REVIEW

Regulators of the RAS-ERK pathway as therapeutic targets in thyroid cancer

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Abstract

Thyroid cancer is mostly an ERK-driven carcinoma, as up to 70% of thyroid carcinomas are caused by mutations that activate the RAS/ERK mitogenic signaling pathway. The incidence of thyroid cancer has been steadily increasing for the last four decades; yet, there is still no effective treatment for advanced thyroid carcinomas. Current research efforts are focused on impairing ERK signaling with small-molecule inhibitors, mainly at the level of BRAF and MEK. However, despite initial promising results in animal models, the clinical success of these inhibitors has been limited by the emergence of tumor resistance and relapse. The RAS/ERK pathway is an extremely complex signaling cascade with multiple points of control, offering many potential therapeutic targets: from the modulatory proteins regulating the activation state of RAS proteins to the scaffolding proteins of the pathway that provide spatial specificity to the signals, and finally, the negative feedbacks and phosphatases responsible for inactivating the pathway. The aim of this review is to give an overview of the biology of RAS/ERK regulators in human cancer highlighting relevant information on thyroid cancer and future areas of research.

Key Words

- thyroid cancer
- RAS/ERK
- GEF/GAP
- scaffold
- MKP

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An overview of thyroid cancer

Thyroid cancer is the most common of the endocrine malignancies and its incidence has been steadily rising during the last four decades (Lim *et al.* 2017). Approximately 90% of all thyroid carcinomas derive from thyroid follicular cells, a monolayer of epithelial cells that renders the follicles and fulfills the main function of the gland – the synthesis and secretion of thyroid hormones (Colin & Gerard 2010). A second cellular type specific for the thyroid is the parafollicular or C-cell, which is responsible for calcitonin production and gives rise to medullary thyroid carcinoma (MTC). In this review, we will discuss only those carcinomas arising from the follicular cells, since they represent the vast majority of

malignant lesions of the thyroid gland. Papillary thyroid carcinoma (PTC) is the most frequent histotype of thyroid tumor, with an incidence of ~80% (Hay *et al.* 2002), followed by follicular thyroid carcinoma (FTC), which represents 10–15% of all tumors. Both carcinomas can be classified as well-differentiated thyroid carcinomas (WDTC), which have a low proliferative index and mutational density and, consequently, a good prognosis. The current treatment for these cancers is removal of the gland (thyroidectomy) followed by radioiodine and thyroid hormone replacement therapy, which has proven a very effective approach with 5- to 10-year survival rates of 90–98%. However, a subset of those WDTC

progress to very aggressive, albeit rare (1–6%) variants termed the poorly differentiated (Volante *et al.* 2007) and anaplastic (Smallridge *et al.* 2009) thyroid carcinomas (PDTC and ATC, respectively), which are characterized by an extremely high proliferation rate and mutational burden, local and disseminated metastases, and a dismal outcome, with a respective mean survival of 3.2 and 0.5 years. PDTC is a transitional step in the progression to ATC and presents intermediate characteristics in terms of differentiation and aggressiveness. Although some kinase inhibitors of the ERK (extracellular signal regulated protein kinase) pathway have been approved for the treatment of advanced thyroid carcinomas refractory to radioiodine uptake (Carneiro *et al.* 2015), the therapeutic response to these drugs is only temporary and relapse of the tumor due to the emergence of resistance is common. Additionally, systemic inhibition of the ERK pathway generates toxicity, especially in highly proliferative tissues. Currently, there is a lack of effective treatments for advanced thyroid carcinomas.

Thyroid cancer is essentially an ERK-driven carcinoma. *BRAF* (rapidly accelerated fibrosarcoma type-B) mutations are found in up to 60% of PTC, followed by mutations in the different *RAS* (rat sarcoma) isoforms (10–15%) with a strong bias for *NRAS*, which are specially associated with FTC and the follicular variant of PTC (FV-PTC). Genetic rearrangements leading to ectopic expression of chimeric tyrosine kinase receptors (TKRs) such as *RET* (rearranged during transfection), *ALK* (anaplastic lymphoma kinase) and *NTRK* (neurotrophic receptor tyrosine kinase) can also be found in both PTC and FTC, with a higher frequency in children than in adults, and may be related to radiation exposure. In addition, genetic rearrangements in *PAX8/PPARγ* (paired box8/peroxisome proliferator-activated receptor gamma) are also found at high rates in FTC and FV-PTC and in an exclusive manner with the aforementioned mutations, and hence, it is also considered an oncogenic driver. While the underlying transforming mechanism is poorly understood, it might be related to *PPARγ* loss of function (Kroll *et al.* 2000). The high overall incidence of these mutations, their clonal nature and exclusive occurrence, provides strong evidence for the requirement of RAS/ERK activation at the onset of WDTC.

The initial *RAS* or *BRAF* mutation is central to the subsequent behavior of the tumors they give rise to. *BRAF* mutations confer strong activation of ERK, whereas *RAS* mutations provide weaker ERK activation, but allow activation of other relevant effectors involved in tumorigenesis such as PI3K (phosphatidylinositol

3-kinase) or *RAC1* (ras-related C3 botulinum toxin substrate 1) (Cancer Genome Atlas Research Network 2014). These differences in signaling likely explain the intrinsic features of *BRAF*- and *RAS*-driven tumors – papillary pattern of growth, lower differentiation state and lymphatic dissemination with lymph node metastases in the former *versus* follicular growth pattern, higher differentiation state and hematogenous spread with distant metastases in the latter.

While there is no doubt that the aforementioned genetic lesions are driver mutations, they do not seem to confer aggressive properties to WDTC *per se* – most of these tumors are confined to the connective tissue capsule that encloses the gland, grow very slowly, present a silent genome in terms of mutation density and retain, to some extent, a differentiated state. This begs the question – how do these indolent tumors progress to overwhelmingly malignant advanced thyroid carcinomas?

Work from The Cancer Genome Atlas (TCGA) Network on PTC has greatly helped in identifying the oncogenic drivers responsible for PTC initiation (Cancer Genome Atlas Research Network 2014) and, together with other pan-genomic studies including large cohorts of patients with PDTC or ATC (Kunstman *et al.* 2015, Landa *et al.* 2016), has provided a model of progression based on genomic instability and a lack of genome control. From this large cohort of PTC, only seven genes were found mutated at a significant rate: *BRAF*, *N*, *K*, and *HRAS*, *EIF1AX* (eukaryotic translation initiation factor 1A X-linked), *CHEK2* (checkpoint kinase 2) and *PPM1D* (protein phosphatase, Mg²⁺/Mn²⁺ dependent 1D). The last two encode for proteins involved in DNA repair and were found at a very low prevalence (1.2%). Interestingly, alterations in these genes occurred concomitantly with ERK pathway mutations. An additional eight genes related to DNA repair were also found mutated (6.5% overall) in a mutually exclusive manner, without reaching significance. Furthermore, the study identified mutations in 57 epigenetic regulatory genes. The bulk of PTC (72.9%) presented a silent genome in terms of somatic copy-number alterations (SCNA) but, interestingly, 9.9% of the tumors showed a 22q arm-level deletion that contains the *CHEK2* and *NF2* (neurofibromin 2) loci and was largely associated with FV-PTC. Loss of heterozygosity in this region has been related to enhanced RAS signaling through a mechanism involving the loss of function of the tumor suppressor *NF2* and activation of the Hippo pathway effectors YAP1 (Yes-associated protein 1) and TEAD (TEA domain transcription factor) (Garcia-Rendueles *et al.* 2015). A hypothesis derived from these

reports is that mutations activating the RAS/ERK axis, together with alterations in genes controlling the health and/or the epigenetic state of the genome, could be responsible for the subset of WDTC that progress to advanced thyroid carcinomas. A model of thyroid cancer progression including the most relevant data is depicted in Fig. 1.

The high rates of *BRAF* and *RAS* mutations in PDTC and ATC are strong indicators of their origin from WDTC. They are also found in an exclusive manner, reflecting their clonal origin and also indicating that a single mutation in one member of the pathway is sufficient not only for the onset of WDTC, but also for the progression to the aggressive variants. Mutations in genes related to DNA repair, epigenetic regulation and chromatin remodeling are also enriched in advanced tumors, again underscoring their involvement in tumor progression (Landa *et al.* 2016).

Mutations in other genes that synergize with RAS/ERK activation in conferring tumor aggressiveness can be found at high rates in advanced thyroid tumors. *TERT* (telomerase reverse transcriptase) promoter mutations can be found at relatively low rates in PTC, but are enriched in PDTC and ATC in a progressive manner (9, 40 and 73%, respectively) (Cancer Genome Atlas Research Network 2014, Landa *et al.* 2016). Furthermore, they are frequently found together with *RAS* and *BRAF* mutations, conferring aggressiveness and poor prognosis. Mutations in the

TERT promoter generate *de novo* binding sites for ETS (E-twenty-six) transcription factors, which are activated by RAS/ERK signaling (Horn *et al.* 2013, Huang *et al.* 2013), explaining the synergy with *BRAF* mutations. This hypothesis has been recently demonstrated in human thyroid tumor-derived cell lines, where FOS activation by *BRAF*^{V600E} increased the expression of the ETS transcription factor GABPB to selectively activate mutant *TERT* promoter (Liu *et al.* 2018). Increased *TERT* expression allows repeated cycles of cell division without shortening of telomeres, resulting in tumor cell immortality (Nakamura *et al.* 1997).

