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Rho GTPases: Anti- or Pro-neoplastic Targets?

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Abstract

Rho GTPases are critical signal transducers of multiple pathways. They have been proposed to be useful anti-neoplastic targets for over two decades, especially in Ras-driven cancers. Until recently, however, few *in vivo* studies had been carried out to test this premise. Several recent mouse model studies have verified that Rac1, RhoA, and some of their effector proteins such as PAK and ROCK, are likely anti-cancer targets for treating K-Ras-driven tumors. Other seemingly contradictory studies have suggested that at least in certain instances inhibition of individual Rho GTPases may paradoxically result in pro-neoplastic effects. Significantly, both RhoA GTPase gain- and loss-of-function mutations have been discovered in primary leukemia/lymphoma and gastric cancer by human cancer genome sequencing efforts, suggesting both pro- and anti-neoplastic roles. In this review we summarize and integrate these unexpected findings and discuss the mechanistic implications in the design and application of Rho GTPase targeting strategies in future cancer therapies.

Keywords

Rho GTPases; tumorigenesis; tumor suppression; anti-neoplasia target; rational drug design

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Conflict of Interest

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Introduction

Rho GTPases are a family of signaling proteins that belong to the Ras GTPase superfamily.[1] Mammalian Rho GTPases include 22 members,[2] of which the most-studied are RhoA, Rac1, and Cdc42. These proteins relay intracellular signals by acting as tightly regulated molecular switches (Figure 1a).[3] The GTPase-activating proteins (GAPs) facilitate the slow intrinsic GTP-hydrolysis reaction to turn off signaling, while the guanine nucleotide exchange factors (GEFs) catalyze the GTP-loading reaction to turn on Rho GTPase signaling. An additional level of regulation is provided by Rho GDP dissociation inhibitors (GDIs) which bind to the GDP-bound Rho GTPases, inhibiting GDP dissociation and sequestering them away from the active intracellular membrane sites. RhoGDIs can also prevent degradation of prenylated Rho GTPases when in the inactive state.[4] This dynamic cycle of GTP-loading/GTP-hydrolysis is essential for proper Rho GTPase signaling function. Alterations of this process may result in changes in Rho GTPase regulated cell functions including cell morphogenesis, adhesion, migration, cytokinesis, polarity, proliferation, and survival (Figure 1b).[5]

RhoA, Rac1, and Cdc42 are thought to be positively involved in cancer cell growth and potential anti-cancer targets in tumor initiation and metastasis.[6, 7] Evidence that they are pro-neoplastic emerged over 20 years ago with studies showing that constitutively active RhoA and Rac1 mutants possess weak transforming activity.[8–10] Dominant negative forms of Rho GTPases could block K-Ras or other oncogene-driven transformation of fibroblasts, suggesting an interconnection between Rho and Ras signaling pathways. Additional evidence for Rho and Ras crosstalk includes that RhoA or Rac1 could transform fibroblasts synergistically with oncogenic Raf[8] and a shared connection between RhoA and Ras with serum response factors (SRFs)[11] that bind to serum response elements and induce the expression of genes important for cell cycle progression, growth, division, and differentiation. A number of Ras effector pathways had been found to lead to Rho GTPase activation.[12, 13] For example, Ras binds to and activates T-lymphoma invasion and metastasis-inducing protein 1 (TIAM1), a GEF for Rac1 that may activate Rac1 to contribute to Ras transformation.[14] PI3Ks, one of the main effector pathways of Ras, can also activate Rac1 via the PIP_3-dependent Rac Exchanger (P-Rex) family of Rac GEFs.[15]

Although tremendous efforts have been dedicated to further implicating individual Rho GTPases in subsequent studies,[16–20] limited information from primary human cancers or mouse cancer model studies was available until recently. Several key findings came to light in the past several years yielding more complicated implications related to the role of individual Rho GTPases in several cancer types. In parallel, small molecule targeting of Rho GTPases has progressed in preclinical studies, making available lead chemical probes that have translational values. It is thus timely to review recent findings of Rho GTPase signaling in cancer biology, and refresh the implications in rational targeting of Rho pathways in neoplasia.

Rho GTPases as pro-oncogenic signal transducers

Rho GTPases and signaling molecules that activate their activity – such as GEFs – have traditionally been considered as oncogenic, and therefore potential anti-neoplastic

targets.[21–23] Indeed, in the past two decades, numerous studies have demonstrated the roles of multiple Rho GTPases in tumorigenesis. Much of the earlier work used loss- or gain-of-function mutant Rho GTPase overexpression in cancer cell or fibroblast cell lines *in vitro*, and the results supported the pro-proliferation and pro-metastasis functions of RhoA, Rac1, and Cdc42. In agreement with this, overexpression or hyper-activation of many Rho GTPases have been implicated in various human cancers and have been extensively reviewed elsewhere.[24, 25] Recent cancer genetic studies by whole-genome sequencing have identified a growing list of recurrent mutations in Rho GTPases (reviewed in Ref.[25, 26]), which were previously thought to be rare. Further, an intimate involvement of Rho GTPases in modulating multiple cell types in the tumor microenvironment, which have active roles in angiogenesis, chemotaxis, and inflammatory responses, also strongly implicates their pro-neoplastic roles.[7, 27]

Adding to the understanding of Rho GTPase signaling pathways and functions characterized by biochemical and cell biological approaches, *in vivo* cell-type specific and cancer-type specific functions of mammalian Rho GTPases have been delineated recently by murine conditional-knockout genetic models,[28–30] including in Ras-driven cancer models (Table 1). These mouse genetic models and human cancer genetic findings of “hot-spot” mutations of Rho GTPases further invigorate the interests.

