SYSTEMATIC ANALYSIS OF RHO GTPases PATHWAY ALTERATIONS IN CANCER

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“Tell me and I forget, teach me and I may remember,

involve me and I learn”

(Bejamin Franklin)

ABSTRACT

RHO GTPases are core signaling proteins involved in the regulation of different cell processes, such as cell survival, proliferation and migration, among others, that are essential for tumor cells acquisition of cancer hallmarks. Most of these proteins are molecular switches tightly regulated by three subfamilies of proteins. The RHO GEFs promote the exchange of GDP for GTP, and subsequent GTPase activation, while RHO GAPs convert a GTPase into an "efficient" GTPase, increasing GTP hydrolysis and promoting its deactivation. The third subfamily of RHO GDIs can play a dual role: on one side they prevent GTPases activation by RHO GEFs, but, on the other side, they avoid their degradation in the proteasomal. RHO GTPases and their activators, the RHO GEFs, have been historically associated with pro-tumorigenic functions in cancer. In this sense, a widely accepted archetype in the field sustains that RHO GTPases and GEFs alterations in cancer will end up in the hyperactivation of the pathways that depend on their activity, with positive outcomes for tumor cells fitness. Conversely, since RHO GAPs activity can antagonize this process, they were classically associated with a tumor suppressor activity. However, while this paradigm is still valid in many cases, the outcomes obtained from the latest whole-genome and whole-exome sequencing of thousands of human tumors demonstrate that this functional prototype is not extendable to all cases. Firstly, we discovered that human tumors present both gain- and loss-of-function mutations in elements from this pathway, including the RHO GTPases and GEFs. Secondly, we found out that these mutations were not developed at high frequencies across cancer cells. Thirdly, we realized that the overexpression of GTPases and GEFs, proposed as an alternative mechanism that would explain the spurious activation of this pathway in cancer, it is not as a general process as originally thought. Collectively, all these observations challenge the long-held functional archetypes for

RHO proteins in cancer cells. In this work, we have carried out a systematic study on the somatic mutations, transcriptomic alterations and copy number variations that 486 genes ascribed to the RHO GTPases pathway present in more than 10,000 human-derived tumors from 33 different cancer types. To that end, we have applied different bioinformatic algorithms, and we developed a new one, to the analysis of multi-dimensional molecular data obtained from the Cancer Genome Atlas (TCGA) and Gene Expression Omnibus (GEO) databases. Then, we validated some of our results in *in vitro* and *in vivo* cancer models of KRAS-mutated lung adenocarcinoma, one of the deathliest cancer types in our days.

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1. RHO GTPases pathway

1.1 Family of RHO GTPases

The Rho family of GTPases is one of the subfamilies ascribed to the RAS superfamily of small G proteins. The human genome encodes 18 RHO GTPases that can be classified according to their structure and homology into the CDC42 (CDC42, RHOQ and RHOJ), RAC (RAC1, RAC2, RAC3 and RHOG), RHOA (RHOA, RHOB and RHOC), RHOD (RHOD and RHOF), RHOH, RHOU (RHOU and RHOV) and RND (RND1, RND2 and RND3) subfamilies [1]. Along with them, some studies also included the RHOBTB (RHOBTB1, RHOBTB2 and RHOBTB3) and MIRO (RHOT1 and RHOT2) subfamilies of GTPases, despite their highly divergent structure, subcellular localization and biological activity [2] **(Fig. I)**.

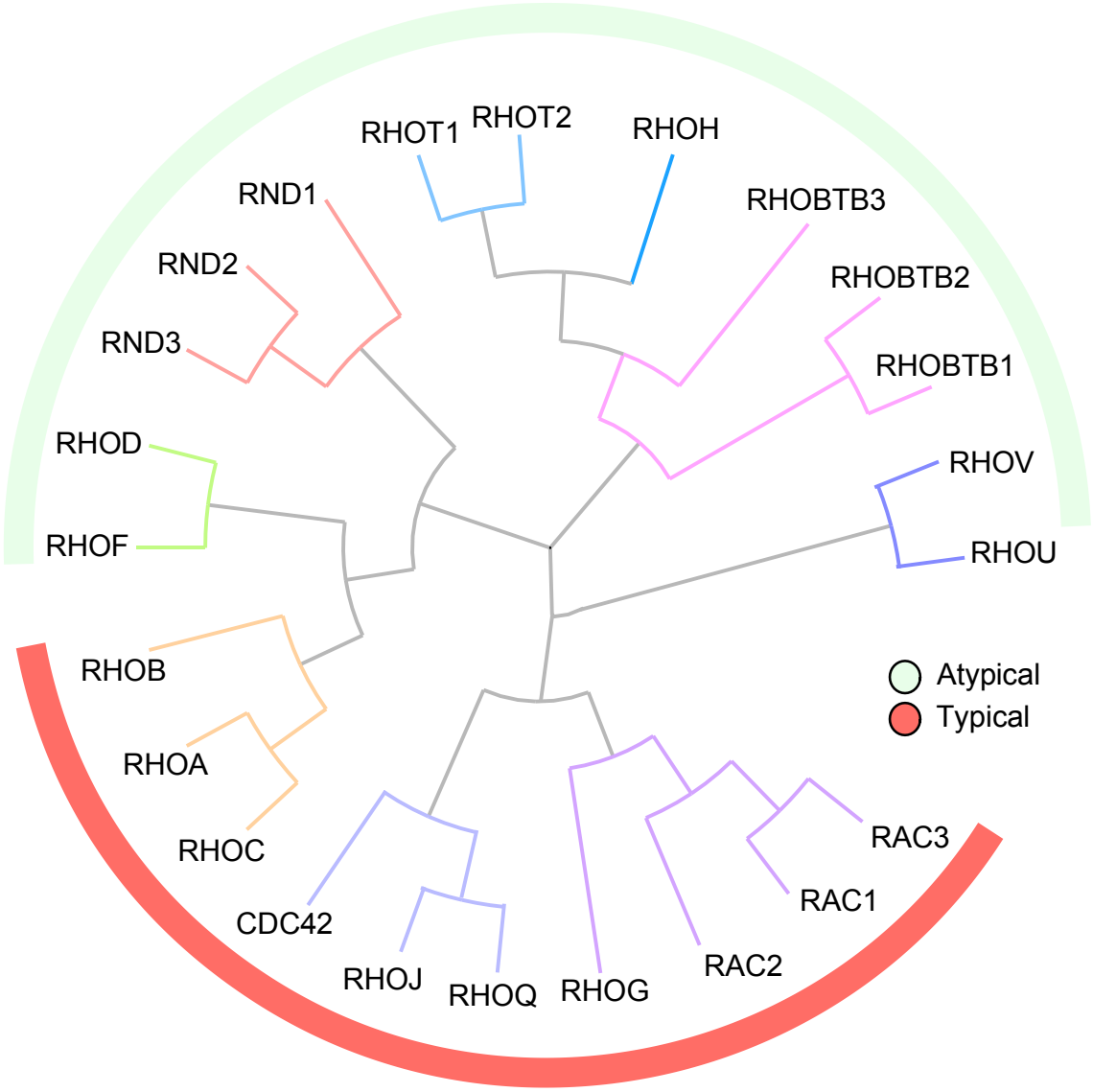

FIGURE I. The human family of RHO GTPases

The RHO GTPase family consists of 23 genes in humans, and it is subdivided into 9 subfamilies, represented in this phylogram by the color of each branch. Beyond their structure, the proteins encoded by these genes can be classified as typical or atypical depending on how their activity is regulated. Typical RHO GTPases are molecular switchers that change from an inactive GDP-bound to an active GTP-bound state, while atypical proteins have amino acid alterations that preclude the GDP/GTP cycle. The unrooted phylogenetic tree is based on CLUSTALW alignment of RHO GTPases amino acid sequences. Splicing variants of these proteins are not included.

Besides their structure and homology, RHO GTPases can be also classified according to how their activity is regulated [3]. Typical RHO GTPases (members from

CDC42, RAC, RHOA and RHOD subfamilies) function as molecular switches by oscillating from an active GTP-bound to an inactive GDP-bound state [4]. This cycle is regulated by three subfamilies of proteins. Firstly, the RHO GTPases guanosine exchange factors (RHO GEFs) promote the exchange of GDP for GTP, and subsequent GTPase activation. Since GTPases hydrolyze GTP very slowly, a second subfamily of proteins is necessary to catalyze this reaction. The RHO GTPases activating proteins (RHO GAPs) interact with active RHO GTPases to promote GTP hydrolyzation, and their inactivation. The third subfamily of proteins plays a dual role. On one side, RHO GDP dissociation inhibitors (RHO GDIs) prevent RHO GEF-mediated GTPase activation; on the other side, they avoid RHO GTPases from proteasomal degradation, keeping a pool of them ready to be delivered and activated at specific subcellular domains [5, 6]. Unlike them, atypical RHO GTPases (members from RHOH, RHOU, RND, RHOBTB and Miro subfamilies) have amino acid substitutions at their RHO switch domains that preclude the GTP/GDP cycling. These GTPases are mainly found in their GTP-bound active state in cells.