Mutations in *EIF1AX* are rare (1.5%) but occur widely in PTC (Cancer Genome Atlas Research Network 2014). Interestingly, these mutations appear exclusively with established driver mutations such as *BRAF*, *RAS* or *RET/PTC*, pointing to *EIF1AX* as a novel driver of WDTC. This exclusivity is lost in advanced thyroid tumors, where *EIF1AX* mutations are found at higher rates – 11% in PDTC and 9% in ATC – and, when found together with *RAS* mutations, result in an increased risk of recurrence and poor prognosis (Landa *et al.* 2016). The underlying mechanism has been linked to enhanced activation of the PI3K/mTOR (mechanistic target of rapamycin) pathway (Krishnamoorthy *et al.* 2019).

Mutations in different components of the PI3K pathway are also found in PDTC (11%) and particularly in ATC (39%), but with a very low prevalence in PTC

Figure 1

Model of thyroid cancer progression. An initial oncogenic trigger frequently induces mutations in the driver genes *BRAF* and *RAS*, which lead to the onset of papillary thyroid carcinoma (PTC) and follicular thyroid carcinoma (FTC), respectively. Those carcinomas are differentiated, and present a slow growth, a low mutational background and an excellent prognosis. However, they promote genomic instability. Secondary mutations in genes related to DNA repair (*CHEK2* and *PPM1D*) and epigenetic regulation increase the mutational burden and alter general gene expression, conferring a hypermutator phenotype. Additional mutations in the *TERT* promoter, *EIF1AX*, *TP53* or components of the PI3K pathway allow progression to advanced and metastatic carcinomas, with increased aggressiveness and growth rate and progressive loss of genome control and differentiation. *TERT* and *BRAF*, in blue, and *RAS* and *EIF1AX* mutations, in pink, synergize to promote progression to advanced thyroid carcinomas.

(4.5%), suggesting that they might represent late genetic events related to tumor progression (Cancer Genome Atlas Research Network 2014, Landa *et al.* 2016; reviewed in Riesco-Eizaguirre & Santisteban 2016).

The dependency of thyroid carcinomas on RAS/ERK activation has been exploited clinically by the use of inhibitors of the different kinases of the pathway. BRAF and MEK (MAP/ERK kinase) inhibitors have been approved for the treatment of RAIr (radioactive iodine-refractory) advanced thyroid carcinomas (Carneiro *et al.* 2015), supported by the encouraging results obtained in mouse models. The efficacy of these inhibitors is due to their capacity to impair ERK activation (Nagarajah *et al.* 2016). However, since they are administered systemically, this is often accompanied by undesirable side effects on highly proliferative tissues such as skin and the intestinal epithelial barrier. A necessary balance is thus required to reach an effective therapeutic threshold. Despite initial encouraging results, a lasting response to these drugs is, however, infrequently seen because of the emergence of resistance mechanisms that reactivate the pathway, leading to tumor relapse. This reflects the complexity of the RAS/ERK pathway, with multiple upstream regulators, downstream effectors, regulators that provide specific locations, multiple positive and negative feedback loops and crosstalk with other signaling pathways. Understanding the dynamics of activation, the mechanisms that confer specificity to the pathway, and their association with the tumorigenic process provides the basis for the inhibition of specific targets that may be essential for the establishment of the primary lesion or its dissemination. Theoretically, this selective approach would be less likely to trigger resistance mechanisms, and undesired toxic effects.

The RAS/ERK pathway

Mitogen-activated protein kinase (MAPK) pathways are evolutionary-conserved signaling cascades that link extracellular and internal stimuli to the control of multiple cellular processes both in physiological and pathological conditions, including cell adhesion, proliferation, survival, metabolism, migration, invasion, differentiation and gene transcription. Six different MAPK modules are known to exist in mammals. We will focus here on the RAS/ERK axis (Fig. 2), owing to its relevance for human and thyroid carcinogenesis.

In a prototypical scenario, a growth factor binds to its TKR, which undergoes auto-phosphorylation,

allowing the binding of adaptor proteins that recruit RAS exchange factors to the membrane where they activate RAS proteins. There are four main RAS isoforms encoded by three independent genes, *N*, *H* and *KRAS* (*KRAS* has two splicing variants, *KRAS4A* and *4B*), with overlapping and specific functions, ubiquitous expression and high sequence homology except for the carboxy-terminal hypervariable region (HVR), which is thought to determine specificity in signaling and functions (Prior & Hancock 2012). RAS proteins use the HVR to bind to specific lipid microdomains of the plasma membrane and intracellular organelles.

All RAS proteins function as molecular switches that cycle between a GDP-bound, inactive state and a GTP-bound active state (Bos *et al.* 2007). Activation is facilitated by GEFs (guanine-nucleotide exchange factors)

Figure 2

Overview of the RAS/ERK pathway. Upon growth factor binding, the tyrosine kinase receptor undergoes autophosphorylation creating binding sites for adaptor proteins that recruit RAS proteins to the membrane, allowing interaction with GEFs that promote GDP to GTP exchange and activation. GAPs promote inactivation by increasing intrinsic RAS-GTPase activity. Activated RAS induces the sequential activation of the kinase catalytic core formed by RAF, MEK and ERK, in a process dependent on dimerization. Activated ERK elicits a dramatic response both in the nucleus and cytoplasm, interacting with a myriad of effectors. Spatiotemporal activation is modulated by scaffold proteins of the pathway and negative regulation is driven by ERK-dependent expression of MKPs that dephosphorylate ERK in the nucleus and cytoplasm. Single arrows mean activation; blunt ends mean inactivation; double arrows indicate transition between two states; dotted lines indicate changes in subcellular localization.

that promote GDP release from RAS proteins, allowing the incorporation of GTP. RAS affinity for GDP and GTP is similar, and binding to GTP is determined by the 10-fold higher cellular concentration of the latter. The GEF is released by conformational changes induced by GTP binding, and interaction with effectors proceeds. Inactivation is catalyzed by GAPs (GTPase-activating proteins) that increase intrinsic RAS GTPase activity, leading to GTP hydrolysis.

In human cancers, specific missense RAS mutations at codons 12, 13 or 61 decrease the affinity toward GAPs, leading to a constitutively GTP-bound active state (Prior *et al.* 2012). RAS is mutated in one-third of all human carcinomas (Bos 1988), with a relative frequency of 85%, 12% and 3% for *K*, *N* and *HRAS*, respectively. Accordingly, their inhibition has been the focus of intense research for more than three decades. Strategies directed at RAS membrane binding, and to the synthesis of GTP analogs or molecules designed to bind the RAS catalytic pocket, have been unsuccessful, and the inhibition of downstream effectors is currently the main subject of research (Cox *et al.* 2014). Once activated, RAS promotes downstream activation of up to 11 families of effectors, and six of them have been implicated in RAS tumorigenic effects (Pylayeva-Gupta *et al.* 2011).

Possibly the best studied RAS effectors involved in human cancer are the RAF proteins, a family of serine/threonine kinases composed of three members in mammals –ARAF, BRAF and CRAF (RAF1) – which are located at the origin of the three-tiered cascade that leads to ERK activation. Insights gained from gene-knockout studies in mice have shown that all three RAF isoforms fulfill both overlapping and non-redundant functions (Pritchard *et al.* 1996, Wojnowski *et al.* 1997, Huser *et al.* 2001, Mikula *et al.* 2001). BRAF presents the highest basal kinase activity (Mason *et al.* 1999) and is the most frequently mutated isoform in human cancer (Davies *et al.* 2002). Indeed, more than 40 different activating mutations in BRAF are known, although the most frequent is the V600E substitution in the activation loop, which accounts for 90–95% of all BRAF mutations (Dankner *et al.* 2018). CRAF and ARAF have been also found mutated in tumors but a much lower rate. RAF proteins are activated in an intricate and not well-understood process that involves phosphorylation and de-phosphorylation of different residues, conformational changes and formation of dimeric complexes (Lavoie & Therrien 2015). Several drugs targeting BRAF^{V600E} have been approved for the treatment of metastatic melanoma and RAI-resistant thyroid carcinomas. Although the response rate is initially high,

relapse of the tumors is largely inevitable due to innate and acquired drug resistance mechanisms (Mandal *et al.* 2016).

There are two major effectors described for RAF proteins – MEK1 and MEK2 – that are respectively activated by phosphorylation on Ser218/Ser222 and Ser222/Ser226 in the activation segment (Kyriakis *et al.* 1992). Once activated, MEKs activate their only known substrates, ERK1 and ERK2, also by phosphorylation, on Thr202/Tyr204 and Thr185/Tyr187, respectively, in the TEY (Thr-Glu-Tyr) motif at the activation loop (Ray & Sturgill 1988, Roskoski 2012). More than 200 effectors have been described for ERK proteins, both in the nucleus and cytoplasm, reflecting their ability to control many different aspects of cell biology (Yoon & Seger 2006, Zaballos & Santisteban 2017).