RhoA

A previous Drosophila study by a forward genetic screen had identified genes that cooperate with oncogenic Ras to drive eye hyperplasia/tumorigenesis.[31] Interestingly, many positive hits in the screen belonged to the Rho GTPase pathway including Rho1, Rac1 and RhoGEF2, which all enhanced Ras-driven tumorigenesis in a JNK-dependent manner. The positive influence of RhoA in cancer was strengthened by two recent reports exploring therapeutic targets in K-Ras-driven murine models of lung cancer. The first report found that K-Ras-mutant cancer cells, but not K-Ras wild-type cancer cells, were vulnerable to perturbations in a GATA2 regulated set of signaling pathways including Rho signaling, IL-1/NF-κB signaling, and Nrf1/proteasome function.[32] Using a mutant K-Ras non-small cell lung cancer (NSCLC) mouse model, the authors further showed that the adenomas could be induced to regress by combination treatment with proteasome and Rho signaling inhibitors. In the second paper, study of a lung adenocarcinoma model in mutant K-Ras; Cdkn2a-null mice found that RhoA signaling was upregulated in adenocarcinomas and downstream FAK (focal adhesion kinase) was a key for malignant phenotypes in both lung adenocarcinoma cell lines as well as in a mouse model.[33] Additionally, studies of RhoA contribution to gastric cancer implicate RhoA activity as a permissive signal for G1-S-transition of the cell cycle progression through INK4 family members.[34]

Recent human cancer genetic studies utilizing whole genome sequencing have found recurrent RhoA mutations in gastric cancer (GC),[35–38] peripheral T cell lymphoma (PTCL),[39–42] adult T-cell leukemia/lymphoma (ATLL),[43] Burkitt Lymphoma (BL),[44] and head and neck cancer.[45] Some of them appear to be gain-of-function while a significant portion are loss-of-function, and their functional significance has yet to be causally defined. Consistent with a pro-cancer progression role, duplication of exons in ROCK, a RhoA

effector, presumably resulting in a gain-of-function, has also been described in lung adenocarcinoma.[46]

RhoC

RhoC has long been associated with cancer cell invasion and metastasis.[18] The first mouse model to address the role of RhoC in metastasis *in vivo* was that of Hakem and colleagues where they produced a constitutively RhoC-null mouse that, surprisingly, showed no abnormal phenotype at its basal state.[47] To assess the effect of RhoC on metastasis, the authors used the MMTV-PyVT transgenic mouse [Mouse Mammary Tumor Virus (MMTV) driven Polyoma Virus middle T antigen (PyVT)], which developed mammary tumors that metastasized to the lung with high penetrance. In this genetic background, RhoC loss led to dramatically fewer metastases to the lung, and the resulting mammary tumor cells showed reduced invasion activity *in vitro*. This evidence correlates with clinical observations of elevated RhoC levels associated with metastatic grade in human breast and gastric cancers.[48, 49]

Rac1 and Cdc42

Both Rac1 and Cdc42 were shown to be pro-transformation in early fibroblast studies. Rac1 conditional knockout mouse studies have shown that Rac1 is necessary for K-Ras-driven lung adenoma formation,[50] epidermal papilloma initiation and growth,[51] and DMBA/TPA-induced skin tumor formation.[52] In a K-Ras-driven pancreatic ductal adenocarcinoma (PDA) mouse model, pancreas-specific disruption of Rac1 or p110α, but not p110β, prevented the development of pancreatic tumors, and the loss of transformation was independent of AKT regulation.[53] In a retroviral expression model of MLL-AF9 induced leukemogenesis, Rac2, but not Rac1, is critical to the initiation of acute myeloid leukemia. However, loss of either Rac1 or Rac2 is sufficient to impair survival and growth of the transformed MLL-AF9 leukemia.[54] Induced deletion of Rac1 in endothelial cells suggested that Rac1 is required for embryonic vascular integrity and angiogenesis, representing potential anti-angiogenetic therapeutic targets for cancer.[55] In a mouse model for benign, more differentiated sebaceous skin tumors, epidermis-specific Rac1 activity did not alter tumor incidence and frequency, but suppressed tumor cell differentiation leading to malignant progression of sebaceous tumors.[56] Likewise, conditional deletion of Cdc42 in Ras-transformed fibroblast cells drastically alters cell morphology and inhibits proliferation, cell-cycle progression and tumorigenicity.[57] In a mouse colorectal cancer model, Cdc42 ablation suppressed the malignant progression of early-stage intestinal epithelial cancer cells carrying single APC or beta-catenin mutations.[58]

Rac1 and Cdc42 protein levels have been shown to be upregulated in multiple human cancers. Rac1b, the splice variant of Rac1 that contains a 19 amino acid insert adjacent to the switch II domain resulting in increased signaling and ROS generation, has emerged as a variant that is upregulated in several cancers such as lung, breast, colon and thyroid cancers.[48, 59–62] Also, Rac1b may cooperate with B-Raf oncogenic mutant V600E in promoting tumorigenesis.[61, 63] In a mouse model of K-Ras induced lung adenocarcinoma, expression of Rac1b synergized with oncogenic K-Ras and caused increased proliferation and accelerated tumor growth.[64]

Recent cancer genome sequencing efforts have revealed functionally relevant Rac1 gain-of-function mutations. Two studies utilizing whole genome sequencing of over 120 melanoma samples found a frequent and previously undescribed Rac1 mutation: 5% of melanomas[65] and 9.2% of sun-exposed melanomas[66] contain a recurrent $Rac1^{P29S}$ mutation that increases its GTP-bound active state and binding to effector proteins with enhanced signaling. A similar mutation, P29L, was found in Rac2.[65] Additional activating Rac1 mutations A159V and Q61R were later found in head and neck cancer and prostate cancer, respectively.[67]

GEFs and Effectors

Historically, the positive Rho GTPase regulators, GEFs, and Rho effectors are considered pro-oncogenic and pro-growth, whereas negative regulators such as Rho GAPs are considered tumor suppressing. For instance, the Rac GEF TIAM1 is also a potential effector for H- and K-Ras, and both its upregulation and deletion have been implicated in tumor initiation and metastasis, respectively (reviewed in Ref.[22]). Another Rac GEF, PREX2, is found to be mutated in human melanoma,[68] pancreatic cancer,[69] and colorectal cancer.[70] Certain PREX2 mutations, when expressed in murine melanoma models, increased Rac1 activity, PI3K-AKT pathway signaling and tumorigenesis, along with shortened survival life of the mice.[71] Vav1, a normally hematopoietic cell-specific RhoGEF, has been identified to be mutated in ATLL[72] and lung adenocarcinoma,[73] and found to be involved in novel fusions in PTCLs.[74] An analysis of the genome data set by Kakiuchi *et al.*[35] using CHASM[75] identified a possible driver mutation in ArfGEF1 in gastric cancer.[76] Mutations in DOCK2 and DOCK3, which belong to a different family of Rho GEFs from the Dbl-like molecules, have also been identified recently in colorectal cancer.[70] Interestingly, certain functions of RhoGEFs in tumors could be independent of their nucleotide exchange activity. For example, P-Rex2a can act as a component of the PI3K pathway by interacting with PTEN to suppress its lipid phosphatase activity in tumor cells.[77] Rho GEF H1, a RhoA GEF, is a critical part of a positive feedback loop in the Ras pathway via direct interaction with scaffolding protein Kinase suppressor of Ras 1 (KSR-1).[78]

