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Nras in melanoma: Targeting the undruggable target

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Contents

Abstract

RAS belongs to the guanosine 5′-triphosphate (GTP)-binding proteins' family, and oncogenic mutations in codons 12, 13, or 61 of RAS family occur in approximately one third of all human cancers with N-RAS mutations found in about 15–20% of melanomas. The importance of RAS signaling as a potential target in cancer is emphasized not only by the prevalence of RAS mutations, but also by the high number of RAS activators and effectors identified in mammalian cells that places the RAS proteins at the crossroads of several, important signaling networks. Ras proteins are crucial crossroads of signaling pathways that link the activation of cell surface receptors with a wide variety of cellular processes leading to the control of proliferation, apoptosis and differentiation. Furthermore, oncogenic ras proteins interfere with metabolism of tumor cells, microenvironment's remodeling, evasion of the immune response, and finally contributes to the metastatic process. After 40 years of basic, translational and clinical research, much is now known about the molecular mechanisms by which these monomeric guanosine triphosphatase-binding proteins promote cellular malignancy, and it is clear that they regulate signaling pathways involved in the control of cell proliferation, survival, and invasiveness. In this review we summarize the biological role of RAS in cancer by focusing our attention on the biological rational and strategies to target RAS in melanoma.

Keywords: NRAS; Melanoma; Prognostic; Predictive; Resistance

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The importance of RAS signaling as a potential target in cancer is emphasized not only by the prevalence of RAS mutations, but also by the high number of RAS activators and effectors identified in mammalian cells that places the RAS proteins at the crossroads of several, important signaling networks.

After 40 years of basic, translational and clinical research, much is now known about the molecular mechanisms by which these monomeric guanosine triphosphatase-binding proteins promote cellular malignancy, and it is clear that they regulate signaling pathways involved in the control of cell proliferation, survival, and invasiveness. In this review we summarize the biological role of RAS in cancer by focusing our attention on the biological rational and strategies to target RAS in melanoma.

1. Historical perspective on NRAS in cancer and a focus on melanoma

Thirty years ago a pioneering study demonstrated that small fragments of DNA from human cancer-derived cells could induce malignant characteristics in mouse fibroblasts [1]. The cellular homolog of an oncogene found in the Harvey rat sarcoma retrovirus (H-RAS) was identified as the DNA sequence responsible for such malignant transformation. A new step in tumor biology had been put in place: this was the first demonstration that human tumors contained activated oncogenes, related to those picked up by retroviruses from their host genomes [2,3]. Gene sequencing revealed that the difference between the wild-type (wt) human H-RAS gene and the oncogenic form found in tumors was a single point mutation. Subsequently, three RAS genes and corresponding proteins were described: N-RAS (neuroblastoma-RAS), H-RAS and K-RAS (Kirsten-RAS) [4–6].

RAS belongs to the guanosine 5′-triphosphate (GTP)-binding proteins' family. When acted upon by specific factors, such as extracellular ligands that bind specific membrane receptors, these proteins cycle between an activated and inactivated form, RAS-GTP and RAS-GDP, respectively [7]. Activation requires dissociation of protein bound GDP, a process that is accelerated by guanine nucleotide-exchange factors (GEFs). This switch-on process involves the reversible exchange of GDP for GTP. The switch-off process is entirely different and involves hydrolysis of GTP to GDP, the guanosine triphosphatase (GTPase) reaction, which is basically irreversible. This process is accelerated by GTPase activating proteins (GAPs) (Fig. 1a).

In physiological conditions, RAS proteins are tethered to the inner cell membrane, coupling growth factor receptors to downstream signaling pathways and regulate important cellular functions such as cell growth, proliferation, and survival. Much is now known about the molecular mechanisms by which these monomeric guanosine triphosphatase-binding proteins promote cellular malignancy, and it is clear that they regulate signaling pathways involved in the control of cell proliferation, survival, and invasiveness.

Mutations at positions 12, 13, or 61 of the H-RAS, N-RAS, and K-RAS impair the GTPase activity of the carrier RAS proteins and lock them into a constitutively activated state in which they elicit downstream effectors, even in the absence of ligands that bind specific membrane receptors [8]. This peculiar oncogenic activation – disabling the enzymatic activity – differentiates RAS from other oncogenic kinases such as EGFR or B-RAF, which are typically mutated to produce a hyperactive enzyme.

The importance of RAS signaling as a potential target in cancer is emphasized not only by the prevalence of RAS mutations, but also by the high number of RAS activators and effectors identified in mammalian cells that places the RAS proteins at the crossroads of several, important signaling networks (Fig. 1b).

The first RAS effector identified is the RAF serine/threonine kinase [9–12]. Activation of RAF initiates a phosphorylation cascade that progresses through MEK and ERK (p42/p44 MAPK), and ultimately leads to fine adjustments in downstream targets that regulate cell proliferation, survival, and differentiation [13]. A second RAS effector is the p110 catalytic subunit of the phosphatidylinositol 3-kinase (PI3K) [14]. Phosphorylation of phosphatidylinositol by PI3K brings the AKT serine/threonine kinase to the plasma membrane, where it becomes activated and transmits downstream signals to regulate cell survival, protein synthesis, and metabolism [15]. RAF and PI3K are also commonly mutated in melanoma, suggesting that these proteins might be the primary oncogenic effectors of RAS signaling [16].

Interestingly, while mutations in RAF and RAS are generally mutually exclusive, this is not the case for PI3K mutation. These biological differences suggest that endogenous levels of activated RAS do not efficiently activate PI3K signaling, while RAS and RAF mutations appear functionally equivalent. Another explanation is that the RAS/RAF double mutation is lethal for the cell whereas RAS/PI3K are not.

Since oncogenic mutations in codons 12, 13, or 61 of RAS family occur in approximately one third of all human cancers with N-RAS mutations found in about 15–20% of melanomas, RAS and the signaling pathways under its control have been kept firmly in focus as therapeutic targets (Fig. 2). However, after 40 years of research, many problems remain open. First, what has prevented the development of drugs against RAS?