ERK activation is tightly controlled by at least 15 scaffold proteins. By definition a scaffold is a protein that binds simultaneously to at least two members of the same signaling pathway. Scaffold proteins fine-tune signals by regulating their amplitude, intensity and timing, while isolating them from interference with other signaling pathways. They also provide spatial selectivity to the signals by confining them to specific subcellular localizations (Calvo *et al.* 2010). Signal flux through the ERK pathway is shut down, or attenuated, by immediate negative feedback loops, and by phosphatases that inactivate the different kinases of the cascade in a gradual manner through dephosphorylation of the activating residues (Keyse 2000).

Most of these spatiotemporal regulators are not usually found mutated in human cancer, although alterations in their expression are commonly associated with different tumor types. The idea of targeting protein–protein interactions in the RAS/ERK pathway, rather than kinase activities, has been proposed as an alternative therapeutic strategy and several approaches have already proven successful at the preclinical level (Garcia-Gomez *et al.* 2018). ERK dimerizes upon phosphorylation of the activation loop, which is essential for extranuclear signaling, and preventing this process with small-molecule inhibitors impairs activation of cytoplasmic but not nuclear ERK effectors and decreases tumor growth of melanoma cells in a xenograft mouse model (Herrero *et al.* 2015). Interestingly, ERK dimerization inhibitors are unaffected by resistance mechanisms that impair BRAF and MEK inhibitors (Herrero *et al.* 2015). This illustrates how targeting a protein–protein interaction can yield therapeutic effects.

The RAS/ERK pathway offers multiple potential therapeutic targets. We will next describe the main RAS

GAPs and GEFs, the scaffold proteins of the pathway and the negative feedback mechanisms involved in the inactivation of this important signaling cascade (Fig. 2), which could be exploited for therapeutic intervention, with special attention to data pertaining to thyroid cancer.

RAS GEFs and GAPs

All small GTPase cycles between an inactive (GDP-bound) state and an active (GTP-bound) state. GTPases possess several structural features to accomplish these functions (Fig. 3A). They harbor a 'G domain' in the N-terminal region, composed of five distinctive G-boxes, G1–G5. G1, also known as P-loop (Saraste *et al.* 1990), is a purine nucleotide binding region. The G2 box is necessary for the functioning of the switch I region that plays an important role in the interaction with effector proteins (Spoerner *et al.* 2001). The function of the G3 box is related to the union and orientation of the catalytic Mg^{2+} ion and the reactive water molecule, acting as a sensor for the conformational change of the switch II region. The G4 region makes hydrogen bond contact with the guanine ring, which confers specificity for guanine

nucleotides. Finally, the amino acids in the G5 box form indirect associations with the guanine nucleotides, which stabilize this union (Dever *et al.* 1987, Colicelli 2004). In addition, RAS shares two common domains important for the correct activation and function of the GTPase – the switch I and switch II regions. These domains change their structure in response to the transition from the GTP-bound to the GDP-bound state (Fiegen *et al.* 2006). Finally, a HVR is located at the C-terminus. As its name suggests, this region is the least homologous among the members of the family. The HVR contains the information necessary for all post-translational modifications that RAS proteins undergo during their maturation and is necessary for RAS binding to membranes, thereby dictating the protein localization within the cell (Wennerberg *et al.* 2005).

RAS GEFs

Under physiological conditions, the RAS activation cycle is exquisitely regulated by GEFs and GAPs (Fig. 3B). There are specific GEFs and GAPs for each GTPase family (Fig. 4), but the mechanisms of action are mostly quite similar (Vigil *et al.* 2010). GEF proteins catalyze nucleotide dissociation from the GTPase by sterically displacing the

Figure 3

G-protein domains and activation cycle. (A) Functional domains of small G proteins. From the N-terminus, residues 1–166 comprise the G domain, containing the consensus sequences for the GDP/GTP-binding motifs. There are two regions inside the G domain important for the conformational change in the GDP/GTP cycle, termed switch I and switch II. The C-terminal region contains a hypervariable region, which determines plasma membrane anchoring. (B) The Ras GTPase cycle. The RAS proteins cycle between an inactive (RAS-GDP) and an active (RAS-GTP) state, regulated positively by GEFs (guanine exchange factors) and negatively by GAPs (GTPase-activating proteins).

Figure 4

Representative RAS GEFs and RAS GAPs. The catalytic domain, either the GEF or the GAP domain, is highlighted in orange. (A) Domain structure of RAS GEFs. The different domains are as follows (in order of appearance): HF (histone folding motif), DH (Dbl-homology), PH (pleckstrin homology), REM (ras exchange motif), PR (proline-rich domain), IQ (calmodulin-binding domain), EF (Ca^{2+} binding domain), C1 (DAG-binding C1 domain), Ras-association (RA), PDZ and cNMP (cAMP/cGMP binding (cNMP-BD) domains. (B) Domain structure of RAS-GAPs. The different domains are as follows (in order of appearance). SH2 and SH3 are domains with homology to the regions 2 and 3 of Src, PH (pleckstrin homology), C2 (high-affinity Ca^{2+} -dependent phospholipid binding domain), CSRD (cysteine and serine-rich domain), SEC14 (a lipid-binding domain) and BTK (Bruton's tyrosine kinase motif).

Mg²⁺ ion and triggering conformational changes on the switch I and switch II regions and the P-loop, which promotes the dissociation of the GDP nucleotide and, subsequently, the release of the GEF itself. Once the GEF is released, the tendency to recover the native conformation restitutes RAS nucleotide affinity and because the cellular concentration of GTP is 10–50-fold higher than that of GDP, the replacing nucleotide will be usually GTP. The incorporation of GTP induces a conformational change in the switch I and II regions, exposing the effector domain and thereby facilitating its interaction with effector molecules (Hall *et al.* 2002).

In mammals, four different GEF families are known to promote nucleotide exchange in RAS: SOS, RASGRF, RASGRP and CNRAS (Fig. 4A). The SOS (son of sevenless) family is composed of two members, SOS1 and SOS2, similar to the RASGRF family (RASGRF1 and 2), whereas the RASGRP family has four isoforms and CNRAS is unique. All four families have a CDC25 domain in common, which was initially described in *Saccharomyces cerevisiae* (Broek *et al.* 1987) and harbors the catalytic activity responsible for nucleotide exchange. Also within this region is the REM (RAS exchange motif), which is important for stabilizing binding to RAS (De Hoog *et al.* 2001). In addition to these motifs, a broad range of domains can also be found in the different GEF families. These mostly serve regulatory roles, orchestrating interactions with proteins and ions and playing important roles in cellular sub-localization (Pham *et al.* 2000, Rojas & Santos 2006).

RAS GEFs in cancer

Since GEFs are the major contributors to small GTPase activation under physiological conditions, it is hardly surprising that their deregulation can contribute to human neoplasia. Strikingly, whereas GEFs for RHO GTPases often appear mutated and behave as *bona fide* oncogenes in human neoplasias, this is rarely the case for RAS GEFs (Bustelo 2018). For instance, in hereditary gingival fibromatosis type 1, a pre-neoplastic condition, a cytosine insertion in exon 21 of the *SOS1* gene causes an overgrowth of the gingiva (Hart *et al.* 2002). Similarly, it has been reported that missense mutations in *SOS1* have a causal relationship with Noonan syndrome, a developmental disorder characterized by short stature, facial dysmorphism, congenital heart defects, skeletal anomalies and augmented tumor frequency (Tartaglia *et al.* 2007). This finding suggested a possible role for *SOS1* as an oncogene. However, further sequencing endeavors in a broad spectrum of primary malignancies determined

that *SOS1* missense mutations were present only in an insignificant number of cases (Swanson *et al.* 2008), arguing against the role of *SOS1* as a driver oncogene in human cancers.

While not directly involved in cellular transformation, RAS GEFs are clearly involved in other processes relevant to carcinogenesis. For example, RASGRF1 expression correlates with that of matrix metalloproteinase-9 (MMP9) and with enhanced metastatic capabilities of human melanoma cells (Zhu *et al.* 2007). A similar role has been proposed for RASGRF2 in controlling migration and invasion by modulating MMP9 through SRC/PI3K and NFκB pathways (Lu *et al.* 2018). RASGRF2 has been implicated in metastasis and invasion also in melanoma cells by regulating the transition between amoeboid and mesenchymal movement *via* inhibition of CDC42 (cell division cycle 42) (Calvo *et al.* 2011). A RASGRF2-GM130 (Golgi matrix protein 130 kDa) complex inhibiting CDC42 signaling has also been implicated in cellular polarity and is lost in breast and colorectal tumors (Bascieri *et al.* 2014). In another context, low expression of RASGRF2 is associated with aberrant methylation patterns in human non-small-cell lung cancer (Fernandez-Medarde & Santos 2011).

