HHS Public Access

Author manuscript

Semin Cell Dev Biol. Author manuscript; available in PMC 2017 October 01.

Published in final edited form as:

Semin Cell Dev Biol. 2016 October ; 58: 60–69. doi:10.1016/j.semcdb.2016.07.012.

The role of wild type RAS isoforms in cancer

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Abstract

Mutationally activated RAS proteins are critical oncogenic drivers in nearly 30% of all human cancers. As with mutant RAS, the role of wild type RAS proteins in oncogenesis, tumour maintenance and metastasis is context-dependent. Complexity is introduced by the existence of multiple RAS genes (*HRAS*, *KRAS*, *NRAS*) and protein "isoforms" (KRAS4A, KRAS4B), by the ever more complicated network of RAS signaling, and by the increasing identification of numerous genetic aberrations in cancers that do and do not harbour mutant RAS. Numerous mouse model carcinogenesis studies and examination of patient tumours reveal that, in RAS-mutant cancers, wild type RAS proteins are likely to serve as tumour suppressors when the mutant RAS is of the same isoform. This evidence is particularly robust in KRAS mutant cancers, which often display suppression or loss of wild type KRAS, but is not as strong for NRAS. In contrast, although not yet fully elucidated, the preponderance of evidence indicates that wild type RAS proteins play a tumour promoting role when the mutant RAS is of a different isoform. In non-RAS mutant cancers, wild type RAS is recognized as a mediator of oncogenic signaling due to chronic activation of upstream receptor tyrosine kinases that feed through RAS. Additionally, in the absence of mutant RAS, activation of wild type RAS may drive cancer upon the loss of negative RAS regulators such as NF1 GAP or SPRY proteins. Here we explore the current state of knowledge with respect to the roles of wild type RAS proteins in human cancers.

Keywords

RAS; wild type; isoform; RASGAP; mutant; cancer

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1. Introduction

Due in part to the prevalence of oncogenically activating mutations in RAS, it has often been assumed that wild type RAS alleles do not contribute significantly to RAS-mediated oncogenesis or tumour maintenance. Several factors have revealed this assumption to be overly simplistic and rendered it demonstrably false. First, both mouse model chemical carcinogenesis studies and the development of genetically engineered mouse models of cancer driven by mutationally activated RAS alleles have focused attention on the roles of the wild type counterparts in the presence of mutant RAS proteins (e.g., wild type KRAS in the presence of mutant KRAS). Second, renewed attention is now being paid to the fact that the term "RAS" represents not a single protein but three distinct genes (KRAS, NRAS and HRAS) and 4 distinct proteins (KRAS4A, KRAS4B, NRAS and HRAS) has directed attention to the roles of wild type RAS isoforms other than the mutated RAS allele (e.g., wild type KRAS in the presence of mutant NRAS). Third, amplification of RAS is being "rediscovered" as a potentially key cancer driver, along with the notion that gene dosage and protein expression levels have significant consequences that may differ at tumour initiation versus progression. Fourth, the increasing emphasis on cancer signaling as a complex network rather than as a series of partially connected linear pathways, and the continuing identification of new genetic aberrations via deep sequencing of patient tumours, have redirected attention to the critical role of RAS activity in cancers driven by the loss of negative regulators of RAS such as NF1 and other RAS GAPs, and the negative regulatory Sprouty (SPRY) family proteins.

1.1 RAS proteins are small GTPases that are deregulated in cancer

RAS proteins, the founding members of the RAS superfamily of small GTPases [1], are located at the center of a highly complicated signaling network that controls many aspects of fundamental normal cellular processes, including cell differentiation, survival and proliferation [2, 3]. RAS proteins are also key contributors to human oncogenesis [3–5]. In particular, point mutations that result in chronic activation of RAS are found in ~30% of all human cancers. Normally, RAS proteins are positively regulated by guanine nucleotide exchange factors (GEFs) such as SOS1 that facilitate exchange of GDP for GTP to promote the active state [6], and negatively regulated by GTPase activating proteins (GAPs) such as p120GAP and NF1 that catalyze hydrolysis of GTP back to GDP and thus promote restoration of the resting state [7]. Oncogenic mutations typically render RAS proteins relatively independent of the GEFs and/or insensitive to the GAPs that modulate the functions of wild type RAS. RAS activity can also be enhanced in cancers by amplification of oncogenic or wild type RAS and by escape of wild type RAS from regulation following gain of upstream inputs such as receptor tyrosine kinases (RTKs) or GEFs, or loss of downstream negative regulators such as GAPs or of feedback from SPRY/SPRED proteins.

1.2 RAS proteins exist as multiple isoforms

The human genome is home to three RAS genes (HRAS, NRAS, and KRAS), which together encode four RAS proteins (HRAS, NRAS, splice variants KRAS4A and KRAS4B). *HRAS*, located on chromosome 11, was the first isoform characterized in human cancers, and studies of this isoform dominated RAS research for many years [2]. The initial

underlying assumption was that RAS proteins are functionally redundant and thus interchangeable, which turned out to be an oversimplification that resulted in numerous wrong turns in the RAS field [4]. *KRAS*, residing on chromosome 12, encodes two splice variants at the 4th exon, KRAS4A and KRAS4B, the latter of which has long been regarded as the major isoform expressed in human cells. However, a recent study showed that KRAS4A can be expressed at levels comparable or near to that of KRAS4B [8], implying a need to consider both of these differentially regulated KRAS proteins. A third RAS isoform, not previously identified in any retrovirus studies, came to light a year after *KRAS*, and was designated *NRAS* (chromosome 1), because it was discovered in human neuroblastoma-derived DNA [2]. These proteins share an overall 82–90% amino acid sequence identity, in which the regions required for nucleotide binding and effector interactions are essentially identical (Figure 1A). However, they differ significantly in the hypervariable region at their C-termini (Figure 1A), which are critical for their respective lipid modifications, and thus determine their distinct membrane binding and trafficking kinetics [9]. Each RAS isoform thus shares overlapping but distinct localizations at the plasma membrane and on endomembranes [10]. This allows RAS engagement of different pools of activators and effectors, contributing to isoform-specific signaling properties [11, 12].