1.2 RHO GTPases regulation

1.2.1 Transcriptional and post-transcriptional mechanisms

The expression of best-characterized classical RHO GTPases (CDC42, RAC1 and RHOA) does not vary much between tissues. Nonetheless, there are others that do have a specific expression pattern in each tissue. For example, *RAC2* GTPase is mainly expressed in the hematopoietic tissue, meanwhile *RAC3* is mainly detected in brain cells. Both GTPases play tissue-specific roles in physiological conditions. RAC2 is involved in the regulation of neutrophils 3D migration and polarity acquisition [7-10], and RAC3 is necessary during neuronal development [11-13]. Besides, some RHO GTPases expression is regulated at the transcriptional level under certain conditions. For example,

the expression of *Rnd3* GTPase is induced after Raf activation in cells [14], as well as *RhoU* expression is increased by Wnt-1 and Notch1 signaling [15, 16]. Moreover, RHO GTPases also undergo post-transcriptional regulation. This mechanism is mainly exerted by microRNAs, short non-coding RNA sequences that directly modify mRNAs translation in cells [17-20].

1.2.2 Post-translational mechanisms

1.2.2.1 C-terminal prenylation

The covalent addition of a lipid in their C-terminal domain is one of the most recurrent regulatory post-translational modifications that both typical and atypical RHO GTPases undergo [2, 5, 21]. The cysteine (C) residue of their C-terminal CAAX domain is prenylated, by either farnesyl or geranylgeranyl isoprenoid lipids, followed by the proteolytic removal of the three C-terminal amino acids (AAX), and the ensuing methylation of the recently exposed α-carboxyl group of the C-terminal isoprenylcysteine residue [2, 5, 22-24]. This post-translational modification occurs in the cytosol, and it is crucial to translocate RHO GTPases to the plasma membrane, and/or to different endomembranes [25]. In addition to the farnesyl or geranylgeranyl modification in the CAAX motif, some RHO GTPases are post-translationally modified by the addition of other lipids (e.g., palmitate) [26] in residues outside this CAAX domain (e.g. RAC1 and RHOB) [27-29]. Moreover, some atypical RHO GTPases do not present the CAAX motif at their C-terminus. For example, RHOU and RHOV present a CFV domain that undergoes palmitoylation, instead [5, 30]. Although, not biochemically confirmed yet, several studies have suggested that RHOBTB1 and RHOBTB2 lack of any C-terminal aminoacidic domain susceptible to be prenylated or palmitoylated [3, 31, 32]. Due to that, the activity of these GTPases can be observed diffused across cells cytoplasm, instead of being exclusively attached to membranes.

1.2.2.2 Ubiquitination and SUMOylation

Another common post-translational modification on RHO GTPases is the ubiquitination on lysine residues [33-35]. Although the covalent addition of a ubiquitin protein is a common flag for proteasomal degradation, it can also modify their subcellular localization and activity [36]. RHO GTPases can be ubiquitinated in their active and inactive states, a process that has been proposed as an important regulatory mechanism of their local activities through cells. CDC42, RAC1 and its splicing short isoform RAC1B, RHOA, RHOB and the atypical RHOBTB2 are the most frequently modified RHO GTPases in this sense [5, 35-39]. Like ubiquitination, SUMOylation consists in the addition of one SUMO protein to RHO GTPases lysine residues. Until now, only RAC1 has been reported to be SUMOylated near to its C-terminus [40].

1.2.2.3 Phosphorylation

RHO GTPases can be also phosphorylated at different serine, threonine and tyrosine residues [3, 5]. In general, RHO GTPases phosphorylation seems to promote a negative effect on their activity. For example, the PKA-mediated RHOA phosphorylation in the Ser188 residue promotes its binding to RHO GDIs with higher affinity [41, 42]. Similarly, the phosphorylation of RND3 by ROCK1 or PKC kinases at 7 different Set/Thr residues increases its stability, while it promotes its translocation to the cytosol and reduces its activity [43, 44]. Lastly, RAC1 phosphorylation at Ser71 by AKT, or at Thr108 by ERK kinases, triggers a decrease in its GTP binding capacity without affecting its GTPase activity, and the inhibition of its interaction with PLCγ, so inducing its translocation to the nucleus, respectively [45-47].

1.2.2.4 Spatiotemporal activation

Another layer in the regulation of RHO GTPases activity, and probably the most difficult to decipher, is the spatiotemporal control of their activity [21, 48, 49]. RHO signaling in

cells requires of many RHO GTPases, regulatory and downstream effector proteins operating simultaneously. Therefore, its activation must be precisely controlled in the right place (subcellular domain) and at the right time. RHO GDIs and their ability to extract GTP-bound RHO GTPases from the membranes were proposed to play crucial roles in this process [6, 50], as well as the steady-state distribution of RHO GEFs and GAPs in, virtually, all cellular components [22, 49, 51]. The impact that these and other mechanisms have on RHO GTPases spatiotemporal activation has been well-studied in specific cell contexts. For example, focal adhesions are hubs of RHO signaling with different composition of regulatory proteins. The nascent focal adhesions in cell periphery are preferentially enriched on RAC1 GEFs, whereas mature ones in the center of the cell are enriched on RAC1 GAPs [52-55]. Like focal adhesions, some cellular structures, many never associated with RHO GTPases activity before, were found mainly hosted by RHO GAPs. This has been proposed to prevent the activity of diffused-away RHO GTPases from their desired site of action [56]. Something similar happens during cell spreading and migration. Meanwhile the front of advance is enriched on activated RAC1, cell rear show higher activation of RHOA signals [57-63]. Finally, transcriptional mechanisms also play important roles in this spatiotemporal regulation. This is the case, for instance, of some RHO GEFs and GAPs, which expression is specifically associated with particular processes. For example, the mRNA levels of the RHO GEF *ECT2* and the RHO GAP *RACGAP1* are upregulated during the G2/M phase transition, to promote RHOA activation at the central ring of actin (ECT2), and to prevent RAC1 activity at this position during the final phases of the cell division (RACGAP1) [64, 65].

1.3 Typical RHO GTPases GDP/GTP cycle

Fully processed and post-translationally modified RHO GTPases are mostly locked in an inactive state, and into stoichiometric complexes with RHO GDIs [5, 21]. Upon certain stimulations, RHO GDIs can dock GTPases at specific membrane subregions, at which typical RHO GTPases undergo the GDP for GTP exchange [5, 21]. Firstly, RHO GDIs release the sequestered RHO GTPase upon being phosphorylated by SRC, PKC or PAK kinases [5, 21], and, secondly, the RHO GEFs promote the guanosine nucleotides exchange. In addition to their phosphorylation, the participation of several lipids as RHO GDI-releasing factors has been also reported within specific signaling complexes [21, 66-68]. Once activated, RHO GTPases can interact with a myriad of downstream effector proteins to transduce their signal. Finally, GTP-bound RHO GTPases are inactivated by RHO GAPs, and sequestered back by RHO GDIs, closing the cycle (**Fig. II**).