Several factors have hampered the development of therapies that are able to inhibit RAS in a specific and effective way: (1) the high affinity of RAS for GTP; (2)

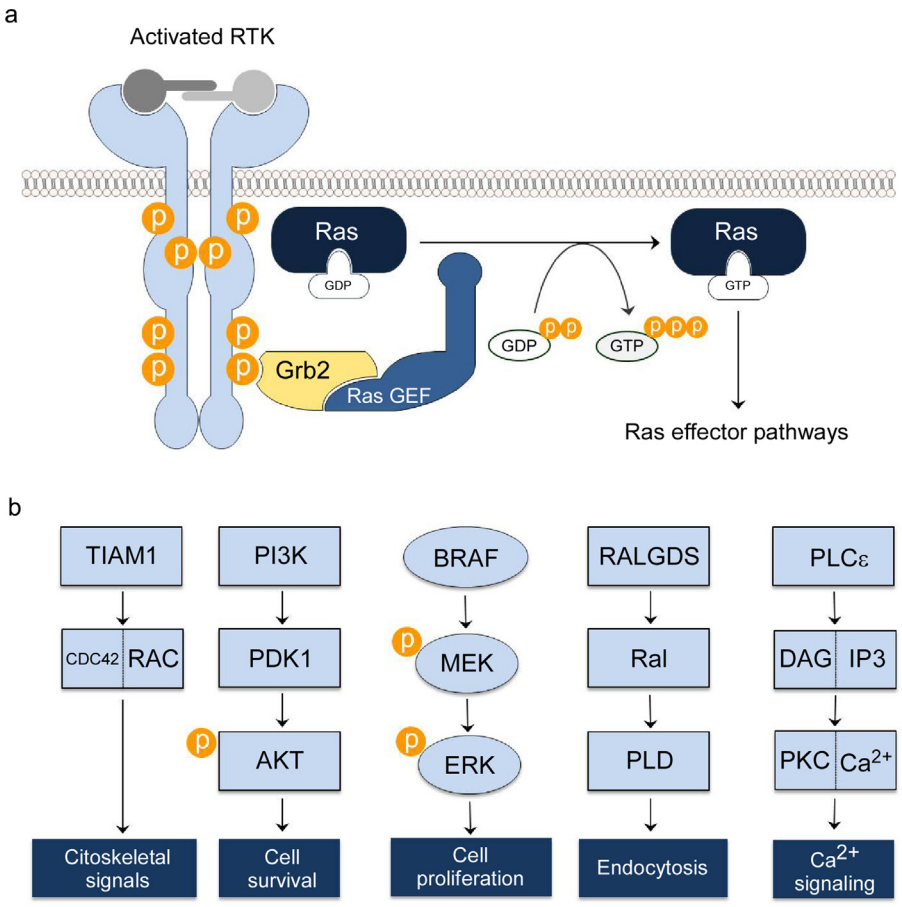

Fig. 1. (a) Mechanism of RAS activation. Receptor tyrosine kinase (RTK)-mediated activation requires dissociation of protein bound GDP, a process that is accelerated by guanine nucleotide-exchange factors (GEFs). This switch-on process involves the reversible exchange of GDP for GTP. The switch-off process is entirely different and involves hydrolysis of GTP to GDP, the guanosine triphosphatase (GTPase) reaction, which is basically irreversible. This process is accelerated by GTPase activating proteins (GAPs). (b) Effectors identified in mammalian cells that place the RAS proteins at the crossroads of several, important signaling networks. (TIAM1: T-cell lymphoma invasion and metastasis 1; PI3K: phosphoinositide 3-kinase; PDK1: phosphoinositide-dependent kinase-1; ERK: extracellular regulated kinase; RALGDS: RAL guanine nucleotide dissociation stimulator; PLD: phospholipase D; PLCε: phospholipase Cε; PKC: protein kinase C).

high intracellular concentrations of GTP; (3) the attempt to inhibit farnesylation, a key posttranslational modification step of RAS that is essential for RAS function, through the farnesyltransferase inhibitors (FTIs), was ineffective in clinical trials; (4) targeting mutant N-RAS with siRNA is still limited to preclinical models because of the significant challenge in delivering antisense oligonucleotides in vivo.

In this review we summarize the biological role of RAS in cancer by focusing our attention on the biological rational and strategies to target RAS in melanoma. For this purpose, we performed an extensive "Medline" and Cancerlit literature review (1995–2012). Various combinations of search terms were used depending on the requirements of the database being searched. These terms included "RAS", "MAPK", "target therapy", "MEK" in combination with "cancer patients", "melanoma", "incidence", "pathogenesis", "management", "cancer", "tumors", "resistance", "trials", "prospective", "phase", "retrospective". In addition, we manually researched all relevant review articles and the references of the retrieved papers. Finally, trials were excluded if relevant data could not be extracted.

2. Biological functions of NRAS

Ras proteins are crucial crossroads of signaling pathways that link the activation of cell surface receptors with a wide variety of cellular processes leading to the control of proliferation, apoptosis and differentiation (Fig. 3). Furthermore, oncogenic ras proteins interfere with metabolism of tumor cells, microenvironment's remodeling, evasion of the immune response, and finally contributes to the metastatic process.

2.1. Cell proliferation

Three decades ago Feramisco et al. demonstrated that oncogenic, mutated forms of ras proteins when introduced

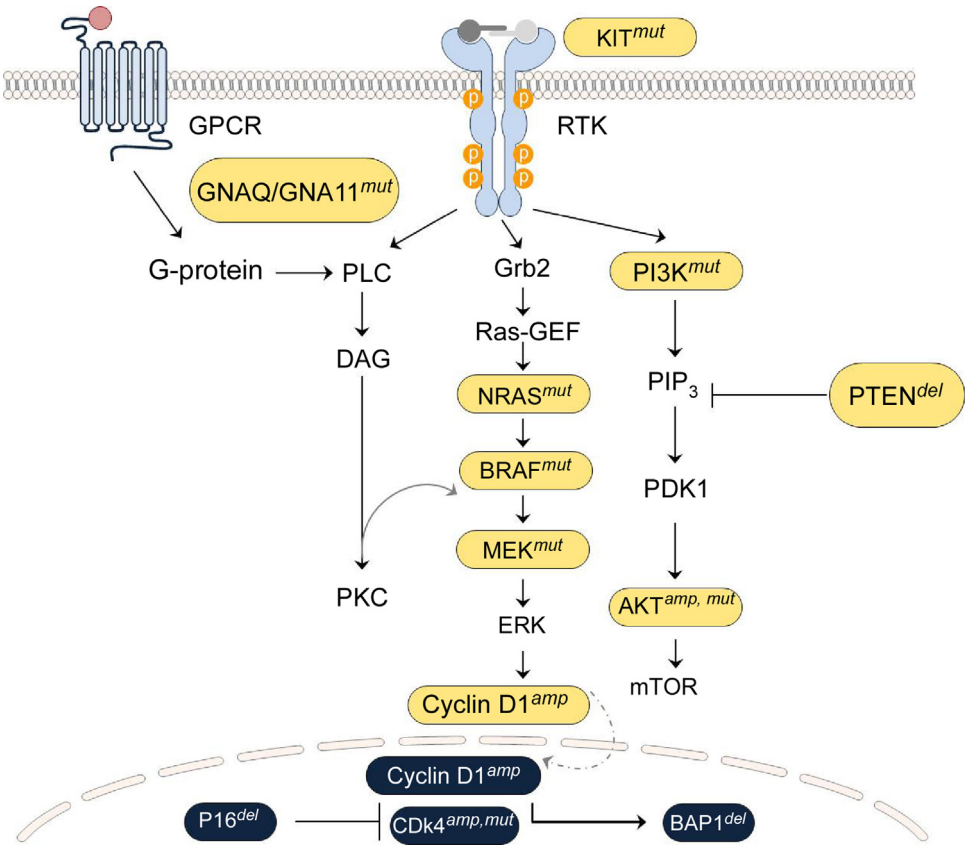

Fig. 2. Potential therapeutics targets in melanoma.