1.3 RAS signaling pathways form a complex network subject to feedback regulation

RAS proteins transmit signals from a vast variety of inputs through numerous downstream effector pathways. The best-characterized of these, which have well-validated contributions to RAS pro-proliferation, -survival and -metastasis functions in cancer, are the canonical RAF-MEK-ERK and PI3K-AKT-mTOR kinase cascades, as well as the RAL-RALGEF and TIAM1-RAC small GTPase cascades [4, 5]. Detection of RAF-MEK-ERK and/or PI3K-AKT-mTOR activity is often used as a surrogate for "RAS pathway activation" in cancers, whether or not they harbour mutant RAS. In addition, the RASSF family of tumour suppressors are RAS effectors that directly link RAS to pro-apoptotic pathways [13] as well as to a plethora of other functions important in cancer (see Donninger, Clark et al., this issue). These are not linear, independent pathways, but rather a complex network of interconnected, scaffolded nodes, with both positive and negative feedback loops. Key negative feedback mechanisms, such as the DUSP MAPK phosphatases and Sprouty negative regulators, are described in section 3.3.

1.4 RAS isoforms are functionally distinct

Although it has been challenging to reproducibly identify isoform- and mutation-dependent signaling differences directly in human tumours as has been done in model cell lines, typically by ectopic expression of mutant RAS [14–16], numerous studies have repeatedly demonstrated that RAS proteins are not all created equal [10, 17–23] (and many others). Although highly similar in structure, wild type RAS isoforms carry out overlapping but still very distinct functions [17, 20]. For example, genetic knockout studies in mice suggested that KRAS is the most important RAS isoform during development, since *Kras*-ablated mice die during embryogenesis [24, 25]. And although replacement of *Hras* into the *Kras* locus led to live births at the expected Mendelian ratio, the adult mice displayed cardiac abnormalities [26], indicating that HRAS was unable to completely replace KRAS function. In contrast, *Nras* gene function was shown to be dispensable for normal mouse development,

growth, and fertility [27], yet was later found to be important for antiviral immune response and T-cell function in mice [28]. Abrogration of *Hras* did not result in any developmental defects [29], but reduced the numbers of papillomas formed after 12-O-tetradecanoylphorbol-13-acetate (TPA) treatment compared with wild type littermates [30]. Strikingly, *Hras*(−/−)/*Nras*(−/−) double knockout mice were viable, and displayed normal growth, fertility, and neuronal development [29]. Nevertheless, both wild type NRAS and KRAS were required for SV40 T Ag-induced transformation in mouse embryonic fibroblasts [18], during which they performed unique functions by engaging different signaling pathways. Specifically, wild type NRAS regulated cell adhesion through RAF and RhoA, whereas KRAS coordinated cell motility through AKT and Cdc42 [18].

1.5 RAS isoforms are oncogenically mutated and/or amplified in human cancers in distinct patterns

Among the evidence that the three RAS genes are distinct from each other are the biased frequency and distributions of RAS mutations in human cancers. Analysis of the COSMIC database (http://cancer.sanger.ac.uk/cosmic) shows (Figure 1B) that KRAS is by far the isoform most frequently mutated (85%) across human cancers. whereas NRAS is the second most frequently mutated (11%) and HRAS the least (4%). Another bias is the preferential mutation of a specific RAS isoform in a given tumour type [4]. For example, although rare in cancers overall, HRAS is the predominantly mutated isoform in bladder cancer (56% of all RAS mutations) and in head and neck squamous cell carcinoma (85%). NRAS, although rarely found in the pancreatic ductal adenocarcinomas or lung adenocarcinomas where KRAS mutations predominate (100% and 96%, respectively), is the major oncogenic RAS isoform in cutaneous melanoma (95%) and acute myeloid leukemia (59%). A third bias is the codon-specific mutation signature of each RAS isoform [31]. Most RAS mutations (98%) occur at the hotspots of codons G12, G13, or Q61 (Figure 1B). Thus, when KRAS is oncogenically mutated, both the KRAS4B and KRAS4A splice variant proteins expressed from the mutant allele are mutant. The vast majority (83%) of KRAS mutations occur at G12, whereas 63% of NRAS mutations occur at Q61 (Figure 1B). Yet Q61 mutations account for nearly 90% of all NRAS mutant cases of cutaneous melanoma, whereas G12 mutations are more prominent in hematopoietic cancers. It is presently unknown whether the roles of wild type RAS proteins are related to particular RAS mutations, although there is some evidence that this may be the case [32, 33]; see section 2.1.1.

As discussed further in section 3.1, to induce and maintain a tumour, RAS activity must be optimally regulated so as to provide sufficient RAS signaling for transformation but not so much as to prompt oncogene-induced senescence [33–38]. Thus, RAS expression is sometimes differentially regulated by amplification, by mRNA stability, and even by altered translation due to codon bias [39]. As described in section 3.1, amplification of the wild type RAS isoforms is distinctly distributed across tumour types (cBioportal.org) [40, 41]. These observations are strong evidence for distinct functionalities of RAS isoforms and support the idea that wild type isoforms may also play distinct roles in RAS-mutant and RAS wild type cancers.

2. Wild type RAS isoforms in RAS mutant cancers: tumour suppressors or tumour promoters?

The interplay between oncogenic and wild type RAS isoforms greatly affects tumour development and maintenance, and metastasis. Studies of wild type RAS can be divided into two broadly distinct categories: 1) those that assess the wild type counterpart of the oncogenic RAS isoform, and 2) those that assess the remaining two wild type RAS isoforms in the presence of the oncogenic isoform. The results of such studies have led to very distinct conclusions regarding the roles of wild type RAS isoforms in the context of mutant RAS, examples of which are shown in Figure 2.

2.1. Wild type alleles of the cognate mutant RAS isoform

Many mouse model carcinogenesis studies, in which cancers were initiated by chemical carcinogens or genotoxic insults that cause RAS mutations, identified loss of heterozygosity (LOH) resulting in loss of the cognate wild type *Ras* allele. LOH at Kras has frequently been observed in genetically engineered mouse models of mutant RAS-driven cancers. Likewise, RAS mutant human cancers often display LOH of the mutant *RAS* gene and loss of the wild type allele. These results suggest that the cognate wild type RAS serves a tumour suppressive function and must be lost in order for tumours to form and progress.

