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c-Myc regulates RNA splicing of the A-Raf kinase and its activation of the ERK pathway

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Abstract

A-Raf kinase can inhibit apoptosis by binding to the pro-apoptotic MST2 kinase. This function relies on expression of hnRNP H, which ensures the correct splicing of a-raf mRNA needed to produce full-length A-Raf protein. Here we show that expression of hnRNP H and production of full-length A-Raf is positively controlled by c-Myc. Low c-Myc reduces hnRNP H expression and switches a-raf splicing to produce A-Rafshort, a truncated protein. Importantly, A-Rafshort fails to regulate MST2 but retains the Ras binding domain such that it functions as a dominant negative mutant suppressing Ras activation and transformation. Human colon and head and neck cancers exhibit high hnRNP H and high c-Myc levels resulting in enhanced A-Raf expression and reduced expression of A-Rafshort. Conversely, in normal cells and tissues where c-Myc and hnRNP H are low, A-Rafshort suppresses ERK activation such that it may act as a safeguard against oncogenic transformation. Our findings offer a new paradigm to understand how c-Myc coordinates diverse cell functions by directly affecting alternate splicing of key signaling components.

Keywords

hnRNP H; c-Myc; A-Raf; splicing; MST2; Ras

Introduction

The family of Raf protein kinases, which comprises A-Raf, B-Raf, and Raf-1, is at the apex of the three-tiered Raf-MEK-ERK/MAPK pathway that regulates many fundamental cellular functions, including proliferation, differentiation, transformation, apoptosis and metabolism (1). Raf kinase activation is initiated by binding to activated Ras GTPases at the cell membrane, which triggers a complex series of activation events that comprise interactions with proteins and lipids as well as coordinated de-phosphorylation and phosphorylation events (2, 3). A-Raf is the least studied member of the Raf kinase family (4). In general, A-Raf seems to be regulated similar to Raf-1, with binding to activated Ras initiating the

growth factor induced activation of A-Raf. However, A-Raf is a poor MEK kinase with barely measurable catalytic activity, which is due to unique non-conserved amino acid substitutions in the N-region (5). Independent of kinase activity, A-Raf constitutively binds mammalian sterile 20-like kinase (MST2) and suppresses MST2 activation and induced apoptosis (6).

The ERK pathway is frequently activated in cancer, often due to activating mutations in Ras (7, 8) or B-Raf (9, 10). By contrast, Raf-1 is rarely mutated (11), and to date no oncogenic A-Raf mutations were found. However, elevated A-Raf expression levels have been observed in a number of malignancies including astrocytomas (12), pancreatic ductal carcinoma (13), angioimmunoblastic lymphadenopathies (14), head and neck squamous cell carcinomas, and colon carcinomas (6, 15).

One way to regulate protein expression and activity is alternative splicing. For B-Raf several different splice forms are known. Two variable exons, 8b and 10, allow for the generation of four distinct isoforms (16, 17). While the presence of exon 10 enhances the basal kinase activity and affinity to MEK, exon 8b has the opposite effect (16). Thyroid carcinomas express B-Raf splice variants that lack the N-terminal auto-inhibitory domain resulting in constitutively active B-Raf variants, suggesting that alternative splicing regulation is a pathophysiological mechanism for oncogenic B-Raf activation (18). An alternative Raf-1 splice form lacking exon 3 was reported in lung cancer, however the functional consequences are unknown (19). Recently, two alternative murine A-Raf splice forms were described, DA-Raf1 and DA-Raf2, which contain the Ras binding domain (RBD) but lack the kinase domain due to pre-terminal stop codons (20, 21). DA-Raf1 and DA-Raf2 bind to activated Ras, but due to the lack of a kinase domain cannot transduce a signal and act as dominant-negative antagonists of the Ras-ERK pathway. Thus, DA-Raf1 is a positive regulator of myogenic differentiation by inhibiting activation of the ERK pathway (20). In another cellular environment, DA-Raf2 binds and colocalizes with the ARF6 GTPase on tubular endosomes and acts as dominant negative effector of endocytic trafficking (21). We recently reported that expression of the full length A-Raf protein requires the expression of the splice factor hnRNP H, which is upregulated in several tumours including colon and head and neck cancers (6, 15) We showed that hnRNP H upregulation ensures the expression of the mature *a-raf* mRNA thus allowing the sufficient production of full-length A-Raf protein to counteract MST2-mediated apoptosis.

Here, we report that *hnRNP H* is a direct transcriptional target of c-Myc, which stimulates its expression. The proto-oncogenic transcription factor c-Myc is a key regulator of various cellular processes such as cell growth, proliferation, apoptosis, and differentiation (22, 23). Recent studies suggest that c-Myc regulates about 15% of all annotated genes by direct transcriptional activation (24, 25). Deregulated and elevated expression of *c-myc* has been shown for a wide range of cancers and it is estimated that c-Myc is involved in 20% of all human cancers (26). We show that hnRNP H maintains the expression of full-length A-Raf protein by suppressing alternative splicing of the *a-raf* mRNA. This novel splice form, A-Raf_{short}, incorporates intronic sequences, and generates a 171 amino acid protein, which lacks the kinase domain. While A-Raf_{short} fails to regulate MST2-mediated apoptosis, it is a potent inhibitor of ERK signaling and cellular transformation by binding and blocking activated Ras. A-Raf_{short} expression levels were reduced in several cancer entities, suggesting that A-Raf_{short} acts like a tumour suppressor protein in these tumours.

Materials and Methods

Cell lines

HeLa, GHD-1, HCT116, and NIH3T3 cells were cultured in standard DMEM containing 10% fetal calf serum (FCS). Cell lines were either purchased from Cancer Research UK or ATCC and were authenticated by the European Collection of Cell Cultures (ECACC). GHD-1 is a self-established cell line from a hypopharynx HNSCC tumour (27).

Transfections

Transient transfections were conducted with Lipofectamine 2000 reagent (Invitrogen, Paisley, UK) or the Nucleofector system (Lonza Cologne, Cologne, Germany) according to the manufacturers' instructions.

Focus Assays

Focus assays were conducted as described previously (28). Briefly, NIH 3T3 cells were transfected with Lipofectamine (Invitrogen) and allowed to grow to confluence. The plates were incubated for 12-15 days. Then, cells were fixed, stained with Giemsa, and the foci were counted.

