heregulin) (#396-HB-050/CF, R&D). Following stimulation, media were changed after 2-3 days, and cells grown for 5-6 days, before fixation with 10% paraformaldehyde in Phosphate Buffered Saline (PBS) for at least 1 hour. Cells were washed once with water, once with 60% isopropanol, and stained with Oil Red O solution for one hour followed by three washes with water. Oil Red O (Sigma-Aldrich) stock solution was prepared by dissolving dye in isopropanol (0.35% w/v), and filtered through 0.22 µm filter and kept at room temperature. For cell staining, Oil Red stock solution was mixed with water in 6:4 ratio, and filtered through 0.22 µm filter one hour after precipitates were formed. Lipid accumulation in MCF7 cells was quantified by extraction of the Oil Red dye with 100% isopropanol and measuring light absorbance of extracted dye at 500 nm by a standard plate reader.
**Focus Formation Assays**

Focus assays were conducted as described previously (21). NIH 3T3 cells were transfected with Lipofectamine (Invitrogen) and allowed to grow to confluence. The plates were incubated for 21 days, fixed, stained with Giemsa, and the foci were counted.

**Immunofluorescence and confocal microscopy**

HeLa cells were plated on coverslips and co-transfected the next day with FRB-A-Raf-flag and Myr.-2xFKBP-HA. 24 h post transfection, cells were treated with 500nM AP21967 for 1 h and fixed with 4% paraformaldehyde for 10 min at room temperature. After fixation, cells were permeabilized with 0.1% Triton X-100 in PBS/1% BSA for 2 min. Nonspecific binding was blocked by incubation with PBS/1% BSA for 30 min at room temperature. Incubation with flag antibody (M2, 1:200) (Sigma Aldrich) was done at room temperature for 1 h followed by extensive washes in PBS. Coverslips were incubated with Alexa-488-conjugated secondary antibody (Molecular Probes) for 30 min followed by several washes in PBS. The coverslips were mounted on glass slides using Vectashield containing DAPI. Cells were imaged on a Zeiss confocal laser-scanning microscope (LSM 510 META). Images were acquired following excitation with 364 and 488 lasers followed by 385-470 and 505-530 nm BP (Band Pass) filters for DAPI and Alexa488, respectively, with a Plan-Neoflur 40X/0.5 or 63X NA objective.

MCF7 cells stably expressing either EGFP or A-Raf-EGFP were imaged on a Zeiss confocal laser-scanning microscope (LSM 510 META). Images were acquired following excitation with the 488 laser followed by 505-530 nm BP (Band Pass) filters for EGFP, respectively, with a Plan-Neoflur 40X/0.5 or 63X NA objective.
Statistical analysis

Significance levels were determined by two-tailed Student t-test analyses.

Supplementary Materials

Fig. S1. A-Raf localizes to the plasma membrane during epithelial differentiation.

Fig S2. A-Raf localisation does not influence proliferation, MAPK signaling dynamics, and oncogenic transformation.

Fig. S3. Rapalogue-mediated dynamic re-localization of A-Raf.

Fig S4. Stable overexpression of A-Raf does not influence proliferation and MAPK/AKT signaling dynamics.

Fig S5. Expression of the scaffold KSR2 is lost during Heregulin-mediated differentiation of MCF7 cells.

References


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Figure Legends

Figure 1: A-Raf localizes to the plasma membrane during epithelial differentiation.

(A) A-Raf expression was detected in carcinoma tissues of HNSCC, and (B), non-malignant stratified, non-keratinized squamous epithelia of the same tissue origin. Immunohistochemistry was performed on cryosections of human tissue specimens.

Figure 2: Plasma membrane-localized A-Raf loses its ability to inhibit MST2-mediated apoptosis.
(A, B) HeLa cells were transfected with either wildtype A-Raf or A-Raf fused with the CAAX motif from KRas4b.

(C) Subcellular fractionation demonstrated the localisation of A-Raf and MST2 by western blot. MEK1/2 and ATPase were used as subcellular markers for the cytoplasm (C) and membrane (M) fractions, respectively.