2.1.1 Evidence for a tumour suppressor function—Early studies of the functions of wild type RAS in transformation and oncogenesis focused on the wild type counterpart of the mutant isoform, and most concluded that wild type RAS plays tumour suppressive roles. For example, Spandidos and colleagues reported that transfection of the normal human H-ras1 (HRAS) gene suppressed the transformed morphology and tumourigenic phenotypes of rat 208F fibroblasts transformed with the human T24 H-ras1 (HRAS) oncogene [42, 43]. Rarely, HRAS-transformed fibroblasts escaped suppression by wild type HRAS and eventually formed tumours in nude mice, but in these cases, wild type HRAS expression was markedly reduced. Balmain and colleagues also found loss of endogenous wild-type *Hras* at high frequencies in *Hras* mutant skin tumours induced by carcinogens [44], which induce distinct *Ras* mutations depending on the particular chemical insult [45]. Pellicer and colleagues observed that carcinogen-induced *Nras*-mutant thymic lymphomas undergo LOH at *Nras* and lack the wild type *Nras* allele [46], supporting a tumour suppressive role for wild type NRAS in the presence of mutant NRAS. Zhang and colleagues found that wild-type *Kras2*, the murine *KRAS* gene, suppressed the formation of chemically induced lung tumours that harboured *Kras2* mutations [47]. Of note, this study utilized a $Kras2^{+/-}$ mouse model that harbours heterozygous loss of wild type KRAS. In an $Nras^{-/-}$ thymic lymphoma mouse model induced by the MNU carcinogen, which can provoke mutations in both *Nras* and *Kras*, loss of the wild type *Kras* allele was observed upon MNU-induced mutation of endogenous *Kras*, suggesting that loss of both wild type *Nras* and *Kras* isoforms more efficiently supported malignant transformation [48]. Further, adding back wild type *Nras* produced tumour suppressive effects whether or not oncogenic *Nras* was also added back [48]. More recently, Balmain and colleagues also reported tumour suppressive functions of wild type HRAS and KRAS in experimental models of HRAS-driven non-melanoma skin cancer and KRAS-driven lung cancer, respectively [49]. Surprisingly, wild type *Kras4A* was

identified as the main mediator of both the oncogenic activity of mutant KRAS and the suppressor activity of wild-type *Kras* [50]. Additionally, a role for wild type *Kras* gene dosage was uncovered in selecting the specific mutation induced by urethane. Wild type mice carried mostly Q61R *Kras* mutations, while those from *Kras* heterozygous mice carried mostly Q61L mutations [32]. In the same urethane-induced lung cancer model, Counter and colleagues found that optimizing codon usage of wild type *Kras* not only decreased the number of tumours but resulted in mutation switching from Q61R or Q61L to the weaker G12D [33]. It is interesting to speculate that these results imply differential effector utilization by these point mutants, whether at the level of specificity and/or efficiency, thereby requiring a greater or lesser degree of functional wild type RAS for tumour growth.

To test directly whether loss of the wild type RAS allele in spontaneous RAS mutant cancers is due to a tumour suppressor role of the wild type counterpart of oncogenic RAS, Bergö and colleagues used the *Kras2LSLMx1-Cre* (KM) mouse model to study *Kras* G12D induced leukemia. They found that expression of mutant *Kras* promoted proliferation and inhibited differentiation of early T-cell progenitors, but that all T-cell acute lymphoblastic leukemia (T-ALL) tumours identified in bone marrow-transplanted mice displayed loss of the wild type *Kras2* allele as an obligate defect. Restoration of wild type KRAS (human KRAS4B) abolished the development of T-ALL but not myeloid proliferative neoplasms (MPN), further supporting a tumour suppressive role of wild type *Kras* in lymphoid cells [51]. In a related study of $Kras^{G12D}$-induced leukemia, Zhang and colleagues found that genetic or epigenetic loss of wild type *Kras* expression promoted tumour growth and worse survival, although they observed preferential induction of MPN rather than T-ALL [52]. Consistent with this, they observed hyperactivation of cytokine signaling, e.g., through GM-CSF. Surprisingly, they also detected upregulation of RAS-GTP levels of each RAS isoform - including KRAS - upon loss of wild type KRAS, which occurred via gene deletion of the wild type allele. The mechanisms proposed in each of these studies to explain the differing results of the other with respect to myeloid versus lymphoid specificity likely cannot both be true simultaneously, but in any case, both studies revealed a tumour suppressive role for wild type *Kras* in $Kras^{G12D}$-driven leukemias.

Whether wild type NRAS also has a tumour suppressor function in mutant NRAS-driven hematopoietic cancers, similar to that seen with wild type KRAS in mutant KRAS-driven cancers, is currently unclear. A study in an (endogenous locus) $Nras^{G12D}$-driven model similar to the KM mice above, performed by Shannon and colleagues, revealed that loss of wild type *Nras* induced neither tumour suppressive nor tumour promoting activities [53], as described further in section 2.1.3. However, two different carcinogen-induced thymic lymphomas did display LOH of wild type *Nras* in the presence of *Nras* codon 61 mutations [46, 48]. In carcinogen-induced B-cell lymphomas, wild type *Nras* apparently served a tumour promoting function [54]; see section 2.1.2. Whether these differing results are due to distinct requirements for loss of wild type NRAS in different tumour types or in the context of G12 versus Q61 NRAS mutations, or due to some other factor(s), remains to be determined.

In addition to these mouse model studies, further evidence for a tumour suppressor function of wild type RAS in the context of the cognate mutant RAS isoform is provided by the

frequent findings of LOH and homozygously mutant RAS in established and patient-derived human cancer cell lines, patient-derived xenografts and primary tumours [55–58]. The implication is that allelic loss of the cognate wild type RAS is necessary for these tumours to become established and/or to progress. For example, as a followup to their study in chemically induced mouse lung cancers cited above [47], Li, Zhang, You and colleagues examined human lung adenocarcinomas and large cell lung cancers and found allelic loss of wild type KRAS in every tumour that harboured mutant KRAS [55]. In a comprehensive examination of 92 KRAS mutant cancer cell lines focusing on KRAS mutant pancreatic, lung and colon cancers, Gazdar and colleagues determined that mutant specific allelic imbalance (MASI) is both widespread and often accompanied by copy number gain (CNG) [58].