Regarding the RASGRP family, RASGRP3 contributes to the malignant phenotype of prostate cancer cells as its downregulation inhibited cell proliferation, impeded cell migration and induced apoptosis (Yang *et al.* 2010a). Suppression of RASGRP3 in melanoma cells has been linked to the inhibition of cell proliferation and decreased tumor xenograft growth in immunodeficient mice. Moreover, overexpression of RASGRP3 in human primary melanocytes caused alterations in cellular morphology, enhanced cell proliferation and promoted tumorigenesis in a mouse xenograft model (Yang *et al.* 2011). Aberrant expression of RASGRPs has been detected in hematopoietic malignancies, such as acute myeloid leukemia (AML) (Ksionda *et al.* 2013). RASGRP4 has transforming potential in fibroblast or epithelial cells, and when overexpressed in myeloid cells in the presence of phorbol esters, RASGRP4 promotes cytokine-independent growth (Reuther *et al.* 2002, Ksionda *et al.* 2013). A possible role for RASGRP4 in leukemogenesis has been suggested by the finding that RASGRP4 overexpression in a mouse bone marrow transplantation model results in AML-like characteristics (Watanabe-Okochi *et al.* 2009).

To date, there is scarce evidence for the involvement of RAS GEFs in thyroid malignancies. However, this likely reflects an understudied field rather than a discordant behavior compared with other tissues.

RAS GAPs

Even though RAS proteins exhibit an intrinsic capacity for hydrolyzing GTP, this is a slow reaction, of about 30 min ($\sim 4 \times 10^{-4}$ /s) (Trahey & McCormick 1987). RAS GAPs accelerate this reaction by several orders of magnitude (Allin *et al.* 2001). Structural studies have demonstrated that all hitherto known GAPs function in a similar fashion – by inserting a highly conserved arginine residue, the arginine finger, into the RAS active site (Ahmadian *et al.* 1997). This structural change results in an augmented net positive charge of the pocket, facilitating the nucleophilic attack undertaken by the reactive water molecule, associated with the conserved Gln61 residue, thereby enabling the cleavage of the gamma-beta-phosphoanhydride bond in GTP. This mechanism of action also provides an explanation for the constitutive activity and oncogenicity of RAS mutants harboring mutations in Gln61, based on their unresponsiveness to GAPs (Scheffzek *et al.* 1997, Vetter & Wittinghofer 2001).

Mammals express five families of GAPs, of which the best characterized are RASA1/p120GAP, NF1 and GAP1 (Fig. 4B). All of them possess a highly conserved RAS-GAP catalytic domain where the arginine finger is located (Fig. 4). Also included in this region is the alpha7 variable loop, which determines the specificity of RAS-GAP binding and the invariant FLR (phenylalanine-leucine-arginine) motif, which forms a scaffold that stabilizes RAS switch regions, contributing to the stimulation of the GTPase activity (Ahmadian *et al.* 2003, Rojas & Santos 2006). In addition, RAS GAPs also have a number of different domains, including CSRD (cysteine and serine-rich domain), PH (pleckstrin homology), Sec14 lipid-binding domain, SH2 (Src homology 2) and SH3 domains. All these domains are involved in regulatory functions, mostly involving protein–protein and protein–lipid interactions (Rojas & Santos 2006).

RAS GAPs in cancer

In contrast to the limited evidence on RAS GEFs participation in cancer, the involvement of GAPs in carcinogenic processes is well established.

Fibroblasts deficient for RASA1/p120GAP exhibit hyperactivation of the RAS/ERK pathway, but not abnormal cell proliferation. Similarly RASA1-deficient mice do not have an increased incidence of tumors (Henkemeyer *et al.* 1995, Koehler & Moran 2001), which suggests that RASA1/p120GAP may be involved in RAS downregulation in physiological processes other than

mitogenic signaling. Accordingly, RASA1 mutations in human tumors are scarce, at best (Friedman *et al.* 1993).

In contrast to RASA1, neurofibromin 1 (NF1), another GAP for RAS, is clearly implicated in carcinogenic processes, acting as a tumor suppressor. Loss-of-function mutations in *NF1* underlie neurofibromatosis type I syndrome, characterized by elevated RAS-GTP levels and an increased incidence of tumors (Yang *et al.* 2006). Furthermore, somatic inactivation of *NF1* is often detected in a broad spectrum of tumors, including lung carcinoma (Ding *et al.* 2008), several leukemias (Boudry-Labis *et al.* 2013), ovarian (Sangha *et al.* 2008) and breast (Dischinger *et al.* 2018) cancer and glioblastoma (Parsons *et al.* 2008). The implication of *NF1* in melanoma has also been widely studied after the discovery of a homozygous *NF1* deletion in 1993 (Andersen *et al.* 1993). Since then, several studies have reported a significant frequency of somatic *NF1* mutations in melanoma (Nissan *et al.* 2014, Krauthammer *et al.* 2015, Ratner & Miller 2015). In the case of thyroid cancer, *NF1* mutations are found at a very low prevalence in PTC (0.5%) in tumors lacking any other known driver mutation (Cancer Genome Atlas Research Network 2014). Interestingly this increases up to 9% in ATC (Landa *et al.* 2016). Furthermore, *NF1* mutations have been associated with acquired resistance to HRAS inhibition in an advanced thyroid cancer mouse model (Untch *et al.* 2018).

More recently, another RAS GAP, DAB2IP (DAB2 interacting protein), has been reported to act as a tumor suppressor in prostate cancer (Wu *et al.* 2014) and hepatocellular carcinoma (Zhang *et al.* 2012). In this respect, reduced *DAB2IP* expression as a consequence of aberrant promoter methylation has been shown in prostate (Chen *et al.* 2003), gastrointestinal (Dote *et al.* 2005), lung (Yano *et al.* 2005) and breast (Dote *et al.* 2004) cancers. Another RAS GAP with a tumor suppressor role in some types of cancer is RASAL1, a calcium-regulated GAP. *RASAL1* is silenced through CpG methylation in multiple tumors including thyroid, colorectal and hepatocellular tumors (Xing 2007, Ohta *et al.* 2009, Calvisi *et al.* 2011). Thyroid tumor cells, both of follicular and papillary origin, express low levels of EBP1 (ERBB3-binding protein 1), which interacts with ERBB3 (erb-b2 receptor tyrosine kinase 3) receptors, acting as a tumor suppressor. Overexpression of EBP1 resulted in enhanced expression of *RASAL1* and attenuation of tumor cell growth, whereas *RASAL1* knockdown abrogated the effects of forced EBP1 expression on cell growth, migration and invasiveness of thyroid tumor cells (Liu *et al.* 2015).

Scaffold proteins

A variety of extracellular stimuli converge on the MAPK pathway to elicit a broad spectrum of cellular responses. To achieve signal diversity and specificity, the spatiotemporal flux through the pathway is organized by scaffold proteins, which recruit different components of the MAPK pathways to form complexes that optimize signal transmission (Fig. 5).

Saccharomyces cerevisiae Ste5p was the first MAPK scaffold to be identified, and was shown to tether three kinases, Ste11 (MAP3K), Ste7 (MAP2K) and Fus3 (MAPK) in the mating response to pheromones (Choi *et al.* 1994). A number of mammalian scaffolds controlling ERK activation have now been identified. In this section, we describe a few of the most representative scaffold proteins of the RAS/ERK pathway (Fig. 6), with special attention to their role in human cancer.

Kinase suppressor of RAS

Kinase suppressor of RAS (KSR) is probably the best characterized of the ERK scaffolds in mammals. It was initially described in *Drosophila melanogaster* and *Caenorhabditis elegans* as a positive regulator of Ras (Kornfeld *et al.* 1995, Therrien *et al.* 1995). Two different isoforms have been identified in mammals, KSR1 and KSR2, which share 61% amino acid identity. KSR1 contains five different domains, termed CA1–CA5. The N-terminal

CA1 domain contributes to BRAF and membrane binding, CA2 is a proline-rich domain with no described function, CA3 is a cysteine-rich domain that mediates membrane binding and CA4 presents a motif for interaction with ERK. Finally, the CA5 region includes a putative kinase-like domain, although it is still unclear whether it actually displays kinase activity (Frodyma *et al.* 2017). Single knockout mice *Ksr1* and *Ksr2* show different phenotypes, indicating that the proteins fulfill independent functions (Lozano *et al.* 2003, Costanzo-Garvey *et al.* 2009). *Ksr1*^{−/−} mice are fertile and develop normally, whereas *Ksr2*^{−/−} mice present reduced fertility and spontaneous obesity. Interestingly *Ksr1*^{−/−} mice are resistant to Ras-driven tumorigenesis.

KSR1 assembles RAF, MEK and ERK at lipid rafts through a tightly regulated mechanism. In resting cells, KSR1 is found in the cytoplasm in complex with the 14-3-3 protein and the E3 ubiquitin ligase IMP (impedes mitogenic signal propagation). Cytoplasmic retention of KSR1 depends on c-TAK1 phosphorylation on Ser392 and Ser297, which provides docking sites for 14-3-3 (Muller *et al.* 2001). Upon growth factor stimulation, RAS induces proteasome-mediated IMP degradation and PP2A-mediated KSR1 dephosphorylation on Ser392 (Ory *et al.* 2003, Matheny *et al.* 2004), leading to the release from 14-3-3 and relocation to the plasma membrane where KSR1 mediates MEK activation by RAF (Lavoie *et al.* 2018). Upon activation, ERK phosphorylates KSR1 in different residues, inducing its release from the plasma membrane and impairing signal transduction (Mckay *et al.* 2009).