(D) Flag-tagged A-Raf or Raf-1 constructs were expressed in HeLa cells. Following serum-starvation or stimulation with FCS, protein complexes were immunoprecipitated with anti-flag antibody, and proteins were detected by immunoblotting with anti-flag or anti-MST2 antibodies. Tubulin served as a protein loading control.

(E) Equal amounts of flag-tagged A-Raf constructs were expressed in HeLa cells. Following serum-starvation, lysates were probed for the indicated proteins by immunoblotting.

(F) Equal amounts of flag-tagged A-Raf constructs were expressed in HeLa cells as in (E). Following serum-starvation and treatment with 10µM etoposide 24 h post transfection, the proportion of apoptotic cells was assessed on measuring DNA fragmentation by flow cytometry 72 h post transfection. Error bars indicate SD of 3 individual experiments.

Figure 3: Dynamic re-localization of A-Raf induces MST2-mediated apoptosis.

(A,B) For rapalogue-induced recruitment of A-Raf to the plasma membrane, HeLa cells were co-transfected with the A-Raf recruitment fusion construct (FRB-A-Raf-flag) and myristoylated tandem FK506-binding protein domain (Myr.-2xFKBP-HA).

(C) 24 h post transfection, cells were treated with the 500nM AP21967 as indicated for 1h, and subcellular fractionation demonstrated the localisation of A-Raf, MST2,
and FKBP by western blot. MEK1/2 and ATPase were used as subcellular markers for the cytoplasm (C) and membrane (M) fractions, respectively.

(D) HeLa cells were co-transfected with FRB-A-Raf-flag and Myr.-2xFKBP-HA and 24 h post transfection, cells were treated with the 500nM AP21967 as indicated. Protein complexes were isolated with anti-HA, anti-flag, or anti-MST2 antibodies, respectively, and proteins were detected by immunoblotting with anti-HA, anti-flag, or anti-MST2 antibodies. Tubulin served as a protein loading control.

(E) HeLa cells were co-transfected with FRB-A-Raf-flag and Myr.-2xFKBP-HA and 24 h post transfection, cells were treated with the 500nM AP21967 and 10µM etoposide as indicated. Protein complexes were isolated 24 h later with anti-flag antibody and proteins detected by immunoblotting with anti-HA and anti-flag antibodies. Lysates were probed for expression of PUMA, MST2, activated MST2 (Thr180), activated ERK1/2, PARP and Caspase 3 cleavage. Tubulin served as a protein loading control.

Figure 4: A-Raf relocalizes to the plasma membrane during mammary differentiation.

(A) Heregulin-mediated differentiation of MCF7 cells. MCF7 cells were seeded in standard 24-well plates (0.4x10^6/well) and serum-starved (1% FCS) 24 hours prior to stimulation with either 10nM EGF or 2nM Heregulin. After 6 days, cells were fixed and stained with Oil Red. Shown are bright field images of indicated cells.

(B, C) MCF7 cells were seeded in standard 24-well plates (0.4x10^6/well) and serum-starved (1% FCS) 24 hours prior to stimulation with either 10nM EGF or 2nM
Heregulin. After 6 days, A-Raf fused to EGFP or EGFP alone were visualised in live cells using confocal microscopy. Scale bar, 20μm

**D** Cells from (A) were lysed and probed for expression of A-Raf, EGFP, activated ERK1/2, MST2, and activated MST2 using western blot. ERK1/2 served as a protein loading control. * endogenous A-Raf, ** EGFP-A-Raf

**Figure 5: Re-localization of A-Raf during mammary differentiation induces MST2-mediated apoptosis.**

(A) MCF7 cells stably expressing EGFP-A-Raf or EGFP alone were seeded in standard 24-well plates (0.4x10⁶/well) and serum-starved (1% FCS) 24 hours prior to stimulation with either 10nM EGF or 2nM Heregulin. After 6 days, subcellular fractionation demonstrated the localisation of A-Raf-EGFP, EGFP, and MST2 by western blot. MEK1/2 and ATPase were used as subcellular markers for the cytoplasm (C) and membrane (M) fractions, respectively. * endogenous A-Raf, ** EGFP-A-Raf

(B) MCF7 cells were seeded in standard 24-well plates (0.4x10⁶/well) and serum-starved (1% FCS) 24 hours prior to stimulation with either 10nM EGF or 2nM Heregulin. After 6 days, subcellular fractionation demonstrated the localisation of endogenous A-Raf and MST2 by immunoblot. MEK1/2 and ATPase were used as subcellular markers for the cytoplasm (C) and membrane (M) fractions, respectively.

