



Faculty of Medicine

Deciphering the dual function of RhoGDI2 in cancer biology

Thesis presented by

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Abstract

Rho GTPases are highly conserved members of the Ras superfamily. They are best known to organize the actin and microtubule cytoskeleton and, thereby, to define cell shape and migration, among other characteristics. Their activity is regulated by several activators and inhibitors. To date, only three human Rho guanine dissociation inhibitors (GDIs) have been identified: RhoGDI1, RhoGDI2, and RhoGDI3. Initially, RhoGDI2 was thought to be specific to immune cells, but it has since been found in various other cell types and tissues, including cancer cells. RhoGDI1 and RhoGDI2 are implicated in multiple human cancers, making them attractive targets for cancer research. While RhoGDI1 has been studied extensively, much less is known about the physiological function(s) of RhoGDI2.

Despite sharing a high structural similarity with RhoGDI1, RhoGDI2 is able to display several specific functions. It has been suggested that its roles probably extend beyond the passive maintenance of RhoGTPases and it was described to have both pro- and anti- tumor properties depending on cancer type and stage.

Our goal was to understand the dual role of RhoGDI2 in cancer, and to elucidate the differences between RhoGDI1 and RhoGDI2.

By immunoprecipitation followed by Mass Spec analysis, we observed that RhoGDI2 potentially interacts with many centrosomal and dynein proteins. Using osteosarcoma cancer cells as experimental models, we then demonstrated that siRNA-mediated suppression of RhoGDI2 expression significantly reduced cell proliferation, an effect that was not observed when RhoGDI1 was silenced. Searching for the underlying mechanisms, we found that RhoGDI2 silencing promoted mitotic errors and centrosomal abnormalities. It also induced supernumerary centrosomes and chromosome congression defects while inhibiting ciliogenesis. As Rho GTPases are also implicated in ciliary defects, we checked the implication of some key Rho GTPases in the modifications observed in RhoGDI2-silenced cells. We showed that silencing of RhoGDI2 promoted the activity of RhoA, but not that of Rac1 or Cdc42, and that the silencing of RhoA rescued the ciliary defects observed upon RhoGDI2 silencing. Furthermore, knocking down RhoGDI2 in NK-92 natural killer cells significantly reduced their tumor-killing abilities, suggesting a critical role in immune synapse formation.

Taken together, these findings point to a previously unrecognized function of RhoGDI2 in centrosomal organization and immune cell activity, positioning RhoGDI2 as both a regulator of cancer cell proliferation and anti-tumor immunity. This multifunctional involvement in cellular processes highlights RhoGDI2 as a compelling target for advancing our understanding of cancer biology and developing more precise therapies.

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1. Introduction

1.1 The Rho GTPase family

Rho GTPases are a family of small GTP-binding proteins (Figure 1) that play crucial roles in regulating various cellular processes, including cytoskeletal dynamics, cell adhesion, cell cycle progression, and gene expression. This family consists of several members, with RhoA, Rac1 and Cdc42 being the most studied and best characterized. All members share downstream effector proteins and cellular functions. The dysregulation of Rho GTPase signaling has been implicated in various diseases, including cancer, neurodegenerative disorders, and cardiovascular diseases ¹⁻⁴. Therefore, understanding their precise regulation and functions is important for unravelling the molecular mechanisms underlying normal cellular physiology and disease.

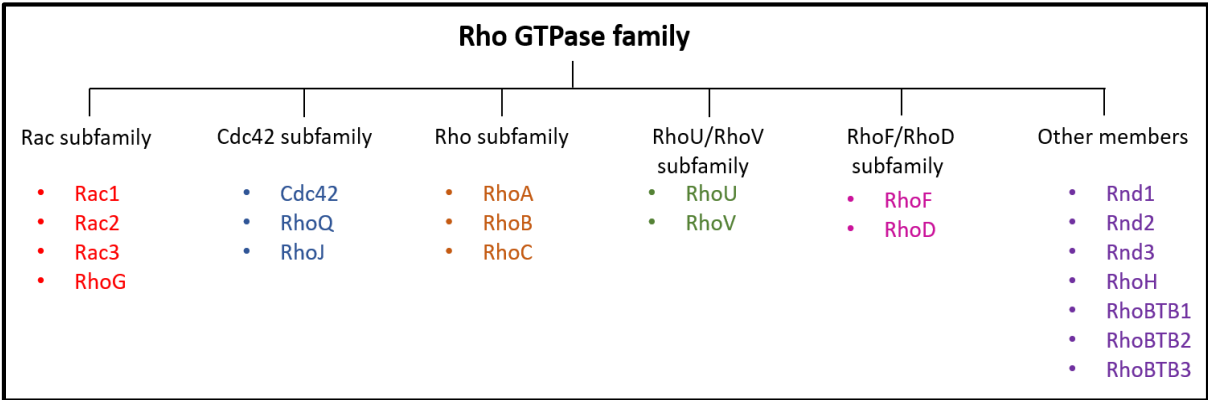


Figure 1: The RhoGTPase family

Rho GTPases, a branch of the Ras superfamily, consist of 21 members in mammals. They are typically categorized into subfamilies based on sequence and functional similarities, including the Rac (Rac1, Rac2, Rac3, and RhoG), Cdc42 (Cdc42, RhoQ, and RhoJ), Rho (RhoA, RhoB, and RhoC), RhoU/RhoV (RhoU and RhoV), and RhoF/RhoD (RhoF and RhoD) subfamilies. Additional members include Rnd1-3, RhoH, and RhoBTB1-3. Among them, RhoA, Rac1, and Cdc42 remain the most extensively studied due to their central roles in cytoskeletal dynamics, cell cycle progression, and tumorigenesis.

The regulation of Rho GTPases is a highly intricate and tightly orchestrated process critical for maintaining cellular homeostasis and coordinating a multitude of cellular processes (Figure 2) ³. GTPases serve as molecular switches, toggling between an active GTP-bound state, and an inactive GDP-bound state. The dynamic equilibrium between these states is carefully controlled by a variety of regulatory proteins. Guanine Nucleotide Exchange Factors (GEFs) facilitate the exchange of GDP for GTP, a process notably triggered by extracellular signals such as receptor tyrosine kinases (RTKs) and

G protein-coupled receptors (GPCRs). The activation of GEFs contributes to the spatial and temporal regulation of Rho GTPases within the cell.

Conversely, GTPase-activating proteins (GAPs) terminate Rho GTPase signaling by accelerating the intrinsic hydrolysis of GTP to GDP, thereby converting the active GTP-bound state into its inactive form. This negative regulation prevents excessive or prolonged Rho GTPase activity. Guanine nucleotide dissociation inhibitors (RhoGDIs) further regulate inactivity by sequestering GDP-bound Rho GTPases in the cytoplasm. By shielding the GTPases from the membrane, RhoGDIs restrict access to guanine nucleotide exchange factors (RhoGEFs) and thus limit inappropriate or premature activation. RhoGDIs also facilitate trafficking of Rho GTPases to specific cellular compartments and protect them from ubiquitylation-dependent degradation, underscoring their dual role as both negative regulators and stabilizers of classical Rho GTPase signaling.

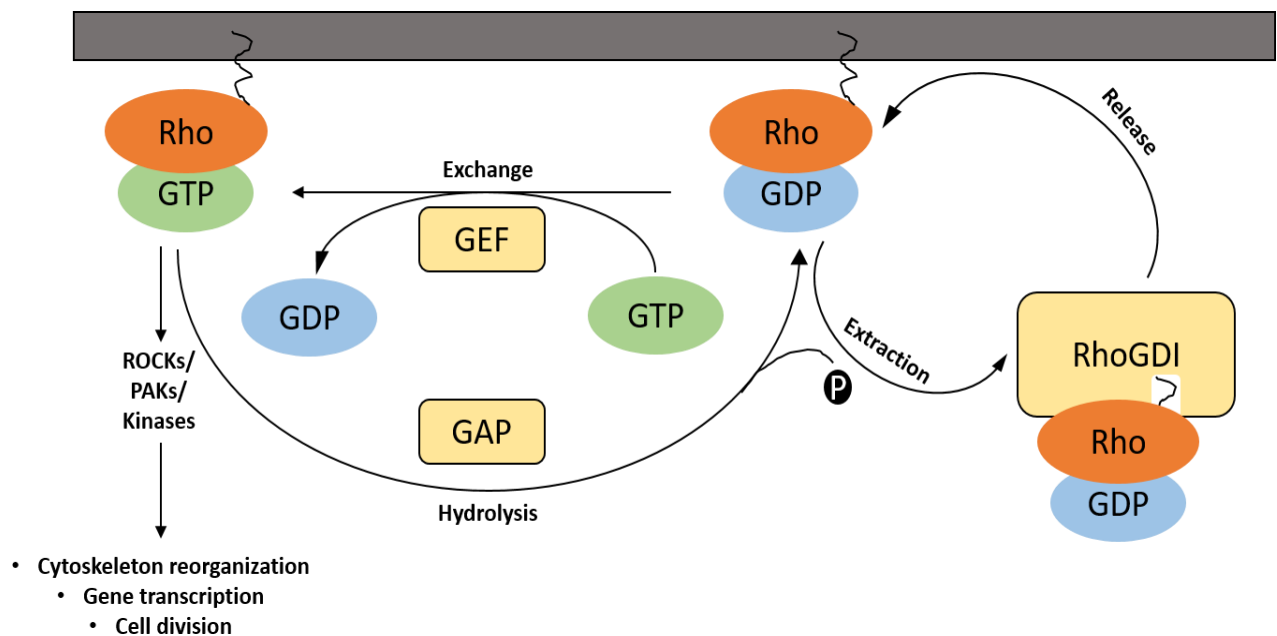


Figure 2: Schematic diagram of the Rho GTPase regulatory cycle

A Rho GTPase is maintained inactive when bound to GDP. Activation occurs when Rho guanine nucleotide exchange factors (RhoGEFs) promote the release of GDP, allowing GTP to bind. The GTP-bound form adopts an active conformation that exposes effector-binding domains, enabling interaction with downstream targets such as Rho-associated coiled-coil kinases (ROCKs), PAK family kinases, and other regulators of cytoskeletal dynamics. Inactivation is mediated by Rho GTPase-activating proteins (RhoGAPs), which stimulate GTP hydrolysis to GDP. The resulting inactive GDP-bound GTPase can then be sequestered by Rho guanine nucleotide dissociation inhibitors (RhoGDIs), preventing both membrane association, which is key for the activation process, but also ubiquitylation-dependent degradation. Through these cyclical transitions, the spatial and temporal activity of Rho GTPases is tightly regulated within the cell. (figure adapted from Tripathi, Colige & Deroanne, 2023)

1.2 Crosstalk between signaling pathways and RhoGTPases

Amongst some classical signaling pathways, extracellular signals, such as growth factors or environmental cues, activate cell surface receptors, leading to the activation of downstream signaling pathways. These pathways converge on the activation of GEFs, which catalyze the exchange of GDP for GTP on Rho GTPases, thereby activating them (Figure 3).

RTKs, such as epidermal growth factor receptor (EGFR) and platelet-derived growth factor receptor (PDGFR), can activate Rho GTPases through the recruitment of GEFs. This activation, in turn, also influences cytoskeletal dynamics and cell migration in response to growth factor signaling^{2,5}. The Wnt signaling pathway, a key regulator of development and tissue homeostasis, also intersects with Rho GTPases. Downstream of Wnt signaling, Rho GTPases can be activated, influencing processes such as cell polarity, migration, and tissue morphogenesis^{6,7}.

Mitogen-Activated Protein Kinase (MAPK) signaling pathways, including the Extracellular Signal-Regulated Kinase (ERK) pathway, also influence Rho GTPases. ERK activation can lead to the phosphorylation and activation of RhoGEFs, affecting Rho GTPase activity and downstream cellular responses^{2,5,8,9}. Stress-activated MAPK pathways, such as c-Jun N-terminal Kinase (JNK) and p38 MAPK, are also nodes of crosstalk. Rac1 and Cdc42 can regulate the activation of JNK and p38, impacting cellular responses to stress and inflammation^{2,3,10}.

Additionally, the Phosphoinositide 3-Kinase (PI3K) and Akt signaling pathway, crucial for cell survival, growth, and migration, also intersects with Rho GTPases, particularly Rac1. Rac1 can activate PI3K, leading to Akt activation and influencing cell survival and migration^{2,8,10}. GPCRs can also activate Rho GTPases through Gα12/13 and Gαq subunits, leading to the activation of RhoA and subsequent cytoskeletal changes. Conversely, Rho GTPases can also regulate the activity of GPCRs, forming a feedback loop^{2,5,11}.

Rho GTPases, including RhoA, Rac1, and Cdc42, play pivotal roles in cellular functions, and their activities are modulated by interactions with various signaling cascades. One significant axis of crosstalk involves integrin signaling. RhoA activation, for instance, promotes the formation of focal adhesions, reinforcing cell adhesion to the extracellular matrix. This integration with integrin signaling is crucial for cell adhesion and migration processes¹²⁻¹⁴. Lastly, mechanical forces can also modulate Rho GTPase activity. For instance, mechanical stretching or forces acting on the cell membrane can activate RhoA, influencing cellular responses to mechanical cues^{3,9}.

Once activated, Rho GTPases initiate cellular signaling cascades by interacting with downstream effectors that regulate essential cellular processes. Rho GTPases regulate actin

cytoskeleton remodeling through downstream effectors such as Rho-associated coiled-coil containing kinases (ROCKs) and PAK family serine/threonine kinases (PAKs), orchestrating cell motility and morphology changes^{2–4} (Figure 3).

Moreover, Nuclear Factor- κ B (NF- κ B) signaling, essential for inflammation and immune response, is also influenced by Rho GTPases, especially Rac1 and Cdc42, where they can impact NF- κ B activation through regulation of I κ B kinase (IKK) complexes^{2,15,16}.

This complex crosstalk between Rho GTPases and various signaling processes underscores the sophisticated regulatory network that cells employ to respond effectively and specifically to diverse extracellular signals and environmental cues. Feedback mechanisms, both positive and negative, are integral to the regulatory network governing Rho GTPases. These feedback loops contribute to maintaining the duration and intensity of Rho GTPase signaling within optimal ranges. Dysregulation of these crosstalk mechanisms can contribute to various diseases, emphasizing the importance of comprehending the integrated signaling landscape for therapeutic insights.

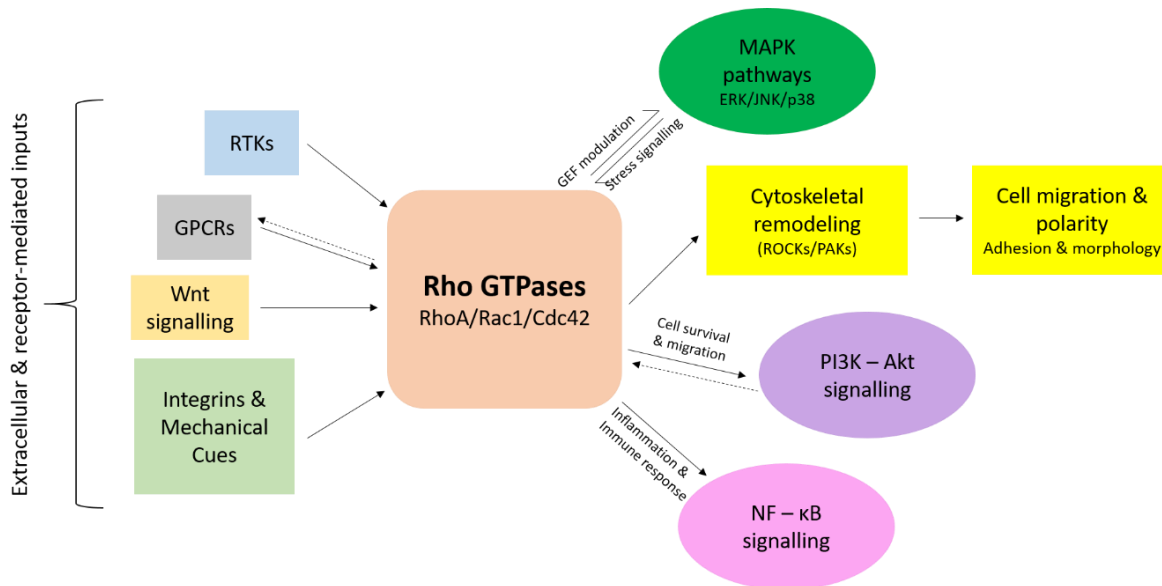


Figure 3: Schematic representation of crosstalk between Rho GTPases and major cellular signaling pathways

Rho GTPases (as RhoA, Rac1 and Cdc42) function as central signaling hubs integrating diverse extracellular and receptor-mediated inputs, including receptor tyrosine kinases (RTKs), G protein-coupled receptors (GPCRs), Wnt signaling, and integrin-mediated mechanical cues. Beyond regulating cytoskeletal remodeling through downstream effectors such as ROCKs and PAKs, Rho GTPases engage in extensive crosstalk with major signaling pathways, including MAPK (ERK, JNK, and p38), PI3K–Akt, and NF- κ B signaling. These reciprocal interactions coordinate key cellular processes such as cell migration, polarity, adhesion, morphology, survival, and inflammatory responses. Dashed arrows indicate feedback regulation. The figure provides a conceptual overview of signaling integration rather than detailed molecular mechanisms.