Semi-quantitative RT-PCR

RNA from human tissues was isolated using the RNeasy 24 cell lysis system (Bertin Technologies, Montigny-le-Bretonneux, France). Total RNA from tissues and cell lines was isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany) and cDNA was generated using the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen, Paisley, UK) according to the manufacturers' instructions.

Immunoprecipitations

Immunoprecipitations were conducted as described previously (6) with the following immobilized antibodies: Monoclonal mouse anti-HA tag antibody 3F10 (Roche Diagnostics, Mannheim, Germany), monoclonal mouse anti-flag antibody M2 (Sigma, Taufkirchen, Germany), polyclonal goat anti-human MST2 antibody sc-6211 (Santa Cruz, Santa Cruz, US), monoclonal mouse anti-human Ras antibody sc-29 (Santa Cruz, Santa Cruz, US).

MST2-kinase activity assay

MST2 kinase activity was measured by in-gel assays as described before (29).

Apoptosis assays

Apoptosis was determined as described previously (6) by measuring subgenomic DNA.

Statistical analysis

Significance levels were determined by two-tailed Student t-test analyses. Due to the non-normal distribution of the expression analysis data (RT-PCR), results are given as the median with the interquartile range (IQR). For comparison of hnRNP H and A-Raf isoform expression between sample groups, we used the Wilcoxon signed-rank test. All tests were two-sided and results considered significant if $p < 0.05$.

Results

HnRNP H regulates A-Raf isoform selection

We reported recently, that the splice factor hnRNP H is necessary for the proper expression of the mature A-Raf mRNA (6). Here we show that, when hnRNP H is depleted a novel, alternatively spliced A-Raf mRNA species appears at the expense of the mature mRNA.

Depletion of hnRNP H decreased the levels of mature A-Raf mRNA and full-length protein levels, while causing the appearance of a new mRNA species, which yielded a larger PCR product (Fig. 1A). Sequencing revealed that introns two and four of the *a-raf* gene were included while introns one and three were spliced out (Figure S1 and S2). This alternative *a-raf_{short}* mRNA encodes 171 amino acids that are only partially related to the A-Raf_{wt} protein sequence due to the intronic inclusions. PANTHER database entries for the full-length generic and the alternative mRNAs/proteins are hCT20300/hCP44398 and hCT2257035/hCP1885829, respectively. The cognate A-Raf_{short} protein lacks the C-terminal two-thirds of A-Raf_{wt} including the kinase domain because of the presence of a stop codon at nucleotide position 716 in intron 4 (Fig. S2). Pre-terminated mRNAs are commonly prone to nonsense-mediated decay (32). However, endogenous A-Raf_{short} protein was detectable (Fig. 1B) suggesting a physiological function for A-Raf_{short}. Furthermore, downregulation of hnRNP H caused a reduction in the expression of the full length A-Raf_{wt} protein with a concomitant increase in the expression of the A-Raf_{short} protein (Fig. 1B), confirming the results of the mRNA expression at the protein level. Pre-incubation of primary antibodies with the A-Raf_{short} peptide used for immunisation resulted in a complete loss of detection by the A-Raf_{short}⁻, but not by the HA-specific antibody (Fig. 1C).

Alternative splice variants of *a-raf* differ in function

Full-length A-Raf prevents apoptosis by sequestering and inactivating the proapoptotic kinase MST2 (6). In contrast, A-Raf_{short} did not interact with flag-tagged MST2 or endogenous MST2 (Figs. 2A,B). Consequently, A-Raf_{short} was neither able to suppress endogenous MST2 kinase activity (Fig. 2C), nor apoptosis in response to hnRNP H knock-down, as measured by the percentage of cells with a subG1 DNA content, the cleavage of PARP and caspase 3 (Fig. 2D).

A-Raf_{short} negatively regulates the Ras-ERK pathway

The truncated A-Raf_{short} contains the Ras-binding domain (RBD) including novel amino acids derived from intronic sequences but lacks the S/T rich domain and the kinase domain. This structure suggested that A-Raf_{short} might act as dominant-negative Ras antagonist. Overexpression of A-Raf_{short} in HeLa, GHD-1, and HCT116 cancer cells (Fig. 3A), reduced cell numbers (Fig. 3A), possibly by inhibition of Ras-ERK signalling. In serum-stimulated HeLa cells overexpression of A-Raf_{short} leads to a decrease in pERK levels 3-fold while having no effect in quiescent cells (Fig. 3B-C, Fig. 4A). In contrast, phosphorylation levels of Akt were unchanged after A-Raf_{short} overexpression (Fig. 3C) suggesting that A-Raf_{short} selectively antagonizes Ras-ERK signalling leaving Ras-PI3K-Akt signalling unaffected. While increased expression of A-Raf_{short} led to a decrease in ERK activity, depletion of A-Raf_{short} using an isoform-specific siRNA had the opposite effect, i.e. increasing activating phosphorylation levels of ERK (Fig. 3D). In serum-stimulated HeLa cells, knockdown of A-Raf_{short} increased pERK by 30%, while having no effect in quiescent cells (Fig. 3D).

A-Raf_{short} interacts with Ras and antagonises Ras transformation

In order to act as a Ras antagonist, A-Raf_{short} should interact with activated Ras. We transfected HeLa cells with A-Raf_{short} or full length A-Raf (A-Raf_{WT}) and performed co-immunoprecipitations showing that both proteins interact with Ras in serum-stimulated cells

(Fig. 4A). The same results were obtained with endogenous A-Raf_{short} and Ras (Fig. 4B). Furthermore, experiments where different amounts of A-Raf_{short} and A-Raf_{WT} were co-transfected, showed that A-Raf_{short} efficiently competes with A-Raf_{WT} for binding to activated Ras (Fig. 4C). These data confirm that A-Raf_{short} acts as a physiological negative regulator of Ras-ERK signalling.