(C) MCF7 cells stably expressing EGFP-A-Raf or EGFP alone were seeded in standard 24-well plates (0.4x10⁶/well) and serum-starved (1% FCS) 24 hours prior to stimulation with either 10nM EGF or 2nM Heregulin. After 6 days, lysates were probed for expression of A-Raf, MST2, activated MST2 (pMST2), activated ERK1/2, and cleavage of Caspase 8 by western blot. * endogenous A-Raf, ** EGFP-A-Raf
Figure 6: A-Raf induces Heregulin-mediated differentiation of MCF7 cells.

(A) MCF7 cells stably expressing EGFP-A-Raf or EGFP alone or control cells were seeded in standard 24-well plates (0.4x10^6/well), and serum-starved (1% FCS) 24 hours prior to stimulation with either 10nM EGF or 2nM Heregulin. After 6 days, cells were fixed and stained with Oil Red, which was extracted with isopropanol, and quantified measuring light absorbance. Error bars indicate SD of 4 individual experiments.

(B) MCF7 cells stably expressing EGFP-A-Raf or EGFP alone were seeded in standard 24-well plates (0.4x10^6/well), co-transfected with either control siRNA or human A-Raf siRNA as indicated, and serum-starved (1% FCS) 24 hours prior to stimulation with either 10nM EGF or 2nM Heregulin. After 6 days, cells were fixed and stained with Oil Red, which was extracted with isopropanol, and quantified measuring light absorbance. Error bars indicate SD of 4 individual experiments.

(C) MCF7 cells were seeded in standard 24-well plates (0.4x10^6/well), transiently transfected with control plasmid (pcDNA3), wild type A-Raf or a kinase-dead version of A-Raf (KD), and serum-starved (1% FCS) 24 hours prior to stimulation with either 10nM EGF or 2nM Heregulin. After 6 days, cells were fixed and stained with Oil Red, which was extracted with isopropanol, and quantified measuring light absorbance. Error bars indicate SD of 4 individual experiments.

Figure 7: The scaffold Kinase Suppressor of Ras 2 (KSR2) regulates A-Raf localization and MST2 activity.
(A) V5-tagged A-Raf was expressed in MCF7 cells. Following serum-starvation or stimulation with FCS, protein complexes were immunoprecipitated with anti-V5 antibody, and proteins were detected by immunoblotting with anti-V5, anti-MST2, anti-KSR1, and anti-KSR2 antibodies. Tubulin served as a protein loading control.

(B) MCF7 cells were seeded in standard 24-well plates (0.4x10^6/well) and serum-starved (1% FCS) 24 hours prior to stimulation with either 10nM EGF or 2nM Heregulin. After 6 days, lysates were probed for expression of hnRNPH, A-Raf, KSR1, and KSR2. ERK1/2 served as a protein loading control.

(C) Left panel - MCF7 cells were transfected with either control siRNA or KSR2 siRNA as indicated and probed for A-Raf, MST2, pMST2, KSR1, and KSR2 by western blot. Tubulin served as a protein loading control. Middle panel - Upon transfection with either control siRNA or human KSR2 siRNA, endogenous A-Raf was immunoprecipitated and proteins detected by immunoblotting with anti-A-Raf and anti-MST2 antibodies. Right panel - Upon transfection with either control siRNA or human KSR2 siRNA, subcellular fractionation demonstrated the localisation of endogenous A-Raf and MST2 by immunoblot. MEK1/2 and ATPase were used as subcellular markers for the cytoplasm (C) and membrane (M) fractions, respectively.