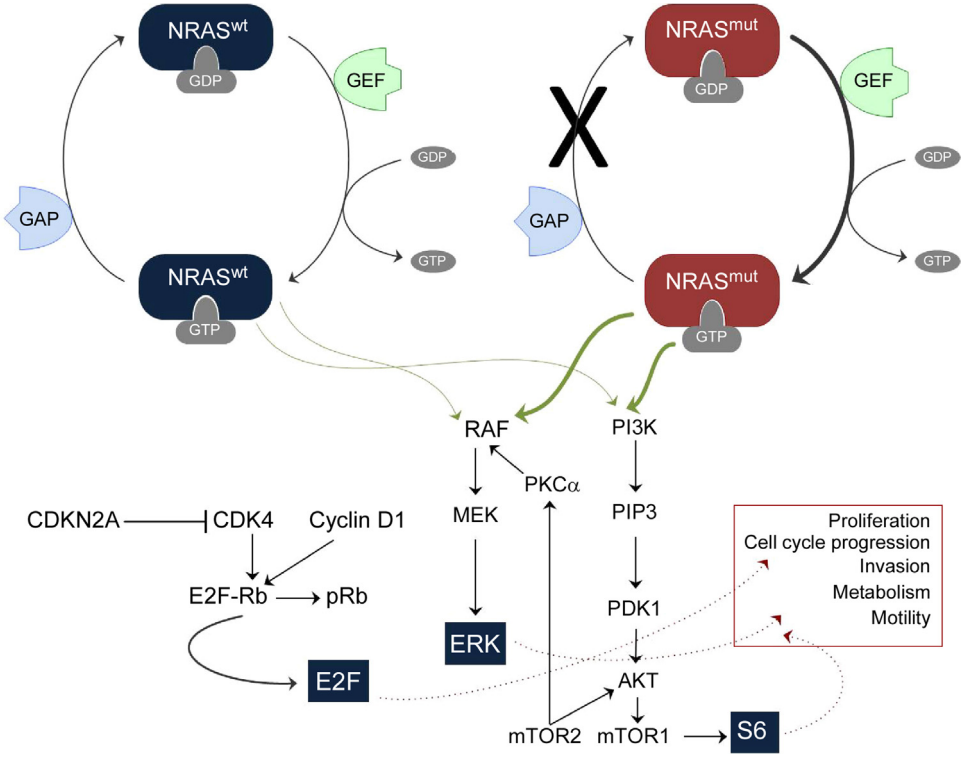

Fig. 3. Ras proteins are crucial crossroads of signaling pathways that link the activation of cell surface receptors with a wide variety of cellular processes leading to the control of proliferation, apoptosis and differentiation.

by microinjection into a variety of somatic cells determine dramatic morphological changes followed by transient cell proliferation [17]. Proliferation is a check and balances process, being the result of different stimuli, that elicit or inhibit cell cycle [18]. Oncogenic RAS fuels cell proliferation through four distinct biological mechanisms that carry the balance of different stimuli to hang on the side of the cell cycle: upregulation of growth factors, expression of growth factor receptors, upregulation of integrins that promote proliferation and downregulation of anti-proliferative signals. These complex and still unclarified mechanisms lead to activation of several transcription factors such serum response factor (SRF), JUN, activating transcription factor 2 (ATF2) and nuclear factor-κB (NF-κB) [19,20]. In turn, these factors trigger the expression of cyclin D1 [21]. The expression of the G1 cyclin seems a crucial determinant of RAS-induced transformation. It has been reported that cyclin D1-deficient mice are resistant to developing epithelial tumors that are induced by the HRAS oncogene. Pharmacological interference with cyclin D1 or cyclin-dependent kinase inhibitors (CKIs), such as p27 and p21, which would otherwise associate with and inhibit cyclin-dependent kinases (CDKs), could be an exciting avenue of cancer research in the coming years.

2.2. Suppression of apoptosis

Oncogenic RAS may have both pro-apoptotic and anti-apoptotic functions. The anti-apoptotic function of oncogenic RAS is mediated by several effector pathways, including the RAS–PI3K and the RAS–RAF pathway. Both pathways have been implicated in phosphorylating and inactivating the pro-apoptotic protein BCL-2-associated agonist of cell death (BAD). There is evidence that RAS is implicated in both the development and maintenance of melanoma. In experimental models, melanoma genesis and maintenance are strictly dependent upon expression of HRas V12G and on the opposite HRas V12G down-regulation results in clinical and histological regression of primary and explanted tumors [22]. The initial stages of regression involved marked apoptosis in the tumor cells and host-derived endothelial cells. These data clearly support the hypothesis of an oncogenic RAS-driven erosion of the apoptotic pathways and its contribution to melanoma development.

2.3. Metabolism

RAS-driven activation of MAPK and PI3K effector pathways stimulate mTOR activity which, in turn, up-regulates the hypoxia-inducible factor 1α (HIF1α), which is well recognized for its ability to stimulate a glycolytic shift [23]. RAS dependent upregulation of HIF1α enhances the transcription of the glucose transporter GLUT1, thus conferring cells with an increased capacity to take up glucose. In addition, oncogenic RAS leads to an increase in the levels of key glycolytic enzymes [24]. Thus, oncogenic RAS directly contributes to metabolic reactions that stimulate the use of glucose as an anabolic substrate in producing building material for cellular growth. Oncogenic RAS interfaces with cellular metabolism and this interaction increases ultimately the glycolytic rate and cellular viability, supporting tumor growth in vivo [25].

2.4. Remodeling the microenvironment

RAS activation sustains pro-angiogenic processes through modulation of endothelial growth factors levels, enhancement of local inflammation and stromal remodeling [26]. RAS upregulates VEGFA via multiple effectors, including, HIF1α, cyclooxygenase 2 (COX2) and prostaglandins' production [27]. Furthermore, RAS-mediated production of pro-inflammatory cytokines, such as IL-6 and IL-8, has emerged as another contributor to the induction of angiogenesis [28]. Finally, upregulation of matrix metalloproteinase 2 (MMP2), MMP9 and urokinase-type plasminogen activator (uPA) has been described [29].

2.5. Evasion of the immune response

Oncogenic RAS can disrupt antitumor immunity by essentially two mechanisms: first, by reducing the surface expression of antigen-presenting major histocompatibility complexes (MHC) on tumor cells, resulting in decreased immunogenicity of the RAS-transformed cells [30]. Second, by overcoming host-protecting adaptive immune responses [31]. Upon oncogenic RAS expression, the recruitment of immunosuppressive regulatory T cells and myeloid-derived suppressor cells at tumor site may lead to a compromised antitumor immune response [32].