Finally, Su and colleagues created a new mouse model [56] that mimics the development and progression of human pancreatic ductal adenocarcinomas (PDA), a disease in which mutational activation of KRAS occurs with >95% frequency. KRAS mutation is one of the earliest events and is sufficient to cause the development of pancreatic intraepithelial neoplasia (PanINs), whereas progression to PDA requires additional mutations over time [59], such as loss of the tumour suppressors p16/Ink4A/Cdkn2a or p53. In human PDA, loss of p16/CDKN2A is the event next most frequent to KRAS mutation [60]. Accordingly, Su and colleagues generated a mouse model with tissue-specific conditional loss of p16 (but not p19/Arf) and mutational activation of KRAS (*p16*$^{flox/flox}$*;LSL-Kras*^{G12D}*;Pdx1-Cre*) that faithfully recapitulates development of PanIN, PDA and widespread metastasis [56]. They observed progressive LOH at *Kras* during the progression from PanIN to PDA to metastasis, such that only 3 of 17 representative cell lines derived from PDA metastases from these mice retained wild type *Kras*, whereas only 3 of 17 lines derived from primary tumours lacked it. The same LOH was observed in microdissected metastases. Further characterization showed that lines derived from primary tumours with LOH at *Kras* had more aggressively transformed phenotypes with respect to growth in low serum, wound healing migration ability, and anchorage-independent colony formation, supporting the idea that loss of wild type *Kras* enhances properties important for metastasis. Extending these studies to human cancer cell lines, whole-genome analyses showed LOH at KRAS (chromosome 12p) in 37% of lines derived from primary tumours and 80% of those derived from metastases. LOH was not found elsewhere, indicating that only wild type KRAS was lost, but not wild type NRAS or HRAS, and that loss of wild type KRAS was highly selected amongst other possible tumourigenic and metastasis-promoting alterations. Moreover, mutant KRAS was not amplified, indicating that loss of wild type KRAS was the primary alteration in KRAS abundance and the key reason for the functional differences observed. Collectively, these results strongly support a tumour suppressive role for wild type KRAS beyond initiation and extending to a role in metastasis suppression.

2.1.2 - Evidence for a tumour promoting function—Conversely, there is also evidence for a tumour promoting role of wild type RAS in the context of the cognate mutant RAS. By comparing signaling events in the isogenic pair of colorectal cancer cell lines HCT-116 (KRAS$^{G13D/WT}$) and Hke3 (KRAS$^{-/WT}$) with or without siRNA-mediated silencing that selectively targeted wild type KRAS, Matallanas *et al.* demonstrated that

mutant KRAS activates the pro-apoptotic MST2 pathway, whereas wild type KRAS antagonizes this activation [61]. This indicates that, in these colorectal cancer cells, wild-type KRAS supports rather than suppresses mutant *KRAS*-induced transformation. Collectively, while the vast majority of studies demonstrate a tumour suppressive role of the wild-type RAS allele in tumours driven by oncogenic activation of the cognate RAS isoform, it is also true that tissue and/or cellular specificity can influence the role that wild-type RAS plays in tumours.

2.1.3 - Evidence that wild type RAS is neither tumour suppressive nor tumour promoting—Still other studies argue that wild type RAS does not necessarily always display either tumour suppressing or tumour promoting activities. In a mouse model of *Nras*-driven AML, mutational activation of $Nras^{G12D}$ was insufficient to produce cancer, but required increased gene dosage of the activated allele ($Nras^{G12D}/Nras^{G12D}$) [53]. However, the loss of the wild type *Nras* allele in this homozygous mutant model was neither tumour suppressive nor tumour promoting, as ectopic expression of wild type NRAS or KRAS neither inhibited myeloid transformation by $Nras^{G12D}$ nor reduced the growth of progenitor cells expressing $Nras^{G12D}$ [53]. Additionally, despite somatic deletion of the wild type allele, expression of Nras was normal or elevated in AML blasts, a result of gene duplication of the oncogenic $Nras^{G12D}$ allele. Further, examination of human cancer cell lines in the Cancer Cell Line Encyclopedia [62] revealed that NRAS expression was elevated in NRAS-mutant cancer cell lines compared to those lacking RAS mutations [53]. Collectively, these results suggest that loss of the wild type protein in these cancers is secondary to a need for increased dosage of oncogenic RAS rather than a need to lose a tumour suppressive function of wild type RAS.

2.2 Wild type alleles of the non-mutant RAS isoforms

Most of the studies that focused on wild-type RAS isoforms that are not the cognates of the oncogenically mutated isoform, but rather that are alleles of the non-mutant RAS isoforms (e.g., NRAS or HRAS in KRAS mutant cancers), have largely but not always revealed tumour promoting functions. For example, in a study aimed at understanding the mechanism of FTI (farnesyl transferase inhibitor)-mediated radiosensitization in cell lines that express oncogenic KRAS, wild type HRAS, although not wild type NRAS, contributed to radiation survival in most of the KRAS mutant pancreatic and colorectal carcinoma cell lines treated [63]. Similarly, ectopic expression of KRAS G12V in the colorectal cancer cell line Caco-2 increased both the expression and activity of endogenous HRAS, and silencing of HRAS showed that oncogenic KRAS partly exerted its effects, such as enhanced invasiveness, through wild type HRAS [64]. On the other hand, Coffey and colleagues demonstrated that the presence of an oncogenic *KRAS* allele increased GTP-bound wild type NRAS in two human colorectal cancer cell lines, HCT116 and DLD-1, compared to their isogenic counterparts in which the mutant *KRAS* allele was disrupted by homologous recombination [65]. Although one might predict that this increased activation would enhance the anti-apoptotic functions of wild type NRAS [66], they found instead that the presence of mutant KRAS sensitized the cells to apoptotic insults, possibly by altering the interactions of NRAS with gelsolin [65]. However, since the study did not include silencing or other disruption of

the endogenous NRAS, it is not clear whether the altered NRAS:gelsolin interaction also impaired tumourigenicity.

A detailed mechanistic insight into how wild type isoforms might provide tumour promoting functions was first provided by Lim, Counter and colleagues, who showed that activation of eNOS (endothelial nitric oxide synthase) promotes C118 *S*-nitrosylation and activation of endogenous wild type HRAS and NRAS proteins in KRAS-mutant cells, suggesting that an oncogenic RAS^{mut}-PI3K–AKT-eNOS-RAS^{WT} pathway is required for both tumour initiation and maintenance [67]. Replacing C118, which is conserved among the RAS isoforms, with non-nitrosylatable C118S into wild type HRAS or NRAS inhibited xenograft tumour formation by KRAS-mutant human PDA cell lines, whereas the same C118S replacement into KRAS did not. This indicates that wild type but not oncogenic RAS proteins are the key target of eNOS in this context. The authors pointed out that activation of the *other* wild type RAS proteins by eNOS may thus serve as an important means to diversify RAS signaling beyond that of oncogenic RAS. In agreement with this speculation, they found that loss of wild type HRAS did not inhibit oncogenic $HRAS^{G12V}$-mediated oncogenesis in TtH cells expressing either scramble or *HRAS* shRNA in addition to RNAi-resistant oncogenic $HRAS^{G12V}$. As indicated in section 2.1, the wild type counterparts of oncogenic RAS proteins are often lost in human cancers [55–58], suggesting that, in contrast to the non-counterpart wild-type isoforms, these play a tumour-suppressive role.