Oncogenic Ras is a well-described activator of Ras-mediated ERK signalling and induces transformation in mouse fibroblasts (33, 34). As A-Raf_{short} interacted with activated Ras, and abrogated ERK activation, we asked whether oncogenic Ras-induced transformation was inhibited by A-Raf_{short}. NIH3T3 cells were transfected with activated Ras mutants (H-RasV12, K-RasV12, N-RasV12) and co-transfected with A-Raf_{short} and tested for the ability to generate foci of transformed cells (Fig. 4C,D). A-Raf_{short} significantly decreased foci numbers with all three Ras isoforms suggesting that A-Raf_{short} can inhibit transformation by all three Ras members. In order to prove that A-Raf_{short} is acting directly on Ras and not on downstream effectors, NIH3T3 cells were co-transfected with A-Raf_{short} and the viral Raf oncogene (vRaf), which lacks the Ras binding domain and transforms cells independently of Ras (33). A-Raf_{short} had no significant effect on v-Raf induced foci numbers, showing that A-Raf_{short} is inhibiting activated Ras and not the downstream kinase Raf (Fig. S3A, B). We also tested whether the function of A-Raf_{short} differs from other known A-Raf splicing isoforms. In colony formation assays, daRaf1 and daRaf2, like A-Raf_{short}, significantly decreased foci numbers with all three Ras isoforms suggesting that these isoforms have overlapping functions (Fig. S3C, D).

c-Myc regulates A-Raf isoform selection via hnRNP H

hnRNP H is over expressed in several carcinoma entities and regulates *a-raf* splicing (6). We asked therefore, which process is responsible for the expression of hnRNP H. As hnRNP H was found as a target gene of the proto-oncogene c-Myc in microarray experiments (24) we tested this hypothesis experimentally in more detail. Depleting c-Myc from HeLa cells using specific siRNAs reduced hnRNP H protein expression levels and, in parallel, decreased levels of A-Raf_{WT} and increased levels of A-Raf_{short} (Figure 5A). Increased cell confluence triggered a similar response, i.e. decreasing levels of c-Myc, hnRNP H, and A-Raf_{WT} while increasing levels of A-Raf_{short} (Fig. S4). These results suggest that a concerted response of c-Myc, hnRNP H and A-Raf isoform expression is part of the physiological programme how cells respond to different growth conditions. To ascertain that this response was coordinated by c-Myc, we transfected HeLa cells with MycER^T (35). MycER^T is a chimeric protein where c-Myc is fused to a mutated ligand binding domain of the human estrogen receptor. MycER^T is retained in the cytoplasm due to the ER portion binding to Hsp90. Addition of the estrogen analog 4-hydroxytamoxifen (4-OHT) releases MycER^T and triggers its translocation to the nucleus and activation of Myc-induced transcription. In the absence of 4-OHT, expression levels of hnRNP H, A-Raf_{WT}, and A-Raf_{short} were constant over a timecourse of 8 hrs. Upon addition of 4-OHT, hnRNP H and A-Raf_{WT} levels increased, while A-Raf_{short} expression decreased (Figure 5B). Additional knock-down of hnRNP H using specific siRNAs abrogated this effect indicating that c-Myc is regulating A-Raf isoform selection via control of hnRNP H expression. Endogenous activation of c-Myc by EGF stimulation corroborated these results (Figure 5C). While expression levels of hnRNP H, A-Raf_{WT}, and A-Raf_{short} remained stable in starved cells, activation of c-Myc led to increased hnRNP H and A-Raf_{WT} expression, but decreased A-Raf_{short} expression.

Data from the ENCODE project (31) suggested, that c-Myc binds to three regions in the HNRNP1 promoter (Fig. S5). We could identify four putative, non-canonical E-Boxes 957bp (CATGTG), 949bp (CACATG), 530bp (CAGCTG), and 63bp (CAGCTG) upstream of the transcription start site, which coincide with the ChIP-Chip data from ENCODE in six different cell lines (Fig. S5). In order to determine direct interaction of c-Myc with the

HNRNPH1 gene we performed a chromatin immunoprecipitation (ChIP) analysis of the human HNRNPH1 promoter. ChIP indicated that c-Myc was constitutively present at three E-Boxes of the HNRNPH1 promoter region (957bp, 949bp, 530bp) in both serum-starved and stimulated cells. However, c-Myc was recruited to the E-box nearest to the transcription start site only in stimulated cells (Fig. 5D). Collectively, our results show that hnRNP H is a direct target of the proto-oncogene c-Myc and that c-Myc regulates the ERK pathway via hnRNP H and subsequent regulation of A-Raf isoform expression.

The A-Raf_{short} isoform is downregulated in carcinomas

The expression of the proto-oncogene c-Myc is elevated in a plethora of human tumours (22). Additionally, hnRNP H and A-Raf_{WT} were shown to be overexpressed in several carcinoma entities including head and neck carcinomas and colon carcinomas (6). Therefore, we asked if A-Raf_{short} and other A-Raf isoforms are regulated during carcinogenesis and whether their expression is dependent on the expression of the upstream regulators c-Myc and hnRNP H. To this end, endogenous mRNA expression levels of *c-myc*, *hnrnpH*, *a-raf_{wt}*, and *a-raf_{short}* were analysed in a series of human head and neck carcinomas (T, n=17) and adjacent non-malignant tissues (N, n=14) by semi-quantitative RT-PCR (Fig. 6A). At the single-patient level, a median relative expression of 2.3-fold for *c-myc*, 1.7-fold for *hnrnpH*, 1.5-fold for *a-raf_{wt}*, and 0.5-fold for *a-raf_{short}* in tumour specimens was calculated, indicating that *c-myc*, *hnrnpH* and *a-raf_{wt}* are over expressed in carcinomas. In contrast, *a-raf_{short}* appears to be downregulated in tumours.

Additionally, after stratifying patients according to their relative expression levels of *c-myc*, *hnRNP H*, *a-raf_{wt}*, and *a-raf_{short}* mRNA in tumour tissue, a significant number of patients showed high expression of *c-myc* ($\chi^2=7.0$, $p=0.082$), *hnRNP H* ($\chi^2=17.3$, $p=0.0001$) and *a-raf_{wt}* ($\chi^2=24.1$, $p=0.0001$) in tumours while at the same time showing a significant downregulation of *a-raf_{short}* ($\chi^2=7.0$, $p=0.008$).

Comparing the relative expression in normal and tumour tissues (Fig. 6B, Table 1) we found that in tumour tissues the expression of *c-myc* (2.5-fold), *hnRNP H* (1.9-fold), and *a-raf_{WT}* (1.3-fold) was significantly higher than in normal tissues. In contrast, *a-raf_{short}* expression in tumours was significantly downregulated compared to normal tissues (1.6-fold decrease). Pearson's correlation (Fig. 6C) showed a significant correlation between *c-myc/hnrnpH* ($r^p=0.8$; $p<0.001$) and *hnrnpH/a-raf_{wt}* ($r^p=0.7$; $p<0.009$).