2.6. Metastasis

Metastasis is a multi-stage process involving a multitude of cellular activities such as cancer cell motility, intravasation, transit in the blood or lymph vessels, extravasation and growth at a new site. RAS promotes these processes by engaging a diverse and broad platform of effector mechanisms. Oncogenic RAS induces alterations in cell–cell and cell–matrix interactions and the acquisition of a migratory phenotype ultimately contributing to the metastatic process. Oncogenic RAS reduces E-cadherin levels and induces the destabilization of E-cadherin – β-catenin complexes and the β catenin relocalization [33]. In addition, oncogenic RAS contributes to the enhanced motility of tumor cells by affecting changes in the polymerization, organization and contraction of actin; the polymerization and/or stability of microtubules; and the transcriptional regulation of mitogenic gene products [34]. Oncogenic RAS protects tumor cells from matrix deprivation-induced apoptosis, or anoikis thereby contributing to their capacity of migration through the circulatory system [33–35].

3. NRAS in melanocytic cell neoplasms

One of the unresolved issues concerning the oncogenic activation of RAS pertains to whether specific oncogenic outputs are driven by mutations in a particular RAS isoform. This hypothesis is supported by the well-recognized non-random distribution pattern of activated isoforms of RAS among different cancer types.

NRAS mutations have been found in approximately 15–20% of human melanomas while HRAS and KRAS mutations are rare (1%) [36]. A rational explanation for the greater occurrence of NRAS mutations relies on distinct differences between the signaling capabilities of NRAS and KRAS in melanocytes [37]. When the transformation efficiencies of mutant NRAS and KRAS were compared in immortal, non-transformed Ink4a/Arf-deficient melanocytes, it was shown that in contrast to KRAS mutation, NRAS mutation leads to increased cellular proliferation and is more potently tumorigenic [37]. Furthermore, NRAS mediates activation of both MAPK and PI3K/AKT/MYC signaling. Specifically, although both NRAS and KRAS efficiently activate the classical MAPK pathway, only NRAS effectively prevents glycogen synthase kinase3 (GSK3)-mediated phosphorylation of Myc via PI3K/AKT, which results in enhanced activity of endogenous Myc protein [37]. In contrast to KRAS, NRAS and HRAS also show a more potent activation of PI3K/AKT likely due to the fact that both NRAS and HRAS colocalize to lipid rafts, whereas KRAS is excluded from lipid rafts and localizes to the disordered plasma membrane [38], resulting in a less efficient activation or a limited access to a defined subset of downstream effector proteins.

There is a great debate whether specific RAS isoforms dictate specific clinico-pathological melanocytic cell neoplasms. An extraordinarily high NRAS mutation frequency seems to be characteristic of medium-sized ($\geq$1.5 cm) and large-giant congenital nevi whereas common acquired nevi and Spitz nevi have rare NRAS mutations (4.6% and 4%, respectively) [39].

The frequency of NRAS mutations in medium-sized congenital nevi is 64–70% [39–41] and raises to 94.7% in large-giant congenital nevi where it has been recently recognized as the sole recurrent somatic mutation [42]. It has been suggested that NRAS mutations exert stronger growth signals, resulting in the formation of larger nevi than those linked to BRAF mutations [43]. In contrast, small congenital nevi (<1.5 cm) are genetically similar to common acquired nevi and tend to show a lower incidence of NRAS mutations and higher incidence of BRAF mutations [40]. In addition, it has been reported that nevi that display histological features frequently found in nevi present at birth (so-called "congenital pattern nevi") but lack a definitive history of presence at birth showed only 25% of NRAS mutations and 71% of BRAF mutations [44]. NRAS mutations were also found in 48% to 70% of proliferative nodules that developed within congenital nevi early in life, but the presence of such mutations does not seem to confer an increased risk of malignant transformation [44,45].

Recently, different studies have demonstrated that early embryonic/post zygotic somatic mutations in the NRAS gene are implicated in the development of neurocutaneous melanocytosis, a rare congenital disorder, in which affected patients have an increased number of melanocytes in the leptomeninges and the skin, with a large congenital melanocytic nevus usually associated with so-called "satellites" in the vicinity, and childhood melanoma of the central nervous system [46–48]. In line with these observations, recently it has been shown that primary melanoma of the CNS in children carries oncogenic mutations in NRAS, unlike primary melanoma of the central nervous system in adults, in which NRAS is not a common driver oncogene [46].

So-called "dysplastic nevi" do not seem to carry NRAS mutations [49–51]. However, in another study 5/7 "dysplastic nevi" from individuals with a hereditary predisposition to melanoma (who carried germline CDKN2A mutations) were reported to be NRAS mutated and it was suggested that NRAS mutations are implicated during early melanoma development [52]. Overall, given the limited number of cases analyzed and the lack of interobserver agreement for the morphology-based diagnosis of "dysplastic nevi" it is too early to draw significant conclusions. A recent study has shown that nevus-associated melanomas show a similar frequency of BRAFV600-and NRASQ61-mutations compared to published reports of melanomas of the skin in general [53]. Such results do not support the concept that oncogenic BRAF or NRAS mutations play a major role in the development of melanoma from nevi and do not sustain the multistep theory of melanoma progression from a benign melanocytic nevus through "dysplastic nevus" and eventually to melanoma [53].

RAS has been extensively investigated in melanoma and several studies have assessed whether specific RAS isoforms correlate with race, pattern of sun exposure, clinical presentation, and conventional morphological features, which are commonly reported in histopathological reports.

NRAS is mutated in approximately 15–20% of primary cutaneous melanomas in Caucasian patients [54–58]. In black Africans and Asian populations there is a lower frequency (12% and 7.2%, respectively) [59,60]. Patients with NRAS-mutated melanomas were reported to be older in comparison with individuals with BRAF-mutated tumors [61] although in a recent meta-analysis on 31 studies involving 1972 patients, no association between age and NRAS mutations was found [55]. Similarly, no correlation was found between gender and NRAS mutations [58].

In most studies, NRAS mutation was significantly more frequent in melanomas arising in chronic sun-damaged skin [55,62]. The incidence of the NRAS mutation according to tumor site was highest in the extremities (25%), followed by the face or scalp (18%) and trunk (18%) [55,61,63]. NRAS mutations have also been found in conjunctival melanomas (18% frequency) [63], sinonasal melanomas (22%) [65], esophageal melanomas (37.5%), including mutations in exon

1, which is a rare mutation site for cutaneous melanoma [66]. Interestingly, melanoma of unknown primary sites showed NRAS mutations in 32% of cases associated with high somatic mutation rates, high ratios of C>T/G>A transitions, and a 45% of BRAF mutations, collectively indicating a mutation profile consistent with cutaneous sun-exposed melanomas [67].

NRAS mutations are overall more frequently evident in patients with nodular melanoma [55]. From 25% to 31% of NRAS mutations occurred in this melanoma subtype [55,59,68]. A higher incidence of NRAS mutations was found in non-acral fast growing melanomas in comparison with non-fast growing melanomas (26.5 versus 12.1%) [69].

While in some studies NRAS mutated melanomas were reported to be significantly thicker and higher Clark's level than wt tumors [61,62,64,68] other reports could not confirm any association between NRAS mutation and tumor thickness [70,71]. Ulceration was reported to be lower in NRAS-mutated tumors in comparison to BRAF mutated tumors (9.7 versus 22.4%, respectively) [63] but no obvious effect of mutational status on the presence of ulceration was reported by others [68]. Melanomas harboring NRAS mutations have shown greater mitotic rates than BRAF mutant melanomas [63,68].

