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Mutationally activated Rho GTPases in cancer

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The Rho family of GTPases (members of the Ras superfamily) are best known for their roles in regulating cytoskeletal dynamics. It is also well established that misregulation of Rho proteins contributes to tumorigenesis and metastasis. Unlike Ras proteins, which are frequently mutated in cancer (around 30%), Rho proteins themselves are generally not found to be mutated in cancer. Rather, misregulation of Rho activity in cancer was thought to occur by overexpression of these proteins or by misregulation of molecules that control Rho activity, such as activation or overexpression of GEFs and inactivation or loss of GAPs or GDIs. Recent studies, enabled by next-generation tumor exome sequencing, report activating point mutations in Rho GTPases as driver mutations in melanoma, as well as breast, and head and neck cancers. The Rac1(P29L) mutation identified in these tumor studies was previously identified by our lab as an activating Rac mutation in *C. elegans* neuronal development, highlighting the conserved nature of this mutation. Furthermore, this finding supports the relevance of studying Rho GTPases in model organisms such as *C. elegans* to study the mechanisms that underlie carcinogenesis. This review will describe the recent findings that report activating Rho mutations in various cancer types, moving Rho GTPases from molecules misregulated in cancer to mutagenic targets that drive tumorigenesis.

Normal Regulation and Biological Function of Small GTPases

The Ras (Rat sarcoma) superfamily of small GTPases are proteins that function to transmit intracellular signals initiated from extracellular stimuli. Under normal biological conditions, Ras small GTPases are involved in many divergent cellular functions including cytoskeletal reorganization, cell survival and proliferation, transformation, and vesicular trafficking.[1] Rho (Ras homologous) family GTPases are a major subgroup within the Ras superfamily of small GTPases. The Rho family members Rho, Rac, and Cdc-42 are best known for their ability to regulate the actin cytoskeleton, resulting in actin stress fibers, lamellipodial protrusions and filopodial protrusions, respectively.[2-4] Cdc-42 family members also play a role in controlling cell polarity.[5]

Small GTPases function as tightly-regulated molecular switches. When they are bound to GTP, they undergo a conformational change and can engage effectors to promote downstream signaling.[6] The intrinsic GTPase activity of these proteins hydrolyzes GTP to GDP, and when GDP-bound small GTPases cannot engage downstream effectors.[6] GTP/GDP guanine nucleotide exchange factors (GEFs) are positive modulators that facilitate the exchange of GDP for GTP, activating these small GTPases.[7] GTPase accelerating proteins (GAPs) stimulate the hydrolysis of GTP, leaving the GTPase GDP-bound and inactive.[7] In addition to GEFs and GAPs, Rho proteins are also regulated by guanine nucleotide dissociation inhibitors (GDIs). GDIs inhibit Rho proteins in 2 ways. They inhibit GDP nucleotide dissociation, and they also sequester Rho GTPases in the cytosol, rendering them inactive.[8]

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Misregulation of Small GTPases

As well as contributing to normal physiological and developmental processes, Rho GTPases have been found to contribute to pathological processes including cancer cell migration, invasion, metastasis, and inflammation.[9,10] Activating mutations in Ras proteins (such as K-Ras, N-Ras, and H-Ras) are found in 15–30% of human tumors.[11] Oncogenic mutations in Ras are mostly found at 2 hotspots, around codons 12 and 61. The frequency of mutations at these positions varies between the 3 main Ras family members. For instance, in K-Ras 99% of mutations occur at the tandem glycine 12-glycine 13 position (86% and 13%, respectively) and mutations at Q61 occur only 1% of the time. In contrast, for N-Ras about 60% of the mutations occur at Q61. Finally, in H-Ras 54% of mutations occur at G12, 34.5% of mutations occur at Q61, and 9% occur at G13.[11] Mutations that occur at the 61 codon most frequently include Q61K, Q61R, and Q61L. Mutations at the 12/13 position most frequently include G13D, G12D, G12S, G12A, G12R, and G12V (reviewed in[12]). However, until recently (with the notable exception RhoH[13,14]), it was generally thought that Rho proteins were only rarely mutated in human cancers. Rather, misregulation of Rho proteins in cancer was generally found to occur by either overexpression of the Rho GTPase itself or misregulation of GEFs, GAPs and/or GDIs (**Table 1**). There are multiple Rho proteins that are upregulated in several human tumor types including RhoA,[15,16] RhoC,[17] Rac1,[15,16] Rac2,[18] Rac3,[19] Cdc-42,[15,16] Wrch-1,[20] and RhoF.[21] For example, overexpression of Rac1 was detected in breast,[16] lung,[22] oral squamous cell[23] testicular,[24] and gastric carcinoma.[25] RhoA is overexpressed in breast,[16] colon,[16] lung,[16] gastric,[25] head and neck,[26] bladder,[27] and testicular carcinomas.[24,28] Furthermore, Cdc-42 is overexpressed in breast[15] and testicular cancer.[24]