The interaction between oncogenic and wild type RAS proteins is the net result of a multitude of diverse mechanisms. Bar-Sagi and colleagues showed that oncogenic KRAS promotes allosteric stimulation of SOS, a key RAS GEF, and leads to activation of wild type HRAS and NRAS [68]. A later study then provided additional insights into RAS protein signaling networks [69], in which wild type HRAS or NRAS depletion from KRAS mutant cancer cells hyperactivated both ERK-p90 RSK and PI3K-AKT, leading to inhibitory phosphorylation of Chk1 at S280. The resulting inhibition of the G2 DNA damage checkpoint then led to increased sensitivity of KRAS mutant cells to DNA damaging agents such as the topoisomerase I inhibitor irinotecan. Furthermore, oncogenic and wild type RAS isoforms have been reported to be responsible for regulating different aspects of signal transduction, with McCormick and colleagues reporting that oncogenic RAS modulates basal mitogen-activated protein kinase (MAPK) pathway signaling, and wild type isoforms control response to stimulatory growth factor signaling [70].

There are some slim observations that demonstrate a potential need for wild type RAS in NRAS mutant cancer cells. A cell proliferation defect was seen in the NRAS mutant rhabdomyosarcoma cell line RD ($NRAS^{Q61H}$) upon knockdown of wild type HRAS and/or KRAS [70]. In the melanoma cell line SK-MEL-103 ($NRAS^{Q61R}$), knockdown of wild type HRAS enhanced γH2AX levels, indicating DNA damage regulation by wild type HRAS [69]. However, surprisingly little is known about whether NRAS mutant cancers require wild type KRAS or HRAS for tumour initiation and/or maintenance. Our ongoing studies (manuscript in preparation) indicate that at least NRAS mutant melanomas require the wild type versions of both of the other RAS isoforms for full transforming activity.

3. Contribution of wild type RAS to RAS wild type cancers

In some mouse model chemical carcinogenesis studies, gastric or hepatic cancers were promoted by overexpression of wild type Hras in the absence of mutated Ras [71, 72]. Likewise, chemically induced B cell lymphomas were promoted by overexpression of wild type Nras [54]. These results, along with a plethora of studies in which ectopic expression of wild type human RAS isoforms was sufficient to transform rodent fibroblasts to tumourigenicity [2, 3, 5], suggested that overexpression of wild type RAS alone could be sufficient to promote tumourigenicity, albeit with the caveat that too much RAS leads to arrest and senescence. More compellingly, endogenous wild type RAS proteins have been demonstrated to contribute by multiple mechanisms to tumour initiation, progression and maintenance, as well as to metastasis, of human cancer cell lines and patient tumours.

3.1 Amplification of wild type RAS

In most cancer types where RAS is altered oncogenically in some way, analysis of the TCGA data indicates that the vast majority of alterations are somatic missense mutations that increase RAS activation (cBioPortal.org) [40, 41]. However, in some cases, there is amplification of wild type RAS along with oncogenic RAS mutation. For example, in NRAS mutant melanoma, *NRAS* amplifications can co-occur in tumours with *NRAS* mutations [73]. In rare cases, for example in neuroendocrine prostate cancer, a third of all samples examined displayed amplification of RAS but not mutations [73], and in some of these cases, more than one wild type RAS isoform was amplified (cBioPortal.org) [40, 41]. Examples of other tumour types where amplification of wild type RAS but not missense mutation is frequently seen include ovarian serous cystadenocarcinoma [74] and esophageal carcinoma.

3.2 Activation of wild type RAS by gene fusion

RAS mutations are uncommon in prostate cancer [73] despite high levels of RAS pathway activity. Instead, a common mechanism for upregulation of RAS activity is epigenetic loss of the RASGAP DAB2IP [75]; see section 3.3.1. In addition, a rare but completely distinct mechanism of RAS activation has been identified recently. Nearly 50% of prostate cancers harbour gene rearrangements of androgen-driven genes with ETS family transcription factors [76]. Upon searching for additional driver gene fusion events in a panel of prostate adenocarcinoma cell lines, Chinnaiyan and colleagues uncovered a novel gene rearrangement of KRAS in DU145 cells [77]. The ubiquitin-conjugating enzyme UBE2L3 was fused to full length KRAS, generating a UBE2L3-KRAS fusion protein that was ubiquitinated and relatively unstable, yet fully capable of transforming NIH 3T3 mouse fibroblasts. Examination of 62 metastatic prostate carcinomas identified the same fusion in 2 patient tumours [77], thereby validating that this rearrangement, although rare, is also found in clinical specimens of prostate cancer. Whether such RAS-activating fusion proteins are also present in other tumour types, and the precise nature of their contributions to oncogenesis, tumour maintenance and metastasis, have yet to be determined.

3.3 Activation of wild type RAS by loss of negative regulators

Two major mechanisms for negative regulation of RAS activity act either directly at the level of RAS itself (RAS GAPs) or indirectly by negative feedback on RAS activators (SPROUTY and SPRED proteins).

3.3.1 Activation of wild type RAS by loss or suppression of RAS GAPs—RAS GAPs promote hydrolysis of RAS-GTP to RAS-GDP, thereby restoring the resting, inactive state of RAS proteins. It is increasingly appreciated that loss of RAS GAPs is important in cancers driven by RAS pathway activation in the absence of mutations in RAS itself. Of the 14 RAS GAPs identified to date, NF1, RASAL1, RASAL2 and DAB2IP perform critical tumour suppressor functions via their roles in downregulating RAS activity [7, 78, 79].

In particular, the product of the NF1 gene, neurofibromin, is not only involved in the familial cancer syndrome neurofibromatosis type 1 (NF1) through germline mutation, but is also involved in many types of sporadic cancers through somatic mutation. For example, NF1 loss-of-function mutations are commonly seen in melanomas [80], glioblastomas [81, 82], and lung adenocarcinomas [83] and squamous cell carcinomas [84]. These are largely not co-occurrent with RAS mutations, suggesting that the pathways are generally redundant. The co-occurrence of NF1 loss in NRAS-mutant melanoma may be explained by the surprising consequence of activation of wild type KRAS and HRAS, but not NRAS [85] in this tumour type, although this selectivity is not always seen [86]. Loss of NF1 has also been shown to promote resistance to RAS pathway inhibitors by enhancing ERK signaling, and has been validated as a potential mechanism of intrinsic resistance in cell culture as well as of acquired resistance in patient tumour samples [85–87].