In conclusion, the retrospective nature of the studies and the heterogeneity of patients' populations may explain the different results obtained so far, and it should be acknowledged that phenotypic-genotypic correlations in melanoma is still a work in progress.

4. NRAS: prognostic or predictive biomarker in melanoma? a critical analysis of current literature

The prognostic and predictive significance of NRAS in melanoma is still a matter of intense debate.

A biomarker is, by definition, an objectively measured and evaluated parameter that provides information on the natural history of a specific disease, its pathogenic process or on pharmacological responses to a specified therapeutic intervention. A prognostic biomarker provides information on overall cancer outcome, regardless of therapy. In the medical literature two types of prognostic biomarkers have been reported: biomarkers that give information on recurrence in patients who receive curative treatment and those that correlate with the median overall survival (OS) in patients with metastatic disease. According to a NIH Consensus Conference, a clinical useful prognostic marker must be a proven independent, significant factor, that is easy to determine and interpret and has therapeutic consequences [72].

Prognostic biomarkers that provide information on the risk of relapse are important not only to better stratify patients in clinical trials but also to spare many patients the treatment-related toxicity without compromising survival. A biomarker with predictive value gives information on the effect of a therapeutic intervention in a patient. Two types of predictive biomarkers have been reported: (1) upfront and (2) early predictive markers. The first can be used for patient selection and the second provides information early during therapy. The latter biomarker is less useful than the former because does not provide reliable and useful information to select the best strategy to be adopted before starting therapy.

4.1. Is NRAS a prognostic biomarker in melanoma?

Several studies have been carried out to examine whether mutations in NRAS confer different pathological features and clinical behavior. The effect of these mutations on clinical outcome remains uncertain [59,61,73,74]. Table 1 summarizes most important studies on the prognostic role of NRAS in melanoma [63,68,74–79,61,80–83]. The majority of these studies have been retrospective in nature, and most of them included patients with recurrent or metastatic disease.

When OS was measured from the time of primary tumor, NRAS mutations were found to have no impact on OS [59,63,61]. Akslen et al. evaluated 51 primary nodular melanomas. In this retrospective study NRAS mutation was found in 27% of patients [82]. RAS mutation was not associated with tumor cell proliferation by Ki-67 expression, tumor thickness, microvessel density, or vascular invasion, and there were no differences in patient survival [82].

In an attempt to correlate BRAF and NRAS mutational status with features known to influence tumor behavior, including age, gender, Breslow depth, Clark level, mitotic rate, the presence of ulceration, and AJCC staging, Ellerhorst et al. performed a study on 223 microdissected primary melanomas [63]. Patients whose tumors carried either mutation presented with more advanced stages compared to patients with wt tumors, and specifically, were more likely to have Stage III disease at diagnosis. BRAF and NRAS mutations did not influence survival. Furthermore, in this study survival did not differ between Stage III patients whose primary tumors do or do not carry mutations, even though the mutated tumors tended to produce larger volume nodal disease [63].

Recently, Devitt et al. reported data obtained from a prospective cohort of 249 patients [67]. When compared to wt NRAS patients, multivariate analysis of melanoma-specific survival identified NRAS mutations as an adverse prognostic factor. However in the multivariate analysis, there was no evidence that NRAS mutation was neither an independent predictor of relapse free survival (RFS) nor of OS [68].

However, in two studies where OS was measured from the time of biopsy of advanced disease, NRAS mutations were associated with improved OS when compared to tumors with BRAF mutations or both BRAF/NRAS wt tumors [73,74].

Mann et al. performed a comprehensive clinicopathological assessment of fresh-frozen macroscopic nodal metastases and the preceding primary melanoma, somatic mutation profiling, and gene expression profiling to identify determinants of outcome in 79 melanoma patients [81]. The authors found that the absence of BRAF mutation or

Table 1
Summary of most significant studies addressing the prognostic significance of NRAS mutations in melanoma.

RAS mutation and melanoma prognosis							
Author	Patients no.	Stage	Site of primary melanoma	Genes	Exons	PFS	OS
Demunter (2001) [75]	81	All stages	Skin	NRAS	1	$p = 0.0130$	–
Omholt (2002) [78]	72	All stages	Skin	NRAS	2 3	–	NS
Houben (2004) [76]	174	All stages	Skin	BRAF	15 11	NS	NS $p = 0.02$[a]
				NRAS	1 2		
Akslen (2005) [81]	57	All stages	Skin	BRAF	15 11	–	NS
				NRAS	2 1		
Edlundh-Rose (2006) [79]	219	NA	Skin	BRAF	15 11	–	NS
				NRAS	2		
Ugurel (2007) [73]	109	III IV	Skin Mucosa Occult NA	BRAF	15 11	–	$p = 0.006$
				NRAS	2 1		
Ellerhorst (2010) [62]	223	I–III	Skin	BRAF NRAS	15 2	–	NS
Devitt (2011) [67]	244	I–III	Skin	BRAF	15	–	$p = 0.04$ (MSS)
				NRAS	3		
Jakob (2012) [77]	667	All stages	Skin Mucosa Uvea Occult	BRAF	15	–	$p = 0.004$
				NRAS	1 2		
Mann (2012) [80]	79	III	Skin	BRAF NRAS	15 2	–	NS
Bucheit (2013) [61]	438	IV	Skin Mucosa Soft parts Occult	BRAF	15	–	NS
				NRAS	1 2		
Birkeland (2013) [74]	85	III IV	Skin Mucosa Uvea Occult	NRAS	3	$p < 0.01$	$p < 0.001$
Ekedahl (2013) [82]	203	IV	Skin	BRAF NRAS	15 2	–	$p = 0.25$

OS: overall survival; PFS: progression free survival; MSS: melanoma-specific survival; NS: not significant.
[a] OS from metastasectomy.

NRAS mutation was independently associated with better survival. Furthermore, a 46-gene expression signature with strong overrepresentation of immune response genes was predictive of better survival; in the full cohort, median survival was >100 months in those with the signature, but 10 months in those without.

Recently, in a retrospective study, Jacob et al. tested for NRAS 677 patients with metastatic melanoma to identify

Table 2
Studies reporting on RAS mutations as predictive biomarkers in melanoma.

RAS as predictive biomarker in melanoma										
Author	Patients no.	Stage	Site of primary melanoma	Genes	Mutations	Drug (s)	OS	PFS	TTP	CCR/CB
Banerji (2008) [83]	6	NR	NR	BRAF	V600E	17-AAG	NR	–	NR	NR
				NRAS	G13D	17-AAG				
Joseph (2012) [84]	208	IIIc IV	NR	BRAF	V600	HD IL2	NS NS	NS NS	–	– $p=0.05$
				NRAS	G12 G13 Q61	HD IL2				
Birkeland (2013) [74]	85	III IV	Skin Mucosa Uvea Occult	NRAS	G12 G13	DTIC	$p<0.001$	NS	–	NS
Patelet (2013) [85]	18	III IV	Skin	BRAF	V600E R603	S. +/−DTIC, TXT, E. or T.	NR	–	NS	NS
				NRAS	Q61R Q61K G12S	S. +/−DTIC, TXT, E. or T.				