Additionally, we assessed endogenous mRNA expression levels of *c-myc* and *a-raf_{short}* in a series of 29 human Dukes B colon carcinomas and autologous adjacent non-malignant tissues by semi-quantitative RT-PCR (Fig. 6B, Table 1). Similar to the results observed in head and neck carcinomas, at the single-patient level a median relative overexpression of 6.4-fold for *c-myc* and 0.85-fold for *a-raf_{short}* was observed in tumour specimens. Furthermore, comparing the relative expression in normal and tumour tissues, *c-myc* expression was significantly higher in tumours than in normal tissue. In contrast, *a-raf_{short}* expression was significantly downregulated in tumours compared to normal. Importantly, we observe similar trends in the corresponding A-Raf protein isoform levels, in a limited number of autologous tissue samples from head and neck carcinomas (n=3). While A-Raf_{short} protein is downregulated in carcinomas by trend ($p=0.08$), expression of A-Raf_{WT} prevails in carcinomas ($p=0.02$) (Fig. S6).

The other two human A-Raf isoforms, daRaf1 and daRaf2, were found to be barely detectable at the mRNA level in the human tissues investigated (semi-quantitative RT-PCR, 1/89 samples, data not shown). Expression of these isoforms was only investigated in mouse tissues and their expression/regulation in human tissues is not known.

Discussion

Alternative splicing occurs in more than 90% of genes (36), and is considered as a key regulatory process by which a common pre-mRNA transcript leads to different mature RNAs, thus producing diverse and even antagonistic functions (37). This greatly expands information content and versatility of the transcriptome generating tissue, stage, and development specific gene expression patterns (38). Tumour suppressors are often inactivated by splicing in cancer, whereas oncogenes are inactivated by alternative splicing during normal differentiation (39, 40). Components of the splicing machinery, such as hnRNP proteins and other RNA binding proteins, have been found altered in tumours and can contribute to cancer cell survival and invasiveness (41).

We showed previously that the splicing factor hnRNP H is overexpressed in colon and head and neck cancers, and promotes the correct splicing of the *a-raf* mRNA that encodes the wildtype full-length A-Raf protein, which binds to and inhibits the pro-apoptotic kinase MST2 (6). In this previous work we also showed that overexpression of A-Raf_{WT} can overcome the effects of siRNA-mediated knockdown of hnRNP H. hnRNP H can regulate alternative splicing of Bcl-X (42) and a neuron-specific variant of Src (43). In both cases, hnRNP H binds to G-rich RNA stretches (44), similar to sequences found in the intron sequences included in A-Raf_{short}. Although hnRNP H favours the production of the pro-apoptotic Bcl-X_s isoform (42), this splicing event does not prevent the A-Raf mediated rescue from apoptosis resulting from hnRNP H overexpression (6).

A-Raf_{short} differs from DA-Raf1 and 2, which encompasses the uninterrupted RBD and adjacent cysteine rich domain. DA-Raf1 and 2 were not expressed in head and neck tissues and in human colon specimens except for one out of 89 samples (data not shown). This fits the current understanding of alternative splicing as usually only two isoforms of a given number of potential isoforms are predominant at the same time in a given tissue (36). Intron inclusion is a rare event in alternative splicing (36) and, in combination with pre-terminal stop codons, these transcripts are commonly prone to nonsense-mediated decay (32). However, sequence-specific Northern blotting showed that *a-raf_{short}* mRNA exists in normal human tissues such as brain, placenta, kidney, pancreas, lung, and spleen (data not shown). The resulting A-Raf_{short} protein is expressed both in cultured cells and in human tissues at low but stable levels, which is consistent with recent findings that such pre-terminated mRNAs typically express low levels of protein (45).

Functionally, A-Raf_{short} inhibits ERK pathway activation by competing for binding to activated Ras. This was un-expected, as intron 2 is inserted into the RBD between glutamine 29 (66 in Raf-1) and lysine 47 (84 in Raf-1), which together with arginine 52 (89 in Raf-1) form a functional epitope in Raf-1 that determines the affinity to Ras (46). However, the binding of A-Raf_{short} to Ras was GTP dependent and of similar affinity as full-length A-Raf, suggesting a functionally relevant interaction. The functionality of this interaction was corroborated by the finding that A-Raf_{short} behaved as a dominant negative mutant, which suppressed Ras mediated transformation and ERK activation. In contrast, A-Raf_{short} cannot bind to and inhibit MST2 pro-apoptotic signalling. Our finding that the expression of A-Raf_{short} is reduced in cancers correlating with the overexpression of hnRNP H and increased expression of full-length A-Raf protein also suggest that alternative *a-raf* splicing is a pathophysiological mechanism that tumours use to evade apoptosis.

We show that one mechanism regulating hnRNP H levels is via the proto-oncogene c-Myc. As suggested by data from the ENCODE project (31) we show that c-Myc binds directly to a non-canonical E-box in the hnRNP H1 gene promoter in a mitogen-dependent way. Knockdown of c-Myc decreased levels of hnRNP H and subsequent A-Raf_{short} splice form

selection. Activation of c-Myc had the opposite effect. Interestingly, c-Myc also stimulates the expression of other members of the hnRNP family, hnRNP A1, hnRNP A2, and hnRNP I (47). These hnRNP proteins promote the alternative splicing of pyruvate kinase resulting in the expression of the embryonic isoform, PKM2, which is almost universally re-expressed in cancer, and stimulates aerobic glycolysis (48). Thus, c-Myc can regulate two splice events via hnRNP proteins that enhance survival via A-Raf mediated MST2 inhibition and switch metabolism to the aerobic glycolysis typical for cancer cells. Additional crosstalk between these two pathways may exist at the protein level as A-Raf was reported to bind to and regulate PKM2 function (49).