1.3 Regulators of Rho GTPases

Rho GTPases are tightly controlled by three major classes of regulatory proteins: guanine nucleotide exchange factors (RhoGEFs), GTPase-activating proteins (RhoGAPs), and guanine nucleotide dissociation inhibitors (RhoGDIs). Together, these regulators coordinate the cycling of Rho GTPases between inactive and active states, ensuring precise spatial and temporal control over downstream signaling ^{10,17}.

RhoGEFs (~85 identified in humans) activate Rho GTPases by catalyzing the release of GDP, allowing GTP binding. This exchange step is the primary rate-limiting event in Rho GTPase activation, making GEFs essential initiators of Rho-driven pathways such as actin remodeling, cell adhesion, and migration. RhoGAPs (~66 identified) perform the opposing function by accelerating the intrinsic hydrolysis of GTP to GDP, thereby terminating Rho GTPase signaling. Through this activity, GAPs prevent excessive or prolonged activation, preserving signaling fidelity and timing, and preventing aberrant cytoskeletal dynamics ^{10,18,19}.

In stark contrast to the expanded families of GEFs and GAPs, the number of human RhoGDIs remains limited, with only three members identified to date: RhoGDI1 (RhoGDI α), RhoGDI2 (D4-GDI), and RhoGDI3 (RhoGDI γ). RhoGDIs bind and stabilize the inactive, GDP-bound form of Rho GTPases, sequestering them in the cytoplasm, preventing spontaneous membrane association, and shielding them from proteasomal degradation ^{18,20,21}. By restricting access to GEFs and GAPs, RhoGDIs provide an additional layer of control over Rho GTPase localization and activity. Collectively, these regulatory proteins form an integrated network that maintains the dynamic balance of Rho GTPase signaling within the cell and ensures appropriate responses to environmental and intracellular cues ^{17,18,22}.

The N-terminal domain of RhoGDIs inhibits GDP-to-GTP exchange by interacting with the switch regions of GDP-bound Rho GTPases, while the C-terminal domain extracts them from the membrane, keeping them inactive until activation signals are received ^{10,17,18,23}. All three inhabit the cytoplasm, with RhoGDI1 exhibiting ubiquitous expression ^{24,25}. Initially considered to be specific to hematopoietic cells, RhoGDI2 has later been identified in various other cell types and tissues, including cancer cells ^{26,27}. Although less extensively studied than RhoGDI1 and RhoGDI2, RhoGDI3 displays a distinct expression pattern and subcellular localization. It is enriched in tissues such as brain, lung, pancreas and testis, and shows a strong association with the Golgi apparatus. RhoGDI3 can interact with several Rho GTPases, including RhoB and RhoG, and has been detected at lower levels in certain immune cell populations ²⁸. However, its functional contribution to immune regulation remains

comparatively limited and is less well defined than that of RhoGDI2. Therefore, the subsequent sections will focus primarily on RhoGDI2, whose immunological roles are more clearly established.

The activity of RhoGDIs is intricately interconnected with other regulators of Rho GTPases, including GEFs and GAPs. The balance between these regulators determines the overall activation status of Rho GTPases and, consequently, cellular responses. Dysregulation of RhoGDIs and their associated Rho GTPases has been implicated in various diseases, including cancer and neurodegenerative disorders ^{29–35}.

1.4 RhoGDI2 and RhoGDI1

RhoGDI1, the best studied member of the RhoGDI family, was initially identified in rabbit intestine and bovine brain cytosol in 1989 ³⁶, and stands as the prototype of RhoGDIs. Corresponding human cDNA was subsequently isolated, and a homologous RhoGDI protein was also discovered in yeast ²⁴. In 1993, RhoGDI2 was characterized by Leffers et al. where its predominant expression was revealed in hematopoietic cells ³⁷.

As reported above, RhoGDI1 and 2 limit the activation of RhoGTPases but also play a protective role, preventing the proteasomal degradation of interacting Rho GTPases ^{21,23,38}. As expected, given their diverse functions, both RhoGDI1 and 2 are implicated in various biological processes such as actin cytoskeletal organization, cell migration, cancer, and immune response ^{3,10,12,17,25}. This section will further explore differences in their functions in detail.

RhoGDI1 and RhoGDI2 are associated with various human cancers, exhibiting modifications of their expression (Table 1), although their involvement in disease progression has not been demonstrated.

Cancer	RhoGDI	Regulation	Reference(s)
Breast cancer	RhoGDI1	Up	39
	RhoGDI2	Up	40,41
		Biphasic	31,42
Hepatocellular carcinoma	RhoGDI1	Down	43
	RhoGDI2	Up	44
Bladder cancer	RhoGDI1	Down	45
	RhoGDI2	Down	30
Ovarian cancer	RhoGDI2	Down	29,46

Hodgkin's lymphoma	RhoGDI2	Down	47,48
Gastric cancer	RhoGDI2	Up	49,50
Pancreatic cancer	RhoGDI2	Up	51–53
Melanoma	RhoGDI2	Up	54
Lung cancer	RhoGDI2	Down	55,56
Osteosarcoma	RhoGDI2	Down	57,58
Leukemias	RhoGDI2	Down	59,60

Table 1: Expression of RhoGDI1 and RhoGDI2 in human cancers, as compared to control healthy tissues

Biphasic: up and then down.

Mouse models have been instrumental in elucidating the specific functions of RhoGDI1 and RhoGDI2. Yin *et al.* used RhoGDI2-null mice and observed normal lymphoid development and immune responses but identified de-regulated Rac1 and Cdc42 activities, defective actin polymerization, and impaired lymphocyte spreading and migration *in vitro* ⁶¹. On the other hand, RhoGDI1-null mice exhibited kidney and reproductive system abnormalities in adulthood. Double-knockout mouse models of RhoGDI1 and RhoGDI2 revealed overlapping and synergistic roles in lymphocyte migration and development, reflecting partial functional redundancy in the spatial regulation of Rho GTPase activity rather than additive inhibitory effects ^{62,63}.

Although RhoGDI1 is primarily cytoplasmic, RhoGDI2 has been reported to localize both in the cytoplasm and at the nuclear envelope, with additional enrichment in perinuclear regions. This localization does not arise from a classical nuclear localization sequence; rather, it appears to depend on cell-type-specific interactions with nuclear membrane-associated partners. The presence of RhoGDI2 at the nuclear periphery suggests potential functions beyond cytoskeletal regulation ^{17,23}. Via high-throughput experiments, numerous proteins outside the Rho GTPase family have been identified to interact with either RhoGDI1, RhoGDI2, or both. By examining databases such as Uniprot (www.uniprot.org) and Biogrid (www.biogrid.org), interactions with proteins like ubiquitin-fold modifier 1 (UFM1), small ubiquitin-like modifier 4 (SUMO4), U2 small nuclear RNA auxiliary factor 2 (U2AF2), and DEAD box polypeptide 58 (DDX58) were extrapolated for both RhoGDIs. Importantly, many of these candidate interactors are multi-compartmental proteins that shuttle between cellular pools. Therefore, their reported interaction with RhoGDI1/2 does not in itself constitute evidence for nuclear localization of RhoGDI1, and may reflect indirect, transient, or context-dependent associations. In contrast, experimentally validated interactions reveal more distinct patterns, RhoGDI1

interacts with Cullin3, EWS RNA-binding protein 1, ezrin, moesin, and radixin, while RhoGDI2 interacts with RhoGEF Vav1, acyl-CoA thioesterase 7, B cell CLL/lymphoma 6, and cadherin1. These distinct protein interactions may contribute to the functional differences observed between RhoGDI1 and RhoGDI2.

Structural differences between RhoGDI1 and RhoGDI2 may contribute to their distinct functional characteristics. RhoGDI1 and RhoGDI2 share the conserved RhoGDI fold and overall domain architecture, with sequence conservation enriched in the C-terminal GDI domain and divergence concentrated in the extreme N-terminal regulatory segment (Fig. 4A). AlphaFold models predict high confidence for the conserved GDI core but lower confidence for parts of the regulatory arm (orange in Fig. 4B), consistent with a flexible/disordered N-terminal region. Together, these observations suggest that isoform-specific variation is primarily localized to the N-terminal regulatory segment and may modulate protein interactions and regulation.

Both RhoGDI1 and RhoGDI2 form complexes with classical Rho GTPases, including RhoA, RhoC, Rac1, Rac2, Rac3, RhoG and Cdc42^{3,20,64,65}. However, the interaction potency of RhoGDI2 with Cdc42 is notably 10 – 20 times lower than that of RhoGDI1. Platko et al. identified a single residue (Ile 177 in RhoGDI1/Asn 174 in RhoGDI2) as responsible for this discrepancy in their affinity for Cdc42⁶⁶. These structural disparities suggest that while the isoforms maintain a fundamental inhibitory mechanism, the specific interactions and affinities with Rho GTPases may vary, contributing to the isoform-specific functions observed in cellular processes and disease contexts. Understanding these structural distinctions is key to elucidate the nuanced roles of RhoGDI1 and RhoGDI2 in cellular regulation.

Introduction

A

P52565	MAEQEPTAEQLAQIAAENEDEHSV	NYKPPAQKSIQEIQLDKDDESLRKYKEALLGRVA	60
P52566	MTEKAPPH--VEEDDDDELDSKL	NYKPPQKSLKELQEMDKDDESLIKYKKTLLGDGP	57
	::*	:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:	
P52565	VSADPNVPNVVVTGLTLVCSSAPGP	LELDLTGDLSEFKKQSFVLKEGVEYRIKISFRVNR	120
P52566	VVTDKPAPNVVVTGLTLVCESAPGP	ITMDLTGDLKALKETIVLKEGSEYRVKIHFKVNR	117
	::*:*:*:*:*:*:*:*:*:*:*:	:*:*:*:*:*:*:*:*:*:*:*:*:*:	
P52565	EIVSGMKYIQHTYRKGVKIDKTDY	MVGSYGPRAEVEFLTPVEEAPKGMLARGSYSIKSR	180
P52566	DIVSGLKYVQHTYRTGVKVDKATF	MVGSYGRPEEVEFLTPVEEAPKGMLARGTYHNKSF	177
	::*:*:*:*:*:*:*:*:*:*:*:	:*:*:*:*:*:*:*:*:*:*:*:*:*:	
P52565	FTDDDKTDHLSWEWNLTIKKDWKD		204
P52566	FTDDDKQDHLSEWNLSIKKEWTE		201

RhoGDI1 Phospho-sites

RhoGDI2 Phospho-sites

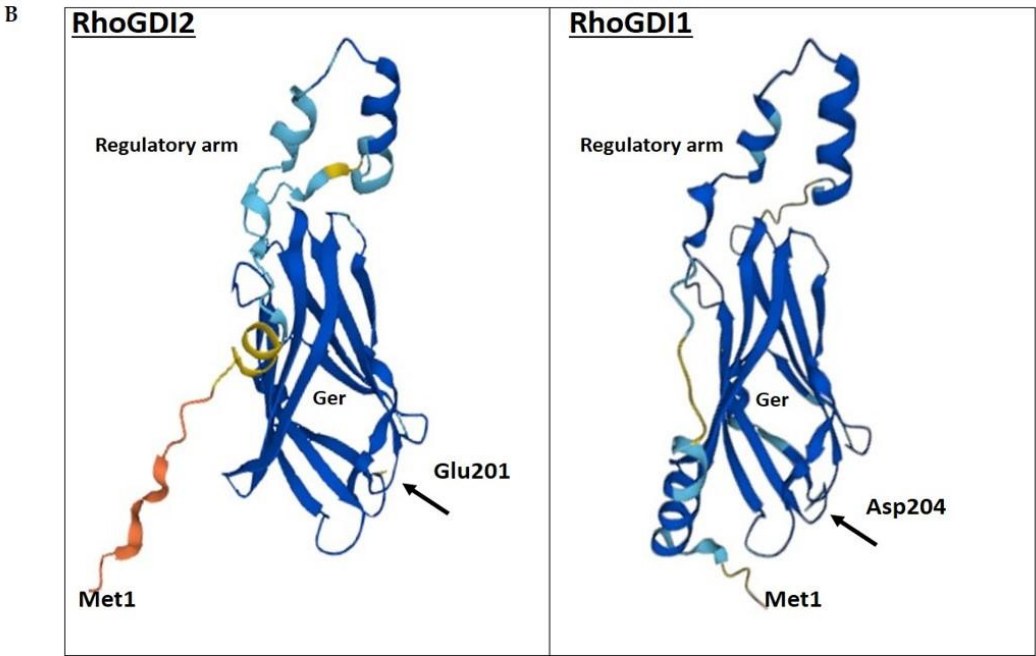


Figure 4: Comparison of the primary and tertiary structures of human RhoGDI1 and RhoGDI2

A. The protein sequences, with accession numbers P52565 for human RhoGDI1 and P52566 for human RhoGDI2, were aligned, highlighting identical residues with asterisks, while substitutions for amino acids with highly similar or somewhat similar characteristics were denoted by double and single dots, respectively. The divergent extreme N-terminal domains are enclosed in the red box. RhoGDI2 undergoes phosphorylation at Y24, S31, and Y153 by $\beta 2$ integrin-related kinases Src, c-Abl, and Syk in response to PSGL-1 antibody ligation. In contrast, phosphorylation of human RhoGDI1 occurs at T7, S34, T91, Y156, and S174 by Polo-like kinase 1, PKC, $\beta 2$ integrin-related kinases $\alpha v\beta 8$, and NIMA (Never In Mitosis A). Reported phosphorylation sites are highlighted on the alignment.

B. Predictions of the 3D structure for RhoGDI1 and RhoGDI2 were obtained from the AlphaFold project (AF-P52565-F1 and AF-P52566-F1, respectively). The color code represents confidence in the 3D structure, with dark blue indicating the highest confidence and orange representing the lowest confidence. The "Ger" label denotes the pocket accommodating the geranylgeranyl moiety of the Rho GTPases.

1.5 Regulatory functions of RhoGDI1 and RhoGDI2

Both RhoGDI1 and RhoGDI2 regulate Rho GTPase activity, but they differ in expression patterns and functional specialization. RhoGDI1 is ubiquitously expressed and serves as the primary housekeeping regulator of Rho proteins, supporting cytoskeletal organization/dynamics and basal cellular functions. In contrast, RhoGDI2 has more specific roles, particularly in immune and hematopoietic lineages. The following section focuses on RhoGDI2, which is of particular relevance to this research given its reported roles in immune/hematopoietic lineages and its emerging functions in cancer-related contexts.

RhoGDI2 serves as a crucial regulator in cellular signaling pathways by modulating the activity of Rho GTPases (Figure 5). As it is involved in the inhibition and sequestration of many Rho GTPases, it impacts a multitude of cellular processes like cytoskeletal dynamics and immune response.

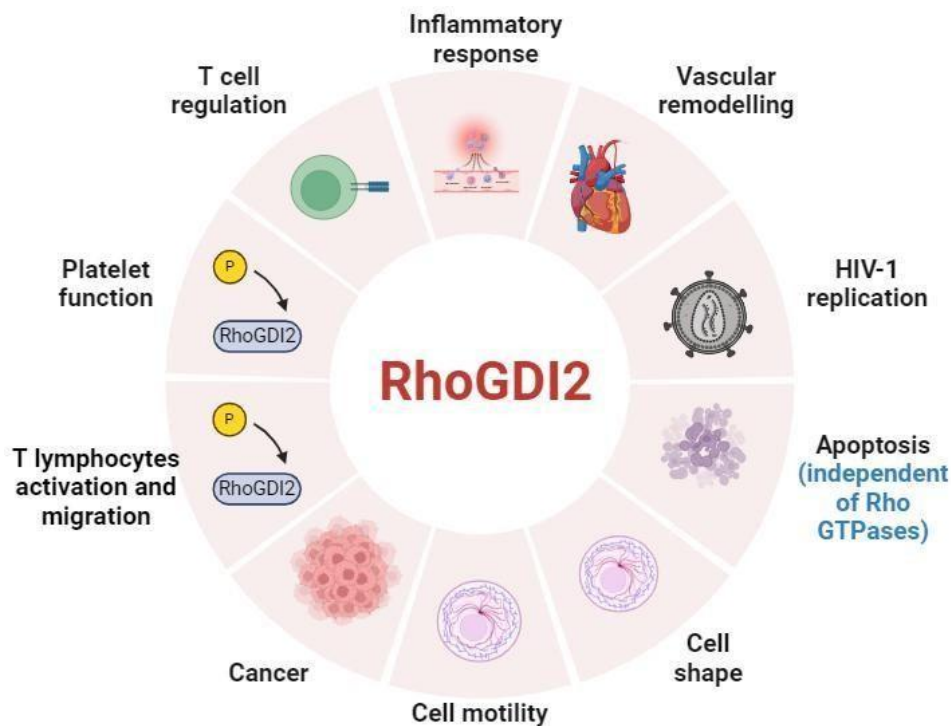


Figure 5: Primary functions of RhoGDI2 in the human body

RhoGDI2 plays a pivotal role in diverse biological processes, influencing actin cytoskeleton organization and thereby impacting cell shape and movement. Additionally, it contributes to immune response mechanisms, being phosphorylated by kinases (Src, c-Abl, and Syk) at Y24, S31, and Y153. Phosphorylated RhoGDI2 plays a crucial role in T cell lymphocyte activation and migration, contributing to T cell regulation and inflammatory responses. RhoGDI2 also plays a role in vascular remodeling by facilitating the migration of smooth muscle cells and is implicated in HIV-1 replication and apoptosis. Notably, in various cancers, RhoGDI2 is involved in all above mentioned processes in a Rho GTPase-dependent manner, except apoptosis where it is involved in a RhoGTPase-independent manner. (Created with BioRender).