With regards to effector proteins, abrogation of ROCK signaling has been shown to have a mortality benefit in murine models of leukemia, hepatocellular carcinoma, as well as breast and lung carcinomas.[79–81] Deletion of both ROCK isoforms, Rock1 and Rock2, but not individually, blocked tumor formation in mouse models of non-small cell lung cancer and melanoma, suggesting indispensable yet redundant roles for ROCK1/2 in cell cycle progression and tumorigenesis.[82] ROCK inhibitors may have high potential for treating cancer and other physiological conditions, and they are being investigated in clinical trials for human diseases including solid tumors.[83] While multiple ROCK inhibitors target the ATP binding pocket, classic inhibitors such as fasudil and Y27632 are generally not ROCK isoform selective and also interfere with other AGC kinases such as protein kinase N (PKN) kinases (also known as PRKs), another effector of Rho, with slightly lower potency.[84] Considerable efforts are being devoted to developing new ROCK inhibitors with higher potency and selectivity.[81] Studies of other downstream proteins such as RhoA effector formins have yielded positive results with regards to inhibiting cancer cell motility and progression.[85] Both Rac1 and Cdc42 directly bind to and activate p110β, a subunit of PI3K, via the Rho Binding Domain of p110β.[86, 87] The Rac1/Cdc42 effector PAK family kinases

(p21-activated kinase), known to phosphorylate important cell signaling proteins such as Bcl-2, MEK, and Raf1 and is a part of the MAPK, JNK and NF-κB pathways, have been found to be upregulated in human cancers.[88] Deletion of PAK1 led to a dramatically reduced tumorigenesis and tumor progression in a K-Ras-induced skin cancer model,[89] and inhibition of PAK1 attenuated tumor growth and metastasis in a model of pancreatic adenocarcinoma.[90] These crucial effector pathways of Rho GTPases appear consistently involved in pro-oncogenic signaling.

Rho GTPases with potential tumor suppressing roles

While most Rho GTPases, especially RhoA, Rac1, and Cdc42, along with their signaling components, have been considered pro-neoplastic (exceptions include RhoB,[91] RhoE,[92] and RhoH[93]), recent mouse model and human genomic data has emerged to suggest that RhoA, Rac1, and Cdc42 can also act in a tumor suppressing role under defined conditions. This new information (Table 1; Figure 2) raises the question whether individual Rho GTPases are pro- or anti-neoplastic in a given tumor, and sheds new light into therapeutic targeting strategies of Rho pathways.

RhoA

A recent study of K-Ras-induced hepatic adenoma formation in Zebra fish found that constitutively active RhoA reduced adenoma size and increased animal survival, while dominant negative RhoA resulted in larger adenomas and decreased survival.[94] These observations appear opposite of results from *in vitro* studies of K-Ras transformed fibroblasts by dominant RhoA mutant expression. The authors found that increased neoplasia resulting from dominant negative RhoA was in part due to increased AKT and S6 signaling and upregulation of cyclin D1. This finding is in line with two *in vitro* studies which found RhoA negatively regulated AKT phosphorylation and decreased cyclin D1 levels in endothelial cells and K-Ras-driven adrenocortical cancer cell lines.[95, 96] Another recent study of a murine colon cancer model induced by mutant APC found that simultaneous expression of dominant negative RhoA resulted in larger and more frequent adenomas and decreased survival.[97] Perhaps more intriguing, conditional gene deletion of either RhoA or RhoC alone did not suppress K-Ras^{G12D} induced lung adenoma initiation. Rather, deletion of RhoA alone exacerbated lung adenoma formation, whereas dual deletion of both RhoA and RhoC significantly reduced K-Ras^{G12D} induced adenoma formation.[98] In this context, deletion of RhoA seems to induce a compensatory mechanism that exacerbates adenoma formation, which is at least partly mediated by RhoC.

The strongest evidence that RhoA may have a tumor suppressor role has come to light in human cancer genomic studies (Figure 2) (commented in Ref.[99–103]). A compelling finding is the recent whole exome sequencing of T cell lymphoma in several studies which found that 50.3% – 70.8% of angioimmunoblastic T cell lymphoma (AITL) and 7.7% – 18% of PTCL, not otherwise specified (PTCL-NOS), share a recurrent RhoA^{G17V} mutation. RhoA^{G17V} causes a loss of nucleotide binding, enhanced GEF interaction, and may act as a dominant negative.[39–42] Another study of Burkitt lymphoma in children found recurrent RhoA mutations such as RhoA^{R5Q} which appear to be loss-of-function and predominately

disrupt RhoA interactions with GEFs.[44] Further characterization of RhoA^{R5Q} mutation confirmed its impaired activity using biochemical and cell functional assays.[104] Most recently, a study of adult T-cell leukemia and lymphomas (ATLL) found that ~15% of ATLLs have several recurrent RhoA mutations in the GTP binding pocket, some of which were previously undescribed.[43] Interestingly, some of the recurrent mutations are gain-of-function mutations, while others are loss-of-function or even dominant-negative mutations. These genetic data indicate that both gain- and loss-of-function RhoA mutations may be pro-oncogenic depending on the cell of origin of the ATLL, such that gain-of-function RhoA mutations are pro-oncogenic in Tregs, whereas loss-of-function mutations are pro-oncogenic in T memory cells.

Inactivating RhoA mutations have also been found in solid tumors. A large scale human cancer genetic study of paired normal and tumor tissues across multiple cancer types identified recurrent RhoA mutations at E40 and Y42 in seven tumors (six head and neck, one breast) that are likely to disrupt the interaction of RhoA with effectors.[45] Similar mutations thought to abrogate or modulate RhoA effector interactions have been recently described in gastric cancer.[35–38] RhoA mutation prevalence was estimated at 14.3% – 25.3% in diffuse-type gastric cancer and 3.9% – 5.4% in the whole cohort. RhoA mutations were noted in hotspot sites including Y42, G17, L57, and R5 (Figure 2). SiRNA-knocking down of RhoA in gastric cell lines containing mutant RhoA in Y42 or G17, but not wild-type RhoA, significantly impairs proliferation.[35] Further rescue experiments in cells suggested that Y42C and G17E are gain-of-function mutations that may provide a strong growth advantage.[35] However, biochemical analysis showed that Y42C and L57V are reduced in the active form, suggesting they work in a loss-of-function manner.[36] Indeed, RhoA^{Y42C} has been evaluated in earlier biochemical assays and shown attenuated activation of PKN, but not mDia2 and ROCK1.[105] Most recently, by applying an unsupervised method, ParsSNP, to the gastric cancer genome data set from Kakiuchi *et al.*,[35] Kumar *et al.* confirmed that RhoA^{Y42C} may be a driver mutation.[76] Recent studies of $G\alpha_{13}$-RhoA signaling axis with a tumor suppressor role in Burkitt's lymphoma and diffuse large B-cell lymphoma have begun to causally associate the loss of RhoA signaling with tumorigenesis.[92] It will be important to better define the mechanism of loss-of-function RhoA mutants, to contrast with gain-of-function mutants.