In summary, we propose the following working hypothesis (Fig. 6D): In tumour cells high levels of c-Myc elevate expression of the splice factor hnRNP H shifting the balance of *a-raf* mRNA splicing in favour of producing the full-length A-Raf protein, which is crucial to keep pro-apoptotic MST2 signalling in check. In normal cells c-Myc levels are low, resulting in reduced hnRNP H expression and a shift of *a-raf* splicing towards A-Raf_{short} with the dual effect of relieving repression of MST2 and reducing ERK pathway activity due to Ras blockade by A-Raf_{short}.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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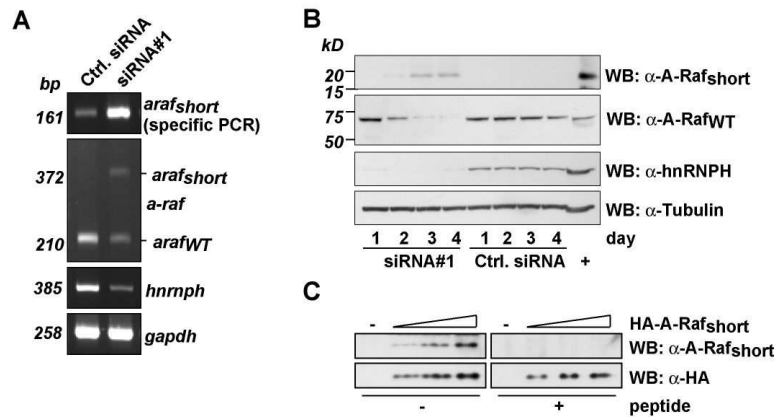


Figure 1: The A-Rafshort isoform expression is induced after hnRNP H knockdown.

(A) *a-raf_{short}*, *a-raf_{WT/short}*, and *hnRNP H* mRNA expression in HeLa cells was analyzed by RT-PCR after transfection with control or hnRNP H-specific siRNA (siRNA#1). Shown is a representative result from two independent experiments. (B) A-Raf_{short}, A-Raf_{WT}, and hnRNP H protein expression was analysed by immunoblotting after transfection with control or hnRNP H-specific siRNA (siRNA#1). As a positive control for A-Raf_{short}, a lysate containing transfected recombinant HA-tagged A-Raf_{short} was used. Shown are the representative results from two independent experiments. (C) **Pre-adsorption of polyclonal A-Raf antibodies with peptides used for immunisation abrogated binding of A-Raf but not HA-specific antibody.** Increasing concentrations of HA-tagged A-Raf_{short} was expressed in HeLa and analysed by immunoblotting using HA- or A-Raf N-terminus specific antibodies. The A-Raf N-terminus specific antibody but not the HA-antibody signal was blocked by adding the A-Raf N-terminal peptide as competitor, showing that the detection of the A-Raf_{short} isoform to be specific. Shown are the representative results from two independent experiments.

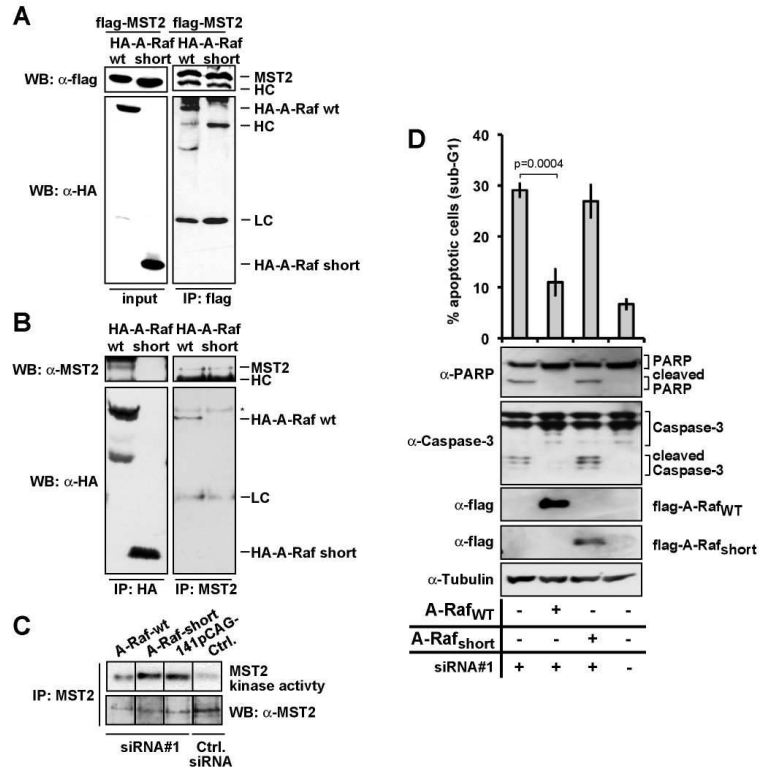


Figure 2: The A-Rafshort protein does not interact with MST2.

(A) Flag-tagged MST2 together with HA-tagged A-Raf_{wt} or A-Raf_{short}, respectively, were expressed in HeLa cells. MST2 and A-Raf isoforms were immunoprecipitated (IP) using flag-tag specific antibodies. Flag-MST2 and A-Raf_{wt}, but not the short isoform A-Raf_{short}, co-precipitated as shown by immunoblotting (WB). Shown are the representative results from three independent experiments. (B) HA-tagged A-Raf_{wt} or A-Raf_{short} were expressed in HeLa cells. Endogenous MST2 and the HA-tagged A-Raf isoforms were immunoprecipitated (IP) with HA-tag or MST2 specific antibodies. MST2 and A-Raf_{wt}, but not A-Raf_{short}, co-precipitated. Shown are the representative results from three independent experiments. (C) **A-Raf_{wt} but not A-Raf_{short} can suppress MST2 kinase activity.** HeLa cells were transfected with hnRNP H (siRNA#1) and control siRNA. Where indicated, HA-A-Raf_{wt}, HA-A-Raf_{short}, or control expression plasmids were co-transfected. MST2 kinase activity was assessed by in-gel kinase assays. (D) **A-Raf_{wt} but not A-Raf_{short} can suppress apoptosis induced by hnRNP H depletion.** Apoptosis was determined in HeLa cells following transfection with hnRNP H-siRNA. Where indicated, cells were co-transfected with A-Raf_{wt}, A-Raf_{short} expression plasmids. The data represent mean percentage of apoptosis with SD of three experiments. Additionally, cells were lysed, and expression of flag-tagged A-Raf, Caspase-3, and PARP was assessed by immunoblotting (lower panels).