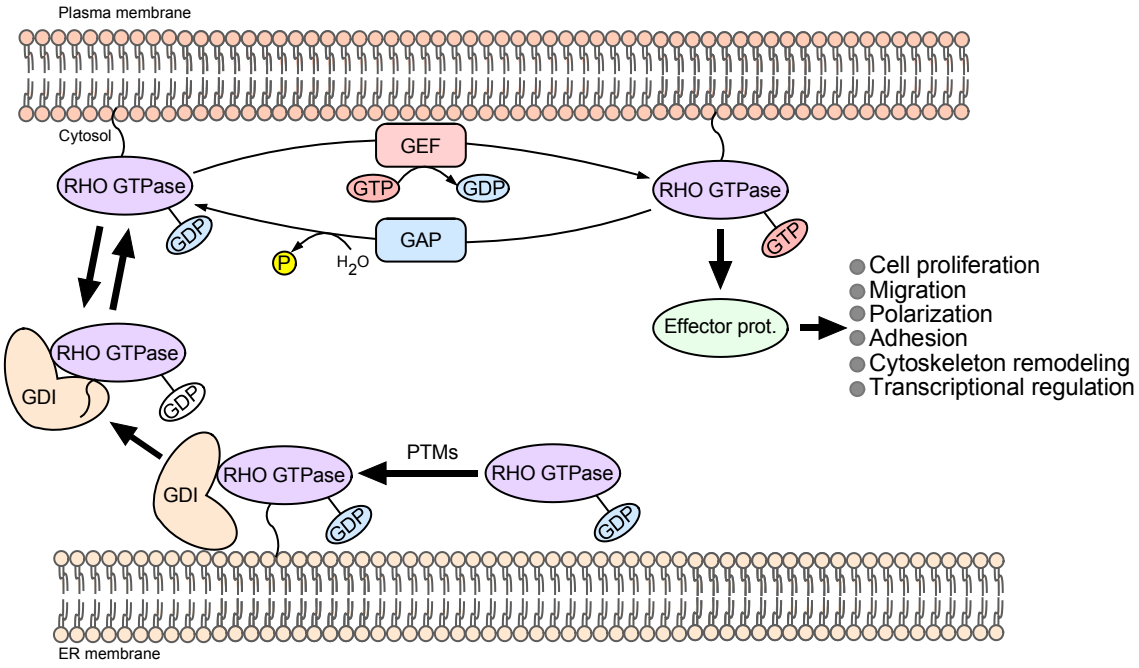

FIGURE II. Typical RHO GTPases cycle

The typical RHO GTPases cycle begins when these proteins are post-translationally modified in cytosolic surface of the endoplasmic reticulum (ER). This consists in the addition of a lipid to their CAAX domain in the C-terminus. Then, RHO GDIs can bind to the RHO GTPase and extract them from the ER membrane. Upon stimulus, the sequestered RHO GTPase is anchored to the inner face of the plasma membrane to initialize the signaling. Firstly, the RHO GEFs promote the exchange of the GDP nucleotide for a GTP one. This step modifies RHO GTPases structure and activates it. Then, RHO GTPase can interact and activate its downstream effectors that will take control over different cell processes. Once activated, RHO GAPs convert the GTPase into an "efficient" GTPase. In this way, the GTP group is hydrolyzed, inactivating the RHO GTPase, that can be then sequestered back from plasma membrane by RHO GDIs, closing the cycle. Figure adapted from [69].

1.3.1 Family of RHO GEFs

The first mammalian RHO GEF identified was a transforming gene from diffuse B-cell-lymphoma cells [70, 71]. This protein contained a region of 240 amino acids that was homologous to a region in *Saccharomyces cerevisiae* Cdc24 protein [72, 73]. Since then, a myriad of new GEFs containing this Dbl homologous (DH) domain were found in the human genome. In addition to the DH domain, all these proteins possess an adjacent C-terminal 100 residues-long pleckstrin homology (PH) domain # [74] # in charge of localizing GEF proteins to the membrane, and to regulate their GEF activity through allosteric mechanisms [70, 75, 76]. Altogether, they conform the Dbl family of GEFs with 73 members **(Fig. III)** [77]. Unlike them, the second family of RHO GEFs do not possess this tandem of DH-PH domains. Instead of that, dedicator of cytokinesis (DOCK) family of GEFs is constituted by 11 structurally conserved proteins with GEF activity mainly for RAC and CDC42 GTPases **(Fig. III)** [78-80]. They can be differentiated by the presence of two domains. The DHR1 domain is responsible for protein translocation to the membrane through its binding ability to phospholipids [78, 81]. The DHR2 domain is responsible for the GEF activity of these proteins [80-85]. Based on sequence homology, we can differentiate four subfamilies between them (DOCK-A, DOCK-B,

DOCK-C and DOCK-D) [74, 80]. The DOCK-A and DOCK-B subfamilies are characterized by the presence of a SRC homology 3 (SH3) N-terminal domain and a C-terminal proline enriched region that bind each other to maintain an autoinhibited state. This autoinhibition is released upon the interaction with the engulfment and cell motility proteins (ELMOs) [85-87]. On the contrary, DOCK-C and DOCK-D subfamilies do not contain the SH3 domain or the C-terminal region, so they lack of this autoinhibition capacity. Instead, these proteins were found regulated by post-translational modifications such as dephosphorylation [88]. Uniquely, DOCK-D GEFs contain a PH domain involved in their membrane translocation [80, 85].

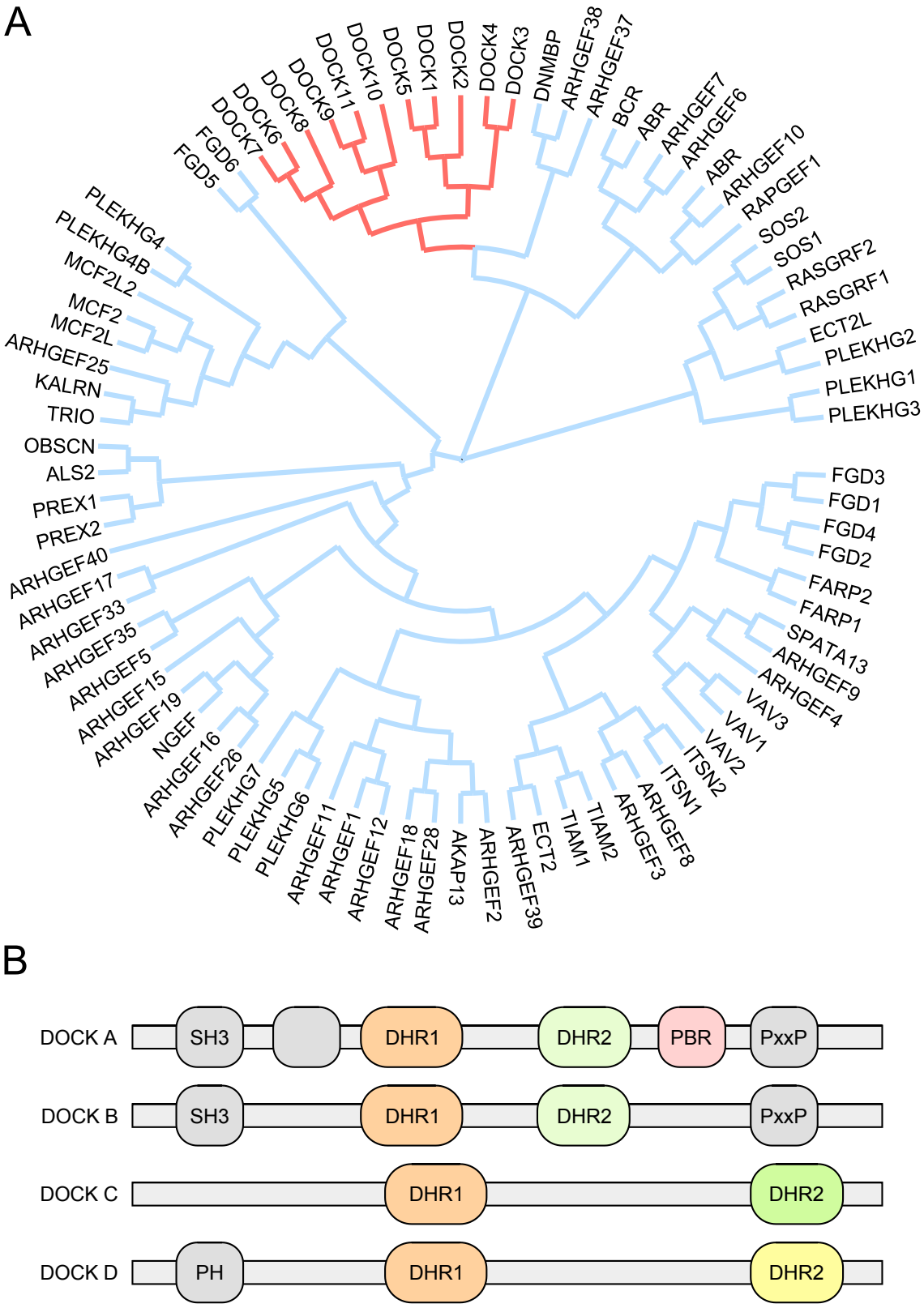

FIGURE III. The human family of RHO GEFs

(A) The RHO GEFs family consists of 81 different proteins that can be subdivided by their structure in 2 subfamilies. The Dbl subfamily (blue branches) consist of 70 GEFs that share a tandem of DH-PH domains in the amino acid sequence. The DOCK subfamily (red branches) consists of 11 GEFs that present the

DHR1 and DHR2 domains instead. The unrooted phylogenetic tree is based on CLUSTALW alignment of RHO GEFs amino acid sequences. **(B)** DOCK GEF subfamily can be subdivided into 4 subclasses that can be distinguished based on their domains. The N-terminus of DOCK-A (DOCK1, DOCK2 and DOCK5) and DOCK-B (DOCK3 and DOCK4) subclasses include a SH3 domain that mediates their interaction with ELMO scaffolding proteins, while in the C-terminus they present a PxxP region that coordinates the interactions with SH3-containing adaptor proteins. Their DHR2 domain determines their exchange activity for GTPases from the RAC subfamily. Unlike them, DOCK-C subclass (DOCK6, DOCK7 and DOCK8) present a DHR2 domain ambivalent for RAC and CDC42 GTPases. Finally, the DHR2 domain of DOCK-D subclass (DOCK9, DOCK10 and DOCK11) is specific to exchange nucleotides in the GTPases from the CDC42 subfamily.