17-AAG: HSP90 inhibitor 17-allylamino-17-demethoxygeldanamycin; DTIC: dacarbazine; CB: clinical benefit: objective response or stable disease recorded 3 months after DTIC treatment; HD IL2: high-dose interleukin 2; CRR: clinical response rate; TTP: time to progression; NR: not reported; S.: selumetinib; TXT.: docetaxel; E.: erlotinib; T.: temsirolimus.

significant associations of mutation with tumor and patient characteristics and with survival from the diagnosis of stage IV disease [78]. Tumor mutation status was associated with the risk of central nervous system involvement at the diagnosis. Patients with NRAS mutations had a median survival of 8.2 months from stage IV diagnosis, which was shorter than the median survival of wt patients (15.1 months). At multivariate analysis, after adjusting for age, sex, metastatic site, serum lactate dehydrogenase level, NRAS mutation was independently associated with decreased OS.

Overall, the results published so far are heterogeneous in terms of patients' selection criteria and methodology. Specifically, difficulties in comparing results arise from the following considerations: (i) most of the available data are retrospective; (ii) patients with different tumor stages have been evaluated; (iii) primary or metastatic sites have been tested; (iv) different tumor histotypes have been included.

Hence, there is no definitive evidence that NRAS mutation is prognostic in patients with limited radically resected disease (stages I–III) or in metastatic setting. Furthermore, most of the observations have been conducted in Caucasian populations with scarcity of data from other geographic areas (e.g. Asian).

4.2. Is NRAS a predictive biomarker in melanoma?

The RAS mutational status does not give information on the effect of a therapeutic intervention in a patient, hence it is not a predictive marker either upfront or as early predictive marker. Table 2 includes studies addressing the predictive significance of NRAS mutations in melanoma [75,84–86].

So far, several different strategies of directly targeting RAS have not resulted in effective therapeutics. There is evidence that some NRAS-mutated cell lines are sensitive to MEK inhibition in vitro [87]. However, in this model, the sensitivity to the MEK inhibitor of N-RAS mutated cells was significantly lower than those harboring BRAF mutation.

The lower activity of MEK inhibitors in N-RAS-mutated in comparison with BRAFV600-mutated melanoma cells may be explained by the complexity of pathways with which RAS interacts within the cell.

It is well known that RAS family members have multiple other targets, such as PI(3)K and RalGDS; these may exert more prominent oncogenic effects in certain tumor subtypes, thereby reducing the requirement for MAPK activation. Hence, single-agent therapeutic strategies may prove insufficient in RAS mutant tumors. Instead, direct RAS inhibitors or combinatorial strategies may be required.

Recently, an oral MEK inhibitor (MEK162) was tested in patients with metastatic melanoma harboring BRAF or NRAS mutations with encouraging results in NRAS mutated patients [88]. In preclinical models MEK162 inhibited growth of *NRAS*-mutated and Val600Glu *BRAF*-mutated melanoma in studies that used in vitro and in vivo models [89].

However, the response rate was reported in only 20% of patients and only in 10% of this population the response was confirmed. Furthermore, the median progression-free survival (PFS) was 3.7 months and the median duration of

response was 7.6 weeks [88]. These data clearly indicate that most of the patients rapidly develop resistance to the MEK inhibitor.

A two-arm, randomized, prospective, open-label, multicenter, phase III study to compare the efficacy and safety of MEK162 (45 mg bis in die) versus dacarbazine (1000 mg/m2 IV every 3 weeks) in patients with advanced (Stage IIIC) unresectable or metastatic (Stage IV) NRAS Q61 mutation-positive cutaneous melanoma is currently underway. The primary end point of the study is progression-free survival, while secondary end point is overall survival ("NEMO trial" NCT01763164).

Another second generation MEK inhibitor, selumetinib, demonstrated marked inhibition of pERK, either in cell lines harboring BRAF mutations as well as in those harboring NRAS mutations [90].

A randomized phase II study comparing the MEK inhibitor Pimasertib (AS703026) with dacarbazine in previously untreated subjects with N-Ras mutated locally advanced or metastatic malignant cutaneous melanoma is currently under way (NCT01693068).

At the time of the publication of this manuscript there are no randomized clinical trials comparing MEK162 with other MEK inhibitors in NRAS mutated melanoma patients.

Recently the development of small molecules that irreversibly bind to a common oncogenic mutant, K-Ras(G12C) has been reported [91]. These compounds rely on the mutant cysteine for binding and therefore do not affect the wt protein. These inhibitors to K-Ras(G12C) subvert the native nucleotide preference to favor GDP over GTP and impairing binding to Raf. These findings are relevant since they reveal, for the first time, a new allosteric regulatory site on Ras that is targetable in a mutant-specific manner.

A subgroup of melanomas with RAS dependence is those with low-activity.

BRAF mutations, such as those found at positions 466, 464 and 597. Cell lines with low-activity BRAF mutations show an impaired activation of MAPK signaling in isolated kinase assays and often harbor concurrent NRAS mutations at positions 12 and 13. It cannot be excluded that NRAS melanoma cells with low activity mutant BRAF may partially explain the sensitivity of a subgroup of NRAS melanoma cells to MEK inhibitors.

In accordance with this hypothesis, Dahlman et al. performed an analysis of BRAF exon 15 in 49 tumors with lack of BRAFV600 mutation and showed that 2 (4%) harbored L597 mutations and other 2 BRAF D594 and K601 mutations [92]. In vitro signaling induced by L597 mutants was suppressed by MEK inhibition. A patient with BRAF L597S mutant metastatic melanoma responded significantly to treatment with the MEK inhibitor, TAK-733. Collectively, these data show clinical significance response to BRAF(L597) mutations in melanoma.

The focus of indirect RAS inhibition has then shifted to interfere with the complex network of activated downstream cascades such as the MAPK, phosphoinositol 3-kinase (PI3K), phospholipid C (PLC), RalGEF.

Posch et al. evaluated the sensitivity of RAS mutated melanoma cells and xenografts to MEK and PI3K inhibitors [93]. NRAS mutated cells were more sensitive to MEK inhibition compared with the PI3K/mTOR cascade inhibition. Combined targeting of MEK and PI3K was superior to MEK and mTOR inhibition in all NRAS mutant melanoma cell lines, suggesting that PI3K signaling is more important for cell survival in NRAS mutant melanoma when MEK is inhibited. However, targeting of PI3K/mTOR in combination with MEK inhibitors is necessary to effectively abolish growth of NRAS mutant melanoma cells in vitro and regress xenografted NRAS mutant melanoma. In this model MEK and PI3K/mTOR inhibition was synergistic. These results indicate that combined targeting of the MEK/ERK and PI3K/mTOR pathways has antitumor activity and could be a valid option in the treatment of NRAS mutant melanoma, for which there are currently no effective therapies.