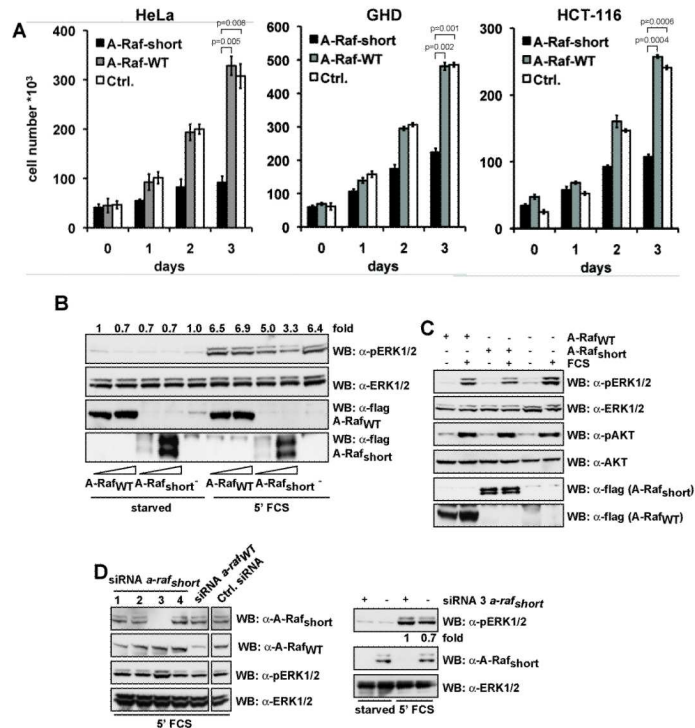


Figure 3: A-Rafshort inhibits Ras-ERK signalling.

(A) Numbers of HeLa, GHD-1, and HCT-116 cells were determined in a time kinetic following overexpression of A-Raf_{short} or A-Raf_{WT}. Results represent the mean with standard deviation (SD) of three independent experiments. (B) **A-Raf_{short} inhibits phosphorylation of ERK.** HeLa cells were transfected with increasing amounts of A-Raf_{short} or A-Raf_{WT}. Cells were serum-starved for 16 hours (0.1% FCS) or serum-stimulated (10% FCS) for 5min after starvation as indicated. Lysates were immunoblotted for expression of the transfected flag-tagged Raf isoforms, phospho-ERK1/2, and ERK1/2 as loading control. (C) **A-Raf_{short} inhibits ERK phosphorylation without affecting AKT phosphorylation.** HeLa cells were transfected with either A-Raf_{short} or A-Raf_{WT}. Cells were serum-starved for 16 hours (0.1% FCS) or serum-stimulated (10% FCS) for 5min after starvation as indicated. Lysates were immunoblotted for expression of the transfected flag-tagged Raf isoforms, phospho-ERK1/2, ERK1/2, phospho-AKT, and AKT. (D) **A-Raf_{short} siRNA#3 specifically downregulates the A-Raf_{short} isoform and increases ERK phosphorylation.** (left panel) HeLa cells were transfected with four different siRNAs for A-Raf_{short} or A-Raf_{WT}. Cells were serum-starved for 16 hours and serum-stimulated for 5min. Lysates were immunoblotted for expression of A-Raf_{short}, A-Raf_{WT}, phospho-ERK1/2, and ERK1/2. Shown are the representative results from two independent experiments. (right panel) HeLa cells were transfected with either A-Raf_{short} siRNA#3 or control siRNA. Cells were serum-starved for 16 hours (0.1% FCS) or serum-stimulated (10% FCS) for 5min after starvation as indicated. Lysates were immunoblotted for expression of A-Raf_{short}, phospho-ERK1/2, and ERK1/2.