DAB2IP is a tumour suppressor of many aggressive cancers, where it is downregulated epigenetically by the histone methyltransferase EZH2 [75], and at the protein level by AKT-SCFBW7-SMURF1 [88]. A large signaling scaffold protein (1189 amino acids), DAB2IP (also known as ASK-interacting protein and by other names) forms part of several cytoplasmic signaling complexes that enable it to modulate numerous signaling pathways that control responses to growth factor signaling, stress, and apoptosis. In addition to its RAS GAP activity, DAB2IP also modulates RAS signaling via its interaction with RALBP1 and other components. In cancers that lack RAS mutations, such as prostate, breast, medulloblastoma, and others, DAB2IP is frequently lost. Cichowski and colleagues demonstrated that, in prostate cancer, loss of DAB2IP results in separate modes of activation of RAS and NF-κB, thereby separately promoting tumour growth and metastasis, respectively [75].

3.3.2 DUSPs, SPROUTY/SPRED—In addition to forward propagation, RAS pathway signaling is also regulated by numerous levels of feedback phosphorylation to provide sensitive and temporal modulation and to restrict signaling output. For example, activation of ERK1/2 induces transcriptional upregulation of negative regulators, including dual-specificity MAP kinase (MAPK) phosphatases (MKPs or DUSPs) and Sprouty (SPRY) proteins. ERK1/2 signaling drives the expression of a variety of DUSP proteins, including the ERK-specific phosphatases DUSP5 (nuclear) and DUSP6/MKP3 (cytoplasmic) [89, 90],

thus providing a straightforward means of controlling its own activity. The importance of DUSPs in controlling RAF-MEK-ERK signaling in cancer is exemplified by the frequent loss of DUSP6 in EGFR- and KRAS-driven non-small cell lung cancers [90] and by the finding that loss of DUSP5 accelerates HRAS-driven skin cancer in mice [91].

SPRY proteins also act as negative regulators of RTK-RAS-RAF-MEK-ERK signaling [92, 93]. Growth factor stimulation leads to tyrosine phosphorylation and plasma membrane translocation of SPRY1 and SPRY2, where they bind the adaptor protein Grb2 to prevent recruitment of the Grb2-SOS complex that couples growth factor stimulation to RAS activation [92]. Loss of SPRY proteins can promote several types of cancer in combination with either the presence of other oncogenes or loss of other tumour suppressors. For example, loss of SPRY1 and SPRY2 in prostatic epithelium hyperactivates RAS-MAPK signaling and, along with loss of the tumour suppressor PTEN, promotes prostatic adenocarcinoma in this RAS wild type cancer [94]. Combined loss of SPRY4, NF1 and p53 promotes acute myeloid leukemias in the absence of KRAS or NRAS (or FLT3 or KIT) mutations [95]. Combined loss of SPRY4 and NF1 would be predicted to upregulate RAS activity, with loss of p53 required to avert oncogene-induced senescence [34]. Although RAS-GTP levels were not evaluated in this study, RAS pathway activation was clearly upregulated, as demonstrated by increased phospho-ERK and phospho-S6 levels [95]. Understanding the negative feedback that occurs or is lost upon downregulation of these regulators of RAS activity is important for predicting the consequences of pharmacologically inhibiting elements of this pathway for cancer treatment, as well as for unraveling the consequences of signaling from wild type RAS isoforms in the presence of oncogenic RAS.

3.4 Activation of wild type RAS by upregulation of positive regulators

Upregulation of positive regulators including increased signaling from overexpressed or oncogenically mutated receptor tyrosine kinases such as EGFR, FGFR, IGF1R, etc. (Figure 3), can also result in increased RAS signaling, often via the best characterized RAS GEF, SOS1. This has been an attractive target of investigation as a node of RAS inhibition through blocking the RAS-SOS1 interaction [4]. However, SOS1 mutations are generally rare in human cancers [96], and are even less frequent in wild-type RAS tumours. Instead, wild type RAS is more frequently activated by upstream mechanisms that transmit signals through SOS1, such as in EGFR-mutated or ALK-translocated lung adenocarcinoma [83]. Aberrant upregulation of the RAS GEF RASGRP1 has been strongly implicated in T-cell leukemogenesis, due to its predominant expression in these cells [97]. Overexpression of RASGRP1 alone is capable of inducing T-cell acute lymphoblastic leukemia (T-ALL) in both murine models and pediatric patients [97]. RASGRP1-overexpressing T-ALL exhibits constitutively high RAS-GTP, mechanistically distinguishing it from $KRAS^{G12D}$-driven T-ALL that lacks constitutive GTP loading of RAS. Kitamura and colleagues showed that aberrantly expressed RASGRP1 cooperated with frequent secondary NOTCH1 gain-of-function mutations to promote mouse T-ALL [98].

4. Concluding remarks

Wild type RAS plays critical roles in both RAS mutant and RAS wild type cancers, in a context-dependent manner. The wild type KRAS isoform largely acts as a tumour suppressor in the context of the cognate mutant KRAS isoform, and its loss facilitates initation and progression as well as metastasis. Support for similar roles of the wild type NRAS isoform in NRAS-driven cancers is more mixed. Some emerging evidence shows that the other wild type RAS isoforms, HRAS and NRAS, may not only be tolerated but even act as tumour promoters of mutant KRAS. The activity of wild type RAS isoforms is also aberrantly upregulated by amplification and by loss of negative RAS regulators such as the DUSP MAPK phosphatases and of NF1 and other RAS GAPs, and by overexpression of positive regulatory GEFs. Ongoing investigations of the contribution of wild type RAS in cancer will certainly reveal additional complexities, some of which may uncover vulnerabilities amenable to cancer treatment.

Acknowledgments

Funding. This work was supported by the National Institutes of Health (CA42978, CA179193, CA175747, CA199235), the Pancreatic Cancer Action Network-AACR, and the Lustgarten Pancreatic Cancer Foundation.