Table 1. Misregulation of Rho proteins in cancer

Type of misregulation	Protein affected	Cancer type	References
Overexpression	Rac1	Breast	16
		Lung	22
		Oral squamous cell	23
		Testicular	24
		Gastric	24,25
	RhoA	Breast	16
		Colon	16
		Lung	16
		Gastric	25
		Head and neck	26
		Bladder	27
		Testicular	24,28
	Cdc-42	Breast	15
		Testicular	24
GEF activation	Tiam1	T-cell lymphoma	29
	MyoGEF	Breast	31
	P-Rex1	Prostate	32
GAP inactivation or underexpression	P190RhoGAP	Glioma	33
	DLC2	Hepatocellular	34
GDI alteration	RhoGDI1*	Various cancer types	36-39
	RhoGDI2*		
Mutational activation	Rac1(P29S)	Melanoma	41,42
		Head and neck	44
		Breast	11
	RHOT(P30L)	Melanoma	42
	RAC2(P29S)	Melanoma	42
	CDC-42(G12V)	Melanoma	42
	MIG-2(S75F)**	?	45

*The data on RhoGDIs are conflicting. **Vertebrates do not have a MIG-2-like GTPase, although MIG-2 may be functionally similar to RhoG. However, this mutation might affect a conserved site and may arise in other GTPases.[50] Rho GTPases are aberrantly regulated in cancer by various mechanisms including overexpression, activation or overexpression of GEFs, inactivation or underexpression of GAPs, and/or alterations in GDI. Recently, Rho proteins have also been found to be mutationally active in various cancer types.

Misregulation of Rho GTPases in cancer can also occur through aberrant changes in GEFs, GAPs, and/or GDIs. For example, Tiam1 (a Rac-specific GEF) was originally identified for its ability to promote T lymphoma invasion.[29] Tiam1 is also overexpressed in breast cancer.[30] A Rho GEF, MyoGEF, has been shown to activate RhoA and RhoC in invasive breast cancer.[31] P-Rex1, a Rac GEF, is upregulated in prostate adenocarcinoma and is also found in lymph node metastases.[32] p190RhoGAP, one of the best-studied Rho GAPs, is a tumor suppressor in gliomas.[33] Another GAP, DLC2 (deleted in liver cancer 2), is underexpressed in a significant number of hepatocellular cancers.[34] The expression of RhoGDIs is also altered in a significant number of cancer types, however, some of the data are conflicting. For example, in breast cancer RhoGDI1 expression is increased or decreased in different studies.[35,36] Furthermore, although changes in RHOGDI1 and RHOGDI2 expression levels have been associated with many cancer types, the results of these changes in expression are varied.[37-40]

Identification of Mutationally Active Rho GTPases

Recently, 2 studies identified mutations in Rho GTPases that likely function as driver mutations in melanoma.[41,42] These studies utilized high-throughput next generation sequencing of the exons (exome sequencing) of 147[41] and 121[42] melanomas and healthy tissue-matched controls. Mutations appearing in multiple melanomas were identified, known as recurrent mutations. Statistical methods comparing the non-synonymous to synonymous mutation rate and loss of heterozygosity at the mutant loci were employed to identify those mutations displaying evidence of positive selection (a high non-synonymous to synonymous ratio and low heterozygosity). Strong positive selection suggests that the mutations were drivers of melanoma formation.

Among the many mutations identified, both studies found mutations in Rac1. Remarkably, the same Rac1 residue, proline 29 (P29), was substituted multiple times in both studies (in 9.2% of melanomas in ref. 41 and 5% of melanomas in ref. 42). While these mutations are likely drivers of melanoma, their low frequency might in part explain why they were not identified until the relatively recent availability of high-throughput exome sequencing techniques. The low frequency also suggests that melanoma formation is a variable process that can occur through multiple genetic pathways, only 5–10% of which involve Rac1(P29).

Both studies explored the implications of this amino acid substitution in Rac1.[41,42] The proline at position 29 is highly conserved among the Rho family GTPases, with the exception of the divergent members RhoBTB1 and RhoBTB2. This position is located in a hydrophobic pocket within the switch I loop. Structural studies determined that Rac1(P29S) is conformationally distinct[41,42] and aligns closely to the hydrogen bonding patterns observed in activated H-Ras,[41] which is predicted to stabilize the GTP-bound form. Biochemical assays showed that Rac1(P29S) was more efficient at

binding the p21-activated kinase (PAK) binding domain (PBD), also suggesting that this mutant is in the activated conformation.[41,42] Although Rac1(P29S) showed increased PBD binding as compared with non-mutated Rac1, it did not bind to PBD as well as a canonically activated version of Rac1, Rac1(Q61L). In sum, these data suggest that Rac1(P29S) is a partially activated mutant of Rac1, which is a driver of melanoma formation.