1.5.1 Actin cytoskeleton

The actin cytoskeleton, consisting of polymerized actin filaments, is central to cellular functions such as cell migration, adhesion, and maintenance of cell shape. This includes the formation of stress fibers, focal adhesions, and lamellipodia, all of which are essential for cell motility and interaction with the extracellular environment ^{67–69}.

The trio of Rho GTPases RhoA, Rac1, and Cdc42 plays a crucial role in modulating actin cytoskeleton organization, dictating cell shape and movement ^{3,19,67,69}. RhoGDI2 has been observed to induce cell rounding in various cell lines upon overexpression, in line with its capacity to inhibit the activity of RhoGTPases ³⁷. Similarly, RhoGDI2 negatively regulates trophoblast migration by inhibiting Rac1 activity. Proper trophoblast proliferation and migration are essential for successful placental development and pregnancy progression ⁷⁰.

1.5.2 Immune response

While traditionally recognized for its role in cytoskeletal dynamics, recent research highlights the diverse immunomodulatory functions of RhoGDI2 in various immune cell types. This is in line with the fact that it is predominantly expressed in immune cells. In this context, RhoGDI2 has been implicated in the regulation of several crucial processes, demonstrating its significance in both innate and adaptive immunity ¹⁰.

mRNA microarray analysis revealed differential expression of RhoGDI2 in chronic chagasic cardiomyopathy (CCC), a condition marked by severe cardiac inflammation leading to heart failure ⁷¹. RhoGDI2 and Rac2 clustered in a gene module enriched for NK cell–mediated cytotoxicity, implicating this axis in CCC pathogenesis ^{72,73}.

RhoGDI2 has also been associated with the regulation of T lymphocyte responses. T cells, crucial effectors of adaptive immunity, undergo dynamic cytoskeletal rearrangements during processes such as migration, activation, and immune synapse formation ^{74,75}. Upon T-cell activation which is commonly induced experimentally using anti-CD3 monoclonal antibodies, RhoGDI2, but not RhoGDI1, was found to interact with Vav1, a RhoGEF highly expressed in immune cells. The co-localization of Vav1 and RhoGDI2 at the immune synapse periphery, the interface between T cells and antigen-presenting cells ^{76,77}, raised questions due to their theoretically opposing roles. This interaction hints at RhoGDI2's potential involvement in regulating hematopoietic-specific Rho GTPases, potentially modulating their activity in an isoform- and context-dependent manner.

Moreover, knocking down RhoGDI2 in Jurkat T cells diminished their adhesive and migratory abilities by reducing Rac1 and Cdc42 activities. This “counter-intuitive” observation may be explained by recent discoveries of RhoGDI2 phosphorylation at Tyr24, Ser31, and Tyr153 (by kinases Src, c-Abl, and Syk). These phosphorylation events clarify why RhoGDI2 specifically influences Rac1 activity: phosphorylation at Tyr24 and Tyr153 enhances RhoGDI2 binding to Vav1, a Rac-specific GEF, thereby modulating Rac1 activation. In contrast, phosphorylation at Ser31 promotes the release of bound Rho GTPases, further affecting Rac1 dynamics^{70,76}. This contrasts with RhoGDI2's anti-migratory effects in various cell types^{56,59,78,79}, emphasizing its diverse regulatory pathways in T lymphocytes. Given its initial observation in B and T lymphocytes, where it is highly expressed³⁷, RhoGDI2 emerges as the predominant RhoGDI in hematopoietic cells, playing a key role in immune response.

The intricate involvement of RhoGDI2 in immune responses highlights its versatility as a regulator in various facets of immunity. Dysregulation of RhoGDI2-mediated processes has implications for immune-related disorders, amongst others. As our understanding of the immunomodulatory functions of RhoGDI2 advances, it opens up avenues for exploring its therapeutic potential in immune-related disorders and underscores its importance as a key player in immune homeostasis.

1.5.3 Other functions

Beyond its established roles in cytoskeletal regulation and cell migration, RhoGDI2 participates in several additional cellular processes. It has been implicated in apoptosis regulation, with cell-type-specific pro- or anti-apoptotic effects^{80–82}. RhoGDI2 also influences vesicular trafficking and Golgi organization, consistent with its perinuclear localization^{83–85}. In immune contexts, RhoGDI2 modulates cytokine production and contributes to inflammatory signaling. Moreover, it has been linked to vascular remodeling and has reported roles in viral processes such as HIV-1 replication¹⁰. These diverse functions highlight the broader regulatory landscape in which RhoGDI2 operates, extending beyond classical Rho GTPase control.

1.6 Dual role of RhoGDI2 in cancer biology

The summary of known or suggested roles of RhoGDI2 in cancer, as described below, is largely drawn from a personal publication ¹⁰.

RhoGDI2 plays different, and sometimes opposite, roles that can regulate tumor progression (Figure 6). Its overexpression has been associated with aggressive phenotypes in various cancer types. Conversely, there are instances where RhoGDI2 has been suggested to exert tumor-suppressive effects.

These studies are going to be summarized below. However, we feel important to mention that many of them suffer from some weaknesses, such as being purely correlative or using antibodies with no proven specificity for RhoGDI2 as compared to RhoGDI1.

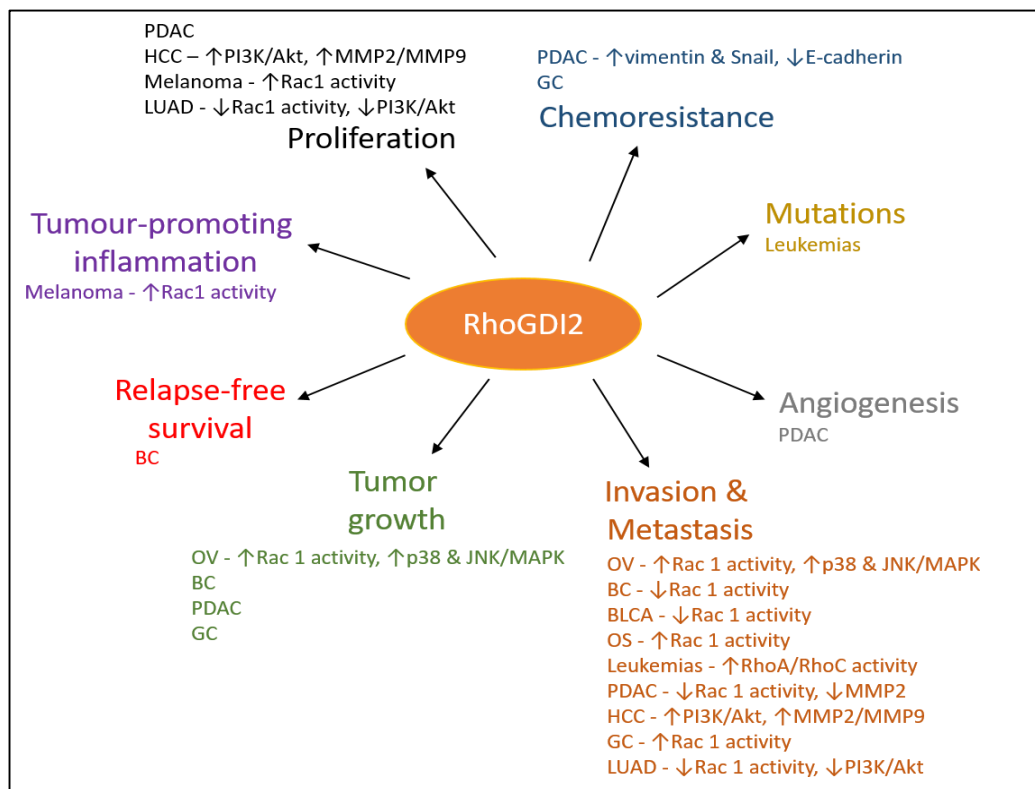


Figure 6: RhoGDI2 in cancer hallmarks

RhoGDI2 appears to affect numerous cancer hallmarks, exerting influence on aggressive cancer phenotypes by directly or indirectly regulating Rho GTPases and interacting with other regulators in the signaling cascade. Its impact encompasses the modulation of cell proliferation, tumor growth, cell migration, invasion, and metastasis. Additionally, RhoGDI2 contributes to chemoresistance, exhibits mutations in certain human cancers, and plays a role in promoting tumor-associated inflammation, angiogenesis, and influencing relapse-free survival across multiple human cancer types. OV – ovarian cancer, BC – Breast cancer, BLCA – Bladder cancer, OS – Osteosarcoma, PDAC – Pancreatic ductal adenocarcinoma, HCC – hepatocellular carcinoma, GC – gastric cancer, LUAD – lung adenocarcinoma

1.6.1 Pro- tumoral role of RhoGDI2 in different human cancers

In hepatocellular carcinoma (HCC) cells, RhoGDI2 is upregulated, contributing (or correlating) to increased proliferation and invasion rates. This effect is attributed to the activation of the PI3K/Akt pathway and elevated levels of matrix metalloproteinase (MMP) 2 and 9. MMPs, crucial in extracellular matrix degradation, play a role in tumor invasion and metastasis while influencing the activities of cytokines and cytokine receptors ⁸⁶.

Recent findings propose that the combined action of traditional Chinese medicines, *Euphorbia Pekinesis* and *Glycyrrhiza glabra*, exert synergistic effects against HCC ascites in mice models. This effect is linked to the downregulation of RhoGDI2 and Fyn-related kinase (Frk), a member of the Src non-receptor tyrosine kinase family. Both Frk and RhoGDI2 participate in the vasopressin-regulated water re-absorption pathway, implicated in fluid retention in cirrhosis and presenting a shared therapeutic target for various anti-ascites drugs ^{87,88}.

In breast cancer (BRCA), RhoGDI2 protein expression is variable, being either upregulated or downregulated depending on the specific cell line, in contrast to the consistent upregulation of RhoGDI1 across all BRCA cell lines ^{40,41,89}. Interestingly, breast cancer tissues demonstrate a stage-dependent biphasic pattern of RhoGDI2 expression, marked by an increase from normal to hyperplasia, followed by a decrease from in situ to invasive lesions ⁹⁰. This dynamic expression profile is associated with an inverse correlation between RhoGDI2 levels and lymph node metastasis ^{31,91}, suggesting a potential role for RhoGDI2 as both a tumor promoter and metastasis suppressor in BRCA.

In HER2-positive (HER2+) breast cancer, the mechanistic basis of RhoGDI2 downregulation has been partially elucidated. Morrison Joly et al demonstrated that the mTORC2/Rictor signaling axis suppresses RhoGDI2 protein expression in HER2-amplified breast cancer cells, thereby relieving inhibition of Rac1 and driving invasive migration and metastasis through two convergent mTORC2-dependent pathways. Clinically, low RhoGDI2 protein expression was associated with shorter relapse-free survival specifically in HER2+ patients, establishing RhoGDI2 as a subtype-relevant prognostic marker in this context. This remains the most mechanistically characterized subtype-specific role of RhoGDI2 in breast cancer to date ⁴².

In triple-negative breast cancer (TNBC), which is defined by the absence of estrogen receptor, progesterone receptor and HER2 expression, the relationship with RhoGDI2 is distinct. Rather than being downregulated, RhoGDI2 protein was found to be overexpressed in TNBC relative to normal breast tissue in a comparative proteomic study ⁸⁹. Functional silencing of RhoGDI2 in TNBC cell lines induced mitochondrial dysfunction and activated caspase-3 and caspase-9, suggesting that elevated RhoGDI2 confers resistance to apoptosis in this subtype. Importantly, RhoGDI2 silencing also

sensitized TNBC cells to cisplatin-based chemotherapy, implicating RhoGDI2 as a mediator of chemoresistance. This pro-survival, pro-tumorigenic role in TNBC is further supported by therapeutic targeting studies wherein HR3119, reported as the first small-molecule ligand for RhoGDI2 with low-micromolar binding affinity ($K_d = 8 \mu\text{M}$), was designed to occupy the protein–protein interaction interface between RhoGDI2 and Rac1, thereby disrupting their binding. The purified (6R)-enantiomer demonstrated a nearly 100-fold affinity advantage over (6S)-HR3119 and engaged RhoGDI2 in cells, suppressing the migration of aggressive breast cancer cells²⁰³. These findings collectively position RhoGDI2 as a pro-tumorigenic factor and potential therapeutic target in TNBC.

By contrast, the role of RhoGDI2 in luminal breast cancer subtypes- luminal A (ER+/PR+, HER2–, low Ki67) and luminal B (ER+, HER2±, high Ki67), which collectively represent the majority of breast cancer cases, remains poorly characterized at the subtype level. Large-scale transcriptomic analyses of unselected BRCA cohorts via the KM Plotter tool (aggregating GEO, TCGA and EGA data) indicate that elevated RhoGDI2 mRNA is associated with reduced overall survival across the broader breast cancer population, a dataset numerically dominated by luminal patients^{42,92} (Figure 7). However, subtype-stratified analyses specifically interrogating RhoGDI2 expression and function in luminal A or luminal B disease are sparse in the current literature. This gap likely reflects the historical research focus on the highest-risk subtypes, HER2+ and TNBC, and represents an unresolved question in our understanding of the full spectrum of RhoGDI2 biology in breast cancer.

Moreover, RhoGDI2 exhibits significant upregulation in pancreatic adenocarcinoma (PDAC) tumor samples, demonstrating a positive correlation with tumor size, differentiation, clinical stage, lymph node metastasis, vascular invasion, and reduced life expectancy (refer to Figure 7). Silencing RhoGDI2 has been shown to diminish the invasiveness of PDAC cells *in vitro* and reduce the expression of MMP 2 (similar to HCC), potentially through the regulation of Rac1 activity^{51,93}. This suggests that targeting RhoGDI2 or its associated pathway could be a promising strategy for this highly aggressive cancer with limited long-term therapeutic options. In addition, IFN- γ was found to suppress RhoGDI2 expression, leading to decreased Rac1 activity and interleukin- 8 (or CXCL8) expression⁵³. IFN- γ is a cytokine primarily produced by NK cells that exerts anti-tumorigenic effects⁹⁴. Notably, the impact of RhoGDI2 on Rac1 activity appears to be highly context-dependent, reflecting its dual role as both a negative regulator and a stabilizing chaperone of Rac1 in different tumour settings.

Furthermore, *in vitro* overexpression of RhoGDI2 in PDAC cells also enhanced proliferation, migration, invasiveness, and apoptotic resistance to Gemcitabine, a chemotherapeutic drug. These effects were attributed to increased Snail expression, a key transcriptional driver of epithelial-to-mesenchymal transition (EMT), along with elevated vimentin, a hallmark mesenchymal marker linked

to cytoskeletal reorganization and enhanced motility. Consistent with EMT progression, RhoGDI2 overexpression also coincided with reduced levels of the epithelial marker E-cadherin. Importantly, silencing Snail reversed these RhoGDI2-induced phenotypes, confirming Snail's central role in mediating the observed changes ^{52,95}.

The RhoGDI2 gene and protein are upregulated in colorectal cancer (CRC) cell lines characterized by high invasiveness and metastatic potential. In CRC tissues, elevated RhoGDI2 expression correlates with poor patient survival. Overexpression of RhoGDI2 enhances CRC cell proliferation, migration, and invasiveness, and these phenotypes have been associated with increased PI3K/Akt pathway activity. Although the mechanistic link remains unresolved, these observations suggest that RhoGDI2 may influence PI3K/Akt signaling indirectly, potentially through its regulation of Rho GTPases ⁹⁶.