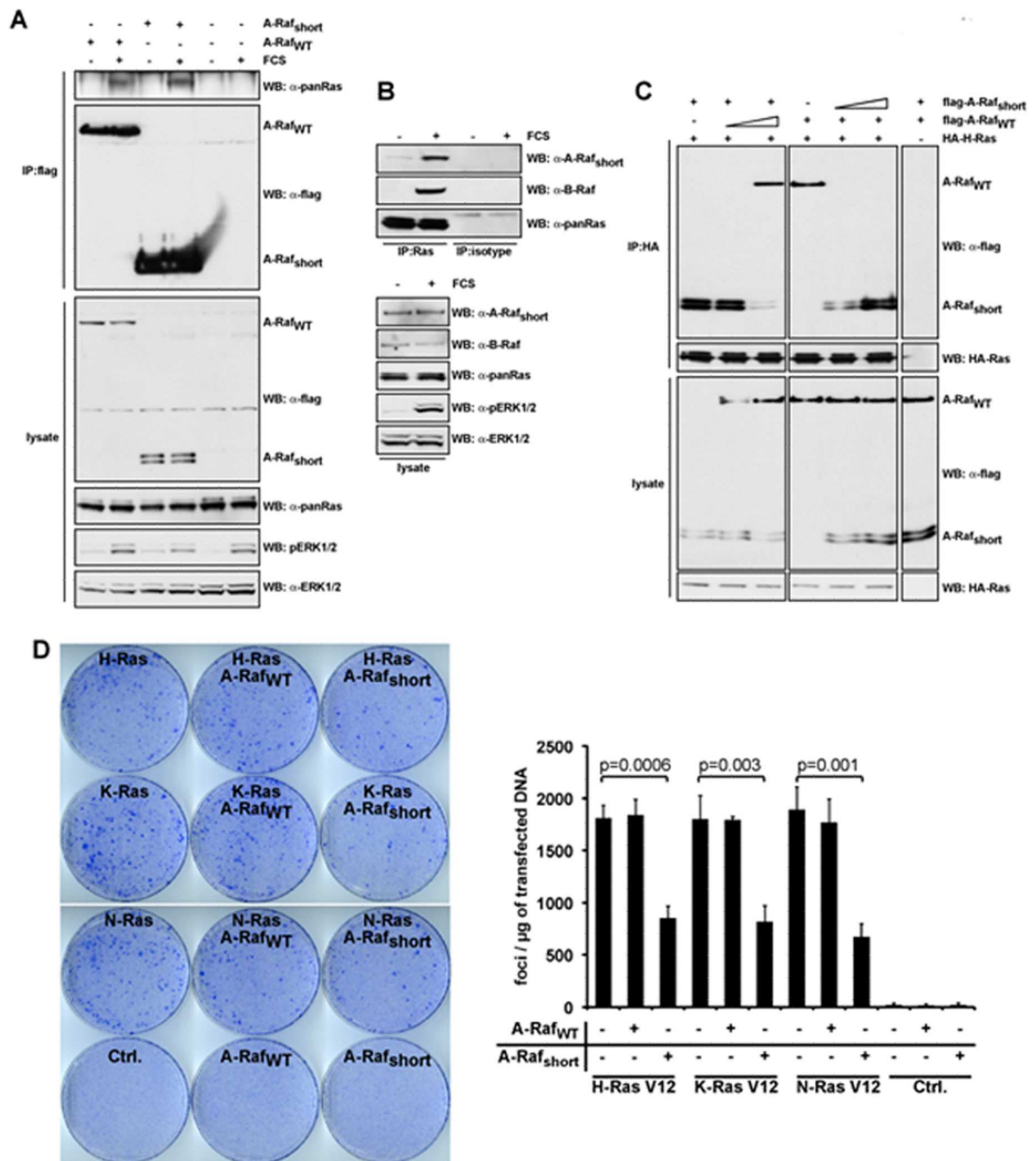


Figure 4: A-Rafshort interacts with activated Ras inhibiting Ras-ERK signalling. (A) Flag-tagged A-Raf_{short} or A-Raf_{WT} were transfected in HeLa cells. Cells were serum-starved for 16 hours (0.1% FCS) or serum-stimulated (10% FCS) for 5min after starvation as indicated. A-Raf isoforms were immunoprecipitated with flag-tag specific antibodies, and analysed by immunoblotting for the interaction with Ras. (B) Endogenous Ras was immunoprecipitated from lysates of HeLa cells, which had been serum starved (0.1% FCS, 16 hours) or treated with full medium (10% FCS) after serum-starvation using a specific antibody for Ras, and analysed by immunoblotting (upper panel). As an isotype control, an antibody specific for GFP was used (middle panel, 'Isotype'). Lysates were immunoblotted

for expression of A-Raf_{short}, Ras, B-Raf, phosphorylated ERK1/2, and ERK1/2 as loading control (lower panel). LC - light chain immunoglobulin. Shown are the representative results from three independent experiments. **(C) A-Raf_{short} binds better to Ras.** Either fixed or increasing amounts of Flag-tagged A-Raf_{short} or A-Raf_{WT} together with HA-tagged H-Ras-V12 were transfected in HeLa cells as indicated. Cells were serum-starved for 16 hours (0.1% FCS) and serum-stimulated (10% FCS) for 5min after starvation. Ras complexes were immunoprecipitated, and analysed by immunoblotting for the A-Raf isoform interaction. **(D) A-Raf_{short} inhibits Ras-induced cellular transformation.** NIH3T3 fibroblasts were transfected with hyperactive Ras constructs. Cells were co-transfected with either A-Raf_{WT} or A-Raf_{short} as indicated (left panel). Numbers indicate average number of foci per microgram of DNA (Ras constructs) (right panel). Error bars represent standard deviation of at least three independent experiments.

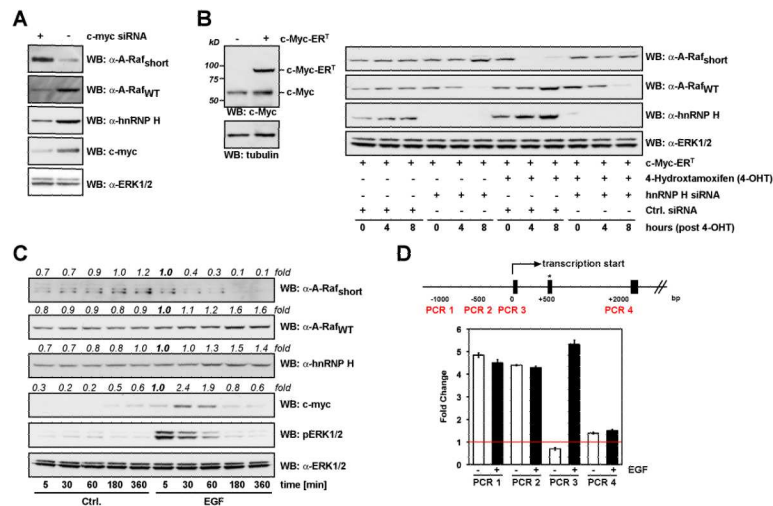


Figure 5: c-Myc regulates hnRNP H expression.

(A) HeLa cells were transfected with either c-myc siRNA or control siRNA. Lysates were immunoblotted for expression of c-Myc, hnRNP H, A-Raf_{WT} or A-Raf_{short}, and ERK1/2 using specific antibodies. (B) HeLa cells were transfected with the 4-hydroxytamoxifen (4-OHT)-inducible chimeric MycER^T, hnRNP H siRNA, or Control siRNA as indicated. Following transfection, cells were serum-starved for 16 hours (0.1% FCS) and MycER^T induced with 4-OHT (100nM). 0, 4, and 8 hours post induction, cells were lysed and lysates immunoblotted for expression of the hnRNP H, A-Raf_{WT} or A-Raf_{short}, and ERK1/2 as loading control. (C) HeLa cells were serum-starved for 18 hours (0.1% FCS) and stimulated with EGF (10nM) as indicated. 5, 30, 60, 180, and 360min post stimulation, cells were lysed and lysates immunoblotted for expression of the c-Myc, hnRNP H, A-Raf_{WT}, A-Raf_{short}, phospho-ERK1/2, and ERK1/2 as loading control. (D) **c-Myc binds to promoter elements of the human HNRNP H1 genomic locus.** c-Myc Chromatin IPs in either serum-starved (18hrs, 0.1% FCS) or EGF-stimulated HeLa cells were analyzed using semi-quantitative Real-time PCR. PCR primer pairs correspond to elements in the human hnRNP H promoter region as depicted. The red line indicates no change in c-Myc-specific enrichment. Shown are the representative results from three independent experiments, error bars represent standard deviations.