1.3.2 Family of RHO GAPs

RHO GAPs activity is essential for RHO GTPases to match the time required for their subcellular functions [22, 75, 78]. The conversion of a GTPase into an "efficient GTPase" depends on a configuration in which the RHO GAP provides several amino acidic residues to the activation of the nucleophilic water molecule that will attack the γ-phosphate of the GTP group [75, 89]. In total, the human genome encodes 66 RHO GAPs **(Fig. IV)**, and 2 proteins (ABR and BCR) that exhibited a dual GEF/GAP activity [51].

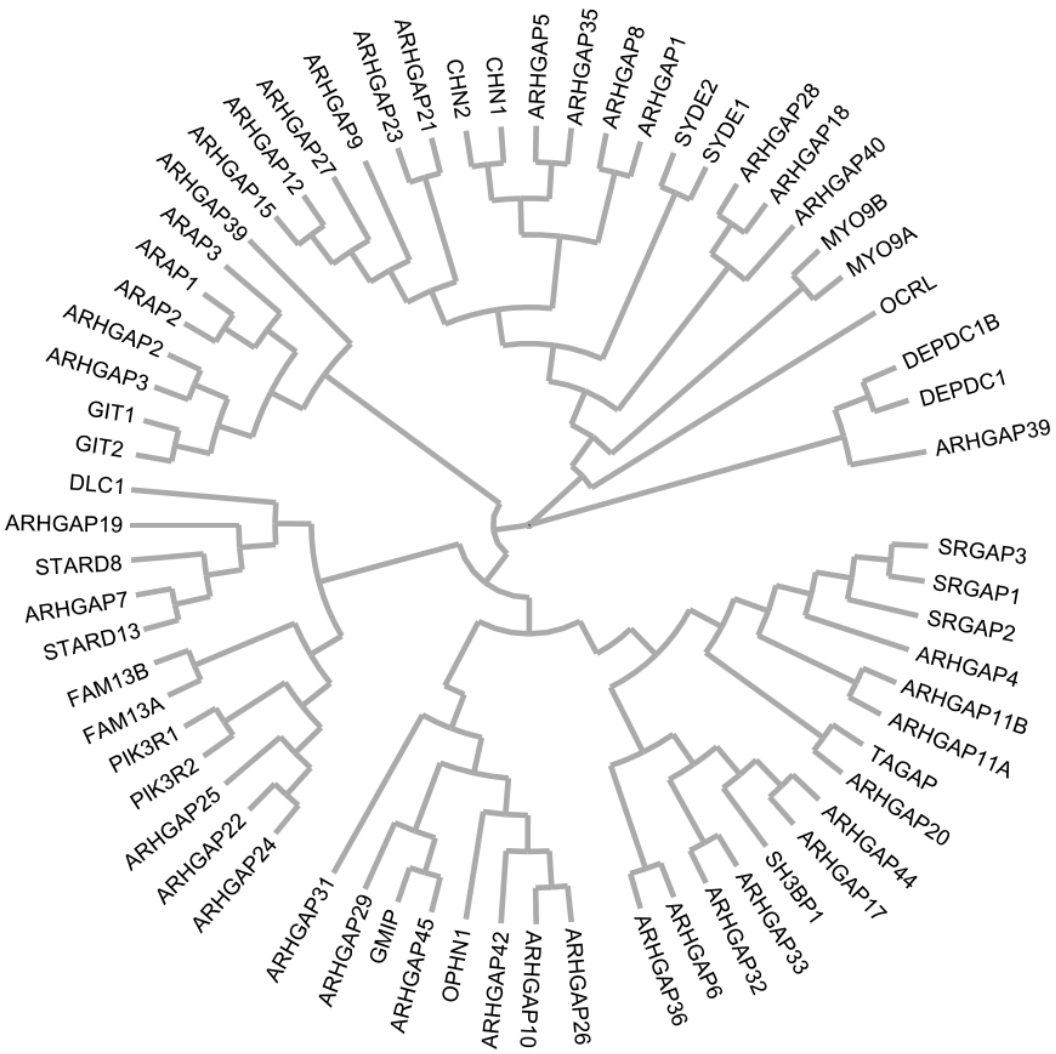

FIGURE IV. The human family of RHO GAPs

The RHO GAPs family consists of 66 proteins with a common catalytic domain of approximately 150 amino acids in length. It contains an arginine residue that is responsible for their catalytic activity. In addition to this domain, they also present others that are necessary for their regulation, subcellular localization, and specific roles in signaling pathways. The unrooted phylogenetic tree is based on CLUSTALW alignment of RHO GAPs amino acid sequences.

1.3.3 Family of RHO GDIs

RHO GDIs family contains 3 members encoded in the human genome, and they are comprised of an N-terminal α-helix bundle that interacts with the switch regions of the GTPase, and a C-terminal β-sandwich domain that binds to its geranyl-geranyl group [78, 90, 91]. RHO GTPases extraction from the membrane by Rho GDIs is plausible to be performed in two steps: first, the α-helical domain binds to the GTPase core, and second,

the β-sandwich domain captures the prenylated C-terminus of the GTPase [78]. However, these contacts between the RHO GDI and the GTPase cannot directly explain how they prevent GDP dissociation or inhibit the GTP binding.

1.3 RHO GTPases proximal interactome

1.4.1 Downstream effectors

The exchange of nucleotides promotes a conformational change in RHO GTPases switch I and switch II effector regions that, in turn, makes possible the interaction of GTP-bound GTPases with their downstream proximal effector proteins. The recognition between RHO GTPases and their effectors depends on structural cues present on the RHO switch regions and, in some of them, other moieties [21]. Although in many cases these cues constraint the type of effectors recognized by each RHO GTPase, protein-protein interaction experiments studying RHO GTPases proximate interactome demonstrate that many of these proteins are highly promiscuous in their interactions. To date, around 60 RHO proximal effectors have been identified. Although they are very heterogeneous in terms of structure and function, we can ascribed them to 5 functional categories [21]: 1) direct regulators of the F-actin cytoskeleton remodeling [92-95]; 2) regulators of protein enzyme complexes [96-98]; 3) protein serine/threonine kinases that belong to the families of mitogen activated kinases (MAPKs) [99-103], protein kinase N (PKNs) [104-106], p21-activated kinases (PAKs) [107-110], Rho-associated protein kinases (ROCKs) [111, 112], atypical protein kinase Cs (aPKCs) [113, 114], myotonic dystrophy kinase-related Cdc42-binding kinases (CDC42BPs) [115-117], citron RHO binding protein kinase (CIT) [118-120], myotonic dystrophy kinase (DMPKs) [121], and myosin light chain kinases (MYLKs) [122], among others; 4) protein tyrosine kinases such as TNK2 (refs); and 5) phospholipid kinases, such as the phosphatidylinositol 3-phosphate kinases (PI3Ks) and

the phosphatidylinositol-4-phosphate 5-kinase (PIP5K), and diacylglycerol kinases (DGKs) [123-125].

In addition to these proximal interactions, proteomic and functional studies have unveiled other kind of proteins that, despite they do not directly interact with RHO GTPases, their activity is modulated by their proximal interactors [126-130]. Among these "distal interactors" we can find 1) protein kinases from different families, such as LIM motif-containing kinases (LIMKs) [131-133], polo-like kinases (PLKs), and ribosomal regulation kinases (RPS6Ks), among others; and 2) proteins from different complexes associated with the regulation of cytoskeleton dynamics [93], such as subunits of the actin related protein 2/3 complexes (ARP2/3), and proteins involved in the formation of the WAVE complex [134].

1.4.2 Marginal interactome

According to the latest proteomic studies, there are more than 180 proteins that present strong protein-protein interactions with at least one RHO GTPase [51]. Unlike previously mentioned downstream effectors, the activity of these proteins is not directly modified by RHO GTPases activation or their downstream effectors, but they are necessary to signal transduction. For example, some of these proteins are chaperones that escorts GTPases from their site of post-translational modification (the ER and Golgi apparatus) to membranes, such as HSPE1, CCT1 or CCT6A. In other cases, they are proteins that take part in the conformation of signalosomes that involve RHO GTPases activity, such as those controlling the organization of cell-cell junctions [53, 135-137], the acquisition of cell polarity [138-142], the regulation of endomembranes trafficking [143], the formation of different membrane structures, such as the lamellipodia, filod [144, 145], and the positive regulation of WASP/WAVE [146] and ARP2/3 complexes [147, 148], among others **(Fig. V)**.