RhoGDI2 exhibits significantly increased expression levels in gastric cancer (GC) samples compared to normal and para-cancerous tissues ⁹⁷. Depleting RhoGDI2 expression diminishes the migration and invasion capacities of GC cells, heightens their responsiveness to chemotherapeutic drugs, and reduces tumor growth in mouse models ^{98–100}. Acting as a scaffold protein, RhoGDI2 facilitates the binding of Rac1 to FilaminA, an actin-binding protein. FilaminA, in turn, promotes Rac1 activation by interacting with Trio, a Rac1-specific RhoGEF, thereby enhancing the invasive capabilities of GC cells. Both RhoGDI2 and FilaminA are implicated in poor prognosis for GC patients. Besides promoting Rac1 binding to FilaminA, the RhoGDI2-Rac1 axis is also implicated in another signaling pathway in GC, as suppressing RhoGDI2 expression hampers the Rac1/Pak1/LIMK1 axis, involved in EMT, cell migration, and invasion in various human cancers ^{49,50,101}.

In contrast to the anti-tumoral roles attributed to wild-type RhoGDI2 in several contexts, oncogenic mutations of RhoGDI2 have been identified in acute myeloid leukemia (AML). Specifically, mutations at positions V68L and V69A were reported in KM3 and Reh AML cell lines. While overexpression of wild-type RhoGDI2 does not affect cell proliferation, expression of mutant RhoGDI2 enhances cellular adhesiveness and invasiveness *in vitro*. Importantly, these pro-invasive phenotypes can be reversed by overexpression of recombinant wild-type RhoGDI2, suggesting a dominant or competitive effect of the mutant protein on shared interacting partners ^{59,82}. These findings indicate that mutated RhoGDI2 can acquire pro-tumoral functions, analogous to gain-of-function mutations observed in classical oncogenes such as RAS ¹⁰².

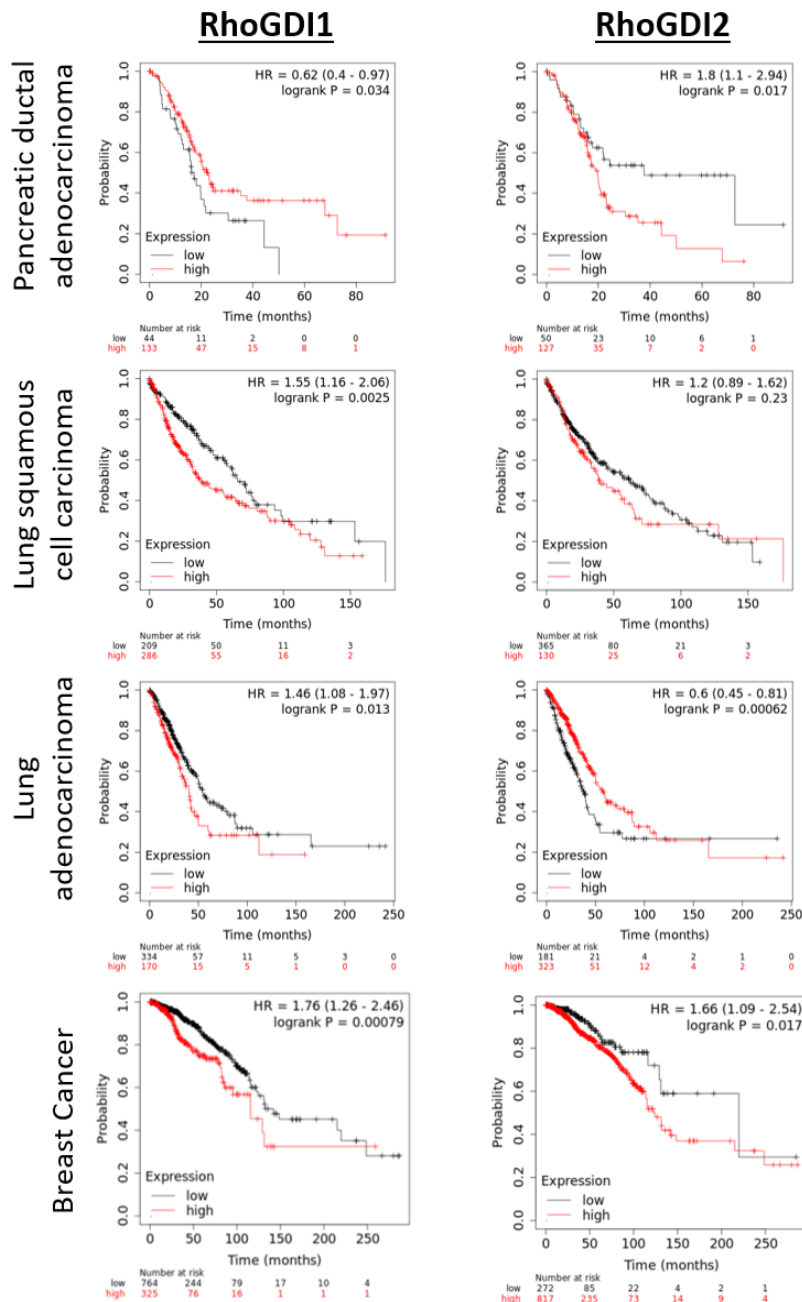


Figure 7: Correlation between patient survival and expression of RhoGDI1 and RhoGDI2 in several cancers

The association between patient survival and the expression of RhoGDI1 and RhoGDI2 in various cancers can be examined using the Kaplan–Meier plotter (<https://kmplot.com>). This tool enables the establishment of correlations between patient survival and the expression levels of any gene in the primary tumor, utilizing data from sources such as GEO, TCGA, and EGA. The presented plots emphasize the dual nature of RhoGDI2 expression in cancer, resulting in either a favorable or unfavorable prognosis. Notably, this duality is observed even in cancers affecting the same organ, as evident in the comparison of curves for lung squamous cell carcinoma and lung adenocarcinoma. The plots also highlight instances where the expression patterns of RhoGDI2 and RhoGDI1 diverge in relation to survival, as exemplified in pancreatic ductal adenocarcinoma, underscoring their distinct roles in cancer progression.

Lastly, depletion of Myo7a reduced RhoGDI2 expression and diminished Rac1 activity in B16 melanoma cells. Notably, re-expression of RhoGDI2 was sufficient to restore Rac1 activity, supporting a functional link between RhoGDI2 levels and Rac1 activation in this context. However, the upstream mechanism by which Myo7a influences RhoGDI2 expression remains unclear and could involve transcriptional and/or post-transcriptional regulation. Together, these data suggest that Myo7a may promote melanoma cell phenotypes at least partly through maintaining RhoGDI2 expression and Rac1 activity⁵⁴. RhoGDI2 (also known as ARHGDIB) is regulated by a proximal promoter and by additional enhancer regions further away. Its expression is known to vary strongly between cell types, especially in immune cells according to the GeneCards RhoGDI2 entry [www.genecards.org, accessed 6 Feb 2026].

1.6.2 Anti- tumoral role of RhoGDI2 in different human cancers

In contrast to its pro-tumorigenic associations, there is evidence suggesting a tumor suppressor-like role for RhoGDI2 in specific cancer types. While RhoGDI1 displays relatively uniform expression across ovarian cancer cell lines, RhoGDI2 levels vary considerably. This variation reflects differences in tumor subtype, intrinsic Rac1 signaling, and stress-responsive pathways.

For example, RhoGDI2 is often downregulated in highly aggressive or high-grade serous ovarian cancer (OV) cells, consistent with a tumor-suppressive role, but is maintained or upregulated in certain endometrioid or clear-cell lines where Rac1 activity and MAPK signaling differ. These context-dependent expression patterns highlight that RhoGDI2's function in OV is not uniform but shaped by the molecular background of each cell type⁴⁶.

This role of RhoGDI2 as a tumor and metastasis suppressor was further validated by a recent study demonstrating that Adenosine, a purine nucleoside known for its anti-tumor effects in diverse human cancers, enhances RhoGDI2 expression in A2780 OV cancer cells and A2780 subcutaneous xenografts in nude mice. Interestingly, Adenosine was found to inhibit tumor growth in these mice, and this inhibitory effect was dependent on RhoGDI2²⁹.

RhoGDI2 has been predominantly linked to a favorable prognosis in bladder cancer (BLCA), where it exerts its metastasis-suppressing effects by inhibiting Rac1 and RhoC activity without affecting primary tumor growth^{30,103–105}. However, recent findings from the Huang lab have unexpectedly revealed consistently elevated levels of RhoGDI2 in most human and mouse BLCA tissues. Three distinct pathways —RhoGDI2/miR-200c/JNK2/Sp1/MMP2, XIAP/Erk/nucleolin/RhoGDI2, and miR-145/Sp1/USP8/AUF1/RhoGDI2— have been identified,

demonstrating that RhoGDI2 promotes BLCA invasion *in vitro* and lung metastasis *in vivo* ^{106–108}. Contrary to its widely acknowledged role as a metastasis suppressor, these results indicate that RhoGDI2 functions as a tumor and metastasis promoter in BLCA, challenging previous observations. Further investigations are warranted to precisely understand the multifaceted role of RhoGDI2 in BLCA.

RhoGDI2 is prominently expressed in acute lymphoblastic leukemia (ALL) cell lines, including Jurkat T-ALL cells. Downregulation of RhoGDI2 enhances CXCL12-driven T-ALL migration. Phosphomimetic mutants of RhoGDI2, created to mimic phosphorylation at Y24 and Y153 (discussed in Figure 4 and 5), rescue the inhibitory phenotype induced by wtRhoGDI2 on CXCL12-mediated ALL migration. Furthermore, phosphorylation of RhoGDI2 at Y24 or Y153 reduces its affinity for RhoA or RhoC, resulting in increased activity of these GTPases. Additional investigations reveal that the phosphorylation of RhoGDI2 is facilitated by non-receptor protein tyrosine kinases Src and ABL1 in response to CXCR4 stimulation by CXCL12 in T-ALL ^{59,76,109,110}. Furthermore, selective downregulation of RhoGDI2 gene expression is observed in Hodgkin's lymphoma compared to B cell non-Hodgkin's lymphoma (B-NHL) cells ⁴⁷.

Silencing RhoGDI2 significantly enhanced the proliferation, migration, and invasion of A549 lung adenocarcinoma (LUAD) cells by elevating Rac1 activity. Rac1, known to promote migratory and invasive capabilities in LUAD, induces cytoskeleton rearrangements. RhoGDI2 overexpression in LUAD cells led to the dispersion of F-actin filaments in the cytoplasm, causing loss of cell-cell adhesion and stress fiber formation. In contrast to PDAC, RhoGDI2 overexpression in LUAD decreased Snail expression and increased E-cadherin expression ^{55,111}. This might explain the anti-tumoral role of RhoGDI2 in LUAD, as suggested by the correlation indicating increased survival for patients with high tumor RhoGDI2 expression (Figure 7). However, lung squamous cell carcinoma showed the opposite trend (also Figure 7), emphasizing the need to define distinct regulatory pathways involving RhoGDI2 in different cancer types.

In addition, unlike in HCC, downregulating RhoGDI2 expression in LUAD increased MMP9 expression and activity, resulting in enhanced migratory and invasive capabilities. Further investigations revealed that, also in contrast to HCC, suppressing RhoGDI2 expression in LUAD also increased PI3K/Akt phosphorylation. This suggests that RhoGDI2 may exert anti-tumoral activities in LUAD by reducing Rac1 activity and deactivating the PI3K/Akt pathway ^{55,56,111}. As a potential explanation, it was demonstrated that Four and a half LIM protein 1 (FHL1) stimulates specifically the expression of RhoGDI2, and not RhoGDI1. FHL1, downregulated in various human cancers, including

LUAD, exhibits anti-tumoral activity ¹¹².

1.7 The actin cytoskeleton, Rho GTPases and cell cycle progression

The actin cytoskeleton is a dynamic and complex network of protein filaments that plays a fundamental role in maintaining cell structure, providing mechanical support, and orchestrating a variety of cellular processes. Actin comprises multiple isoforms (α -, β - and γ -actins). In non-muscle cells, β -actin (ACTB) and γ -actin (ACTG1) dominate; here we mainly refer to β -actin as the major cytoplasmic actin in our cancer cell systems ¹¹³. The organization and regulation of actin filaments contribute to various cellular functions, including cell shape, cell motility, division, and intracellular transport. Polymerization involves the sequential addition of G-actin subunits to the growing filament, forming a double-stranded helical structure known as F-actin. The process is dynamic, with constant assembly and disassembly, allowing the cell to respond rapidly to internal and external cues ^{114–116}.

Lamellipodia and filopodia are actin-based cellular protrusions. Lamellipodia are broad, sheet-like extensions containing a meshwork of actin filaments, contributing to cell motility. Filopodia are slender, finger-like extensions involved in sensing the environment and guiding cell movement ^{68,117}.

Rac1 and Cdc42 are well-established in promoting actin polymerization and cell protrusions ^{15,102}. Rac1 activates the Wiskott-Aldrich syndrome protein-family verprolin-homologous protein (WAVE) complex, facilitating actin polymerization and lamellipodia protrusion. Cdc42 promotes filopodia formation through activation of formin proteins ^{118–121}. RhoA primarily promotes actomyosin contractility via ROCK, but its contribution to migration is context-dependent and can include roles in protrusion initiation in certain settings. RhoA exhibits distinct membrane localization dynamics, predominantly cytosolic except at specific cellular structures such as cell–cell junctions and the cleavage furrow during cytokinesis ¹²².

The cell cycle is a highly regulated process comprising successive phases, named G1 (cell growth), S (DNA synthesis), G2 (preparation for mitosis), and M (mitosis) ¹²³. Progression through these phases is tightly controlled by checkpoints and regulatory molecules, including cyclins, cyclin-dependent kinases (CDKs), and tumor suppressors like p53 ^{124,125}. The actin cytoskeleton and its regulatory factors, such as Rho GTPases and their related proteins, play integral roles in cell cycle regulation. Dynamic changes in the actin cytoskeleton are indeed essential for processes like cell rounding during mitosis, maintenance of cell shape, and cytokinesis ^{8,22} (see figure 8).

RhoA has been implicated in the regulation of cyclin D1, a cyclin important for G1 phase progression ¹²⁶. The transition from G1 to S phase is a critical checkpoint where cells decide whether

to proceed with DNA synthesis. Here, Rac1 and Cdc42 are involved in the control of the G1/S transition, facilitating changes in cell shape and promoting progression through the cell cycle. Rac1 has also been shown to influence the expression of cyclin D1, whereas Cdc42 induces Cyclin E expression through the activation of p70 S6 kinase ^{127,128}.

DNA synthesis takes place during the S phase. Activation of RhoA can stimulate the formation of stress fibers and alterations in cell shape, reduce the expression of cyclin-dependent kinase inhibitors, and drive progression through the S phase of the cell cycle in fibroblasts ^{129,130}. Rho GTPases also contribute to cell proliferation by influencing signaling cascades involved in DNA synthesis. Additionally, they can impact cell survival pathways, influencing the fate of cells entering S phase ¹³¹.

The G2 phase represents the final preparatory stage before mitosis and is crucial for ensuring genomic integrity. During G2, cells complete the synthesis of proteins required for chromosome condensation, spindle assembly, and mitotic entry ¹²³. A major function of this phase is the G2/M checkpoint, which monitors DNA integrity following replication; if damage is detected, signaling pathways involving p53 delay progression to allow repair. Cyclin A and later Cyclin B complexes accumulate and drive the transition toward mitosis once all conditions are met ¹²⁵. In parallel, cytoskeletal components begin to reorganize, establishing the groundwork for the dramatic actin and Rho GTPase-dependent morphological changes that occur during mitosis ¹³².

In mitosis, RhoA is a central regulator where it activates ROCK, leading to the formation of the contractile ring and subsequent cleavage of the cell, i.e. cell rounding ^{22,133}. Moreover, RhoA and Cdc42 have been implicated in spindle orientation, likely through effects on cortical actomyosin tension and polarity pathways that modulate astral microtubule–cortex interactions ^{134,135}.

While the roles of Rho GTPases in orchestrating cytoskeletal rearrangements across cell cycle phases are well-documented, it is equally important to recognize that the relationship is bidirectional wherein cell cycle regulatory machinery directly controls Rho GTPase activity in a temporally coordinated manner. Cyclin-dependent kinases (CDKs) and their cyclin partners phosphorylate upstream regulators of Rho GTPases, including GEFs and GAPs, to modulate their activity in a phase-specific fashion. A well-characterized example is the CDK1–cyclin B-dependent phosphorylation of the GEF Ect2 at mitotic entry, which promotes localized RhoA activation at the cell cortex and at the cleavage furrow, coordinating cytokinesis with mitotic progression ¹³⁴.