KSR1 participates in tumorigenesis in various types of cancer, including skin, colorectal, breast, lung and pancreatic carcinoma. In KRAS-driven pancreatic and lung carcinomas, *KSR1* downregulation by antisense oligonucleotides reduced xenograft tumor growth in nude mice (Xing *et al.* 2003). Also, *Ksr1* depletion inhibited proliferation of HRas-dependent skin tumors in mice (Lozano *et al.* 2003). Conversely, KSR1 has been described as a tumor suppressor in breast cancer, where its expression positively correlates with overall and disease-free survival. Furthermore, KSR1 overexpression inhibited tumor growth in mice, by stabilizing BRCA1 and inhibiting p53 transcriptional activity in a manner independent of ERK activation (Stebbing *et al.* 2015). Finally, expression of KSR1 plays a potential role in chemotherapy response in breast cancer, by enhancing cisplatin-induced ERK activation and cisplatin sensitivity (Kim *et al.* 2005). The role of KSR1 in thyroid cancer remains largely unknown, although *KSR1* downregulation has been associated with decreased NOTCH signaling, downregulation of genes

Figure 5

Functions of scaffold proteins. Scaffold proteins localize different members of the MAPK pathway, optimizing activation and inactivation and providing isolation from other signaling pathways. Through these interactions scaffolding proteins control the amplitude and time of activation of the pathway, and provide specific subcellular localization and points of crosstalk with other signaling pathways.

Figure 6

Scaffolds of the MAPK pathway. Scaffold proteins target MAPK complexes to different subcellular localizations to interact with specific sets of regulators and effectors to control diverse cellular processes. Arrows indicate activation; blunted lines mean inactivation; dashed lines indicate changes in subcellular localization. APS-2-79, WW and DEL22379 are inhibitors of KSR2, IQGAP1 and ERK dimerization respectively, that have been used in preclinical models. Figure created with BioRender.io (Toronto, Canada).

related to oxidative phosphorylation and ERK inactivation in PTC (Lee *et al.* 2015).

Beyond the ERK pathway, KSR2 seems to be involved in the control of energy homeostasis through AMPK activation, favoring the transformed phenotype of tumor cells (Costanzo-Garvey *et al.* 2009, Fernandez *et al.* 2012). In agreement, mutations in KSR2 are associated with obesity, insulin resistance and impaired cellular fatty acid oxidation (Pearce *et al.* 2013).

The mild phenotype of *Ksr1*^{-/-} mice indicates that KSR1 is not essential for the adult, but it is required for RAS-driven transformation, making it an attractive therapeutic target. KSR1 levels can determine the response to RAF inhibitors. It has been described that RAF inhibitors trigger paradoxical ERK activation by inducing BRAF/CRAF heterodimerization in the presence of active RAS (Poulikakos *et al.* 2010). KSR1 competes with CRAF for BRAF binding in the presence of RAF inhibitors, reducing ERK activation and altering the effects of RAF inhibitors (Mckay *et al.* 2011).

In a different therapeutic approach, APS-2-79, a molecule that stabilizes KSR2 in an inactive conformation, impaired MEK phosphorylation by RAF and increased the therapeutic response of MEK inhibitors in RAS-mutant cells (Dhawan *et al.* 2016).

IQ motif-containing GTPase-activating protein

The IQ motif-containing GTPase-activating proteins (IQGAP) are a conserved family of scaffolding proteins comprising three members in most vertebrates: IQGAP1, IQGAP2 and IQGAP3. These are multidomain adapter proteins that interact with up to 100 different protein partners to control a wide range of cellular processes. The different isoforms fulfill independent functions and present specific patterns of tissue expression and subcellular localization (Hedman *et al.* 2015).

The best described functions for IQGAPs have been associated with the regulation of cytokinesis, cell polarity, migration and cell-cell adhesion, through control of the cytoskeleton. This is achieved through binding to F-actin, small GTPases such as RAC1 and CDC42 and proteins involved in cell contacts such as cadherins and catenins (Watanabe *et al.* 2015). Their role as scaffolds of the ERK pathway has also been well established, and it has been reported that IQGAP1 binds BRAF, MEK1/2 and ERK1/2, which is necessary for signaling downstream of epidermal growth factor (EGF) receptor (EGFR) (Roy *et al.* 2004, 2005, Ren *et al.* 2007). The distinct functions of the different isoforms may be partially explained by the differential binding to effectors; for instance,

IQGAP1 can bind ERK1 and 2, whereas IQGAP3 only binds ERK1 (Yang *et al.* 2014).

IQGAP1 has recently been identified as an important PI3K pathway scaffold and may function as a switch between ERK and AKT activation. IQGAP1 binds different proteins involved in the generation of PI3,4,5P₃ (phosphatidylinositol-3,4,5-trisphosphate) from phosphatidylinositol (Choi *et al.* 2016). The main PI3K effectors, AKT and PDK1 also bind IQGAP1, providing a platform for optimal pathway activation. Interestingly, the binding sites for PI3K and ERK components overlap and therefore the binding of the former excludes the binding of the latter. A recent report indicates that FOXO1 (forkhead box O1) is a major determinant of IQGAP1-mediated ERK activation (Pan *et al.* 2017). The FOXO1 transcription factor is regulated by AKT phosphorylation-dependent nuclear exclusion. Cytoplasmic, phosphorylated FOXO1 binds IQGAP1, leading to dissociation of the ERK pathway components from the complex (Pan *et al.* 2017). FOXO1 expression is diminished in thyroid cancer (Zaballos & Santisteban 2013), consistent with its role as a tumor suppressor. Therefore, the possibility exists that FOXO1 downregulation primes ERK over PI3K activation in these tumors.

IQGAP1 is upregulated in multiple types of human tumors, including breast (Jadeski *et al.* 2008), pancreatic (Wang *et al.* 2013), ovary (Dong *et al.* 2006) and PTC (Liu *et al.* 2010), among others. Interestingly, the expression of the protein in different tumors is higher in invasive areas than in the tumor center or in healthy tissue, and increased IQGAP1 membrane localization has been associated with the loosening of cell–cell contacts and cell adhesion required for gastric cancer cell spreading (Takemoto *et al.* 2001). Genetic copy gain of *IQGAP1* is frequent in thyroid cancer and increases in parallel with tumor aggressiveness, whereas *IQGAP1* depletion in human thyroid cancer-derived cell lines prevents invasion and colony formation (Liu *et al.* 2010). High IQGAP1 expression in GBM (glioblastoma multiforme) defines a group of aggressive cells with stem-like properties (Balenci *et al.* 2006). The tumorigenic properties conferred by IQGAP1 establish this protein as an attractive therapeutic target, an idea that has been explored in a skin carcinoma mouse model (Jameson *et al.* 2013). In this study, *Iqgap1*-null mice were resistant to *Hras*-driven skin chemical carcinogenesis. IQGAP1 binds ERK1/2 through a conserved WW domain, and a peptide containing the IQGAP1 WW motif inhibited RAS- and RAF-driven tumorigenesis and bypassed acquired resistance to BRAF inhibitors (Jameson *et al.* 2013). Interestingly *Iqgap1*^{-/-} mice develop normally

(Li *et al.* 2000) and therefore a high toxicity of IQGAP1-targeted approaches is not expected.

Unlike IQGAP1, IQGAP2 expression is reduced in many different tumors where it is associated with poor prognosis, pointing to its role as a tumor suppressor (Kumar *et al.* 2017). Loss of expression of *IQGAP2* has been related to promoter hypermethylation in ovarian and gastric carcinomas (Jin *et al.* 2008, Deng *et al.* 2016). Furthermore *Iqgap2*^{-/-} mice develop late-onset hepatocellular carcinoma (Schmidt *et al.* 2008), supporting a role for IQGAP2 in tumor suppressive functions. Interestingly, loss of *Iqgap2* expression was accompanied by an increase in *Iqgap1* expression and activation of the Wnt/bcatenin pathway. In thyroid cancer, nuclear bcatenin is observed in cells that harbor RAS but not BRAF mutations and contributes to tumor progression (Sastre-Perona *et al.* 2016); however, a possible involvement of IQGAPs has not yet been addressed.

Paxillin

Paxillin is a multidomain adapter protein associated with focal adhesion (FA) sites – the points of connection between transmembrane integrins and components of the extracellular matrix (ECM) – where it is essential for FA turnover required for cell adhesion, migration and invasion. Growth factors and integrin-dependent adhesion to ECM induce paxillin phosphorylation at multiple sites, providing docking elements for proteins involved in FA dynamics such as FAK, SRC, talin and vinculin, among others. FAK and SRC phosphorylate paxillin in different tyrosine residues, allowing downstream activation of signaling pathways (Deakin & Turner 2008). Paxillin has been described as an ERK scaffold owing to its ability to bind CRAF, MEK1 and ERK1/2 and to mediate ERK-induced cell spreading in response to hepatocyte growth factor. In resting conditions, paxillin is associated with MEK at FA. Upon hepatocyte growth factor stimulation, SRC phosphorylates Tyr118 on paxillin allowing ERK binding followed by RAF recruitment and pathway activation (Ishibe *et al.* 2003, 2004). In turn, activated ERK phosphorylates paxillin on several serine residues, leading to disassembly of the complex and ERK-paxillin nuclear translocation where both proteins cooperate to induce gene transcription in prostate cancer (Sen *et al.* 2012). Activation of intracellular pathways mediated by paxillin ultimately leads to the regulation of RHO family proteins at FAs allowing the control of cytoskeleton dynamics necessary for cell movement (Chen *et al.* 2005). Thus, by serving as a docking platform for FA-associated kinases,

phosphatases and other proteins, paxillin links cell adhesion to different intracellular signaling complexes and the control of the cytoskeleton to promote cell migration and invasion.