In addition to Rac1(P29), other mutagenic changes in Rho GTPases can contribute to melanoma. An equivalent P29 mutation in Rac2 was discovered as a melanoma driver,[42] although at a lower frequency than Rac1(P29). Furthermore, a similar mutation at this proline was observed for RHOT1, RHOT1(P30L),[42] further highlighting the importance of this residue as a possible hot spot for mutations in Rho family GTPases. Because this residue is highly conserved between the Rho family proteins, this mutation may occur in other Rho proteins in other cancer types. This study also identified the canonically activating G12V mutation in Cdc-42 as a driver mutation in melanoma.[42] This mutation is a well-characterized activating mutation, and is a frequently occurring Ras mutation in various cancer types.[43] There is also emerging evidence that Rho proteins may be mutationally activated in other forms of cancer. For example, the RAC1(P29S) mutation has been reported in a head and neck tumor,[44] as well as a breast tumor.[11] These data, taken together with the 2 recent papers citing Rho family mutations in melanoma, suggest that mutations in Rho proteins may occur in various cancer types, and further work will need to be done to identify these mutations.

Previous Evidence of Mutationally Activated Rho GTPases in *C. elegans*

While Rac1(P29S) is a newly discovered melanoma driver mutation, it was discovered previously by our lab in a different context in *C. elegans*. We used a sensitized genetic background to screen for mutations in *C. elegans* that displayed synthetic lethality with a weak Rac1 mutation.[45] *C. elegans* Rac1 is called CED-10.[46] This screen identified a mutation in *ced-10*/Rac itself at the P29 residue (P29L). We think that we were able to recover this activating CED-10/Rac mutation in this screen because the *ced-10/Rac* locus in the screen already harbored a point mutation in the CaaX prenylation sequence, which reduced its function. Thus, while broad CED-10/Rac activation is lethal to *C. elegans*, P29L in the context of the already-weakened *ced-10/Rac* gene was not lethal.

We concluded that CED-10(P29L) was a gain-of-function mutation.[45] First, CED-10(P29L) displayed defects in axon guidance, whereas loss of function of the *ced-10/Rac* gene did not, suggesting that P29L was not a loss of function mutation. Furthermore, we used a transgenic approach to express CED-10(P29L) specifically in neurons and not in other tissues. This resulted in axon guidance and branching errors that were not seen in equivalent transgenic lines expressing wild-type CED-10/Rac. From these studies we concluded that P29L was a new gain of function mutation in CED-10/Rac. However, CED-10(P29L) is likely a weaker gain-of-function compared with the canonically activating CED-10(G12V), which causes axon guidance errors and branching similar to CED-10(P29L), but also causes robust induction of ectopic lamellipodial and filopodial protrusions that were only weakly observed in the P29L lines. The analysis of the P29S mutation in the melanoma exome sequencing studies[41,42] is consistent with our results and indicates that Rac(P29) mutations result in partial activation of the molecule.

Clues about other Rho family activating mutations might come from the genetic screen in *C. elegans* our laboratory conducted that identified CED-1(P29L).[45] The screen also identified a novel mutation in the MIG-2 GTPase, which is an invertebrate-specific GTPase with structural and functional similarity to Rac and Cdc-42 GTPases[47-49] and might be the functionally equivalent to RhoG.[50] The MIG-2 mutation changes serine 75 to phenylalanine (S75F) near the predicted switch 2 region. Furthermore, a previously isolated allele of *ced-10*/Rac (G60R also near the switch 2 region) caused axon defects on its own, whereas simple *ced-10*/Rac loss of function did not, suggesting that G60R might also be a gain-of-function mutation.[46] A similar genetic and transgenic analysis described for CED-10(P29L) was conducted with MIG-2(S75F) and CED-10(G60R) with similar results,[45] indicating that both of these are new gain-of-function mutations in Rho GTPases. The S75 and G60 residues are conserved in human Rac1 and Cdc-42, suggesting that they might also have a similar role in these molecules. Further studies will reveal the roles of these mutations, if any, in cancer.

Summary

The recent studies using exome sequencing of tumors have revealed that Rho GTPase mutations are likely driver mutations in various forms of cancer. These discoveries change the status of Rho GTPases from molecules misregulated in cancer to mutational target drivers of cancer. The low percentage of tumors harboring these Rho mutations might have precluded their earlier identification until the advent of high throughput sequencing technology. Their relative rarity in melanomas (5–10%) also suggests that melanoma, like other cancer types, is a heterogenous disease that can come about through multiple distinct genetic mechanisms. Therefore, the continued identification of driver mutations in melanoma and other cancers will be of profound importance. This effort can be aided by studies of Rho GTPases in model organisms such as *C. elegans*, in which the Rac(P29L) activating mutation was first identified. Recent work from our laboratory further demonstrates the utility of using *C. elegans* developmental neurobiology as a platform to study oncogenesis, as we have shown that TIAM-1, a Rac GEF, acts downstream of CDC-42 and upstream of the Rac GTPases in a linear pathway that regulates neuronal development and protrusion downstream of the UNC-40/Deleted in colorectal cancer(DCC) receptor molecule.[51] Further work in oncogene and tumor suppressor regulation and function in *C. elegans,* combined with data from other systems, will continue to reveal new insights into the pathological mechanisms underlying tumor formation, maintenance, and metastasis.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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