RhoB and other Rho family members

In contrast to findings suggestive of increased RhoA and/or RhoC expression or activity in many cancers, it has been long known that RhoB is deleted in multiple cancers, including lung cancer.[106–108] Although RhoA, B, and C can all regulate actin stress fibers, cytoskeleton organization, and vesicle transportation redundantly, RhoB differs from RhoA/C in cellular localization and has distinct functions. RhoB is primarily localized to endosomes and regulates cytokine trafficking and cell survival,[109] and has anti-proliferative and pro-apoptotic effects in cancer cells.[91] RhoB is unique in that it can be modified by either a farnesyl or a geranylgeranyl moity, and its prenylation state seems to affect RhoB actions. Whereas farnesylated RhoB can be either pro- or anti-growth in different settings, geranylgeranylated RhoB displays consistent anti-growth activity.[110] RhoB appears to act more than an inhibitory isoform that opposes the effects of RhoA and RhoC signaling.[110] In

agreement with its tumor suppressor role, RhoB deletion was shown to accelerate chemically induced skin tumors in mice.[111] In a recent study, RhoB deletion lowered the risk of UVB-induced skin carcinogenesis, but tumors that did form were preferentially undifferentiated and highly proliferative, suggesting RhoB may promote skin cancer initiation but limits the tumor aggressiveness.[112]

There is evidence that Cdc42, which is typically considered an oncoprotein, can also present a tumor suppressing function as exemplified by the finding that mice with Cdc42-deficiency in blood developed a lethal myeloproliferative disorder.[113] Likewise, mouse with Cdc42 ablated in hepatocytes and bile duct cells developed hepatomegaly soon after birth, and signs of transformation and hepatocellular carcinoma was observed later.[114] Intestinal deletion of Cdc42 resulted in a hyperplasia of intestinal epithelial cells and drastically increased intestine length and thickness.[115] Recently, Rac1 and Cdc42 activities were found to be decreased in human pheochromocytomas, possibly resulting from reduced expression level of two RhoGEFs, ARHGEF1 and FARP1.[116]

GAPs and Other Rho GTPase regulators

Since Rho GAPs act to decrease Rho-GTP species, they are generally considered tumor suppressors by virtual of their capability to downregulate Rho/Rac/Cdc42 activities.[117] For instance, DLC-1 gene, the product of which is a Rac GAP, so named because it is often deleted in liver cancer, is either deleted or methylated in a wide variety of cancers.[118, 119] However, another GAP that regulates RhoA activity, p190GAP, has been found to have upregulated in expression in inflammatory breast cells, and may have a pro-growth role.[120] P190B heterozygous mouse showed reduced tumor penetrance and remarkably delayed tumor onset in MMTV-Neu breast cancer model.[121] Similarly, expression of several RhoGAP genes was increased in basal-like breast cancer (BLBC), and knockdown of two of them, ArhGAP11A and RacGAP1, resulted in significant defects in the proliferations of BLBC cells.[122] In the same study using CHASM[75] to analyze the data set from Kakiuchi *et al.,*[35] a potential driver mutation in one RhoGAP, ArhGAP28, was identified.[76] While the role of individual RhoGAP in specific cancer awaits further characterization, their apparent pro- and anti-cancer cell proliferative functions may be associated with the paradoxical role of their respective substrates, Rho GTPases. Alternatively, RhoGAPs may be associated with the cycling regulation of Rho GTPases. Overexpression of RhoGAPs may allow proper cycling of Rho GTPase substrates under conditions of exacerbated Rho-dependent signaling in cancer cells.

Changes of RhoGDIs expression levels have also been associated with cancer.[123] The changes vary by cancer types (reviewed in Ref.[124]). For example, RhoGDI1 expression is upregulated in colorectal and ovarian cancers,[125–127] but downregulated in brain cancers.[128] Increased expression of RhoGDI2 has been found in pancreatic cancers,[129, 130] while the opposite occurs in bladder cancers.[131] In breast cancer, conflicting results have been found for RhoGDI1 expression,[48, 132] whereas GDI2 seemed to have a biphasic expression pattern.[133] Genetic studies of specific RhoGDI in murine cancer models have been lacking and the full degree of complexity of RhoGDI function and regulation in cancer remains to be appreciated.

Balancing the pro- and anti-neoplastic roles of Rho GTPases in developing targeted therapy

An outstanding question is how to reconcile the pro- and anti-neoplastic effects attributable to individual Rho GTPases. While the majority of *in vitro* studies examining the role of RhoA, Cdc42 and Rac1 support their pro-neoplastic function, it is within the mouse *in vivo* and human genetic findings where the opposite effects arise. How can we reconcile such seemingly paradoxical findings? This may be partly explained by the difference between *in vitro* and *in vivo* experimental systems. First, *in vitro* studies may be biased due to extensive culture resulting in clonal variability of cell lines. The cell lines are well adapted towards rapid growth with reliance on key signaling pathways including Rho GTPases, and could be hypersensitive to perturbations of key signal transduction pathways. In contrast, malignant cells in an *in vivo* environment may be more resilient to cell signaling perturbations and possess a malleable signaling network more plastic for adaptive compensations. Second, Rho GTPases may also affect the tumor microenvironment *in vivo*, which is missing *in vitro*, to either favor or antagonize tumor growth in a cell-type specific manner. Third, manipulating the level of one Rho GTPase in cell lines may affect the level and activity of other endogenous Rho GTPases, thereby, some of the conclusions from *in vitro* studies need to be cautioned. It is increasingly clear that our current understanding of Rho signaling in tumorigenicity is still incomplete.

Analogy to other targeted approaches in compensatory response

In a recent study, RhoA was found to be pro-neoplastic in the absence of RhoC, but anti-neoplastic in the presence of RhoC in murine lung adenoma model.[98] This seemingly paradoxical observation shares an analogy with other targeted therapy situations. A prominent example is the adverse effects of B-Raf inhibitors.[134] Despite the well appreciated pro-neoplastic role of BRAF-activating mutations in metastatic melanoma, clinical trials of the B-Raf inhibitors, vemurafenib and dabrafenib, yielded unexpected results that B-Raf targeting can mediate the related C-Raf activation leading to a relapse of more malignant tumors.[135, 136] This compensatory interplay of B-Raf and C-Raf parallels that of Rho GTPase crosstalk, including the roles of RhoA and RhoC, despite that they may be mediated by distinct mechanisms (Figure 3). Such a compensatory response is not surprising in the context of cancer cell signaling network. In a recently report, inhibition of MEK in K-Ras-mutant lung and pancreatic cancer cells provokes a signaling rebound via FGFR1. Combinational inhibitors of MEK and FGFR1 enhance tumor death *in vitro* and *in vivo*.[137] The compensatory response may reflect another way that Rho family members can contribute to the development of resistance to targeted therapies: the level or activity of one Rho protein can affect the level and/or activity of others.