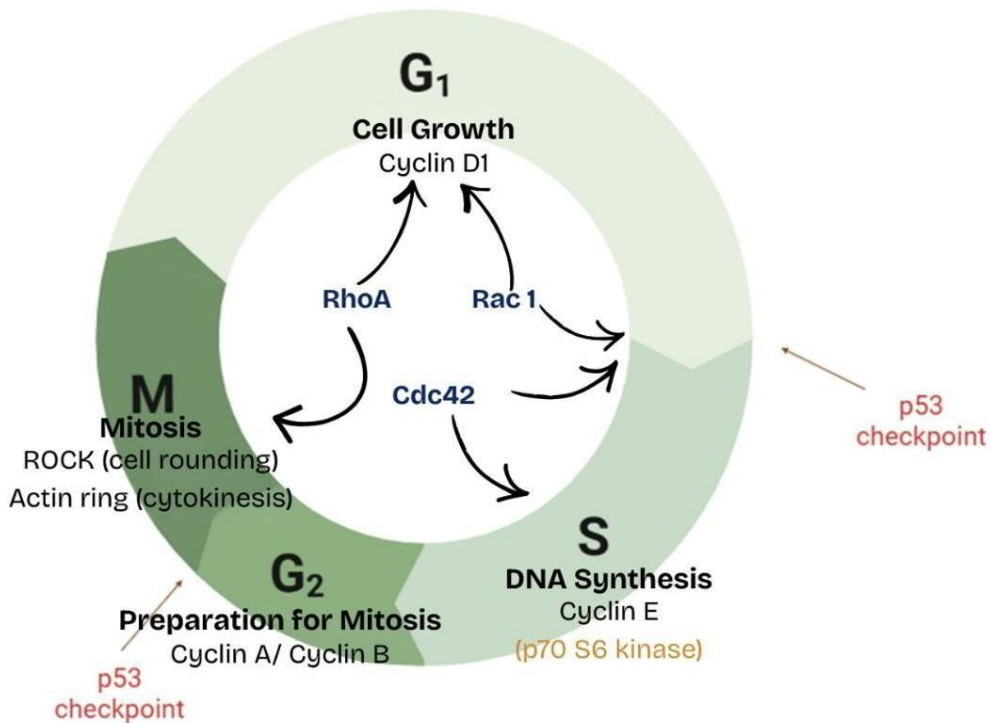


Figure 8: Roles of Rho GTPases in cell cycle progression and cytoskeletal dynamics

The eukaryotic cell cycle comprises G₁, S, G₂, and M phases, coordinated by cyclin–CDK complexes and checkpoint mechanisms. Beyond their established role in mitosis and cytokinesis via RhoA–ROCK signaling, Rho GTPases also regulate earlier cell-cycle transitions. RhoA and Rac1 influence Cyclin D1 expression during G₁, while Rac1 and Cdc42 contribute to G₁/S progression. Cdc42 further promotes Cyclin E expression through activation of p70 S6 kinase, linking cytoskeletal regulation to cell-cycle control. p53-dependent checkpoints ensure genomic integrity at critical transitions. (Created with Biorender)

Conversely, RhoA activity is actively suppressed during S phase to permit centrosome duplication, as sustained RhoA–ROCK II signaling has been shown to be both necessary and sufficient to drive supernumerary centrosome formation ¹⁴⁶. Similarly, Rac1 activity is temporally gated, it promotes G₁/S progression but is downregulated as cells enter mitosis, with its re-emergence at early G₂ mediating PAK-dependent centrosome maturation and mitotic entry ¹⁴⁸. This temporal oscillation of GTPase activity which is governed by the cyclin–CDK machinery, ensures that cytoskeletal reorganization, centrosome duplication, and chromosomal segregation are tightly coupled to cell cycle phase transitions. Disruption of these oscillatory GTPase signals, whether through upstream regulators such as RhoGDIs, is therefore expected to have pleiotropic consequences on cell cycle fidelity.

Understanding the intricate connections between the actin cytoskeleton, Rho GTPases and cell cycle progression is crucial for developing targeted therapeutic strategies. Small molecules that

modulate Rho GTPase activity or actin dynamics are being explored as potential anti-cancer agents, aiming to disrupt the abnormal cellular processes characteristic of cancer cells ³⁵.

1.8 Centrosomes

Centrosomes are key organelles in eukaryotic cells, serving as the primary microtubule-organizing centers (MTOC) and playing a central role in various cellular processes. Comprising a pair of centrioles surrounded by a protein-rich pericentriolar material (PCM) (Figure 9), centrosomes participate in key functions such as organizing the mitotic spindle during cell division, establishing and maintaining cell polarity, and contributing to the overall structural organization of the cell (see table 2) ¹³⁶. Their dynamic nature and regulatory influence on the cytoskeleton make centrosomes essential components for cellular integrity, ensuring the faithful distribution of genetic material and playing crucial roles in diverse cellular functions across different stages of the cell cycle (Figure 10) ¹³⁷.

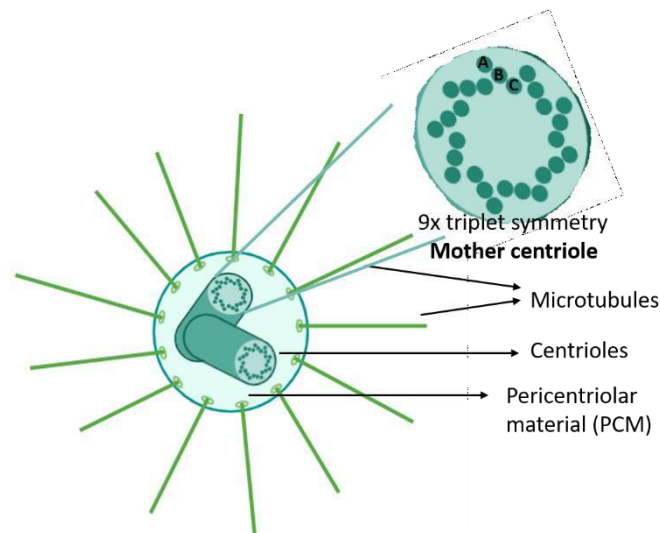


Figure 9: Structural organization of the centrosome and centriole architecture

The centrosome consists of two centrioles, termed the mother and daughter centrioles, embedded within the pericentriolar material (PCM), which serves as the primary microtubule-organizing center in animal cells. An inset highlights the ultrastructural organization of a centriole, composed of nine triplet microtubules arranged in a cylindrical symmetry. This conserved architecture underlies centriole duplication and microtubule nucleation, contributing to mitotic spindle assembly and faithful chromosome segregation during cell division. (Created with BioRender)

Understanding centrosome's regulation is integral to unraveling fundamental aspects of cell biology and has implications for various physiological and pathological processes including cancer.

Protein	Category	Known function	Relevance to centrosome/mitosis
Pericentrin (PCNT)	PCM scaffold	Anchors γ -TuRC, recruits CDK1/cyclins	Essential for microtubule nucleation & spindle formation
γ-Tubulin	Microtubule nucleation	Forms γ -tubulin ring complex	Core of MTOC activity; nucleates microtubules
CEP 152	Centriole biogenesis	Recruits PLK4	Master regulator of centriole duplication
CEP 192	PCM maturation	Scaffold for PCM expansion	Controls mitotic centrosome maturation and spindle formation
CEP 215	PCM assembly	Stabilizes γ -TuRC	Ensures proper spindle pole organization
CEP 104	PCM protein	Centriole cohesion and PCM organization	Centrosome maturation, spindle assembly
Centrin-2 (CETN2)	Centriole stability	Calcium binding	Stabilizes centriole structure
PLK 4	Kinase	Master regulator of centriole duplication	Initiates procentriole assembly
PLK1	Mitotic kinase	PCM expansion & spindle assembly	Coordinates centrosome separation and cytokinesis
Aurora A kinase	Mitotic regulator	Activates spindle assembly	Critical for centrosome maturation
Dynein heavy chain 11, axonemal	Motor protein	Microtubule minus-end motor	Centrosome anchoring, spindle pole focusing, cargo transport
Dynein heavy chain 14, axonemal	Motor protein	Axonemal dynein subunit	Microtubule movement, ciliary/centrosomal association
Dynein intermediate chain 2, axonemal	Motor protein	Dynein cargo-binding component	Ensures spindle positioning and centrosome integrity
Coatomer subunit beta'	Vesicle trafficking protein	Cargo transport & Golgi dynamics	Also localizes to spindle poles during mitosis
Multidrug resistance-associated protein 6	Transporter	Efflux transporter	Reported centrosomal localization in mitosis

Table 2: Key canonical centrosomal proteins and microtubule-associated proteins and their functions

Canonical centrosomal regulators, such as Pericentrin (PCNT) and γ -tubulin, are involved in PCM scaffolding. CEP proteins (CEP152, CEP192, CEP215, CEP104) coordinate centriole duplication and microtubule nucleation. Centrin-2, PLK4, PLK1, and Aurora A localize to centrosomes to control spindle formation and cell-cycle transitions. Motor complexes, particularly dynein subunits (dynein heavy chain 11, dynein heavy chain 14, dynein intermediate chain 2) have established or emerging roles at the centrosome and contribute to microtubule dynamics or spindle architecture. Transporters such as Coatomer subunit beta and Multidrug resistance-associated protein 6 localize to mitotic spindles and potentially facilitate crosstalk or regulate centrosomal homeostasis. PCM- Pericentriolar material; γ -TuRC- γ -Tubulin ring complex; CDK – cyclin-dependent kinase; MTOC – Microtubule-organizing center; CEP – centrosomal protein; PLK-Polo-like kinase.

Centrioles are cylindrical structures within the centrosome, each composed of nine triplets of

microtubules arranged in a cylindrical pattern. The two centrioles in a centrosome are termed the mother and daughter centrioles. The mother centriole serves as a template for the assembly of the daughter centriole during the cell cycle. Centrioles are involved in nucleating and organizing microtubules, contributing to the formation of the mitotic spindle during cell division ^{136,138,139}.

Surrounding the centrioles, the PCM contains an array of proteins, including γ -tubulin, which acts as a nucleating factor for microtubules. Other PCM components include pericentrin, centrosomin, and numerous other regulatory proteins that contribute to the dynamic organization and function of the centrosome. As cells progress through the cell cycle, the centrosomes duplicate, and each pair organizes the microtubules that form the mitotic spindle during mitosis ^{140,141}.

Centrosomes undergo duplication precisely once during the G1 phase of the cell cycle. This process is tightly regulated to ensure the accurate segregation of genetic material during cell division. The duplication starts with the formation of a new daughter centriole adjacent to the pre-existing mother centriole ^{136–138}. During interphase, centrosomes are involved in microtubule nucleation and organization.

Mitosis is initiated with prophase, where it is characterized by increased microtubule instability. Cells develop dynamic microtubule arrays around duplicated centrosomes, which separate to form spindle poles, thus facilitating bipolar spindle assembly. Shortly after, prometaphase follows where the nuclear envelope breaks down, allowing condensed chromosomes to stabilize microtubules from spindle poles. Opposing microtubules create tension and align chromosomes at the metaphase plate where the metaphase spindle microtubules are highly dynamic. During anaphase, spindle poles move apart. Lastly, telophase sees reformation of nuclear envelope around separated chromosomes ^{132,142,143}.

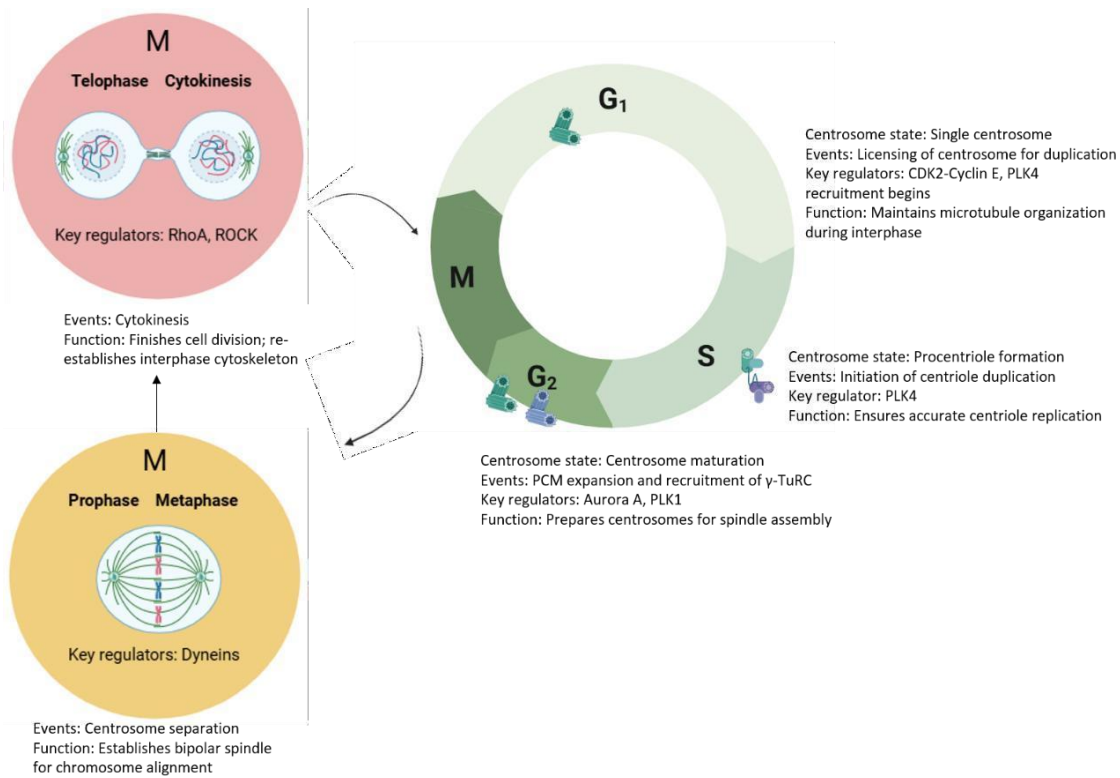


Figure 10: Centrosome cycle dynamics across the eukaryotic cell cycle

The centrosome undergoes a highly regulated series of structural and functional transitions tightly synchronized with the cell cycle. During G₁, cells contain a single centrosome composed of a mother–daughter centriole pair that maintains the interphase microtubule network and becomes “licensed” for duplication. In S phase, procentriole formation marks the onset of centriole duplication, driven by PLK4. Throughout G₂, centrosomes mature through expansion of the pericentriolar matrix and recruitment of γ -tubulin ring complexes, increasing microtubule nucleation capacity in preparation for mitosis. By M phase, the duplicated centrosomes separate to form the two spindle poles, enabling bipolar spindle assembly and accurate chromosome segregation. During cytokinesis, RhoA–ROCK–mediated constriction of the actin ring completes cell division, after which daughter cells inherit a single centrosome and re-enter G₁. (Created with BioRender)

1.8.1 RhoGTPases and centrosomes

Rho GTPases are key regulators of cellular processes, and their influence also extends to the organization and dynamics of centrosomes. RhoA, Rac1 and Cdc42 participate in the regulation of centrosome positioning within the cell (Figure 11). These GTPases influence the organization of the cytoskeleton, including actin filaments and microtubules, which, in turn, impact the movement and positioning of centrosomes^{22,144}.

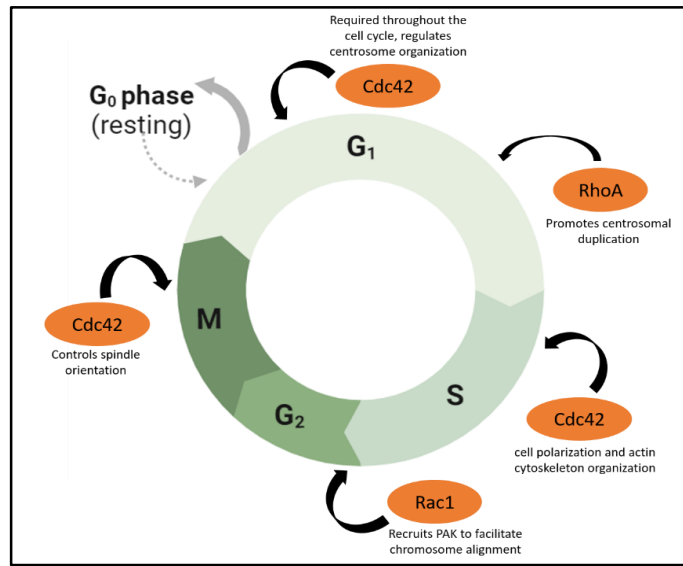


Figure 11: Schematic overview of Rho GTPase involvement during cell cycle progression

The eukaryotic cell cycle is divided into G₁, S, G₂, and M phases, with a reversible exit into the quiescent G₀ phase. Distinct members of the Rho GTPase family contribute to cell cycle-dependent processes. RhoA has been implicated in centrosome duplication and cytoskeletal regulation during G₁ and G₂ phases. Rac1 participates in chromosome alignment during mitosis, in part through recruitment of PAK kinases and regulation of microtubule–actin crosstalk. Cdc42 is required throughout the cell cycle and regulates centrosome organisation, spindle orientation during mitosis, and actin cytoskeleton polarisation during interphase. Together, these pathways coordinate cytoskeletal dynamics and centrosome-dependent events essential for accurate cell cycle progression. (Created with BioRender)

RhoA is associated with the promotion of centrosome duplication^{145,146}. In addition, RhoA/ROCK pathway has also been shown to be relevant in autosomal dominant polycystic kidney disease (ADPKD) patients, leading to dysregulated compartmentalized centrosomal RhoA signaling. This dysregulation involves specific RhoGAPs, including ARHGAP5, -29, and -35¹⁴⁷.