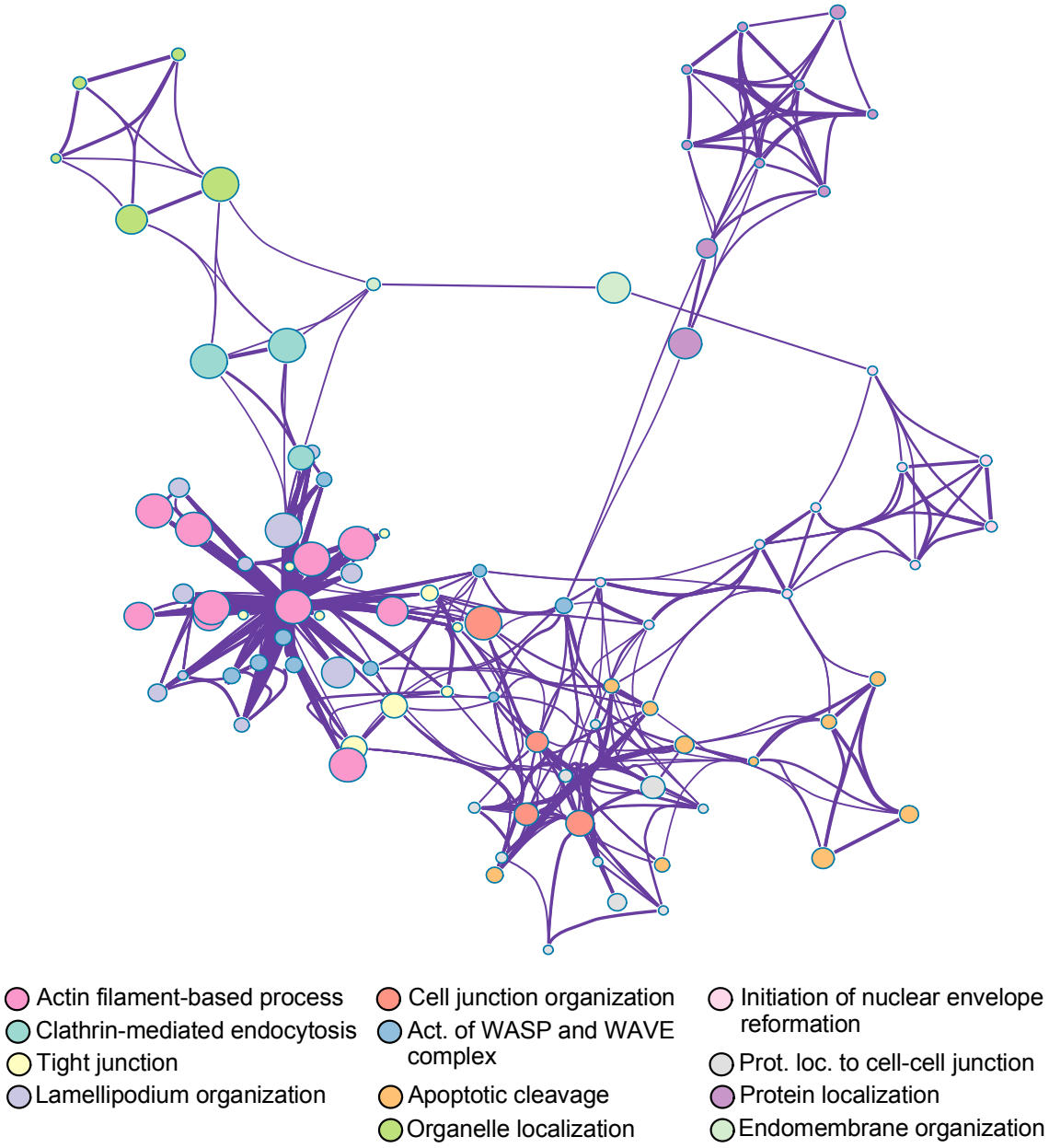

FIGURE V. RHO GTPases marginal interactome

Top biological processes in which proteins from RHO GTPases pathway marginal interactome are enriched on. These proteins are mainly involved in the signalosomes that control membrane and actin cytoskeleton remodeling (WASP and WAVE complexes), endomembrane trafficking, and protein localization to subcellular domains, such as the cell-cell junction. The size of the circles is directly proportional to the number of proteins enriched in each GO term. Each process in the network is represented by several circles that share the same color. Each circle represents one subprocess that is nested in one of the general biological terms that are explained in the legend. The thinness of the edges that connect two circles is

proportional to the number of proteins shared between both subprocesses. This network has been generated using Metascape.org WebApp. Act., activation; Prot., protein; loc., localization.

2. RHO GTPases pathway in cancer

2.1 Pro-tumorigenic functions: a controversial archetype

RHO GTPases are core signaling proteins initially linked with the regulation of cell migration, polarity, and shape due to their central role in the control of F-actin cytoskeleton remodeling [1, 22, 24, 31, 53, 58, 69, 139, 149]. Later, these proteins were associated with a broader range of functions such as the control of cell cycle progression [150-152], cell survival and proliferation [21, 153, 154], as well as to some cell type-specific responses during immune reaction [69, 155-157], angiogenesis [158-160], and neuronal development [11-13, 161-163]. All these functions are essential to maintain normal tissues homeostasis, but they also contribute to tumor cells acquisition of different cancer hallmarks [1, 23, 154]. Due to that, RHO GTPases have been historically proposed as essential factors in cancer cells development of malignant features [23, 164]. This hypothesis was initially supported by the outcomes obtained from gain- and loss-of-function experiments using different *in vitro* and *in vivo* cancer models [77, 149, 164-166]. These experiments revealed two key features about the role of this pathway in cancer. Firstly, they showed that some mutations on Cdc42, Rac1 and RhoA GTPases, as well as several ones in their positive regulators, the RHO GEFs, promote the acquisition of malignant features in fibroblasts and different cancer cell lines. Secondly, they unveiled that the expression of some RHO GTPases and GEFs is necessary to keep tumor fitness. Based on these evidence, the hypothesis that RHO GTPases and GEFs are prominent pro-oncogenic factors became the most accepted archetype in the field, according to which RHO GTPases and GEFs alterations in tumors end up in the hyperactivation of this signaling axis and positive outcomes in tumor fitness. In this functional prototype, RHO GAPs are expected to antagonize the foregoing process [167, 168] according to their function as negative regulators of GTPases activation. This idea

has received further impetus during the last decade. The massive genomic characterization of [169]different human tumors has demonstrated that they develop gain-of-function mutations on some RHO GTPases, such as RAC1 and RHOA [170-189], as well as in their regulators (i.e., the RAC GEFs PREX2 and VAVs proteins) [21, 190-195], and some downstream effectors (i.e., the PAKs [173] and ROCK1 [196, 197]). However, these results also unveiled something else: these mutations are at low prevalence among cancer patients. Facing this data is unavoidable to address the question of [21]: why cancer cells do not consistently develop mutations in this pathway at high frequencies?

One possible answer to this question is that there are other mechanisms that could lead to the spurious activation of this route [21, 24, 198]. For example, a strong emphasis has been placed on the observed upregulation of RHO GTPases expression in different tumors, such as Rac1, in breast, gastric, prostate, and testicular cancers [167, 199, 200]; RhoA and RhoC, in tumors with epithelial origin [167, 200, 201]; and Cdc42 in testicular, colorectal, and non-small cell lung cancers [202-205]. Nevertheless, these results should be taken cautiously for several reasons. Firstly, some of them have been obtained by directly measuring RHO GTPases protein expression, with western blots or immunohistochemical assays, and, in some cases, these high protein levels did not correlate with an increase in their mRNA expression [200, 206-208]. These results could be explained by the use of poorly validated antibodies, whether in terms of isoform specificity or due to the nature of the epitope that it is being actually detected [21]. Secondly, new studies using high-throughput techniques have shown that RHO GTPases are not only overexpressed in cancer. In the last decade, different transcriptomic studies have unveiled that the expression of some of them is significantly repressed in tumor compared to matched healthy tissues (information obtained from Open Targets Platform

database) [209]. Thirdly, the increased abundance of RHO GTPases may [210]not elicit the functional consequences that would be expected. For example, RHO GTPases upregulation could be easily buffered by their association with RHO GDIs in stoichiometric complexes [21]. Alternatively, instead of RHO GTPases expression upregulation, it has been proposed that it is the overexpression of RHO GEFs and downregulation of RHO GAPs the transcriptional changes that could be positively contributing to the hyperactivation of this signaling axis in cancer. In fact, some RHO GEFs have been found overexpressed in different tumors, such as *ARHGEF7 [211]*, *ARHGEF12 [212]*, *DOCK1 [213]*, *ECT2 [214]*, *PLEKHG6 [215]*, *PREX1 [216]*, *TIAM1 [217, 218]*, *VAV1 [219]*, *VAV2* [220] and *VAV3* [77, 221, 222], as well as some RHO GAPs have been described transcriptionally repressed in some others [223-228]. However, the transcriptomic studies performed during the last decade have challenged this archetype once again. RHO GEFs can be found downregulated in tumors, as well as RHO GAPs are overexpressed in patients-derived cancer samples [209]. Altogether, these observations demonstrate that, in addition to their pro-tumorigenic effects, the elements from this pathway can play more variegated roles in cancer than initially expected.