Rational targeting of Rho GTPase signaling

Due to their globular structure, small GTPases such as Rho and Ras have been deemed “undruggable” by traditional drug design approaches. However, there have been advances in the field over the past decade with pre-clinical and clinical progress of several targeting strategies of Rho GTPase pathways. One strategy is targeting the interaction of Rho GTPase

with its GEFs, which has been achieved at the preclinical level in the three prototypical Rho GTPases, i.e. Rac1, RhoA, and Cdc42. For example, NSC23766 is an inhibitor which disrupts the interaction of Rac1 with GEFs such as TIAM1, and CASIN is a Cdc42 inhibitor which disrupts the Rho:GEF interaction.[138, 139] In fact, Rac1 inhibitors are especially desirable in the treatment of many melanomas, as common $Rac1^{P29S}$ mutation in melanoma has been shown to confer resistance to B-Raf inhibitors for the treatment of B-Raf driven metastatic melanomas.[140] The chemical probe Rhosin (also termed G04) was developed to bind between the two important "switch" regions of RhoA and inhibit its interaction with GEFs such as leukemia-associated RhoGEF (LARG). Not only does this probe decrease phosphorylation of myosin and have antineoplastic effects *in vitro*, but importantly it inhibits signaling of RhoC as well.[141, 142] The alternate approach of targeting RhoGEFs rather than Rho GTPase itself has also been useful conceptually for inhibiting Rho activities. For example, the chemical probe Y16 binds to LARG and inhibits Rho GEF activity.[141, 143]

The other major target for disrupting GTPase activity is the activity of downstream effectors. With regard to Rho GTPases, a successful example is the ROCK inhibitors fasudil and Y-27632.[144, 145] Both inhibitors bind the ATP-binding pocket of ROCK and inhibit serine-threonine kinase activity. Fasudil is the only clinically used Rho GTPase pathway inhibitor and is used to treat pulmonary hypertension and cerebral hypertension in Japan, along with a sister compound, ripasudil, being used to treat glaucoma in Japan. Other GTPase effector inhibitors include several developed against the Cdc42 and Rac effector, PAK.[146] Another notable lead inhibitor, Phox-I, inhibits the Rac effector $p67^{phox}$ of the NOX2 enzyme complex which produces Rac-mediated superoxide.[147] Extensive reviews can be found elsewhere describing the development of Rho GTPase inhibitors and strategies to inhibit GTPase signaling including targeting GDIs or post-transcriptional modifications.[24, 148, 149]

Despite the important roles of Rho GTPases in cancer and considerable efforts to target Rho-dependent pathways, no inhibitor of Rho GTPase signaling has yet been used clinically to treat cancer, and the number of clinical trials is still limited. Besides the technical difficulty to target small GTPases *per se*, one major challenge is the lack of knowledge about the actual roles of Rho GTPase-dependent pathways at the organismal level, which is reflected by the recent *human cancer genetic studies*. It will be important to carry out further analysis of the signaling network changes of the gain- and loss-function mutation bearing tumors, particularly the downstream pathways that may better explain possible compensatory effects and selection pressure of the cancer cells under the driver mutations and defined microenvironment. Such comprehensive characterization of Rho GTPase signaling activities will guide the rationale in pharmacological targeting of Rho GTPases for anti-cancer therapy development.

In addition to modulate cancer cell proliferation, survival and migration, Rho GTPase pathways play a role in the resistance of anti-cancer radiotherapy and chemotherapy. For example, the RhoA/ROCK pathway was implicated in cancer cell stemness and radioresistance.[150] Overexpression of RhoGDI1 increased resistance of cancer cells to the induction of apoptosis by chemotherapeutic agents etoposide and doxorubicin,[151] and RhoGDI2 was identified as a key player in resistance to a cyclin-dependent kinases inhibitor.[152] Thus, combined inhibition of Rho signaling with other anti-cancer therapy may

be useful to achieve greater efficacy while reducing potential resistance. To this end, ROCK inhibitor fasudil and MEK inhibitor trametinib cooperatively induced apoptosis in N-Ras mutant melanoma.[153] Using a synthetic lethal drug screen to identify innovative drug combinations to treat K-Ras mutant cancers, Wang *et al*. recently showed that dual inhibition of Rho signaling components polo-like kinase 1 (PLK1) and ROCK leads to synergistic effects to induce apoptosis and cell-cycle arrest *in vitro* and causing potent tumor regressions *in vivo*.[154]

Conclusions and Perspectives

Rho GTPase signaling is important in Ras pathways and other cancer-driven mechanisms. Multiple Rho GTPases haven been found to be potential anti-neoplastic targets in a wide variety of cancers including colon cancer, breast cancer, and leukemia.[7, 155, 156] Fasudil, a ROCK inhibitor, has been used to treat pulmonary hypertension and is now being researched for the treatment of refractory angina.[157–159] Recent studies show that Rho GTPases, RhoA and Cdc42 in particular, may also behave as “tumor suppressors” in certain cancer and defined circumstances. Such a complex, and sometimes paradoxical, interpretation of Rho GTPase functions also applies to their regulators and effectors, and redundant functions between Rho family homologs and interplays of feedback signaling loops may be involved. Dynamic selection pressure upon loss or gain of a Rho GTPase function is likely a contributing factor in the specific tumor context, as evidenced in T cell leukemia and lymphoma.[43] Loss- and gain-of-function mutations of RhoA may endow the tumor cells selective advantage in early vs. late stages of the cancer progression, respectively. Further stringent demonstrations of their causal role and unveiling the underlying mechanism in driving tumorigenesis or tumor suppression in specific cancer types are warranted.