On the other hand, Rac1 associates with centrosomes during early G₂ phase until prometaphase. p21-activated kinase (PAK) is linked to G₂/M-phase centrosomes in a Rac1-dependent manner. PAK1-3 are serine/threonine kinases that act downstream of Rac1 and are essential for chromosome alignment. Inhibiting Rac1 activity suppresses activation of PAK1/2, in late G₂ phase/prophase, resulting in delayed mitotic entry^{148,149}. Lastly, active Cdc42 regulates centrosome

organization and function throughout interphase and mitosis. Cdc42 also controls the spindle orientation during mitosis ^{150–152}.

1.8.2 Different roles of centrosomes

Centrosomes play a central and crucial role in the formation of the mitotic spindle, a dynamic structure essential for the accurate segregation of chromosomes during cell division. As cells enter mitosis, the duplicated centrosomes become the two poles of the mitotic spindle (Figure 10). Microtubules extend from each centrosome, forming an intricate array that constitutes the spindle apparatus. The microtubules form a radial pattern from the centrosomes, creating the spindle fibers that will interact with and segregate the chromosomes ^{153,154}.

These microtubules also attach to the chromosomes at structures called kinetochores, ensuring proper alignment and segregation during later stages of mitosis ¹⁵⁵. The formation of the mitotic spindle is closely monitored by a regulatory mechanism known as the spindle assembly checkpoint (SAC). The SAC ensures that all chromosomes are properly attached to the spindle fibers before allowing the cell to progress to the next stages of mitosis. This checkpoint prevents premature separation of chromatids, promoting chromosomal stability ¹⁵⁶. Once all chromosomes are correctly attached, the spindle fibers contract, pulling the chromosomes apart (Figure 10). The centrosomes, now positioned at opposite poles of the cell, exert forces on the microtubules, directing the movement of chromosomes toward the poles. This process ensures the equal distribution of genetic material to the daughter cells ^{156,157}.

Moreover, centrosomes are integral to the formation and function of primary cilium, specialized microtubule-based protrusions on eukaryotic cell surfaces involved in sensory functions, signal transduction, and various physiological processes. The mother centriole within the centrosome plays a central role in initiating and organizing primary cilia. During the transition from the cell cycle to the G0 phase, the mother centriole transforms into the basal body, which serves as the foundation for the primary cilium's extension from the cell membrane ¹⁵⁸.

Furthermore, centrosomes play a multifaceted role in the immune response. Centrosomes contribute to the function of immune cells, such as cytotoxic T cells and natural killer NK cells. During an immune response, interactions between immune cells and target cells involve the formation of an immunological synapse, a specialized cell-cell junction (Figure 12B). The reorientation of the centrosome and associated microtubules helps guide cellular components, including secretory vesicles and lytic granules, toward the immunological synapse for efficient target cell recognition and

response^{163,164}. Centrosomes also contribute to the migration of immune cells to sites of infection or inflammation. This process is crucial for immune cell trafficking within tissues and effective immune surveillance^{165,166}.

Understanding the involvement of centrosomes in immune responses provides insights into the cellular mechanisms that govern effective immune surveillance and defense against pathogens. Figure 12 highlights the conserved architectural and trafficking mechanisms underlying ciliogenesis and immunological synapse formation, two well-characterized contexts of centrosome polarization.

1.8.3 Centrosome and Ciliogenesis

Ciliogenesis, the process of primary cilium formation, is initiated by docking of the mother centriole to the cell membrane, leading to the assembly of the ciliary axoneme, which is a microtubule-based structure that forms the cilium's backbone (Figure 12A)¹⁵⁹. The mother centriole acts as a template for organizing microtubules within the primary cilium, providing structural support and serving as tracks for intraflagellar transport (IFT) machinery that facilitates the bidirectional movement of ciliary components, including signaling proteins, receptors, and structural proteins, between the cilium base and tip¹⁶⁰. Primary cilia thereby function as specialized sensory and signaling organelles, contributing to cellular homeostasis by sensing and responding to changes in the extracellular environment through mechanosensing, chemosensing, and the transduction of signals linked to cell cycle progression and differentiation^{160–162}. Dysregulation of primary cilia is associated with a broad spectrum of developmental disorders and diseases collectively termed ciliopathies.

Among the signaling pathways coordinated by primary cilia, the Hedgehog (Hh) pathway occupies a central position, as the cilium constitutes the obligate spatial platform for its transduction in vertebrates. In the absence of Hh ligands, the receptor Patched-1 (PTCH1) localizes to the ciliary membrane and suppresses Smoothened (SMO), a G-protein-coupled receptor-like transducer, by preventing its ciliary accumulation. Concurrently, the GLI family transcription factors (GLI1, GLI2, GLI3) are processed into transcriptional repressor forms through partial proteolytic cleavage and are retained in the cytoplasm. Upon binding of Hh ligands (Sonic (SHH), Desert (DHH), or Indian (IHH) hedgehog) PTCH1 is displaced from the cilium, permitting SMO to accumulate in the ciliary membrane and triggering the translocation of full-length GLI complexes to the ciliary tip²⁰⁴. At the tip, GLI proteins are converted to their transcriptional activator forms and subsequently translocated to the nucleus, where they drive expression of target genes including *PTCH1*, *GLI1*, *CCND1* (cyclin D1), and pro-survival

factors. The ciliary tip therefore functions as the critical processing hub where the balance between GLI activator and repressor forms is established; loss of primary cilia in cancer cells uncouples this balance and can contribute to constitutive or aberrant Hh/GLI activity ¹⁶¹.

While primary cilia are broadly conserved across mammalian cell types, their structural properties, stability, and signaling repertoire differ substantially between cells of epithelial and mesenchymal origin, a distinction directly relevant to the interpretation of ciliogenesis data in osteosarcoma models. In polarized epithelial cells, primary cilia are typically long, structurally stable, and orientated apically, where they integrate flow-sensing cues (as in renal tubular epithelial cells), planar cell polarity (PCP) signals via non-canonical Wnt signaling, and growth factor receptor trafficking including that of the EGF receptor. Their formation is coupled to apical junction maturation and is favored by the low-RhoA, low-actin-polymerization state characteristic of quiescent, confluent epithelia ²⁰⁵. By contrast, in cells of mesenchymal origin, including fibroblasts, osteoblastic precursors, and mesenchymal-derived cancer cells, primary cilia are shorter, more transient, and more sensitive to perturbations of the actin cytoskeleton. Mesenchymal cilia are particularly associated with PDGFR- α and TGF- β pathway transduction, both of which regulate mesenchymal identity and motility ²⁰⁶. In this cellular context, F-actin stress fibers organized by RhoA–ROCK signaling actively compete with ciliary growth by anchoring the basal body and restricting axoneme extension; consequently, elevated RhoA activity is a well-established inhibitor of ciliogenesis in mesenchymal cells ^{145,198}.

The assembly, maintenance, and signaling function of the primary cilium depend on IFT, a bidirectional trafficking system driven by two families of molecular motors operating along the axonemal microtubule doublets. Anterograde transport, from the ciliary base toward the tip, is powered by heterotrimeric kinesin-2 in association with the IFT-B complex, delivering structural components, membrane receptors, and signaling molecules to the ciliary tip. Retrograde transport, from tip back to base, is driven by cytoplasmic dynein-2 (IFT-dynein), a motor complex distinct from the cytoplasmic dynein-1 that operates broadly in the cytoplasm ^{207, 208}. Dynein-2 is organized around a dedicated heavy chain (DYNC2H1) together with intermediate, light intermediate, and light chain subunits, and operates in concert with the IFT-A complex to return processed signaling components and IFT machinery to the cell body for recycling. Disruption of anterograde kinesin-2 function produces short, stunted cilia incapable of extending their axoneme, whereas loss of dynein-2 retrograde activity results in the accumulation of IFT particles within a swollen, dysfunctional cilium each representing distinct ciliopathic outcomes ²⁰⁹. Beyond IFT, cytoplasmic dynein-1 contributes to

centrosome positioning and basal body anchoring prior to ciliogenesis, establishing the spatial prerequisites for ciliary docking. The identification of multiple dynein heavy chain subunits as candidate RhoGDI2 interactors in our IP/MS analysis therefore raises the possibility that RhoGDI2 participates in the regulation of motor protein activity at the centrosome–basal body interface ²⁰⁷⁻²⁰⁹.

1.8.4 Centrosomes in cancer

Centrosomal dysfunction is increasingly recognized as a driver of tumorigenesis, contributing to chromosomal instability, aberrant cell division, and malignant progression. Genomic instability is a hallmark of cancer and can contribute to the acquisition of mutations and the development of malignant phenotypes ^{167,168}.

Many cancer cells exhibit centrosomal abnormalities, including centrosome amplification (excess centrosomes) or centrosome clustering, which can lead to the formation of multipolar spindles during cell division inducing errors in chromosome segregation, aneuploidy (abnormal chromosome number) and genomic instability ¹⁶⁹. All of these can contribute to tumor heterogeneity, as cancer cells with different centrosomal configurations can exhibit distinct cellular behaviors, which leads to tumor heterogeneity, a critical factor in cancer progression, responses to therapy, metastatic potential and overall clinical outcomes ¹⁷⁰⁻¹⁷⁴.

In addition, lack of proper checkpoint control can contribute to the survival and proliferation of genetically aberrant cells. Chromosome clustering can perturb microtubule and actin dynamics in ways that disrupt cell polarity or alter mechanical properties, which may promote or impair migration depending on the cellular context. Cancer cells with abnormal centrosomes may display enhanced motility, promoting metastasis to distant tissues ^{170,175}.

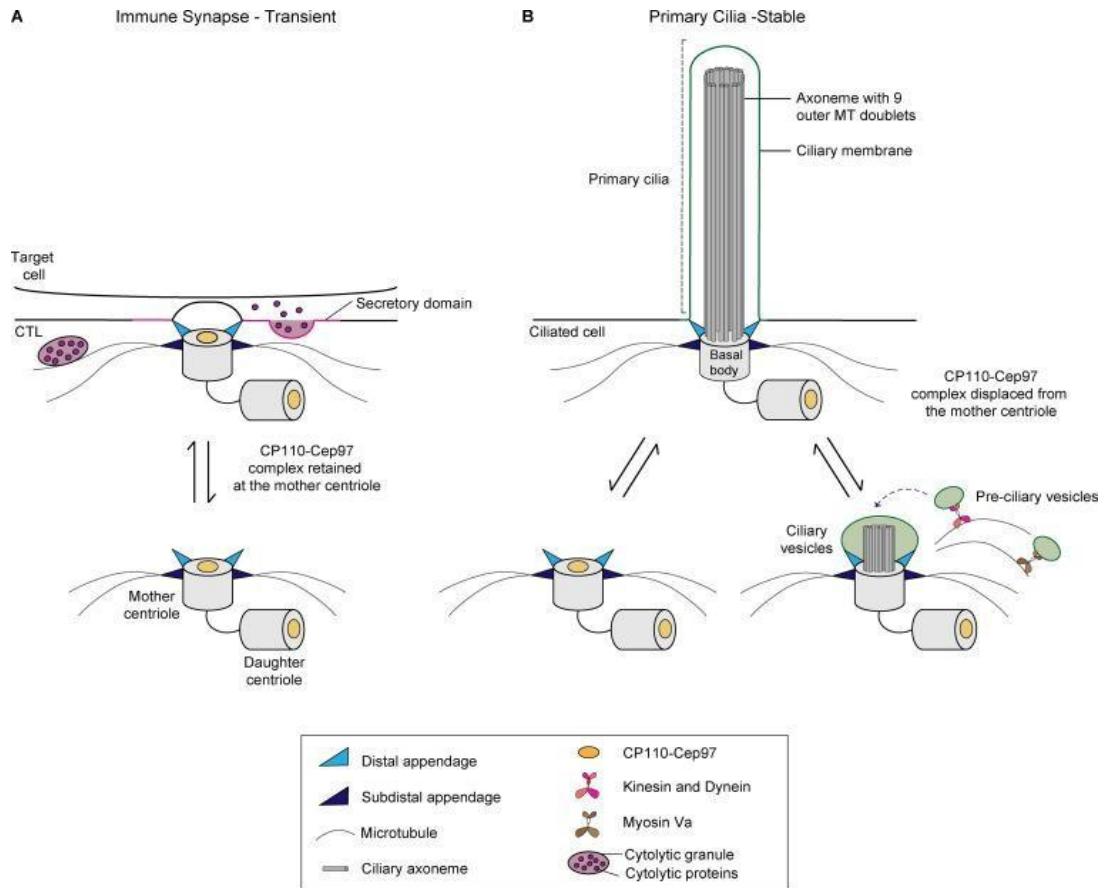


Figure 12: Centrosomal docking: immune synapse vs. primary cilia

A. Immune synapse formation in cytotoxic T lymphocytes (CTLs). Upon contact with a target cell, the mother centriole, the older of the two centrioles forming the centrosome, docks at the plasma membrane via specialised anchor structures called distal appendages. Microtubules extend from the mother centriole, forming tracks along which cytolytic granules are delivered to the contact site. Throughout this process, the CP110–Cep97 complex remains at the centrioles, preventing premature axoneme growth. This docking event is transient.

B. Primary cilia formation. In response to environmental or developmental signals, the mother centriole transforms into a basal body and docks at the plasma membrane, again via distal appendages. Microtubules then extend outward to form the axoneme, the internal scaffold of the cilium. Unlike during immune synapse formation, the CP110–Cep97 complex must be displaced from the mother centriole to allow axoneme extension. This event is relatively stable. MT, microtubule. (Figure taken from Douanne T, Stinchcombe JC, Griffiths GM, 2021)

The association between centrosomal abnormalities and cancer progression has led to the exploration of centrosome-related targets for cancer therapy^{169,171,174}. Targeting centrosomal proteins (CEPs) or pathways involved in centrosome regulation is an active area of research with the aim of developing novel therapeutic strategies to selectively inhibit the growth of cancer cells. Shan *et al* showed that CEP290 knockdown in Hep3B liver cancer cells led to reduced proliferation, migration, and invasion. *In vivo* experiments demonstrated that suppressing CEP290 effectively

inhibited tumor growth ¹⁷⁶.

A recent study showed that overexpression of CEP55 induced spontaneous tumorigenesis and expedited tumors *in vivo*. In mouse embryonic fibroblasts (MEFs), CEP55 overexpression was shown to increase proliferation by influencing multiple cellular signaling networks, including hyperactivation of the PI3K/Akt pathway ¹⁷⁷. In addition, CEP55 is enriched in hepatocellular carcinoma (HCC) tumor cells (as observed in patient HCC tissues and cultured HCC cell lines), and its overexpression has been linked to unfavorable overall survival and increased recurrence ¹⁷⁸.

Moreover, CEP120 overexpression was found to induce centrosome amplification in gastric cancer (GC) *in vitro* and *in vivo* experiments. The study revealed that CEP120 facilitates centrosome amplification and GC progression by enhancing the expression and centrosome aggregation of the deubiquitinating enzyme USP54 ¹⁷².

Lastly, CEP70 emerged as a crucial mediator in breast and pancreatic cancers, influencing various aspects of tumor progression. In breast cancer (BC), CEP70 is up-regulated, promoting cell proliferation, colony formation, migration, and invasion. Additionally, it enhances BC lung metastasis. CEP70 also plays a role in paclitaxel sensitivity in BC and interacting with tubulin to modulate microtubule assembly ^{179–181}. In pancreatic cancer, CEP70 is highly expressed and has been reported to promote cell proliferation, colony formation, and tumour growth, while reducing apoptosis. Moreover, CEP70 dysregulation is associated with centrosome abnormalities, microtubule disorganization, and altered mitotic spindle formation ^{182,183}. Understanding the mechanisms underlying centrosomal dysfunction in cancer provides insights into the molecular basis of tumorigenesis and opens avenues for the development of targeted cancer therapies.

2. Objectives

Rho GTPases are a family of small GTP-binding proteins that regulate various cellular processes essential for cell morphology, motility, and signaling. They orchestrate dynamic changes in the actin cytoskeleton, thereby influencing cell shape and movement. In addition to their roles in cytoskeletal dynamics, Rho GTPases also govern diverse cellular functions such as cell adhesion, proliferation, gene expression, and vesicle trafficking. As of now, approximately 85 RhoGEFs and 66 RhoGAPs have been discovered for nearly 21 members of the Rho GTPase family.

Although only three RhoGDIs have been identified, they are however key players in the RhoGTPases regulatory pathways by their capacity to inhibit RhoGTPases activity while also protecting them from degradation. RhoGDI1 and 2 are the most abundant and widely expressed, and they display high similarities in terms of amino acid sequence and domain composition. Although they share some common and redundant functions, they appear to be differently implicated in cancer progression. While RhoGDI1 has been, and continues to be, the subject of numerous studies, RhoGDI2 remains much less characterized. Our goal was therefore to acquire new knowledge about its various functions, particularly in the context of cancer.