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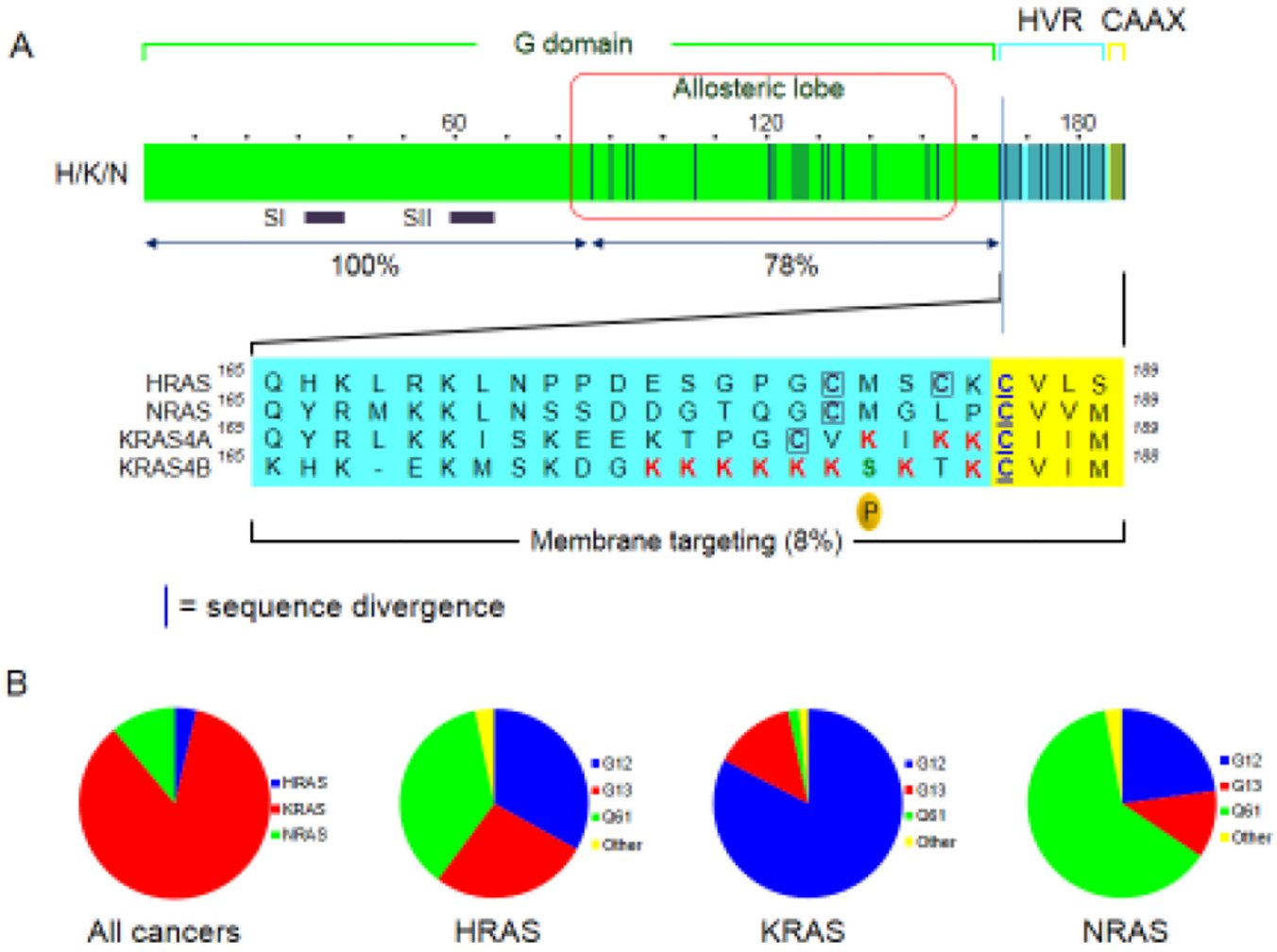

Figure 1. RAS isoforms

A. Sequence identity and divergence between the four human RAS proteins include conformational changes to the switch I (SI; amino acids 30–38) and II (SII; 59–76) regions in the GDP and GTP bound states, hence regulating effector binding affinity. G domain, GTP-binding. HVR, hypervariable region. CAAX, Cysteine, Aliphatic, Aliphatic, Any amino acid. Sites of posttranslational modifications are indicated by underlines and boxes; P, phosphorylation. B. Frequency of missense mutations in the three human RAS genes and at the three hotspots for RAS mutations (codons G12, G13 and Q61).