(D) V5-tagged KSR2 was expressed in MCF7 cells in combination with siRNA-mediated knock-down of A-Raf. Following serum-starvation, protein complexes were immunoprecipitated with anti-V5 antibody, and proteins were detected by immunoblotting with anti-V5, anti-MST2, and anti-ARAF antibodies.

(E) Hypothesis and model for KSR2-mediated regulation of the A-Raf-MST2 complex. In tumour cells and highly proliferating normal cells, high levels of KSR2 ensure mitochondrial expression of A-Raf to keep MST2 in an inactive state thereby
providing an anti-apoptotic milieu. In contrast, in differentiated normal cells, KSR2 expression is lost leading to A-Raf localisation at the membrane, disruption of the complex with MST2, and induction of MST2-mediated apoptosis.
Figure 1

A

B

stratum granulosum

stratum spinosum

stratum basale

surface
Figure 2

A. Diagram showing constitutive membrane and mitochondrial localization of A-Raf WT and A-Raf CAAX.

B. Constructs:
- Flag A-Raf
- Flag A-Raf CAAX

C. Western blot analysis of A-Raf WT (flag) and A-Raf CAAX (flag) with markers for flag, MST2, ATPase, MEK1/2, lysate.

D. Western blot analysis of IP: flag, lysate, showing comparisons between A-Raf WT (flag), A-Raf CAAX (flag), Raf-1 (flag), and Ctrl.

E. Western blot analysis of A-Raf WT (flag) and A-Raf CAAX (flag) with markers for p-MST2, total MST2, flag, Tubulin.

F. Graph showing the percentage of apoptotic cells (sub-G1) with different treatments: A-Raf WT, A-Raf CAAX, Etoposide, and Ctrl.
Figure 3

A

2xFKBP
A-Raf_FB
AP21967
transient
membrane

2xFKBP
A-Raf_FB
AP21967

mitochondria

B Constructs

Myr. 2xFKBP HA
FRB A-Raf flag

C

+ + + - - A-Raf_FB (flag)
+ + + - - Myr.-2xFKBP (HA)
- - + - - AP21967

flag
MST2
HA
MEK1/2
ATPase

D

+ + - A-Raf_FB (flag)
+ + - Myr.2xFKBP (HA)
- + - AP21967

IP: MST2
A-Raf_FB (flag)
MST2

IP: flag
MST2
Myr.-(FKBP)^2 (HA)
A-Raf_FB (flag)

IP: HA
A-Raf_FB (flag)
Myr.-(FKBP)^2 (HA)
MST2

lysat
A-Raf_FB (flag)
Myr.-(FKBP)^2 (HA)
Tubulin

E

+ + + - - A-Raf_FB (flag)
+ + + - - Myr.-2xFKBP (HA)
- - + + + Etoposide

IP: flag
A-Raf_FB (flag)
Myr.2xFKBP (HA)
Tubulin

IP: flag
cleaved Casp.-3
PARP
pMST2 (Thr180)
tot. MST2
Myr.-2xFKBP (HA)
A-Raf_FB (flag)
Figure 4

A

Ctrl.  EGF  HRG

B

Ctrl.  EGF  HRG

EGFP

Ctrl.  EGF  HRG

A-Raf-EGFP

C

HRG

A-Raf-EGFP

D

+  -  -  EGFP  EGFP-A-Raf

** A-Raf

-  -  A-Raf

-  -  EGFP

-  -  EGFP

25  50  75  100

p-MST2

50  75  100

tot-MST2

50  75  100

ERK1/2

37  50  75  100
Figure 7

A

IP: V5

KSR2
KSR1
MST2
V5

lysate

KSR2
KSR1
MST2
V5

Tubulin

0.1 10

% FCS

A-Raf (V5)

B

KSR2
KSR1
A-Raf
hnRNPH
tot ERK1/2

EGF

HRG

C

- +

KSR2 siRNA

KSR2
KSR1
pMST2
MST2
A-Raf
Tubulin

lysate

- +

KSR2 siRNA

MST2
A-Raf

IP: A-Raf

IP: isotype

D

tumour and proliferating normal cells

KSR2

A-Raf

MST2

apoptosis

differentiated normal cells

KSR2

A-Raf

MST2

apoptosis