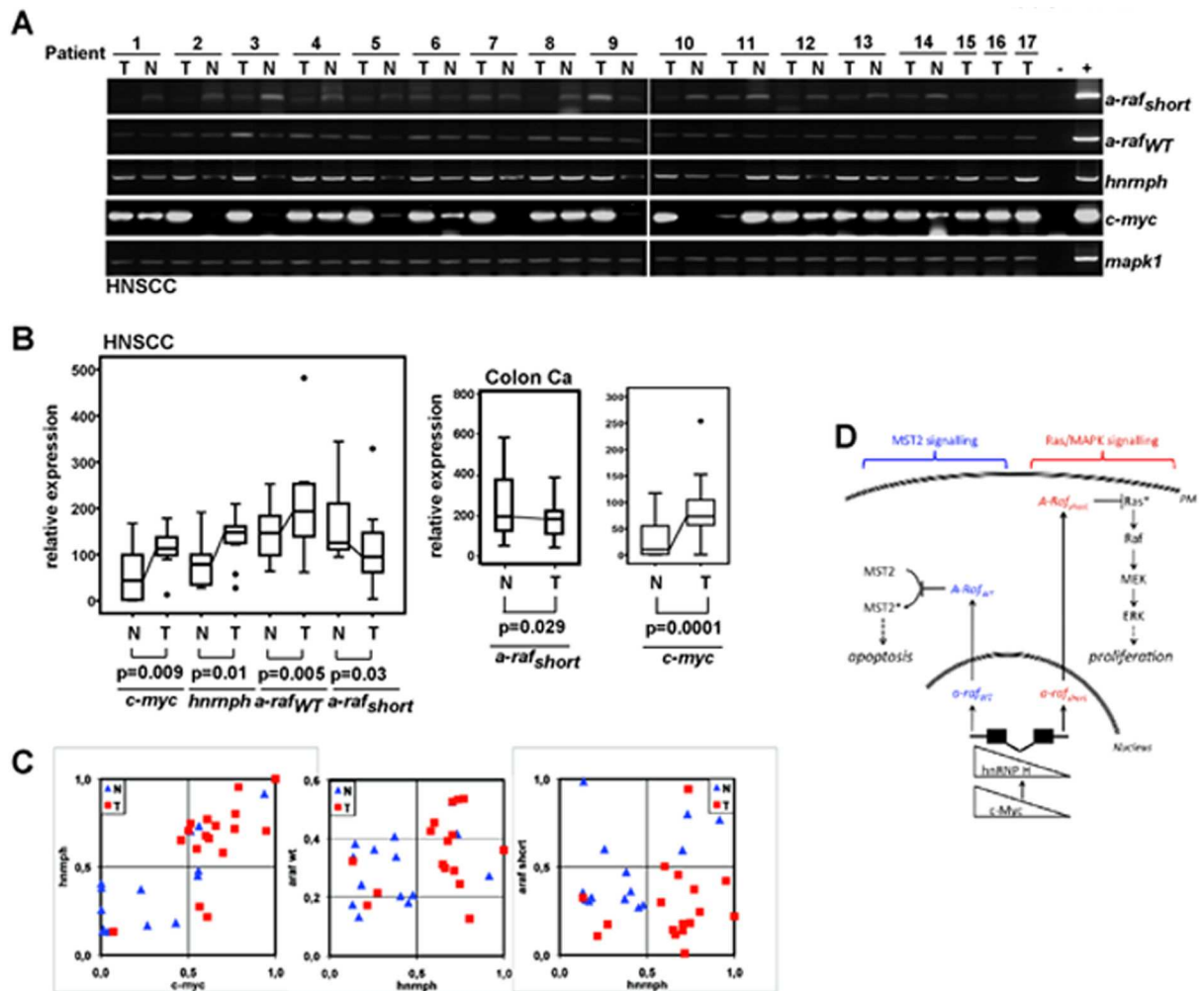


Figure 6: Expression of *c-myc*, hnRNP H, and *a-raf* isoforms in carcinomas and adjacent normal tissues.

(A) The expression of *a-raf_{short}*, *a-raf_{WT}*, *hnRNP H*, and *c-myc* mRNA was assessed in head and neck (HNSCC) tumours (T) and normal adjacent tissues (N) by semi-quantitative RT-PCR. *Mapk1* mRNA levels were determined as a loading control. (B) Relative expression of *c-myc*, *hnRNP H* and *a-raf* isoforms in head and neck tissues (left panel) and in colon carcinomas (n=29) and adjacent normal tissues (n=29) (right panel) according to disease state. Shown are boxplots where boxes indicate the median (line) and IQR, whiskers show the range, and asterisks indicate outliers. (C) Scatterplots are used to illustrate the degree of correlation between *c-myc*/*hnRNP H*, *hnRNP H*/*a-raf_{WT}*, and *hnRNP H*/*a-raf_{short}* relative expression in tumours (red) and normal tissues (blue). (D) **Hypothesis and model for hnRNP H-mediated A-Raf isoform selection.** In normal cells, low levels of the proto-oncogene *c-Myc* and the splice factor hnRNP H ensure increased A-Raf_{short} levels thus keeping proliferation via MAPK signalling in check. Tumour cells show higher expression of both *c-Myc* and hnRNP H thereby enabling sufficient A-Raf_{WT} expression and keeping MST-2 mediated apoptosis in check. Additionally, A-Raf_{short} is downregulated in tumour cells enabling increased proliferation.

Table 1
Relative expression of *c-myc*, *hnRNP H*, *a-raf_{wt}*, and *a-raf_{short}* mRNA in normal and tumour tissues

Shown are the medians with interquartile range (IQR). Fold-change is the median of the tumour tissue expression relative to the corresponding normal tissue.

Tissue	RT-PCR	Normal Tissue	Tumour Tissue	Fold-change	p-value
HNSCC	<i>c-myc</i>	44 [IQR 2-98]	109 [IQR 98-137]	2.5	0.009
	<i>hnrnph</i>	79 [IQR 36-99]	147 [IQR 126-156]	1.9	0.01
	<i>araf_{wt}</i>	147 [IQR 99-181]	173 [IQR 140-218]	1.3	0.005
	<i>araf_{short}</i>	126 [IQR 111-210]	76 [IQR 50-130]	0.6	0.03
Colon	<i>c-myc</i>	30 [IQR 8-64]	62 [IQR 52-73]	2.1	0.0001
	<i>araf_{short}</i>	224 [IQR 157-398]	213 [IQR 144-252]	0.8	0.029