Finally, Johnson et al. reported that patients with NRAS mutated metastatic melanoma achieve increased clinical benefit from immunotherapy compared to those with BRAF/NRAS wt [94].

These data suggest that NRAS mutation status may be a biomarker of response to immunotherapy in metastatic melanoma and that molecularly targeted immunotherapy may be feasible. However a larger, prospective analysis is necessary to validate and expand on these results, including those with BRAF mut and KIT mut metastatic melanoma to draw firm conclusions.

Overall, the above data suggest that: (i) a subgroup of NRAS mutated melanoma may be sensitive to MEK inhibition but in most cases resistance rapidly occur; (ii) a subgroup of NRAS mutated melanoma harbor low activity BRAF mutation, and the meaning of these mutations should be further investigated; (iii) single-agent therapeutic strategies may prove insufficient in RAS mutant tumors. Instead, combinatorial strategies may be required to overcome resistance.

5. NRAS as a mechanism of resistance to BRAF inhibitors in melanoma

A high percentage of patients with BRAFV600E mutant melanomas respond to selective RAF inhibitors but resistance eventually emerges.

Unlike what happens in other tumors where additional mutations eventually occur in the target (EGFR in non-small cell lung cancer, c-KIT in GISTs, BCR-ABL in chronic myeloid leukemia) the early evidence from direct sequencing of BRAF exons suggests that new point mutations are not evident and that BRAFV600E persists.

RAS has been consistently described as a mechanism of resistance to BRAF inhibitors. It is well known that there is a switch in RAF isoform usage depending on whether BRAF or RAS is mutated (Table 3) [95–101]. In melanocytes in

Table 3
Resistance to BRAF inhibitors.

Resistance to BRAF inhibitors				
Author	Patients with acquired resistance to therapy no.	Drug	Mechanism of resistance	NRAS acquired mutations patients no. (%)
Nazarian (2010) [95]	12	Vemurafenib	NRAS codon 61 mutations PDGFRB overexpression	1 (8.3%)
Trunzer (2013) [96]	13	Vemurafenib	Increased pERK levels MEK1 mutations NRAS codon 61 mutations	3 (23%)
McArthur (2011) [97]	11	Vemurafenib	NRAS codon 61 mutations	1 (9.09%)
Poulikakos (2011) [98]	19	Vemurafenib	Increased RAS-GTP levels Increased RAS-independent RAF dimerization	6 (31.6%)
Wagle (2014) [99]	5	Dabrafenib Trametinib	Mutation in MEK2 BRAF splice isoform BRAF amplification	5 (100%)
Van Allen (2014) [100]	30	Vemurafenib Dabrafenib	MAPK pathway Alterations MEK1 Mutations MEK2 Mutations MIFT Amplification	23/45 (51%)
Rizos (2014) [101]	38		Mutation in MEK2 Mutation in MEK1 Mutation in NRAS Mutation in AKT BRAF splice isoform BRAF amplification	3 (8%)

which BRAF is mutated, BRAF is primarily responsible for signaling to MEK and ERK. In presence of RAS mutation an excessive ERK signaling through BRAF and in general MAPK activation would induce cell cycle arrest or senescence through transcriptional up-regulation of proteins such as p21, p27, and $p16^{INK4A}$ [102]. To avoid this, the cells switch to CRAF, which provides weaker signaling and is compatible with tumor progression.

Nazarian et al. demonstrated that high levels of activated N-RAS resulting from mutations lead to significant MAPK pathway reactivation upon BRAF inhibitor treatment [95]. In a series of elegant experiments, knockdown of NRAS reduced growth of the respective BRAF inhibitors resistant cells. On the opposite, overexpression of N-RAS conferred BRAF inhibitor resistance to BRAF inhibitor sensitive parental cell lines.

Recently, Su et al. used cell lines to establish BRAFV600E melanoma clones with acquired resistance to a BRAF inhibitor [103]. The authors confirmed that no second-site mutations could be identified in the BRAF coding sequence. In this model, resistance correlated with increased levels of RAS-GTP, and sequencing of RAS genes revealed a rare activating mutation in KRAS, resulting in a K117N change in the KRAS protein. Elevated levels of CRAF and phosphorylated AKT were also observed. Interestingly, combination treatment with BRAF inhibitor and either a MEK inhibitor or an AKT inhibitor synergistically inhibited proliferation of resistant cells. These data support clinical studies in which combination therapy with other targeted agents are being strategized to overcome resistance.

Trunzer et al. [96] evaluated serial biopsies to study changes in mitogen-activated protein kinase (MAPK) signaling, cell-cycle progression, and factors causing intrinsic or acquired resistance by immunohistochemistry, DNA sequencing, or somatic mutation profiling to a BRAF inhibitor within the BRIM 2 study [104]. In this study 3/13 patients had NRASQ61K co-occurring mutations in tumor samples taken at progression. Combining these findings with those previously reported by Nazarian [95] and McArthur [97], among 36 patients analyzed, five patients (14%) had an *NRAS* mutation in a progressive lesion. This further supports the hypothesis by Nazarian et al. [95] that the *NRAS* mutation is one mechanism of escape from vemurafenib therapy.

Overall, the above reported data suggest that: (1) A concomitant baseline mutation in the upstream NRAS oncogene is rare but may result in early lack of clinical benefit to BRAFi; (2) RAS mutation is a common mechanism of acquired resistance; (3) whether a combination therapy with other targeted agents could overcome resistance remains to be elucidated.

Table 4
RAS target in locally advanced or metastatic melanoma: ongoing clinical trials (www.clinicaltrials.gov accessed January 26, 2014).

Drug	Phase	Trial	Disease(s)	Primary outcome measures
Monotherapy non-randomized				
MEK162	II	NCT01320085	BRAF or NRAS Mutated melanoma	ORR
RAF265	II	NCT00304525	Melanoma	MTD DLT Association mutations in NRAS/clinical response (°)
Selumetinib (AZD6244)	II	NCT00866177	BRAF or NRAS Mutated melanoma	Anti-tumor response
Monotherapy randomized				
Pimasertib versus dacarbazina	II	NCT01693068	NRAS mutated melanoma	PFS
MEK162 versus dacarbazine	III	NCT01763164	NRAS mutated melanoma	PFS
AZD6244 versus temozolamide	II	NCT00338130	Melanoma	PFS ORR* TTD Duration of response Assessment of the efficacy of AZD6244 versus temozolomide BRAF or NRAS MM patients (°)
Combination therapy non-randomized				
BKM120 + MEK162	I	NCT01363232	EGFR mutant NSCLC in PD on EGFR inhibitors Triple negative breast cancer Pancreatic cancer CRC Melanoma NSCLC, with KRAS, NRAS, and/or BRAF mutations	DLT
Trametinib (GSK1120212) + GSK2141795	II	NCT01941927	BRAF wt melanoma	ORR* in patients with either mutated NRAS or wt NRAS/wt BRAF M
RAF inhibitor (BMS-908662) + immunotherapy (ipilimumab)	I	NCT01245556	Melanoma	Toxicity PD will be assessed by evaluating markers of RAS/RAF pathway activity (°)
PI3K/mTOR inhibitor BEZ235 + MEK1/2 inhibitor MEK162	Ib	NCT01337765	EGFR mutant NSCLC in PD on EGFR inhibitors Triple negative breast cancer Pancreatic cancer Colorectal cancer Melanoma NSCLC Other advanced solid tumors with KRAS, NRAS, and/or BRAF mutations	Incidence of DLT
LEE011 + MEK162	Ib	NCT01781572	NRAS mutated melanoma	Incidence of DLT ORR*

MTD: maximum tolerated dose; EAS: ectopic ACTH secreting; wt: wild type; NR: not reported; ORR: overall response rate; PFS: progression free survival; TTD: time to death; DLT: dose limiting toxicity; ORR*: objective response rate; CRC: colorectal cancer; (°): secondary outcome measures.