2.2 Tumor suppressor activities: another brick in the wall

One of the most puzzling discoveries about RHO GTPases pathway in the genomic era was the discovery of both dominant negative and loss-of-function mutations on RHOA gene in patients-derived tumors [21, 176, 177, 179, 181, 185-187, 189, 229-234]. These alterations can be subclassified into three classes. The first class is constituted by mutations that impairs RHOA binding to guanosine nucleotides, such as $RHOA^{G17V}$ hotspot mutation (**Fig. VI)** found in the whole exome sequencing of 50.3-70.8% angioimmunoblastic T-cell lymphoma (AITL) [21, 229, 232, 235], and 7.7-18% of

peripheral T-cell lymphomas, not otherwise specified (PTCLs-NOS) [236, 237], patients. In the second class, there are mutations that alter RHOA binding to downstream effector proteins (**Fig. VI)**. These mutations often target RHOA switch I domain around Tyr37 and Tyr42 residues, impairing RHOA ability to interact with PKNs [105], or Tyr40, preventing ROCK and DIAPH3 activation (**Fig. VI)** [238]. Here, it is important to notice that these mutations generate what is called a "branch deficient signaling" mutant of RHOA protein, since the mutated version can still interact with other downstream effectors. This has been well-exemplified in the case of RHOA Tyr42 mutation which effect on RHOA signaling has been especially difficult to pin down. The initial idea that it was a loss-of-function mutation has been recently discarded. The RHOAY42C mutant in diffuse gastric tumors not only maintains its binding to ROCK and DIAPHs proteins, but increases this joining, incrementing the signaling through these proteins [239, 240]. Lastly, the third subclass contains mutations that can lead to *RHOA* transcriptional repression in cancer. Among them, there is often include the hemizygous deletion of RHOA WT allele in heterozygotic mutated tumors [177]. However, it is important to notice that these hemizygous deletions could be a mechanism in cancer cells to upregulate the expression of the mutated allele instead of downregulating RHOA expression levels [241-244].

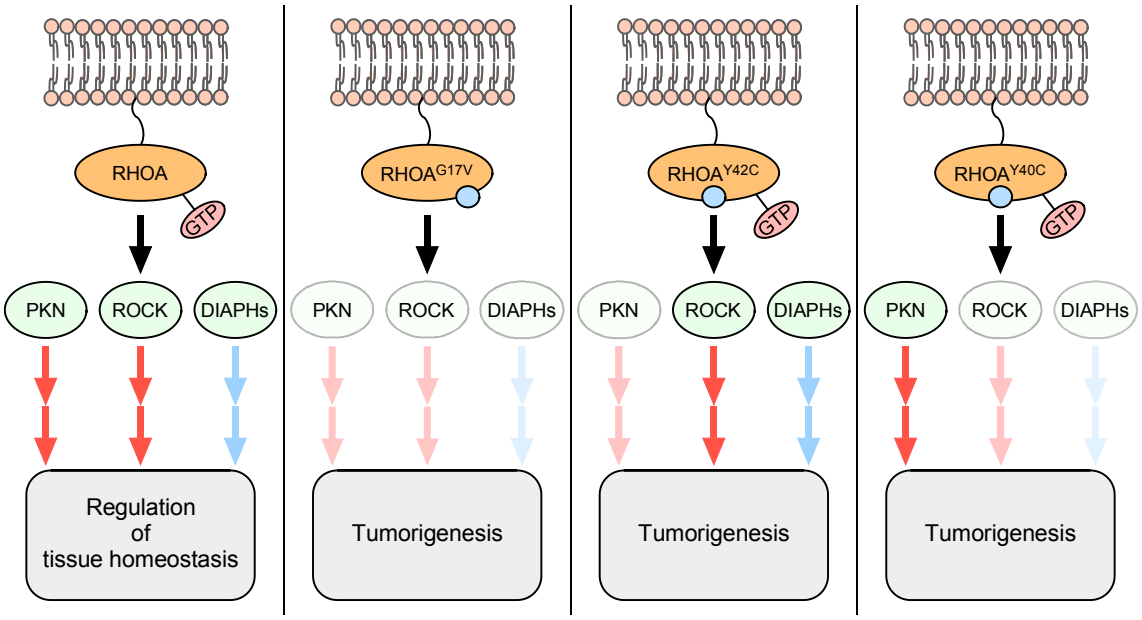

FIGURE VI. Representation of RHOA loss-of-function mutations putative effects in cancer progression

(A-D) Potential effects induced by wild-type **(A)** and mutant **(B-D)** RHOA in tumors. The wild-type version keeps a balance in the signaling pathways initiated by RHOA downstream effectors to maintain homeostasis in healthy tissues. This balance is interrupted in some tumors that present nucleotide-free mutations on RHOA **(B)** or mutant versions defective to interact and activate its some of its downstream effectors **(C and D)**. In the case of the $RHOA^{G17V}$ mutation **(B)**, it impairs RHOA GEFs ability to promote the exchange of GDP for GTP, so activating RHOA in the healthy tissue. The inactivated RHOA cannot bind an activate any of its downstream effector proteins and this depletion on all RHOA-dependent signals contributes to healthy cells transformation and/or tumor progression. Unlike $RHOA^{G17V}$, the mutations that impairs RHOA binding to one or more of its downstream effectors **(C-D)** induce a branch-defective signaling that finally contributes to cancer progression. Adapted from [21].

Nonetheless, RHOA mutations were not the first evidence of potential tumor suppressor activities ascribed to a RHO GTPase. Two decades ago, researchers found that the geranylgeranylated version of the RhoB GTPase promote tumor suppressor activities in fibroblasts and some tumors [245-248]. This GTPase is often found deleted in multiple cancers [246], and its deletion has been found to accelerate the onset of chemically induced skin tumors in mice [249-251]. Along with RhoB, other RHO GTPases have

been associated with tumor suppressor activities, such as RhoE [252] and RhoH [253]. Even Cdc42, mainly considered as a pro-oncogenic protein, have been proposed to play suppressing roles in cancer, as it has been exemplified in the fact that the ablation of its expression in mice correlates with the appearance of hyperproliferative disorders, such as myeloproliferative disorder in blood [254], hepatomegaly in liver [255], and hyperplasia of intestinal epithelial cells in the intestine [256]. In addition to RHO GTPases, there are also evidence that some RHO GEFs can play tumor suppressor activities as well. In addition to the fact that several tumor significantly repress the expression of some of them, there are other evidence in this sense, including 1) the recurrent loss of genes encoding some of them in patients-derived cancer samples, such as *ARHGEF10* and *ARHGEF10L*, [257-259], and 2) the role as double agents in cancer described for some others, such as the members of the VAV subfamily (VAV1, VAV2 and VAV3) [260-262]. These evidence do not support the initially accepted archetype about RHO GTPases pathway as an universal pro-tumorigenic signaling axis in cancer. In turn, they demonstrate that there are many roles played by these proteins that we did not even suspect decades ago they could play. A good example is the fact that some RHO GAPs have been already described associated with pro-oncogenic activities, such as *ARHGAP11,* which silencing correlates with lower proliferation of basal-like breast cancer cells [263], among others [24, 167, 209, 226, 264-269].

2.3 Current perspective and future approaches

All the observations that we have introduced in the previous sections demonstrate one thing: significant advances have been made in the last ten years to understand the modus operandi that RHO GTPases pathway has in cancer. The initial archetype has been supported until the last decade since most of our understanding about the role that this

signaling axis play in cancer came from the study of the three prototypical RHO GTPases (CDC42, RAC1 and RHOA). In fact, if we analyze the number of publications since 1960 associated with each RHO GTPase, these three GTPases has more than 2000 articles, that is four times the number of articles associated with the fourth and fifth most studied RHO GTPases in oncology, RHOC and RHOB **(Fig. VII)**. Moreover, something similar can be observed in the cumulative number of articles associated with RHO GEFs since then **(Fig. VII)**. ARHGEF1 is the most studied RHO GEF in cancer, except for BCR (B-cell receptor). The interest for BCR exploded since 2010 due to its implications in the origin and development of B-cell leukemias and their treatment [270-275] **(Fig. VII)**.