The overarching objective of this thesis was to clarify context-dependent roles of RhoGDI2 in cancer and to define functional differences between RhoGDI1 and RhoGDI2.

To address this, the thesis pursued the following aims:

1. Identify candidate RhoGDI2-associated proteins detected by immunoprecipitation coupled to mass spectrometry.
2. Evaluate the functional consequences of reducing RhoGDI2 expression in cancer cell models, specifically on cell proliferation and mitotic fidelity.
3. Determine whether RhoGDI2 depletion disrupts centrosomal and mitotic processes, including centrosome number and chromosome congression.
4. Assess the impact of RhoGDI2 depletion on ciliogenesis and test involvement of key Rho GTPases.
5. Examine the role of RhoGDI2 in immune effector function, by testing whether RhoGDI2 depletion affects the tumor-killing capacity of NK-92 natural killer cells (as a proxy for immune synapse-dependent cytotoxicity).

Objectives

6. Explore isoform-specific determinants of RhoGDI function, by generating and testing N-terminal domain-swapped chimeric RhoGDI1/2 constructs for their ability to rescue phenotypes observed upon RhoGDI2 silencing.

3. Results

This “Results” section is organized as follows:

- It starts with a careful description the IP/MS experiment (Immunoprecipitation followed by Mass Spec analysis). Indeed, the data that resulted from this proteomic approach were at the origin of this work, even though they are only briefly summarized in our published article.
- The main body of this section consists of our published article.
- Finally, data that have not been published or are still preliminary constitute the last part of this section, in order to provide a current and comprehensive overview of the work.

3.1 Multiple centrosomal proteins potentially interact with RhoGDI2

3.1.1 IP/MS to identify RhoGDI2 interactors.

To identify proteins potentially associated with RhoGDI2, we had first to generate a dedicated cell model in order to circumvent the absence of an efficient specific antibody for RhoGDI2. The coding sequences for RhoGDI2 containing a “M2-tag” at either its N- or its C-terminus were inserted into the pcDNA™4 TO/Zeo (a mammalian expression vector used to drive constitutive expression using a CMV promoter). The reason RhoGDI2 was expressed in two labeling configurations was to minimize the risk of artifacts related to marker position and, potentially, to increase confidence in future results (when the same “ligand” is identified in both experimental conditions).

These constructs (and an empty vector used as negative control) were then transfected into U-2 OS cells (osteosarcoma, of mesenchymal origin). After selection in a 10 % FBS complete culture medium containing 200 µg/mL zeocin, subcloning was performed to isolate clones expressing high levels of “M2-tagged” RhoGDI2, while maintaining a phenotype similar to that of the parental cells. Two clones were selected for further experiments: the “RhoGDI2 M2” and the “M2 RhoGDI2” with the tag at the C- or the N-terminus, respectively. For the “immuno-precipitation” experimental step, 10⁷ cells in monolayers were extensively washed and then lysed (Tris 50 mM, pH 7.0, 150 mM NaCl, protease inhibitors, 0.1 % Triton X100). The extracts were then centrifuged to remove cell debris and the supernatant were then incubated (4°C, 16 hours) with anti-flag M2 agarose beads under agitation. The bead pellets were then washed and the bound proteins were eluted (30% acetonitrile and 100 mM acetic acid, or 100 mM acetic acid alone) to be identified by Mass Spec (Laboratory of Prof M Fillet, CIRM) (see figure 13).

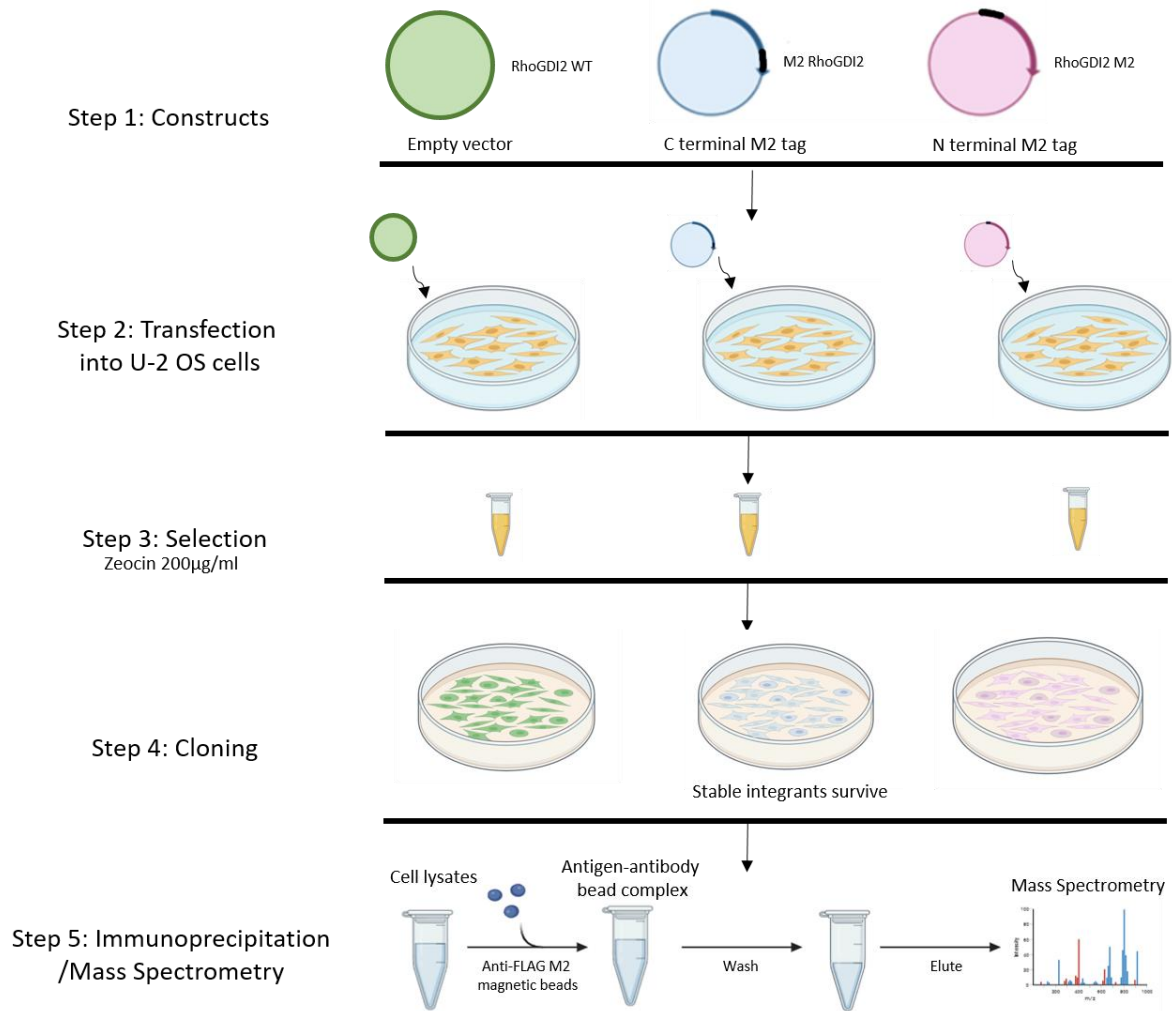


Figure 13. Workflow of RhoGDI2 IP/MS used to identify candidate RhoGDI2-associated proteins

U-2 OS cells were transfected with plasmids encoding N-terminally M2-tagged RhoGDI2 (M2 RhoGDI2), C-terminally M2-tagged RhoGDI2 (RhoGDI2 M2), or an empty vector as a negative control. Stable cell lines were established by selection with 200 µg/ml zeocin, followed by single-cell cloning and amplification. For immunoprecipitation, cells were lysed under native conditions and clarified lysates were incubated with anti-M2 affinity beads. After stringent washing, co-precipitated proteins were eluted by M2 peptide competition and subjected to mass spectrometry (MS). Candidate interactors were defined as proteins enriched in M2-tagged conditions relative to the empty vector control and filtered against the Crapome contaminant repository (716 controls). M2, FLAG-M2 epitope tag; RhoGDI2, Rho GDP-dissociation inhibitor 2; WT, wild-type. (Created with Biorender)

3.1.2 Key RhoGTPases are observed in IP/MS.

Mass spectrometry output was used to generate lists of proteins detected in each immunoprecipitate, together with quantitative or semi-quantitative MS evidence (e.g., spectral counts and numbers of unique peptides) supporting their identification. Additional filtering was made. Only proteins absent in our negative controls (cells not expressing "tagged"-RhoGDI2) and not listed as common contaminants in immunoprecipitation experiments (CRAPome 2.0 – Contaminant Repository for Affinity Purification – Mass Spectrometry data) were taken into account.

The results of this comprehensive analysis revealed the presence of known RhoGDI2 interactors such as Rac1, Rac2, Cdc42, RhoA, RhoB, and RhoC, thus validating the efficacy of the experimental procedure (Table 3). RhoG was also identified for the first time as a potential interacting partner, but only with the RhoGDI2 M2 clone. Confirmatory studies were therefore performed by Western blotting experiment.

Results

RhoGDI2 M2 (C- terminal RhoGDI2)	M2 RhoGDI2 (N- terminal RhoGDI2)	# Spectra	# Unique Peptides	% Coverage	Score	Accession #	Entry_name	Crapome (out of 716)
v	v	86	22	87.5	276.2	P52566	Rho GDP dissociation inhibitor 2	4
RhoGTPases								
v	v	59	11	70.0	146.0	P61586	RhoA	87
v	v	61	11	70.0	141.0	P08134	RhoC	77
v	v	13	3	28.5	37.0	P62745	RhoB	46
v	v	4	4	28.0	54.0	P84095	RhoG	14
v	v	26	9	34.8	99.7	P63000	Rac1	58
v	v	15	4	18.2	46.2	P15153	Rac2	43
v	v	40	6	41.3	83.0	P60953	Cdc42	165
Dynamics of the microtubule network and centrosomes								
v	v	3	3	12.2	21.6	Q9GZS0	Dynein intermediate chain 2	2
v	v	5	4	1.8	36.2	Q8TD57	Dynein heavy chain 3	2
v		2	2	0.8	16.4	Q8WXX0	Dynein heavy chain 7	0
v	v	3	3	1.8	16.9	Q96JB1	Dynein heavy chain 8	2
v	v	4	4	2.4	23.1	Q9NYC9	Dynein heavy chain 9	1
v	v	6	6	3.1	49.9	Q81VF4	Dynein heavy chain 10	0
v	v	4	4	2.4	24.1	Q96DT5	Dynein heavy chain 11	4
v	v	4	3	1.9	28.4	Q6ZR08	Dynein heavy chain 12	6
v	v	3	3	1.7	21.6	Q0VDD8	Dynein heavy chain 14, axonemal	3
v	v	4	3	0.6	18.9	Q9UPN3	Microtubule-actin cross- linking factor 1	113
v		5	4	6.1	30.3	Q5JWR5	Protein-dopey 1	4
v	v	4	4	5.4	27.4	Q5TB80	Centrosomal protein 162	1
v	v	4	4	7.0	23.8	Q8TEP8	Centrosomal protein 192	44
v	v	2	2	5.0.	18.4	Q60308	Centrosomal protein 104	6
v	v	2	2	7.9	19.3	Q5SW79	Centrosomal protein 170	145
v	v	3	3	4.2	21.4	Q8NDM7	Cilia- and flagella- associated protein 43	0
v	v	2	2	6.6.	17.2	Q92674	Centromere protein 1	1
x	v	4	3	4.0	20.5	Q9P265	Disco-interacting protein 2 homolog B	12

Table 3: Interactome of RhoGDI2 identified by co-immunoprecipitation coupled to mass spectrometry (IP/MS)

Proteins co-purified with RhoGDI2 (P52566) from U-2 OS clones with M2 tagged at N- or C- terminal of RhoGDI2 were identified by MS and are listed with the number of spectra, unique peptides, percent sequence coverage, Mascot score, and UniProt accession number. The CRAPome score indicates the frequency of detection across 716 control IP/MS experiments, serving as a measure of non-specific background. Interactors are grouped into functional categories: RhoGTPases (RhoA, RhoB, RhoC, Rac1, Rac2, CDC42), confirming the known biology of RhoGDI2 as a cytosolic chaperone for Rho family GTPases wherein we also identified RhoG for the first time; and dynamics of the microtubule network and centrosomes, comprising dynein heavy and intermediate chains, centrosomal proteins, and associated factors, pointing to a putative interaction of RhoGDI2 with the centrosomal machinery. v: detected in the corresponding cell line; x: not detected.

3.1.3 RhoG co-precipitates with RhoGDI2 in U-2 OS cells

Immunoprecipitations were carried out using an anti-M2 antibody. Cell lysates and immunoprecipitates were analyzed by immunoblotting. Mock immunoprecipitations from U-2 OS cells transfected with empty vector served as negative controls and showed no detectable M2 signal (Figure 14). Endogenous RhoG was not detected in mock IPs, consistent with the IP/MS data. In contrast, RhoG was detected in anti-M2 immunoprecipitates from cells expressing RhoGDI2 labeled with M2 at the C-terminus, and much less in cells expressing RhoGDI2 labeled with M2 at the N-terminus. These data confirmed the efficacy and reliability of our IP/MS experimental procedure and identified RhoG as an additional binding partner of RhoGDI2.

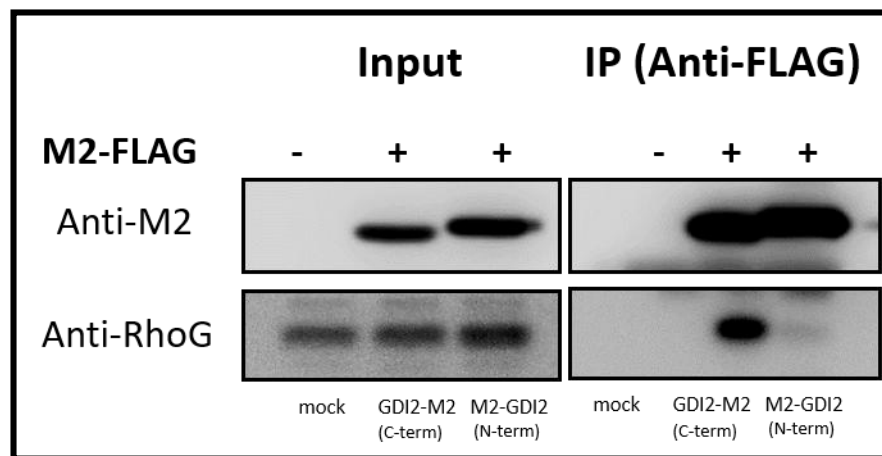


Figure 14: RhoG co-precipitates with RhoGDI2 in U-2 OS cells

U-2 OS cells were left untransfected (mock) or transfected with RhoGDI2 carrying a M2 FLAG tag at the N- or C-terminus. Immunoprecipitations were performed using an anti-M2 antibody. Cell lysates (input) and immunoprecipitates were analyzed by immunoblotting using antibodies against RhoG and M2 (RhoGDI2). Mock immunoprecipitations served as negative controls. Data are representative of three independent biological replicates.

Other factors related to GTPases (such as GEFs and GAPs, and downstream effectors) were also identified in the immune-precipitated fractions. However, the most “enriched” category of factors was related to “tubulin” and its dynamic functioning, which could indicate additional functions of RhoGDI2. Due to its novelty and relevance in cancer, particular attention was then paid to potential tubulin-related roles of RhoGDI2. Description of these original data were recently published (see the next chapter).

3.2 Reduced RhoGDI2 expression disrupts centrosomal functions and promotes mitotic errors

Article

Reduced RhoGDI2 Expression Disrupts Centrosome Functions and Promotes Mitotic Errors

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Abstract

RhoGDI2 is a RhoGTPase regulator that has roles in cytoskeleton organization and cell survival, amongst others. It is differentially expressed in many cell types and tissues, including several human cancers, where its expression has been correlated with either good or bad prognosis. To identify the underlying mechanisms, we knocked down its expression in human cancer cell lines. We observed that repression of RhoGDI2 expression, but not that of the closely related RhoGDI1, significantly reduces their proliferation rate. In parallel, RhoGDI2 suppression induces supernumerary centrosomes and inhibits ciliogenesis. As RhoGDIs are regulators of GTPases, we checked whether key RhoGTPases are involved in these effects. We found that silencing RhoA partially rescued the induction of supernumerary centrosomes and ciliary defects observed upon RhoGDI2 silencing. It was previously shown that RhoGDI2 is strongly expressed in immune cells and that there are striking similarities between primary cilia and immune synapses. Based on this knowledge, we silenced RhoGDI2 in NK cells and could demonstrate that this strongly affects their immune synapse-related cancer cell-killing activity. Altogether, these data suggest novel roles for RhoGDI2 in centrosome functions in human cancer and immune synapses in immune cells, which provides an explanation for its reported dual role in cancer.