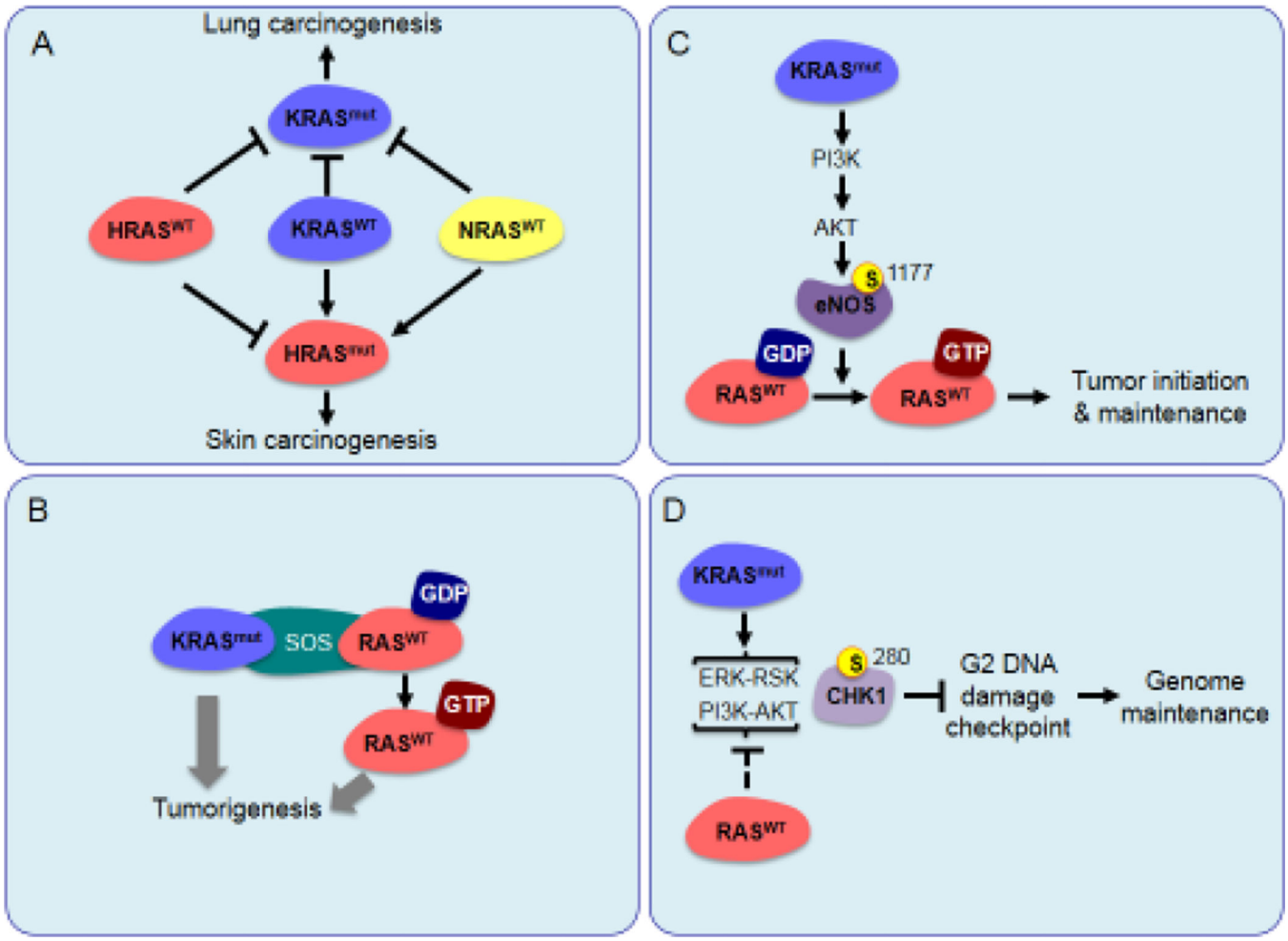

Figure 2. Interplay between wild type and mutant RAS alleles in cancer

(A) Wild type RAS proteins can display either tumour promoting or tumour suppressing functions, depending on context. Some ways in which mutant KRAS promotes tumourigenesis are by (B) allosteric activation of the RAS GEF SOS1 to activate wild type HRAS and NRAS, or (C) activation of eNOS (endothelial nitric oxide synthase) to nitrosylate and activate wild type HRAS. Additionally (D), wild type HRAS and NRAS can block inhibition of the G2 DNA damage checkpoint to promote genome maintenance.

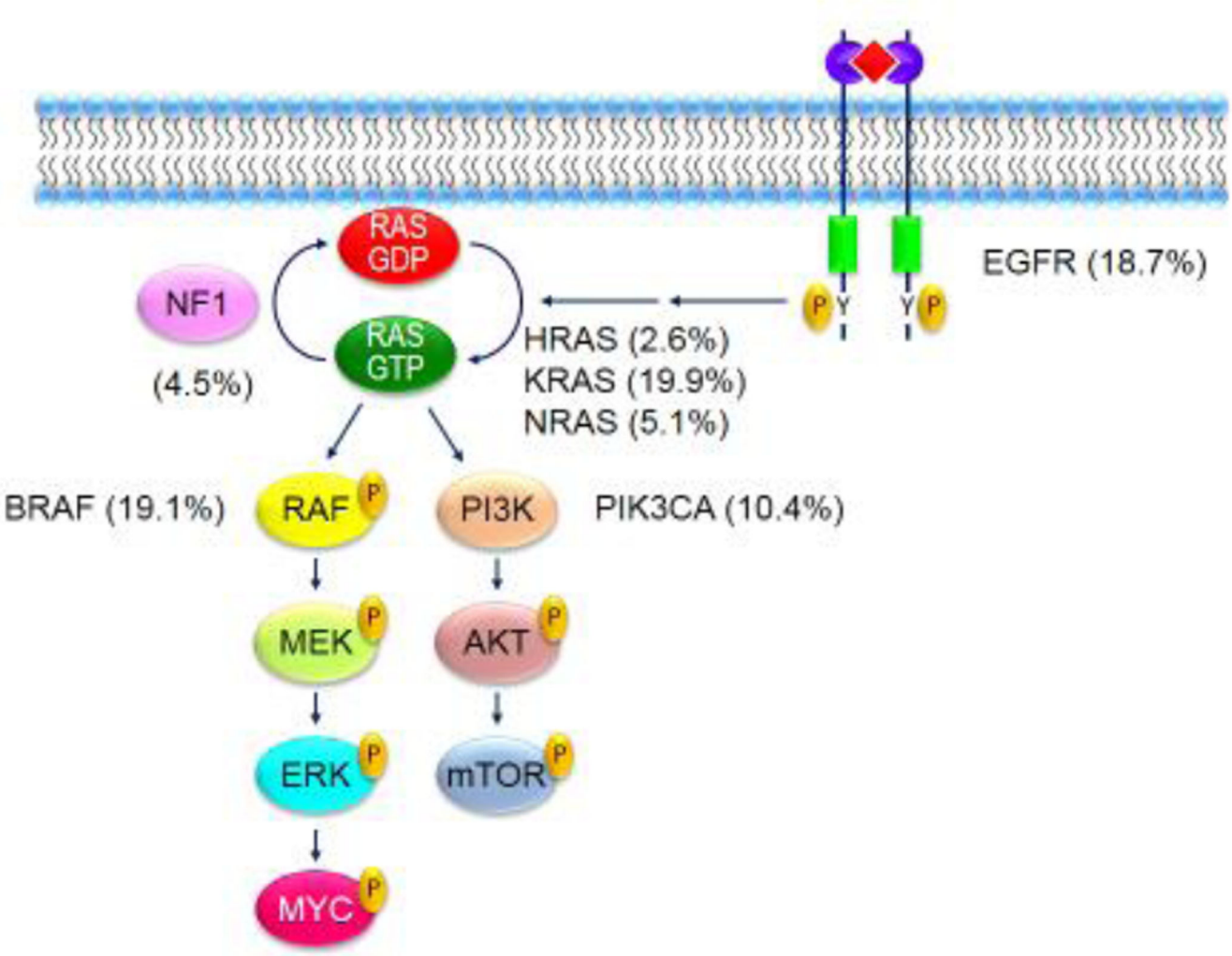

Figure 3. Mutational activation of the RAS signaling network in cancer

RAS function can be activated directly by mutation of RAS or indirectly by mutational activation or loss of components upstream or downstream of RAS. Missense mutation frequencies are indicated in parentheses and were compiled from COSMIC.