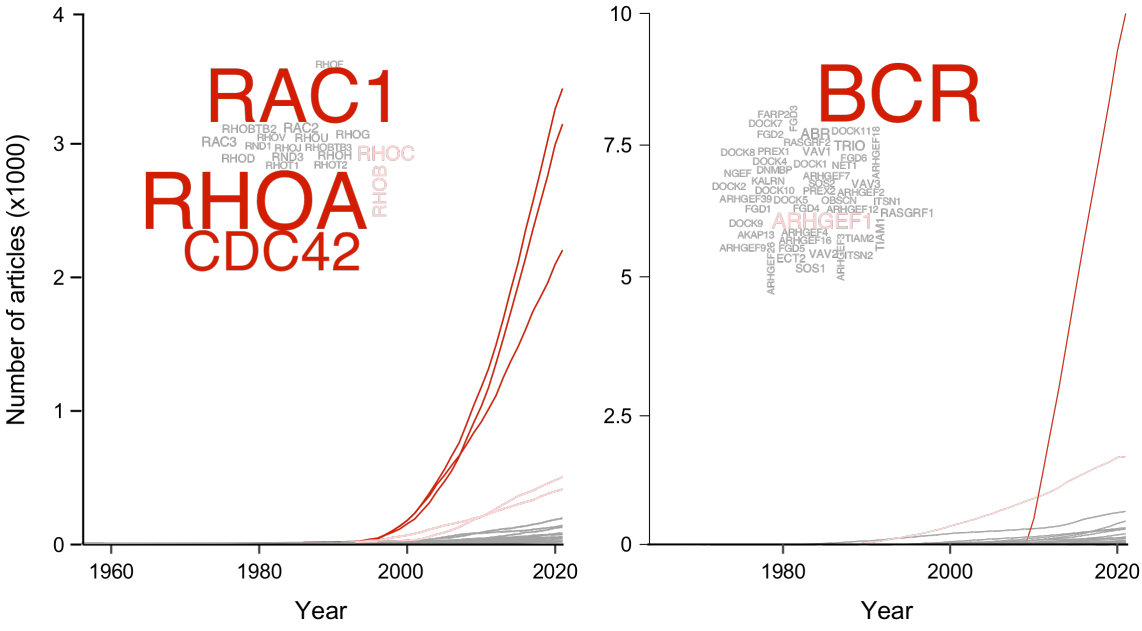

FIGURE VII. Most studied RHO GTPases and GEFs in cancer since 1960

Number of publications indexed in PubMed relating RHO GTPases/GEFs and cancer studies since 1960. Dark red represents the most studied GTPase and GEF until the date, and the GTPases and GEFs associated with more than 50% of their number of articles associated. Light red represents GTPases and GEFs associated with more than 10% of the articles associated with the most studied GTPase and GEF in history, respectively.

However, in the last decade this archetype has been challenged due to the incorporation of modern high-throughput technologies to the study of RHO GTPases pathway. One of the most important milestones in this sense has been the application of proteomic techniques to the study of the RHO GTPases proximal interactome, and RHO GEFs and GAPs specificity [51, 276], creating the most complete repertoire of RHO GTPases pathway interacting proteins. The next-generation DNA and RNA sequencing technologies have been also successfully applied to the analysis of RHO GTPases pathway role in tumors. As previously mentioned, the whole-genome and whole-exome sequencing of thousands of human tumors have unveiled new oncogenic mutations on RHO GTPases pathway. Today, much of this information is available at different databases, such as the Cancer Genome Atlas (TCGA). In addition to the somatic mutations, the TCGA provides access to the transcriptomic, epigenetic and copy number profile of more than 10,000 tumor samples from 33 different cancer types.

In this study, we used the multi-dimensional molecular data obtained from the TCGA database to dissect the alterations that elements in the RHO GTPases pathway present across multiple cancer types. To that end, we applied and developed different bioinformatics algorithms designed to 1) create a complete catalogue of somatic mutations in RHO GTPases pathway, and discern which of them are potentially oncogenic; 2) identify the most recurrently differentially expressed genes in this pathway across multiple tumors; 3) study the real impact that somatic copy number variations have on its dysregulation in cancer; and 4) describe cancer cell lines dependency on the expression of the most deregulated elements in this pathway. Finally, we validated some of the outcomes obtained from this pan-cancer systematic analysis in *in vitro* and *in vivo* cancer biological models. In this work, we opted for the study of KRAS-mutated lung adenocarcinoma tumor models since this is one of the deathliest cancers with a very

narrow spectrum of low-effective therapies. In particular, we evaluated these tumors dependency on the expression of several deregulated RHO GEFs as an approach to discover new vulnerabilities that could be further translated in new therapeutic targets for its treatment.

OBJECTIVES

1. Catalogue all the potential oncogenic mutations that affect RHO genes in TCGA tumors.

2. Identify the most relevant transcriptional alterations in this pathway in datasets from TCGA and GEO databases.

3. Analyze how RHO genes somatic copy number variations can modify their transcription in cancer.

4. Identify new potential targets in cancer among the genes that constitute this pathway.

5. Identify new potential therapeutic targets in KRAS-mutated lung adenocarcinoma tumors among this pathway.

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APPENDIX: RESUMEN EN CASTELLANO

INTRODUCCIÓN

Nuestro genoma codifica 18 proteínas que constituyen la familia de las GTPasas RHO. En función de su homología y estructura, estas proteínas pueden clasificarse en 9 subfamilias: CDC42 (CDC42, RHOQ y RHOJ), RAC (RAC1, RAC2, RAC3 y RHOG), RHOA (RHOA, RHOB y RHOC), RHOD (RHOD y RHOF), RHOH, RHOU (RHOU y RHOV) and RND (RND1, RND2 y RND3). En algunos estudios, se añaden dos subfamilias más, aunque tienen una homología y funciones muy diferentes a las de las proteínas que se incluyen en estas nueve subfamilias. Son las GTPasas de las subfamilias RHOBTB (RHOBTB1, RHOBTB2 y RHOBTB3) y Miro (RHOT1 y RHOT2). Inicialmente, estas proteínas se asociaron funcionalmente con la regulación de la dinámica del citoesqueleto de actina de las células, y de procesos que dependen de esta actividad como son el cambio de forma de la célula, la creación de estructuras de membrana, la migración y el control de la polaridad. Luego, nuevas investigaciones las asociaron con el control de un número mucho más amplio de funciones, como la regulación de la proliferación celular, el control del ciclo celular, e incluso respuestas específicas durante la reacción inmune, la angiogénesis o el desarrollo de las neuronas. Debido a su papel central en la regulación de todos estos procesos, la desregulación de las GTPasas RHO se ha asociado significativamente con diferentes procesos fisiopatológicos, incluido el cáncer. Históricamente, las alteraciones en estas proteínas se han asociado con la adquisición de características malignas en las células tumorales. Estas asociaciones se descubrieron utilizando experimentos de ganancia y pérdida de función tanto in vitro, en células tumorales crecidas en placa, como in vivo, en modelos murinos. Estos experimentos corroboraron que cambios en esta ruta se correlacionaban con mayor malignidad de los tumores. Debido a ello, con el tiempo el arquetipo de que esta ruta ejercía efectos prominentemente protumorales se convirtió en un arquetipo ampliamente

aceptado. Sin embargo, los resultados de los análisis masivos de tumores humanos con nuevas herramientas biotecnológicas de amplio espectro que se han llevado a cabo en la última década han puesto en entredicho este arquetipo. Estos estudios has demostrado que las mutaciones oncogénicas asociadas a las GTPasas RHO en realidad se encuentran en muy baja prevalencia entre los tumores obtenidos de los pacientes. Subsecuentemente, los investigadores han buscado desde entonces qué otros mecanismos pueden llevar a la activación de esta ruta independientes de la mutación y que se puedan dar en un conjunto mucho mayor de pacientes. Una de las posibilidades que se propusieron fue la sobreexpresión de estas proteínas de manera que mayor expresión contribuyese positivamente a un aumento de la señalización de la ruta. Sin embargo, de nuevo los últimos avances en el estudio del transcriptoma de las GTPasas RHO en cáncer ha demostrado que, si bien existe dicha sobreexpresión, en muchos casos estas proteínas también presentan una reducción significativa de su expresión en comparación con el tejido sano. A estos descubrimientos, debemos sumar otros datos que demuestran la presencia de mutaciones y otras alteraciones recurrentes en tumores humanos que contradicen el arquetipo sobre esta ruta. De entre todos ellos, el más paradójico fue el descubrimiento de un amplio rango de mutaciones de pérdida de función asociadas al gen que codifica para la GTPasa RHOA en diferentes tipos de leucemia. La existencia de estas mutaciones, así como todas las evidencias contradictorias que hemos comentado, ponen de manifiesto que esta ruta, en escenarios específicos, podría también tener una función supresora de tumores.