Cell adhesion to the ECM is essential for survival and dissemination of tumor cells. Because paxillin is involved in signal transduction from the ECM, its role in tumor progression has been examined in different types of human carcinomas. Indeed paxillin was identified in fibroblasts as a protein that undergoes multiple tyrosine phosphorylation events upon transformation by *v-Src* (Glenney & Zokas 1989). Increased paxillin expression and phosphorylation is associated with the progression of colorectal (CRC) (Yang *et al.* 2010b), prostate (Sen *et al.* 2012) and lung (Mackinnon *et al.* 2011) carcinomas, among others. In the lung, overexpression of paxillin through genomic amplification is found from the earliest to metastatic stages of lung cancer development (Mackinnon *et al.* 2011). However, this may be tumor dependent, since paxillin expression is low in SCLC (small-cell lung carcinoma) as compared with healthy tissue (Salgia *et al.* 1999). Furthermore, paxillin somatic mutations were reported in 9% of NSCLC (non-small-cell lung cancer); the most frequent of which is the activating mutation A127T, which promoted tumor growth and invasion in a xenograft mouse model (Jagadeeswaran *et al.* 2008, Mackinnon *et al.* 2011). Nevertheless, the relevance of paxillin somatic mutations for tumor progression is controversial and other studies found no mutations in lung and other solid tumors (Pallier *et al.* 2009, Kim *et al.* 2011). In breast cancer, increased paxillin levels correlate with HER2 overexpression and predict the response to chemotherapy, depending on HER2 status (Short *et al.* 2007). Paxillin is also involved in conferring aggressive properties to tumor cells. Invadopodia are cellular protrusions involved in ECM degradation and tumor dissemination, and tyrosine phosphorylation of paxillin modulates invadopodia dynamics through ERK-mediated calpain activation (Badowski *et al.* 2008). Indeed, paxillin phosphorylation at Tyr118 has been shown to control anchorage-independent growth of mouse embryonic fibroblasts (Sachdev *et al.* 2009). Also, in CRC cell lines and mouse models, paxillin increases survival and chemotherapy resistance in a process involving direct interaction and stabilization of Bcl2 (Huang *et al.* 2015). A possible involvement of paxillin in thyroid tumorigenesis has not been addressed.

bArrestins

bArrestins are cytosolic proteins that mediate homologous desensitization of G-protein-coupled

receptors (GPCRs) by inducing their internalization (Rajagopal *et al.* 2010). They have been shown to function as scaffolds for different signaling pathways including ERK, PI3K and NFkB (Dewire *et al.* 2007). Furthermore, bArrestins can activate ERK indirectly through c-SRC activation and promote the transactivation of TKRs. The Gq-coupled PAR2 (proteinase-activated receptor-2) receptor is internalized in a process depending on bArrestin and clathrin-coated vesicles, forming protein complexes that include RAF and ERK. In this process, ERK is activated and retained in the cytoplasm and nuclear mitogenic effects of ERK are prevented (Defea *et al.* 2000). MEK has also been described to be part of this complex and phosphorylates bArrestin2 on Thr383 to recruit and activate ERK upon activation of different GPCRs (Cassier *et al.* 2017).

bArrestins influence tumor progression by controlling cellular processes such as proliferation, apoptosis, migration, invasion and angiogenesis. In CRC cell lines, PGE2 (prostaglandin E2) induces the formation of a signaling complex containing the PGE receptor 4, bArrestin1 and c-SRC, which leads to transactivation of EGFR. A mutant bArrestin1 with reduced binding to c-SRC was unable to promote EGFR transactivation and decreased cell migration *in vitro* and liver metastatic disease *in vivo* (Buchanan *et al.* 2006). Migration of breast cancer cell lines upon activation of the PAR2 receptor depends on both bArrestin1 and 2-mediated ERK activation (Ge *et al.* 2004). In NSCLC cell lines, bArrestin1 translocates to the nucleus in response to nicotine and increases expression of E2F-target genes to promote survival and proliferation (Dasgupta *et al.* 2011). In contrast to the aforementioned tumor-promoting functions of bArrestins, depletion of bArrestin2 in a mouse lung model increased tumor growth and angiogenesis through NFkB activation (Raghuwanshi *et al.* 2008). Conversely, decreased expression of bArrestin2 in hepatocellular carcinoma is associated with poor prognosis and aggressive pathological features (Sun *et al.* 2016). A role for bArrestins in thyroid cancer has not been addressed, although bArrestin1 was reported to be involved in the internalization of the thyrotropin receptor in rat thyroid-derived cell lines (Frenzel *et al.* 2006).

MP1/LAMTOR3

MEK partner 1 (MP1), also known as late endosomal/lysosomal adaptor MAPK and mTOR activator 3 (LAMTOR3), was first identified in a yeast two-hybrid assay using MEK1 as bait. It was further demonstrated that

MP1 also binds ERK1 but not MEK2 or ERK2 (Schaeffer *et al.* 1998). MP1 localizes to the cytoplasmic face of late endosomes through binding to the adaptors p14 (Wunderlich *et al.* 2001, Teis *et al.* 2002) and p18 (Nada *et al.* 2009). In the absence of p14, MP1 is localized in the cytoplasm where it is rapidly targeted for degradation by the proteasome machinery (De Araujo *et al.* 2013). MP1 also controls activation of the mTORC1 complex at late endosomes/lysosomes (Sancak *et al.* 2010). Interestingly, MP1 has been shown to bind to other scaffold proteins such as MORG, which is able to bind CRAF, BRAF, MEK1/2 and ERK1/2 and to mediate pathway activation in response to specific extracellular agonists (Vomastek *et al.* 2004).

MP1 has been associated with the control of FA turnover during migration. Adhesion to fibronectin induces PAK1 (p21-activated kinase) activation of MEK1, in a process mediated by MP1-p14 that ultimately leads to suppression of RHO activity to allow FA turnover and cell spreading (Pullikuth *et al.* 2005). MP1-positive endosomes move along microtubules to the cell periphery to control FA dynamics and migration. Interestingly, MP1 interacts with IQGAP1 and promotes its dissociation from FA, allowing cell migration (Schiefermeier *et al.* 2014).

Consistent with the actions of MP1-mediating cell adhesion, a pro-tumorigenic role of MP1 has been proposed in different human carcinomas. MP1 mediates migration induced by fibronectin adhesion in prostate cancer cell lines, although, surprisingly, it does not depend on PAK or ERK activation and may rather involve paxillin stabilization at FAs (Park *et al.* 2009). In addition, MP1 has been implicated in the control of other tumorigenic processes related to tumor survival and resistance mechanisms. In mesenchymal lung cancer cells, MP1-dependent BCL2 expression is associated with acquired chemo-radioresistance and mediates pro-survival signals through AKT1 activation in ER-positive but not ER-negative breast carcinomas (Marina *et al.* 2012). Furthermore, MP1 expression was associated with poor survival in glioma patients, and was also found to mediate ERK activation downstream of a gain-of-function mutant EGFR, to promote stemness of neural cancer stem cells (Kwon *et al.* 2017). An opposing role for MP1 has been described in mouse embryonic stem cells, where MP1 depletion impairs FGF4-induced differentiation but not proliferation (Westerman *et al.* 2011). Finally, in pancreatic carcinomas, overexpression of PAF (PCNA-associated factor) is associated with increased ERK activation and proliferation, and this effect is mediated by PAF-induced MP1 expression (Jun *et al.* 2013).

IL17RD/SEF

Interleukin 17 receptor D (IL17RD), also known as similar expression to FGF (SEF), was first identified as an antagonist of fibroblast growth factor (FGF) signaling during zebrafish development (Tsang *et al.* 2002). SEF functions as a MAPK scaffold, with the ability to bind MEK and ERK mostly at the Golgi apparatus, but also in early endosomes and the plasma membrane. At the Golgi, SEF inhibits MEK-ERK dissociation and blocks ERK nuclear translocation, and therefore, favors the activation of ERK cytosolic substrates in response to different growth factors and in different cell lines (Torii *et al.* 2004). Upon growth factor stimulation in rat pheochromocytoma PC12 cells, SEF locates at the plasma membrane to interact with EGFR, which increases the stability of the receptor and enhances ERK activation. Interestingly SEF is internalized with the EGFR and is found in early but not late endosomes, thereby favoring recycling *versus* degradation of the receptor (Torii *et al.* 2004). SEF has been implicated in the inhibition of other signaling pathways such as PI3K/AKT, downstream of the FGF receptor (FGFR) (Kovalenko *et al.* 2003) or cytokine signaling through NFκB cytoplasmic retention (Fuchs *et al.* 2012). Upstream of NFκB, a negative regulation of TLR (toll-like receptor)-induced inflammatory response by SEF has also been described and *Il17rd*^{-/-} mice were found to be more susceptible to septic shock (Mellet *et al.* 2015). These data indicate that SEF opposes cytokine signaling, acting at different levels, and suggest an important role for SEF in controlling the crosstalk between ERK and inflammatory signaling. An alternatively spliced isoform of *SEF*, *SEF-b*, presents a more restricted pattern of expression and impairs ERK but not AKT activation upon FGF or platelet-derived growth factor stimulation (Preger *et al.* 2004).