Considering recent findings, development of novel approaches inhibiting individual Rho GTPase activities need a more careful consideration. The timing and balance of downstream signals and possible compensatory feedback mechanisms after effective inhibition of one Rho GTPase must be assessed in order to ensure a beneficial tumor suppressing outcome. For example, the possible redundant and compensatory signaling from RhoC should be considered upon specific targeting of RhoA in tumors from the onset. These considerations suggest that the use of relatively more “promiscuous” drugs that inhibit multiple Rho GTPases such as RhoA/RhoC or further downstream signaling, such as ROCK or PAK, with acceptable toxicity, may provide better efficacies and also be beneficial for reducing potential resistance to the therapy against a single Rho GTPase target. In congruence with this idea, agents such as phytochemical Rocaglamide that inhibits the activities of Rho, Cdc42, and Rac[160] may represent a new class of anticancer drugs. Although inhibitors of Rho GTPase pathways have not been clinically used for cancer treatment, rational targeting of Rho GTPase signaling nevertheless carries significant potentials in anti-cancer drug discovery, especially in future combinatory therapies.

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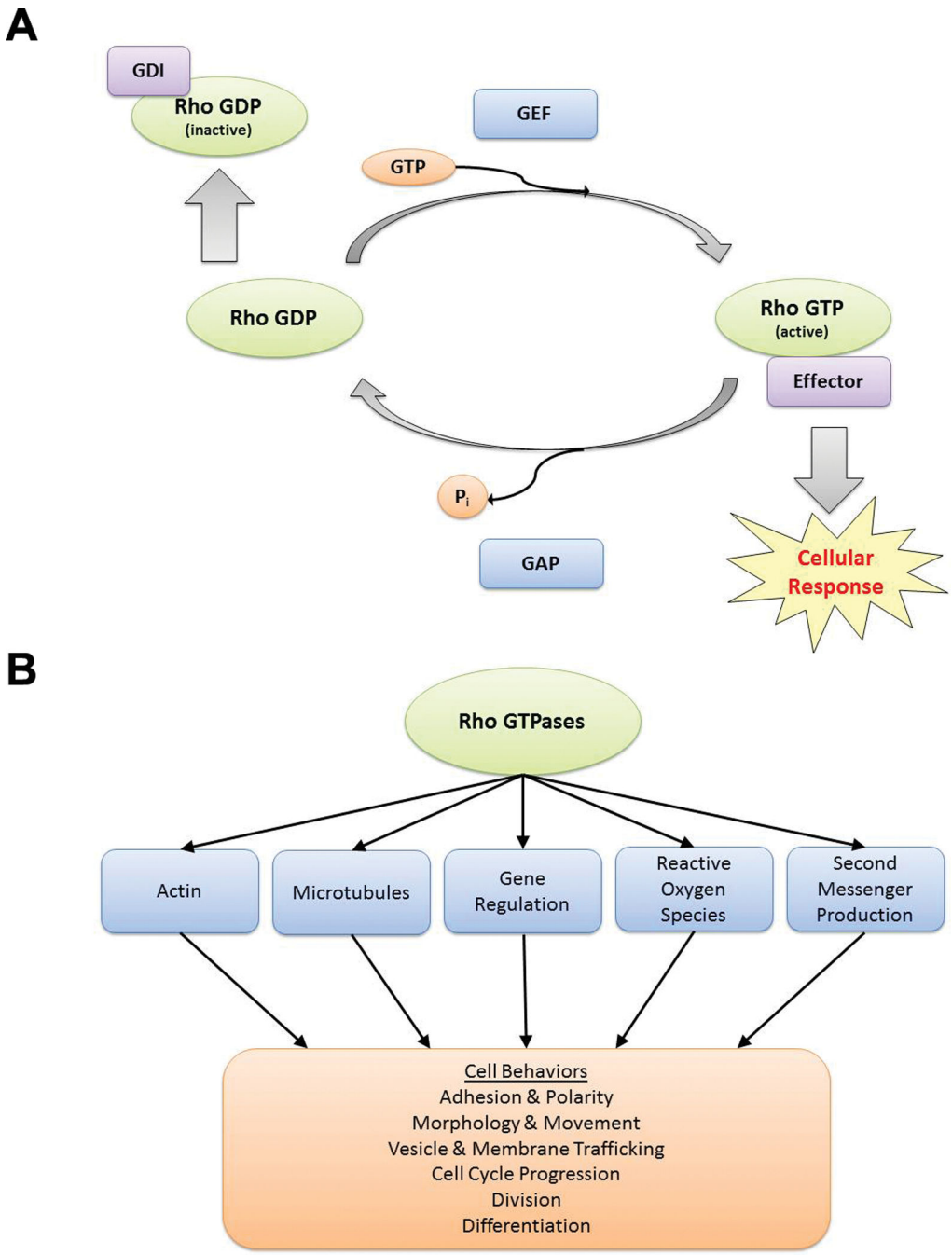

Figure 1.

The GTP-binding and GTP-hydrolysis cycle and signaling functions of Rho GTPases in cells. (**a**) The biochemical model shows the signal regulation of Rho GTPases by GEFs, GAPs, and GDIs cycling in the GTP-bound active, and GDP-bound inactive, states. (**b**) Major cellular processes directly affected by Rho GTPases and the cell behaviors subsequently affected by those processes are depicted.