Para identificar mejor el papel de esta ruta de señalización en cáncer, en este estudio proponemos el uso de datos masivos de secuenciación, tanto de ADN como de ARN, provenientes de más de 10.000 pacientes de 33 tipos de tumor diferentes. Con estos datos, esperamos poder abordar tres objetivos que den respuesta a las principales

preguntas que hoy existen sobre este eje señalizador en el desarrollo de tumores: 1) crear un catálogo completo de las mutaciones en esta ruta y diferencia de entre ellas aquellas que puedan tener un potencial oncogénico, 2) identificar cuáles son los cambios de expresión más relevantes en los pacientes de cáncer, y 3) proponer para su investigación genes cuyas alteraciones puedan tener un papel significativo en el desarrollo de los tumores y que, en un futuro, pudiesen constituir posibles dianas terapéuticas para el tratamiento de esta enfermedad.

RESULTADOS

Análisis de las mutaciones que afectan a la vía de las GTPasas RHO en cáncer

En primer lugar, el análisis de las mutaciones ha demostrado que el 98% de los genes que participan en esta ruta (486 en total) presentan algún tipo de alteración somática en alguno de los 33 tipos de tumores analizados. Sin embargo, entre ellas tan sólo el 10% se presentan en alta prevalencia (en más del 10% de los pacientes analizados). Entre estas mutaciones, ninguna se ha encontrado bajo la acción de la selección positiva, salvo las mutaciones ya conocidas como oncogénicas que afectan al gen PIK3CA. El resto de las mutaciones seleccionadas positivamente están representadas en menos de un 5% de las muestras analizadas y concretamente localizadas en tipos de tumores muy específicos. Algunas de ellas ya son conocidas y su función ha sido estudiada en diferentes modelos biológicos de cáncer. Sin embargo, dos de ellas son mutaciones en el gen RHOB y PIK3R1 bastante recurrentes en pacientes con cáncer de vejiga y endometrial, respectivamente, y sobre las cuales apenas conocemos ninguna implicación funcional en el desarrollo de estos tumores.

Análisis de los cambios transcripcionales en la vía de las GTPasas RHO en cáncer

En segundo lugar, el análisis de los cambios transcriptómicos asociados a esta ruta en cáncer, ha demostrado que el 74% de estos genes están desregulados en alguno de los 20 tipos de tumores analizados. De entre ellos, el 44% son genes cuyo cambio de expresión no sólo es recurrente entre los pacientes de una cohorte, sino también que es recurrente entre los diferentes tipos de cáncer analizados. De entre los grupos de genes que constituyen esta ruta, las GAPs y los GEFs RHO son los que mayor proporción de elementos desregulados presentan en estos tumores, aunque con una diferencia muy destacable. En el caso de las GAPs RHO una gran proporción de ellas están exclusivamente sobreexpresadas y otra reprimidas a lo largo de los tumores analizados. Esto no se encuentra entre los GEFs RHO, el 50% de los cuales pueden encontrarse o bien sobreexpresados o bien reprimidos dependiendo del tipo de tumor que analicemos. Ya que estas proteínas regulan directamente la actividad de las GTPasas, a continuación, decidimos analizar si sus cambios de expresión podrían estar favoreciendo cambios en la señalización de una GTPasa frente a las demás. Para ello, cada GAP y GEF se clasificó en función de su actividad para cada una de las GTPasas prototípicas (CDC42, RAC1 y RHOA), y luego seleccionamos aquellas que son específicas para cada uno. Lo que encontramos fue que la mayor parte de las GAPs que se reprimen a lo largo de estos tumores son específicas para RHOA, al igual que la mayor proporción de los GEFs que se sobreexpresan. Al mismo tiempo, descubrimos que lo contrario pasa con la GTPasa RAC1. En este sentido, estos resultados sugieren que muchos de los tumores analizados podrían regular la actividad de estas GTPasas directamente controlando la expresión de sus reguladores positivos (GEFs) y negativos (GAPs) a nivel transcripcional. Este sería un nuevo mecanismo compartido por una gran parte de los tumores humanos que conllevaría el control de la señalización de estas proteínas. Para saber qué procesos

celulares podrían estar afectados a la vez que estos genes se desregulan transcripcionalmente, estudiamos cómo la expresión de las GAPs y GEFs específicos para cada GTPasa correlacionaba con la expresión de cada uno del resto de tránscriptos celulares. Luego, esta información nos sirvió para identificar en qué procesos se involucraban preferentemente estos genes. De este modo, observamos que el cambio de expresión de los genes reguladores correlacionaba con el cambio de expresión de genes involucrados en las rutas canónicas de señalización de estas GTPasas, sobre todo asociadas al mantenimiento del crecimiento celular, el metabolismo y la respuesta inmune.

Análisis del impacto que los cambios de número de copia tienen sobre la expresión de los genes RHO en cáncer

En tercer lugar, analizamos el papel que los cambios en el número de copia de estos genes podrían tener sobre su expresión en cáncer. Para ello, desarrollamos nuestro propio algoritmo orientado a estudiar estos cambios. Descubrimos que un porcentaje alto de todas estas mutaciones en el número de copia se asociaban a cambios de expresión que no concordaban con los cambios observados entre el conjunto de muestras tumorales y el conjunto de muestras de tejidos sanos. Al mismo tiempo, observamos que el 30% de todas estas asociaciones si tenían esta concordancia, de manera que podrían ejercer un papel relevante en el cambio de expresión que se observa entre las muestras tumorales y los tejidos control. De entre ellos, identificamos 10 genes que presentan estas asociaciones concordantes en un número alto de cohortes, lo que indicaría que sus alteraciones en el número de copia podrían constituir un nuevo mecanismo que conllevase a su expresión diferencial en diferentes tumores. Sin embargo, en todos los casos, estos cambios de

número de copia se supeditaron a la existencia de un oncogén cercano con el que se encontraban co-amplificados o co-deleccionados en los tumores.

Identificación de nuevos GEFs RHO con capacidad pro-tumoral en adenocarcinoma de pulmón KRAS mutado

Por último, dado que los cambios de expresión fueron el tipo de alteración más recurrente entre los genes RHO en todos los tumores, decidimos centrarnos en estos cambios a la hora de elegir un candidato para comprobar su papel oncogénico en modelos biológicos de uno de los tumores con pero pronóstico hoy en día, el adenocarcinoma de pulmón KRAS mutado. Dado que, desde el punto de vista farmacológico, inhibir una molécula es más factible que incrementar su actividad, decidimos centrarnos en los genes RHO que se sobreexpresaban. Dado que los cambios de expresión de las GTPasas y GAPs RHO no siempre correlacionan con los cambios funcionales que se esperarían, decidimos además reducir nuestro campo de búsqueda al subgrupo de GEFs RHO. De entre todos ellos, tan sólo tres se encontraron consistentemente sobreexpresados en estos tumores: *ECT2*, *PLEKHG2* y *VAV2*. Dado que el papel en cáncer ya se conoce desde hace décadas, y que la actividad del segundo como GEF es muy débil, nos decantamos por analizar el papel de VAV2 en este tipo de tumores. Primero, identificamos que su expresión es necesaria para el desarrollo de adenocarcinomas pulmonares en ratones luego de que se indujese la generación de estos tumores a través de la expresión de una versión mutada y oncogénica de la proteína Kras. Luego, eliminamos su expresión en líneas celulares de adenocarcinoma de pulmón KRAS mutados provenientes tanto de ratón como de humano, y comprobamos que la deficiencia en la expresión de *VAV2* correlacionaba significativamente con 1) una reducción en su proliferación, 2) una disminución en su

capacidad de migración y metástasis, y 3) una mayor sensibilidad al tratamiento con cisplatino o compuestos inhibidores de la proteína kinasa MEK.

CONCLUSIONES

- Los tumores humanos desarrollan mutaciones con potencial oncogénico poco prevalentes en la ruta de las GTPasas RHO.
- Los cambios de expresión son una alteración más frecuente en esta ruta de señalización.
- Un porcentaje alto de los genes RHO desregulados transcripcionalmente lo están en un amplio número de tumores diferentes.
- Muchos de estos cambios transcripcionales se suceden a nivel de la regulación de las GTPasas RHO, en sus GEFs y GAPs, siendo muchas de estas proteínas reguladoras específicas de la actividad de las GTPasas RHOA y RAC1.
- VAV2 es uno de los tres GEFs RHO exclusivamente sobreexpresados en una gran parte de los tumores.
- La deficiencia en la expresión de VAV2 conlleva cambios en las células de adenocarcinoma de pulmón KRAS mutados que lo convierten en una posible futura diana terapéutica para su tratamiento.