Downregulation of SEF expression has been observed in several human carcinomas, including endometrial, breast, ovarian, prostate and thyroid carcinomas (Zisman-Rozen *et al.* 2007). Interestingly, decreased SEF expression in prostate cancer was particularly frequent in metastatic tissue (Darby *et al.* 2009) and was shown to increase ERK, p38 and JNK signaling and to promote EMT (Hori *et al.* 2017). SEF has also been related to the inhibition of EMT in breast cancer, and cytokines that promote EMT also reduce SEF expression (He *et al.* 2016). SEF expression is decreased in CRC cell lines and human carcinomas compared with normal intestinal epithelial cells and healthy tissue. Expression of oncogenic RAS in normal intestinal epithelial cells abrogates SEF expression and increases MEK/ERK nuclear localization,

whereas re-expression of SEF in CRC cell lines prevents polyploidization and tumorigenesis (Duhamel *et al.* 2012).

Phosphoprotein-enriched in astrocytes 15

Phosphoprotein-enriched in astrocytes 15 (PEA15) was originally identified in a screen for phosphoproteins that could regulate functions in astrocytes (Araujo *et al.* 1993). Subsequently, PEA15 was found to be widely expressed in most tissues (Danziger *et al.* 1995). PEA15 binds and retains ERK in the cytoplasm, preventing activation of nuclear but not ERK cytoplasmic effectors (Formstecher *et al.* 2001). Furthermore, PEA15 binding to ERK also impairs ERK dephosphorylation, providing a pool of activated ERK in the cytoplasm that can elicit a rapid nuclear response upon ERK release from the complex (Mace *et al.* 2013). The mechanism responsible for maintaining ERK phosphorylation has been explored downstream of FGFR (Haling *et al.* 2010). FRS2 is an adapter protein that links FGFR to RAS/ERK activation. Activated ERK phosphorylates a threonine residue on FRS2, preventing further activation of the pathway. Binding to PEA15 impairs ERK targeting to the plasma membrane and prevents inactivation of the pathway while blocking entry of ERK to the nucleus. Accordingly, PEA15 increases ERK activation in the cytoplasm. PEA15 can also bind cytoplasmic ERK effectors such as RSK2 but not RSK1, promoting its activation by ERK; this interaction also impairs RSK2 nuclear entry (Vaidyanathan & Ramos 2003).

Although the mechanisms involved in the disruption of the PEA15/ERK complex are not completely understood, phosphorylation of PEA15 has been proposed as a crucial step. PEA15 presents two phosphorylation sites that are critical for its function: Ser104 – phosphorylated by PKC and Ser116 – phosphorylated by either CaMKII (calcium/calmodulin-dependent protein kinase II) or AKT (Kubes *et al.* 1998, Trencia *et al.* 2003). Phosphorylation of PEA15 at Ser104/116 prevents ERK binding and increases activation of ERK nuclear effectors (Krueger *et al.* 2005). However, this has been challenged by a report showing that PEA15/ERK-binding affinity does not depend on ERK or PEA15 phosphorylation state (Krueger *et al.* 2005). The phosphorylation state of PEA15 also modulates the apoptotic role of the protein (Renganathan *et al.* 2005). PEA15 contains an N-terminal DED (death effector domain) that can be found in proteins involved in apoptotic processes. Through this domain, PEA15 binds FADD (Fas-activated dead domain protein) impairing the recruitment of caspases and the apoptotic cascade.

This binding requires phosphorylation on PEA15 Ser116. This important switch is responsible for the dual action of PEA15 on tumor progression, acting as a pro- or anti-tumorigenic protein depending on the cellular context. Furthermore, phosphorylation at Ser116 by AKT increases stability of the protein, implicating PEA15 in the pro-survival actions of AKT (Trencia *et al.* 2003).

The anti-tumorigenic actions of PEA15 are associated with ERK sequestration at the cytoplasm, whereas pro-tumorigenic actions are related to its anti-apoptotic functions. Altered PEA15 expression has been described in different human carcinomas. PEA15 expression inversely correlates with malignancy grade of astrocytic tumors (Watanabe *et al.* 2010), and high PEA15 levels are associated with good prognosis in neuroblastoma (Gawecka *et al.* 2012), CRC (Funke *et al.* 2013) and ovarian (Bartholomeusz *et al.* 2008) carcinomas. Expression of PEA15 in cell lines derived from those tumors leads to the inhibition of proliferation, migration, anchorage-independent growth and invasion. By contrast, increased expression of PEA15 has been reported in glioblastoma (Eckert *et al.* 2008), NSCLC (Zanca *et al.* 2008) and renal (Heikaus *et al.* 2008) carcinoma. Importantly, increased PEA15 expression has been found associated with resistance in tumor cells. PEA15 mediates resistance to TRAIL (Zanca *et al.* 2008) and to glucose (Eckert *et al.* 2008) and serum (Funke *et al.* 2013) deprivation in cells derived from different tumor types. In thyroid cancer cell lines, PEA15 is involved in protection from CD95-induced apoptosis through autocrine IL4 and IL10 production, which promotes PEA15 accumulation (Todaro *et al.* 2006).

Negative regulators and phosphatases

The balance between positive and negative feedback signals determines the spatiotemporal activation of the ERK pathway and the outcome of a specific input (Marshall 1995). For instance, in PC12 cells, EGF induces proliferation through transient activation of ERK, whereas nerve growth factor promotes neurite outgrowth by activating ERK in a sustained manner (Traverse *et al.* 1992, Nguyen *et al.* 1993). In many cases, transient *versus* sustained activation is a consequence of negative feedback mechanisms. Most negative feedback mechanisms arise from activated ERK that promotes inactivation of the pathway: (i) directly, through phosphorylation of upstream components of the signaling cascade, rendering them inactive; (ii) indirectly, by inducing the expression of protein phosphatases that promote its dephosphorylation or the dephosphorylation

of upstream kinases. A summary of the main mechanisms involved in negative regulation of the pathway is depicted in Fig. 7.

Direct negative regulation

ERK phosphorylates EGFR in a juxtamembrane Thr669 residue. This reduces signaling and trafficking of the receptor due to impaired dimer cross-activation (Li *et al.* 2008). ERK can also promote the activation of tyrosine phosphatases including CDC25C, which dephosphorylates EGFR at Tyr1068 (Prahallad *et al.* 2012), a residue required for activation. Interestingly, the poor response to BRAF inhibitors in BRAF^{V600E} CRC-derived cell lines has been explained by the loss of this negative feedback regulatory loop in cells with high levels of EGFR. Other TKRs such as FGFR1 are also negatively regulated by ERK phosphorylation (Zakrzewska *et al.* 2013). Downstream of the TKRs, ERK phosphorylation of SOS1 on multiple residues impairs its binding to the adaptor proteins SHC and GRB2, uncoupling from the EGFR and preventing

transmission of the signal (Corbalan-Garcia *et al.* 1996, Porfiri & McCormick 1996, Kamioka *et al.* 2010). CRAF and BRAF are phosphorylated by ERK at several serine residues, impairing interaction with RAS, membrane localization and dimer formation (Ueki *et al.* 1994, Wartmann *et al.* 1997, Dougherty *et al.* 2005). This particular feedback loop is relevant for BRAF^{V600E}-driven carcinomas, since mutant BRAF does not require dimerization for its activation and is therefore insensitive to this negative feedback loop (Pratilas *et al.* 2009). The activity of MEK1 and MEK2 is also negatively regulated by ERK1/2 phosphorylation on Thr292 of MEK1. Interestingly the analogous residue is absent in MEK2; nevertheless, phosphorylation of Thr292 on MEK1 also inhibits MEK2 by an undefined mechanism (Catalanotti *et al.* 2009). Scaffold proteins of the pathway such as KSR1 have also been described to be negatively regulated by multiple phosphorylation events (Cacace *et al.* 1999), leading to membrane dissociation, disruption of the BRAF-KSR1-MEK1/2 complex and impairment of signal transmission (Mckay *et al.* 2009).