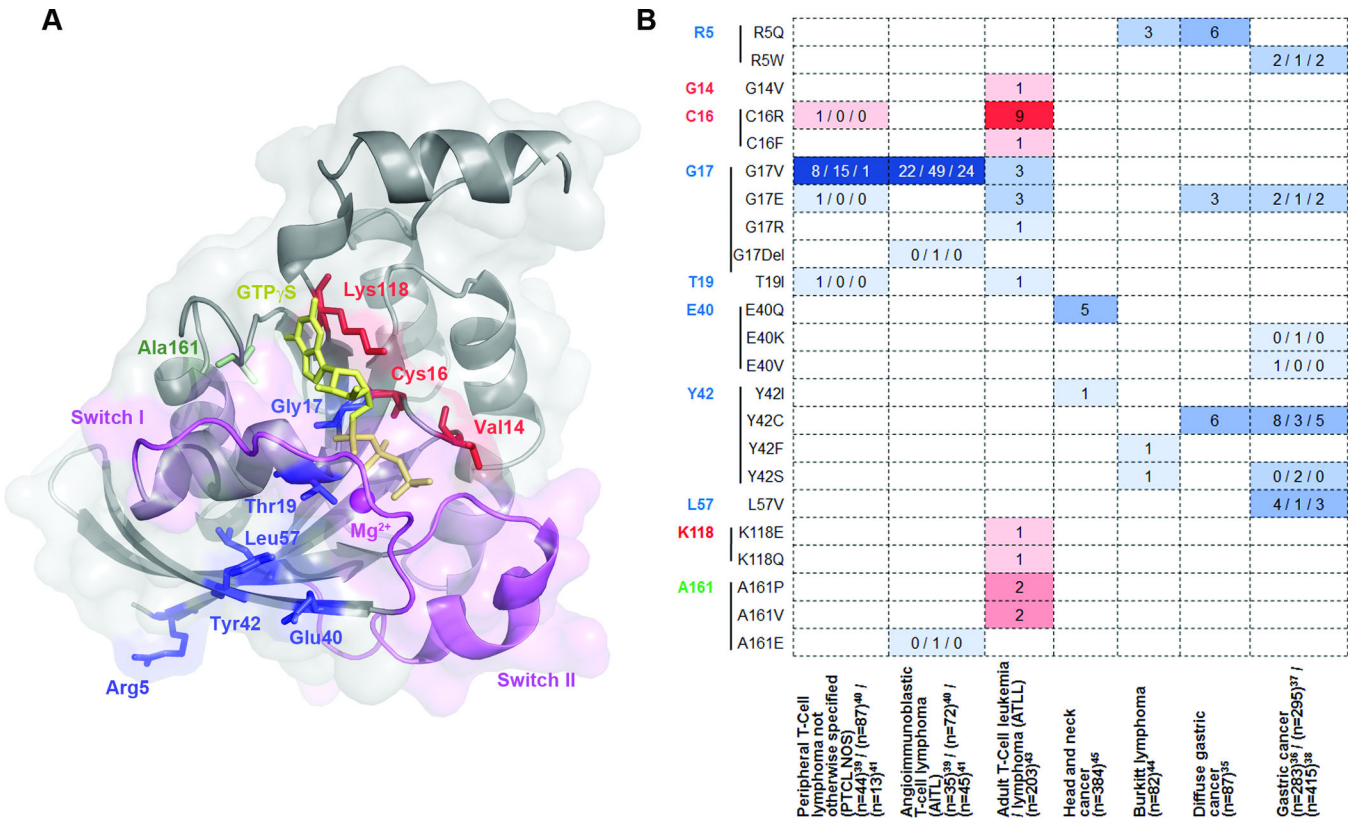

Figure 2.

Recurrent RhoA mutations found in human cancers. (**a**) Most commonly mutated amino acid positions identified in recent reports are mapped to the 3D structure of activated RhoA[161] (PDB#: 1A2B). GTPγS is shown as yellow sticks and the magnesium ion is shown as magenta sphere. Sites for gain-of-function mutations G14 (shown as V14, mutated in the original structure), C16, and K118 are shown as red sticks; sites for loss-of-function mutations R5, G17, T19, E40, Y42, and L57 are shown as blue sticks; while A161 is shown as green sticks as both gain-of-function mutations (A161P and A161V) and lost-of-function mutations (A161E) have been identified. (**b**) The occurrence of the hot-spot mutations are listed across tumor types. Hotspots are ordered by amino acid position and colored in the same scheme as in (**a**). Gain-of-function and loss-of-function mutations are classified based on preliminary biochemical studies and need further characterization. In Palomero *et al.*[39], RhoA mutations other than G17V were identified in a single case each and the authors didn't specify the PTCL subtype. They are included in PTCL-NOS here for simplicity.

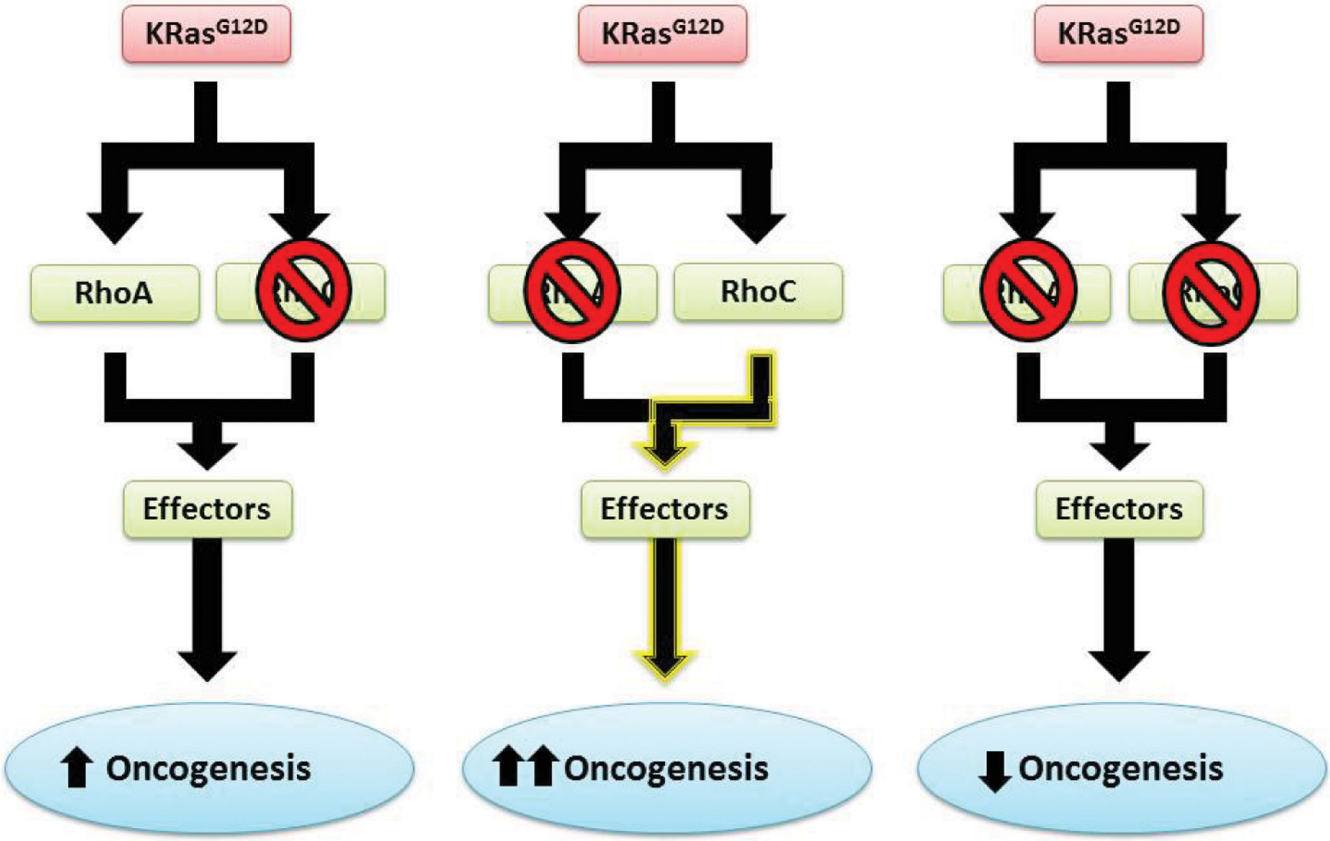

Figure 3.