The importance of RAS in melanoma deserves clinical and biological investigation to optimize treatment of locally advanced and metastatic melanoma. Although, in the last two decades, progress has been slow, there are now a variety of therapeutic strategies that are primed for clinical investigation. Table 4 summarizes ongoing trials in which RAS, and preferentially NRAS, has been selected as a target.

6. Future directions

Thirty years of basic, clinical and translational research have produced a large amount of knowledge pertaining to the RAS oncogene family (Fig. 4). The prevalence of RAS mutations, but also the high number of RAS activators and effectors identified in mammalian cells place the RAS

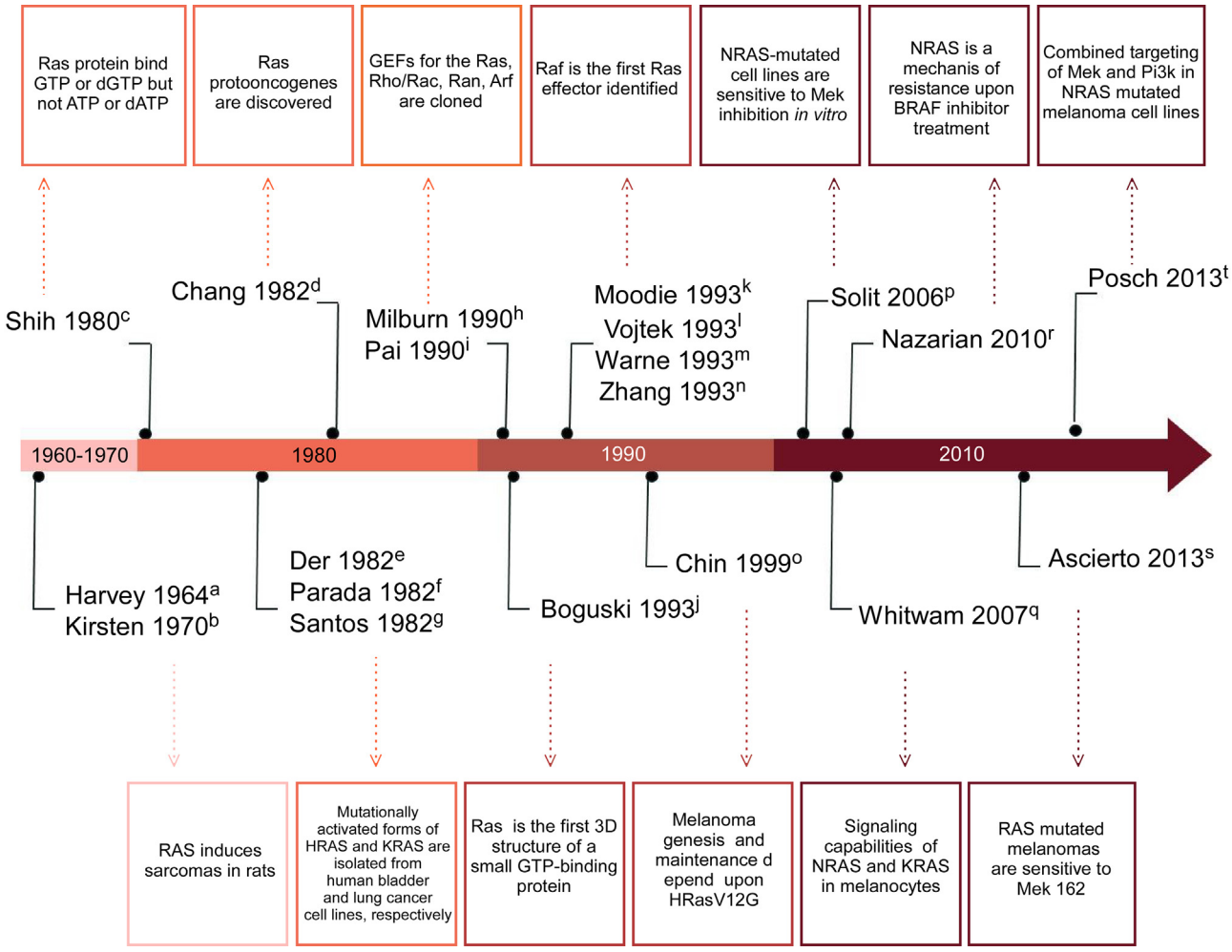

Fig. 4. Timeline of key advances in NRAS clinical and translational research. (a) Harvey et al. (1964) [97]; (b) Kirsten et al. (1970) [98]; (c) Shih et al. (1980) [99]; (d) Chang et al. (1982) [2,3], (e) Der et al. (1982) [4], (f) Parada et al. (1982) [5], (g) Santos et al. (1982) [100], (h) Milburn et al. (1990) [103]; (i) Pai et al. (1990) [102]; (j) Boguski et al. (1993) [101]; (k) Moodie et al. (1993) [9]; (l) Vojtek et al. (1993) [10]; (m) Warne et al. (1993) [11], (n) Zhang et al. (1993) [12]; (o) Chin et al. (1999) [22]; (p) Solit et al. (2006) [86]; (q) Nazarian et al. (2010) [90]; (r) Whitwam et al. (2007) [37]; (s) Ascierto et al. (2013) [87], (t) Posch et al. (2013) [89].

proteins at the crossroads of several signaling networks. Nevertheless, this extensive knowledge has not yet translated into clinically effective therapies for melanomas expressing mutant forms of RAS.

As RAS is mutated in 15–20% of melanomas, priority actions are needed:

1. Future studies should focus on co-extinction strategies other than reinforcing inhibition of MAPK signaling.
2. Inhibition of the activated downstream cascades including MAPK, PI3K, PLC, RAL should be pursued in preclinical and early phase clinical studies.
3. Most of the downstream targets are not tumor specific therapies and bear the risk of severe side effects. Hence, well-designed clinical studies with appropriate pharmacokinetics and pharmacodynamic end point between combination therapies are needed.
4. MEK inhibitors as monotherapy should be validated in prospective, randomized phase III studies.

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