Indirect negative regulation

ERK activation also triggers the expression of different negative regulators that elicit inactivation of the pathway in a delayed manner. The best studied are the dual-specificity MAPK phosphatase (MKP) or dual-specificity phosphatase (DUSP) family, which display both threonine and tyrosine phosphatase activity. DUSPs are divided into families according to sequence homology, substrate specificity and subcellular localization. The first group includes the nuclear phosphatases DUSP1, 2 and 4, which dephosphorylate ERK1/2, p38a and JNK (c-Jun N-terminal kinase), and DUSP5, which is specific for ERK1/2. The second group is composed of DUSP6, 7 and 9, which are ERK1/2-specific cytoplasmic phosphatases. The third group comprises the p38a and JNK cytoplasmic and nuclear phosphatases DUSP8, 10 and 16. All DUSPs possess an N-terminal domain (Tanoue *et al.* 2000) responsible for substrate specificity and subcellular localization and a C-terminal catalytic domain (Keyse & Ginsburg 1993) that provides phosphatase activity. The mechanism of activation of DUSPs differs among members: DUSP1, 2, 4 and 6 are catalytically activated upon ERK binding (Camps *et al.* 1998, Chen *et al.* 2001, Slack *et al.* 2001, Zhang *et al.* 2005), whereas DUSP5 and 10 do not require substrate binding for activation (Tanoue *et al.* 1999, Mandl *et al.* 2005). Furthermore, DUSP5 can bind and sequester inactive ERK2 at the nucleus (Mandl *et al.* 2005), whereas DUSP6 can retain inactive ERK2 in the cytoplasm

Figure 7

Negative feedback regulation. Activated ERK elicits a rapid (red lines) and a late (black lines) negative regulatory response. ERK phosphorylates multiple upstream components of the pathway, impairing signaling. Through activation of nuclear ETS transcription factors, ERK induces the expression of MKPs that dephosphorylate ERK in the nucleus and the cytoplasm, and provide points of crosstalk with other signaling pathways. SPRY expression is also induced, inactivating the pathway upstream of ERK in an undefined manner. Arrows indicate activation; blunted lines mean inactivation; dotted lines indicate changes in subcellular localization.

(Karlsson *et al.* 2004), providing a mechanism for ERK signaling compartmentalization.

ERK activation affects MKPs at multiple levels. Expression of DUSP5 and DUSP6 is triggered by growth factor stimulation, depends on MEK activity and is mediated by ETS transcription factors binding to the DUSP5 and 6 promoters (Ekerot *et al.* 2008, Kucharska *et al.* 2009). MKPs have a short half-life and are rapidly degraded by the proteasome machinery. DUSP6 is targeted for degradation by the proteasome when phosphorylated by ERK on Ser159 and Ser197 (Marchetti *et al.* 2005). By contrast, ERK-dependent phosphorylation of Ser359 and Ser364 on DUSP1 and DUSP4 stabilizes the proteins, delaying ubiquitination and degradation (Brondello *et al.* 1999, Cagnol & Rivard 2013).

The differences in MKP substrate specificity allow ERK to interact with other signaling pathways. For example, ERK phosphorylation of the JNK phosphatase DUSP16 increases the stability of the protein and enhances the inactivation of the JNK pathway (Katagiri *et al.* 2005). MKP expression can also be elicited by other signaling cascades, providing points of crosstalk with the MAPK pathways. In lymphocytes and hepatocytes, TGF β (transforming growth factor β) induces the expression of DUSP4 in a SMAD3-dependent manner, impairing ERK-mediated degradation of BIM (Bcl-2-interacting mediator of cell death) and promoting apoptosis (Ramesh *et al.* 2008). An interesting reciprocal regulation has been described between DUSP6 and FOXO1. FOXO1 is a transcription factor involved in insulin signaling, in which AKT phosphorylation of FOXO1 induces its nuclear export and proteasomal degradation. DUSP6 promotes FOXO1 dephosphorylation, increasing FOXO1 nuclear retention and transcriptional activity (Wu *et al.* 2010). Interestingly, FOXO1 binds and activates the DUSP6 promoter, increasing the levels of the protein (Feng *et al.* 2014).

Both DUSP5 and DUSP6 have been found dysregulated in human tumors, suggesting a role for both in tumor progression. DUSP5 expression is lost in gastric and prostate cancer and confers a poor patient outcome. Also ectopic expression of DUSP5 in gastric cancer cell lines reduced proliferation and colony formation *in vitro* (Shin *et al.* 2013, Cai *et al.* 2015). Deletion of DUSP5 in an HRAS^{Q61L}-skin carcinogenesis mouse model enhanced the development of skin tumors (Rushworth *et al.* 2014). These data indicate a tumor suppressor role of DUSP5 in some tumor types.

DUSP6 expression is upregulated in early pancreatic intraepithelial neoplasia, but is downregulated in invasive PDAC (pancreatic ductal adenocarcinoma) due to

promoter hypermethylation (Furukawa *et al.* 2003, 2005, Xu *et al.* 2005). A similar situation has been described in KRAS-driven lung tumors where DUSP6 expression is lower in more proliferative and aggressive tumors (Okudela *et al.* 2009). Interestingly, intrinsic resistance to TRK inhibitors in ELM4/ALK-driven NSCLC has been associated with the loss of DUSP6 expression (Hrustanovic *et al.* 2015) and prolonged treatment of gastric cancer cell lines with MET (hepatocyte growth factor receptor) inhibitors generates resistance due to loss of DUSP6 expression (Lai *et al.* 2014). By contrast, DUSP6 expression is increased in human glioblastoma, melanoma and PTC, and DUSP6 overexpression in cell lines derived from those tumors confers increased anchorage-independent growth, migration and invasion (Messina *et al.* 2011, Li *et al.* 2012, Degl'Innocenti *et al.* 2013).

A good example for illustrating the opposing roles of the MKPs in tumor progression is leukemia. Decreased expression of DUSP6 in AML is pro-tumorigenic (Arora *et al.* 2012), whereas elevated DUSP6 expression is associated with a worst outcome in pre-B ALL (acute B lymphoblastic leukemia) and is necessary for tumor progression. Indeed, pharmacological inhibition of DUSP6 in mice greatly delays the onset of pre-B ALL and is associated with increased ERK activation, reactive oxygen species production and p53-mediated cell death (Shojaee *et al.* 2015).

In PTC, DUSP5 and DUSP6 are overexpressed in parallel with the degree of activation of the pathway and their silencing in human thyroid tumor cell lines reduces migration, invasion and growth and decreases cell viability depending on the cell line tested (Degl'Innocenti *et al.* 2013, Buffet *et al.* 2017). Interestingly, DUSP6 expression is elevated in PDTC but not in ATC (Degl'Innocenti *et al.* 2013), suggesting that, as in pancreatic and lung carcinomas, expression of MKPs is initially upregulated but then silenced during progression to the most aggressive tumor variants.

MKPs are the most important negative regulators of ERK activation, not only determining the timing of activation of the pathway, but also providing mechanisms of control for ERK-subcellular localization and points of crosstalk with other signaling pathways. Other groups of phosphatases can also dephosphorylate ERK, leading to pathway inactivation (Keyse 2000). A group of phosphatases that catalyzes tyrosine dephosphorylation includes PTP-SL, STEP and HePTP; a second group displays serine/threonine phosphatase activity and includes PP2A and PP2C.

ERK also promotes the expression of the SPRY (Sprouty) family of proteins, which have been implicated

in the negative regulation of the pathway by an yet unclear mechanism that likely takes place at multiple levels upstream of ERK (Masoumi-Moghaddam *et al.* 2014). SPRY proteins antagonize ERK activation in response to most growth factors, although EGF-induced ERK phosphorylation is unaffected (Impagnatiello *et al.* 2001, Sasaki *et al.* 2001) or even increased (Wong *et al.* 2002) by SPRY overexpression in cell lines derived from different tissues. Growth factors depending on ERK activation promote the expression of SPRY (Ozaki *et al.* 2001), though a differential effect on the expression of the different members of the family has also been described in some cell types (Gross *et al.* 2001, Impagnatiello *et al.* 2001).

Downregulation of SPRY expression has been described in several human tumors, including breast, prostate, liver or lung carcinomas (Masoumi-Moghaddam *et al.* 2014). *Spry1*-knockout mice show thyroid hyperplasia due to impaired NF κ B-induced senescence and, when combined with loss of PTEN, show accelerated tumor formation. Unexpectedly, these effects were ERK independent. Decreased SPRY1 expression has also been described in follicular adenomas and FTC (Macia *et al.* 2014). Furthermore, SPRY1 has been proposed as a tumor suppressor in MTC, where SPRY1 expression is frequently silenced by promoter methylation (Macia *et al.* 2012).

Concluding remarks

Spatiotemporal activation of the pivotal RAS-ERK pathway is controlled by GAPs and GEFs that regulate RAS activation, scaffolding proteins that provide subcellular specific localization and optimize signaling and negative regulatory mechanisms involved in the termination of signal transmission. Dysregulation of the expression of the different proteins that participate in ERK signaling dynamics is associated with the progression of human carcinomas and has been shown to frequently mediate resistance to small kinase inhibitors. Despite the essential role that aberrant ERK activation plays in thyroid cancer onset and progression, the functions of these important regulators in the normal physiology of thyroid cells and their alteration in thyroid cancer are largely understudied. Currently, there is a lack of effective treatment options for advanced thyroid carcinomas and the use of small kinase inhibitors of the RAS-ERK pathway has been disappointing, due to the emergence of resistance mechanisms and the toxicity of these systemic treatments. The challenge is to design new therapeutic strategies to overcome these

problems. In this respect, mice depleted for *Iqgap* and *Ksr* develop normally; therefore, a high toxicity is not expected for drugs targeting these proteins. This offers the possibility to generate low toxicity approaches that could be used in combination to increase the efficacy of current small kinase inhibitors.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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