A scheme of the interplay between RhoA and RhoC signaling in K-Ras-driven cancer. The model summarizes the possible effects of RhoA inhibition on K-Ras-driven tumor formation. RhoA loss can paradoxically result in increased oncogenesis through a compensatory elevated RhoC activity, endowing RhoA to behave in a tumor suppressing role. In the absence of RhoC, RhoA is required for oncogenesis, displaying pro-oncogenic signal as an antineoplastic drug target.

Table 1

Function of Rho GTPases in *in vivo* cancer models.

Rho GTPases	Pro- or anti-oncogenic	Model	Remarks	References (year)
RhoC	Pro-oncogenic	RhoC$^{-/-}$; pyV-MT mouse model of mammary tumor	Loss of RhoC did not affect tumor development but decreases tumor cell motility and metastatic cell survival leading to a drastic inhibition of metastasis	Hakem *et al.*[47] (2005)
Rac1	Pro-oncogenic	Rac1$^{flox/flox}$ and LSL-K-Ras^{G12D} mouse, lung infections with adenovirus expressing Cre (Ad-Cre)	Rac1 function is required for tumorigenesis in this oncogenic K-Ras-induced lung cancer model	Kissil *et al.*[50] (2007)
Rac1	Pro-oncogenic	Rac1$^{flox/flox}$; K5-Cre mouse, treated with DMBA/TPA to induce skin tumors	Rac1 is crucial for skin tumor formation and mice with keratinocyte-restricted deletion of Rac1 are resistant to skin tumor formation.	Wang *et al.*[52] (2010)
Rac1	Pro-oncogenic	LSL-K-Ras^{G12D}; K14-Cre:ER; Rac1$^{WT/-}$ mouse model of epidermal papilloma	Active Rac1 level was high in this model and genetic removal of one Rac1 allele significantly impaired K-Ras induced oral papilloma growth	Samuel *et al.*[51] (2011)
Rac1/Rac2	Pro-oncogenic	Transplant of Low density bone marrow from Rac1$^{flox/flox}$; Mx1-Cre or Rac2$^{-/-}$; Mx1-Cre mouse, transfected with retrovirus expressing MLL-AF9 to induce leukemia.	Rac2, but not Rac1, is critical to the initiation of acute myeloid leukemia in this model; however, loss of either Rac1 or Rac2 is sufficient to impair survival and growth of the transformed MLL-AF9 leukemia	Mizukawa *et al.*[54] (2011)
RhoA/Rac1	Pro-oncogenic	Oncogenic Ras driven eye hyperplasia /tumorigenesis in Drosophila (ey-GAL4; UAS-Ras85D^{V12})	Rho1, Rac1 and RhoGEF2 were identified to enhance oncogenic Ras driven tumorigenesis in a genome-wide screen	Brumby *et al.*[31] (2011)
RhoA	Pro-oncogenic	K-Ras$^{LA2\text{-}G12D}$ mouse model of non-small cell lung cancer (NSCLC)	Combined inhibition of the proteasome and ROCK robustly suppresses *K-Ras* mutant tumor growth	Kumar *et al.*[32] (2012)
Rac1b	Pro-oncogenic	LSL-K-Ras^{G12D}; Rosa26-LSL-Rac1b mouse model of lung cancer, lung infection with adenovirus expressing Cre (Ad-Cre)	Expression of Rac1b synergized with oncogenic K-Ras resulting in increased cellular proliferation and accelerated tumor growth.	Zhou *et al.*[64] (2013)
Rac1	Pro-oncogenic	Rac1$^{flox/flox}$; K-Ras$^{(LSL\text{-}G12D)/+}$; Ptf1a$^{cre/+}$ mouse model of pancreatic ductal adenocarcinoma (PDA) with pancreas-specific deletion of Rac1	Pancreas-specific deletion of Rac1 prevented the development of pancreatic tumors.	Wu *et al.*[53] (2014)
Cdc42	Pro-oncogenic	APC$^{min/+}$; Cdc42$^{flox/flox}$; Vil-Cre or Catnb$^{(ex3)/+}$; Cdc42$^{flox/flox}$; Vil-Cre mouse model of colorectal cancer with intestinal epithelial cell specific Cdc42 deletion	Reduction of Cdc42 alleviates the tumorigenicity of mutant intestinal cells carrying single APC or β-catenin mutations	Sakamori *et al.*[58] (2014)
RhoA	Anti-oncogenic	TO(K-Ras^{G12V}/RhoA), TO(K-Ras^{G12V}/RhoA^{T19N}), or TO(K-Ras^{G12V}/RhoA^{G14V}) zebra fish model of hepatocellular carcinoma	Liver enlargement and hepatocyte proliferation induced by tet-on-inducible, liver-specific expression K-Ras^{G12V} was augmented by dominant-	Chew *et al.*[94] (2014)

Rho GTPases	Pro- or anti-oncogenic	Model	Remarks	References (year)
			negative RhoA^{T19N}, but reduced by constitutive-active RhoA^{G14V}.	
RhoA	Anti-oncogenic	APC$^{min/+}$; RhoAT$^{19N/-}$; Vil-Cre$^{tg/-}$ mouse model of colorectal cancer with expression of dominant-negative RhoA^{T19N}	RhoA inactivation contributes to colorectal cancer progression/metastasis, largely through the activation of Wnt/β-catenin signaling	Rodrigues *et al.*[97] (2014)
RhoB	Anti-oncogenic	RhoB$^{-/-}$ mouse with cutaneous squamous cell carcinomas (SCC) induced with UVB	RhoB deletion lowered the incidence of SCC precursor tumors following chronic exposure to UVB.	Meyer *et al.*[112] (2014)
Rac1	Pro-oncogenic	K14-ΔNLef1; K14-Rac1^{Q61L} mouse model of sebaceous adenoma with epidermis-specific active Rac1	Active Rac1 did not change the incidence or frequency of tumors, but could suppress tumor cell differentiation and enable malignant progression of sebaceous tumors.	Frances *et al.*[56] (2015)
RhoA/RhoC	Anti-oncogenic	LSL-K-Ras^{G12D} with RhoA$^{flox/flox}$ or RhoC$^{-/-}$ or both, mouse models of lung carcinoma induced by lung infection of adenovirus expressing Cre (Ad-Cre)	Deletion of RhoA or RhoC alone did not suppress K-RasG12D induced lung adenoma initiation; rather, deletion of RhoA along accelerated lung adenoma formation.	Zandvakili *et al.*[98] (2015)