Keywords: RhoGTPases; centrosomes; primary cilia; chromosome congression; immune synapse



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1. Introduction

The small GTPases of the Rho family (RhoGTPases) are well known for their regulatory roles on the actin cytoskeleton and microtubule organization, which affect several main cellular characteristics and functions like morphology, migration, and mitosis. They cycle between active (bound to GTP) and inactive (bound to GDP) forms [1]. Three types of factors regulate this process. Guanine nucleotide exchange factors (GEFs) activate RhoGTPases by allowing the exchange of GDP for GTP, which, consequently, drives the activation of downstream effectors. GTPase-activating proteins (GAPs) stimulate the intrinsic GTPase activity of the RhoGTPases, hence increasing the hydrolysis of GTP into GDP and, therefore, suppressing downstream signaling. Guanine nucleotide dissociation inhibitors (GDIs) extract the RhoGTPases from the membrane and sequester the GDP-

bound form of GTPases in the cytosol, protecting them from degradation by enclosing their c-terminal prenyl group but also preventing their activation [2–4].

While there are tens of GEFs, GAPs, and downstream effector signaling molecules for RhoGTPases, there are only three RhoGDI [1]. RhoGDI1 (also known as RhoGDI α) is expressed ubiquitously and usually found at high concentrations. It has affinity and interacts with most RhoGTPases. The expression of RhoGDI3 (also known as RhoGDI γ) is usually very low, except in the nervous system. In physiological conditions, RhoGDI2 (also called RhoGDI β , LyGDI, or D4-GDI) is largely expressed by hematopoietic cells [5], although lower expression can be found in most tissues, suggesting it could have roles extending beyond the hematopoietic lineage. It binds to the same RhoGTPases as RhoGDI1, but with some different affinities [6].

In comparison to RhoGDI1, RhoGDI2 has some specific functions. It was notably reported that it can increase the activity of Rac1 via its interaction with the GEF VAV1 [4] but also by promoting interaction between Rac1 and filaminA [7]. This demonstrates that RhoGDI2 regulates specifically different signaling pathways through mechanisms that are not shared with RhoGDI1. The role of RhoGDI2 in cancer has been evaluated but has led to puzzling observations that it can promote or repress tumor progression and/or metastasis, depending on the cancer types and stage [8,9].

Here, we analyzed the role of RhoGDI2 by repressing its expression in two cancer cell lines. We report that its siRNA-mediated silencing reduces cell proliferation and significantly alters primary cilia formation, a cellular function orchestrated by the centrosome. Silencing of RhoGDI2 significantly increased RhoA activity, and RhoA silencing reversed the inhibition of primary cilia formation observed after RhoGDI2 knockdown. Finally, we observed that RhoGDI2 silencing in natural killer cells inhibited their cell killing activity, a function mediated by the immune synapse, a functional structure exhibiting striking similarities with the primary cilium.

2. Materials and Methods

2.1. Reagents and Cells

The U-2 OS and MG-63 human osteosarcoma cell lines were obtained from ATCC. The NK-92 natural killer cell line was a kind gift from the Laboratory of Hematology, GIGA-I3, University of Liege. U-2 OS and MG-63 cells were cultured in DMEM (#L0103-500, VWR, Leuven, Belgium) supplemented with 7% fetal bovine serum (FBS) (#10270-106, Gibco, Fisher Scientific, Merelbeke, Belgium). NK-92 cells were cultured in RPMI 1640 (VWR, #L0498-500) supplemented with 10% FBS, 25 mM HEPES, and 5 ng/mL of human recombinant cytokine IL-2 (#124-10CB-29, Proteintech, Manchester, UK). For experiments with co-cultured NK-92 and MG-63 osteosarcoma cells in Incucyte, the cells were co-cultured in Ham's F-12K medium (Gibco, #21127022) with 7% FBS supplementation. All media were supplemented with penicillin-streptomycin (100 units/mL each) and Fungizone (250 ng/mL), both from Fisher Scientific. Bisbenzimidazole H 33258 was from Calbiochem (Merck, Overijse, Belgium). Ro-3306 was from SelleckChem (#S7747). Mouse anti-M2 FLAG® (#F3165) was from Sigma (Diegem, Belgium). Mouse anti-RhoA (#sc-418) was from Santa Cruz Biotechnology (BioConnect, Huissen, The Netherlands). Mouse anti-Rac1 (23A8) was from Upstate Biotechnology (Sigma). Mouse anti-Cdc42 (610929) was from BD Biosciences (Erembodegem, Belgium). Rabbit anti-ERK1/2 (#M-5670) was from Sigma. The secondary horseradish peroxidase conjugated anti-mouse IgG (#7076) and goat anti-rabbit IgG (#7074) were from Cell Signaling Technology (Bioké, Leiden, The Netherlands). Rabbit and mouse anti- γ -tubulin (#T5192 and #T6557, respectively) and mouse anti- α -tubulin (#T6199) were purchased from Sigma. Rabbit anti-Arl13b (#17711-1-AP) and CoraLite® Plus 488-conjugated Centrin 1 Monoclonal antibody (#CL488-68433) were from Proteintech.

2.2. siRNA Transfection and Nucleofection

siRNAs chemically synthesized, desalted, deprotected, and PAGE-purified were from Eurogentec (Liege, Belgium). The sequence of the siRNAs targeting RhoA, Rac1, Cdc42, and RhoGDI1 and of the control siRNA (siScr1) were described and validated previously [10,11]. The sequences of siRNA used for repressing RhoGDI2 expression were as follows: siRhoGDI2(A) (5'-CAGCUGGGUCCCUUCAATT-3' and 5'-UUGAAGAGGGACCCAGCUGTT-3'); siRhoGDI2(B) (5'-GAGCUGGACAGCAAGCUCATT-3' and 5'-UGAGCUUGCUGUCCAGCUCTT-3'); and siRhoGDI2(C) (5'-GCCUGAAAUACGUUCAGCATT-3' and 5'-UGCUGAACGUUUUCAGGCTT-3'). Each pair of oligonucleotides was annealed at a concentration of 20 μ M in 50 mM NaCl, 1 mM EDTA, 10 mM Tris-HCl pH 7.5. siRNA transfection was carried out as described previously [12]. Briefly, calcium phosphate-mediated transfection was performed overnight (14–16 h) on subconfluent cells at a final concentration of 20 nM siRNA. Cells were washed twice with PBS and once with complete medium, with this last step being defined as time 0 post-transfection.

siRNA knockdown in NK-92 cells was performed using the Lonza 4D-nucleofector® X unit (AAF-1003X, Lonza, Verviers, Belgium) based on the manufacturer's recommendation. Briefly, 5×10^6 NK-92 cells were placed in 100 μ L electroporation solution with 20 nM siRNA and nucleofected using Nucleofector Program CA-137. Post-nucleofection, NK-92 cells were cultured in complete medium supplemented with 5 ng/mL IL-2 and allowed to recover for 24 h.

2.3. Western Blotting

Western blotting was carried out as previously described [13]. Briefly, cells were lysed in SDS-PAGE lysis buffer, and proteins were separated by polyacrylamide gel electrophoresis. Proteins were transferred to a Protran™ nitrocellulose transfer membrane (Perkin Elmer, Mechelen, Belgium). Membranes were then blocked and incubated overnight with diluted primary antibody following the manufacturer's instructions. Membranes were then washed three times, incubated in the diluted secondary horseradish peroxidase-conjugated antibody for 1 h, and revealed by chemiluminescence using a homemade ECL kit. The membranes were re-probed with an anti-Erk1/2 antibody to obtain protein loading normalization data.

2.4. Real-Time Quantitative PCR

Real-time quantitative PCR was carried out as previously described [13]. Briefly, total RNA was isolated using a High Pure RNA Isolation Kit (#11828665001, Roche Molecular Biochemical, Vilvoorde, Belgium) following the manufacturer's instructions. Five hundred nanograms of total RNA were reverse transcribed using SuperScript III Reverse Transcriptase (Invitrogen, Fisher Scientific). Real-time qPCR was performed in a final volume of 20 μ L containing 2 μ L of cDNA (corresponding to 10 ng of total RNA), 300 nM of each primer, and 10 μ L of the qPCR MasterMix Plus for SYBR® Green (Eurogentec) in a StepOne™ Real-Time PCR system (Applied Biosystems, Halle, Belgium). The results were analyzed with StepOne™ Software v2.1 and normalized to the level of the GAPDH transcript. The primers used for qPCRs are listed in Supplementary Table S1.

2.5. Proliferation Assay

Cells were seeded in 24-well costar plates immediately after transfection with siRNA and collected at different time points to determine the DNA content by a fluorimetric technique [14] as previously described [15]. Briefly, cell layers were rinsed three times with saline and homogenized in 600 μ L of PBS by sonication (20 s/well). A total of 100 μ L of each sample was transferred into a 96-well plate and supplemented with 100 μ L of

bisbenzimidazole solution (200 µg/mL bisbenzimidazole, 4 M NaCl, 20 mM NaH₂PO₄, pH 7.4). In each plate, a standard curve of DNA (from 2 µg to 0.03 µg) was included. After plate agitation for 5 min, fluorescence was read in a microplate spectrofluorometer SpectraMax Gemini XS (Molecular Devices, Sunnyvale, CA, USA) with an excitation wavelength of 355 nm and an emission wavelength of 460 nm.

2.6. BrdU Incorporation Assay

For these analyses, the BrdUCell Proliferation ELISA kit (colorimetric) from Abcam (Amsterdam, The Netherlands) (Ab126556) was used. MG63 cells were seeded in 96-well costar plates immediately after transfection with siRNA. A total of 8 h after seeding, BrdU was added to the culture medium, and the cells were maintained in culture for 16 h. Then, the cells were fixed and processed following the manufacturer protocol.

2.7. Immunoprecipitation and Mass Spectrometry Analysis

N-terminal and C-terminal M2-flagged-humanRhoGDI2 sequences were generated by RT-PCR and cloned in pcDNA4/T0. The primers used are listed in Supplementary Table S2. U-2 OS cells were transfected with these vectors and with empty pcDNA4/T0, and resistant clones were selected with Zeocin. 10⁷ U-2 OS ctr or cells expressing either N-terminally or C-terminally flagged RhoGDI2 were lysed. Lysates were immunoprecipitated with anti-M2 FLAG[®] antibody. The immune complexes were then subjected to mass spectrometry analysis following a previously published procedure [16]. The number of replicates per condition is *n* = 2. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE [17] partner repository with the dataset identifier PXD069111.

2.8. GTPase Activity Assay

The assay was carried out as previously described [12,18]. Briefly, cells were chilled on ice and lysed in a lysis buffer (ice-cold buffer containing 1% Triton X-100, 25 mM HEPES pH 7.3, 150 mM NaCl, 4% glycerol, 0.1 mM AEBSF, 4 µg/mL aprotinin). Lysates were centrifuged for 6 min at 16,000 × *g*. Supernatants were immediately frozen in liquid nitrogen and stored at -80 °C until used. An aliquot of each supernatant collected before freezing was denatured in SDS-PAGE lysis buffer to measure the total RhoGTPase content by Western blotting. For pull-down assays, supernatants were incubated for 30 min with 30 µg of GST-CRIB protein containing the Rac binding region or 30 µg of GST-RBD protein containing the RhoA binding region of Rhotekin affinity-linked to glutathione-Sepharose beads. The beads were washed 4 times in lysis buffer and boiled in 60 µL SDS-PAGE lysis buffer.

2.9. Immunofluorescence

Cells were seeded on coverslips in a twelve-well plate at 60% confluence. Cells were treated as described in Section 3 for the respective experiments. Post-treatment, the medium was removed, and cells were permeabilized at 37°C with 0.5% Triton X-100 in 4% paraformaldehyde for 7.5 mins. The permeabilization buffer was aspirated, and cells were washed twice with PBS. Cells were then fixed in 4% paraformaldehyde for 7.5 min, washed twice with PBS, and then blocked for 1 h in PBS supplemented with 5% bovine serum albumin (BSA, Sigma). Immunostainings were performed overnight at 4 °C in PBS+1% BSA (washing buffer) with the indicated antibodies. The coverslips were then washed 3× in washing buffer for 5 min and incubated (for 1 h, in the dark) with fluorescent secondary antibodies diluted in washing buffer (1:1000, goat anti-mouse Alexa-Fluor 488 nm #A21121, or goat anti-rabbit Alexa-Fluor 555 nm #A21429, Fisher Scientific, Merelbeke, Belgium). Cells were washed three times with washing buffer and labelled with a 1:1000 dilution of 4',6-diamidino-2-phenylindole (DAPI) for 20 mins. The

slides were washed once with PBS and once with water and mounted onto coverslips with DAKO fluorescent mounting medium (#S3023, Agilent Technologies, Diegem, Belgium). The coverslips were mounted onto microscope slides, and images were obtained with a Confocal Zeiss LSM880 AiryScan Elyra S1 with a 63x objective.

2.10. Tumor Cell Killing Assay

A total of 2000 target cancer cells (MG-63) were seeded per well of a 96-well plate. The next day, the medium was replaced by 50 μ L of Ham's F-12 K medium (to prevent fluorescence background) containing 20 μ M of Caspase 3/7 green dye (#4440, Sartorius AG, Göttingen, Germany). Effector immune cells (NK-92) (previously nucleofected with either siScr1, siRhoGDI1, or siRhoGDI2) were then added in a 1:1 ratio (final volume of 200 μ L). The plates were then incubated at 37 °C, 5% CO₂ for 30 mins before being placed into the Incucyte Live-Cell Analysis System (Sartorius AG) for 24 h. We repeated scanning at 1 h intervals.

3. Results

3.1. RhoGDI2 Silencing Repressed Cell Proliferation

RhoGDI1 and RhoGDI2 are both expressed in MG-63 and U-2-OS osteosarcoma cells, but at different levels as assessed by RT-qPCR (respectively 22C_T and 27C_T). RhoGDI2 was silenced by RNAi to evaluate its importance for the cell phenotype. As observed by RT-qPCR, RhoGDI2 and RhoGDI1 (used as functional control) expressions were strongly and specifically repressed 48 h after transfection with their respective siRNA in MG-63 and U-2 OS (Figure 1A,B), which lasted for at least 5 days.

The proliferation rate of both cell lines was assessed after 3 and 5 days, as compared to "day 0" (end of siRNA transfection). RhoGDI2 suppression decreased the proliferation rate in both cell lines by approximately twofold as compared to the control (siScr1). Remarkably, the silencing of RhoGDI1 had no significant effect (Figure 1C,D), clearly demonstrating that RhoGDI2 has specific functions that are not shared with RhoGDI1.

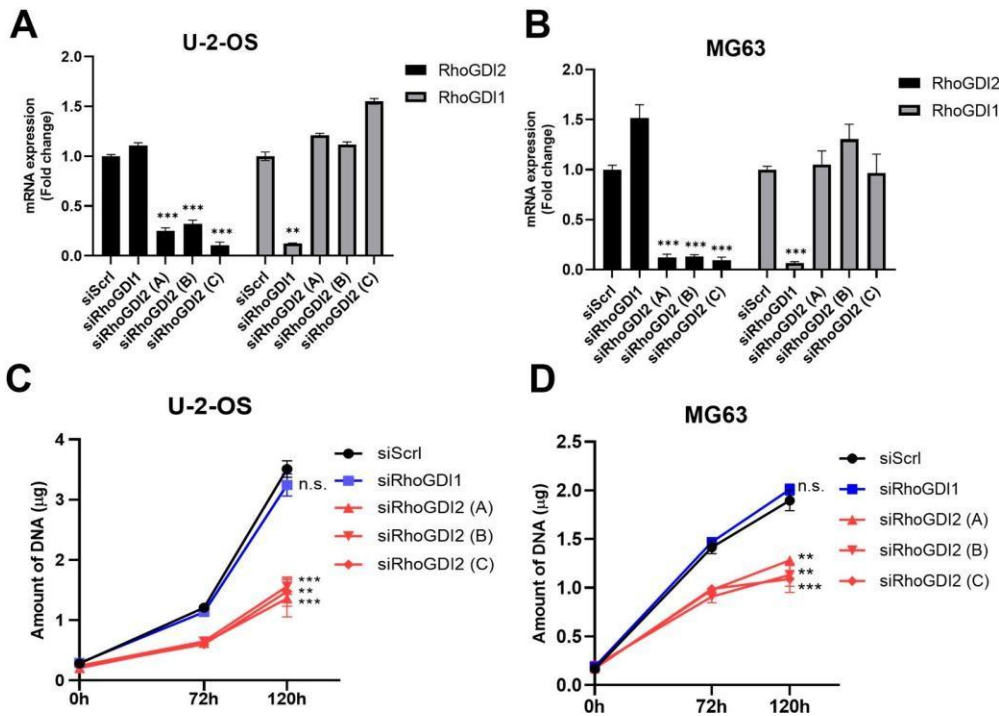


Figure 1. Cont.

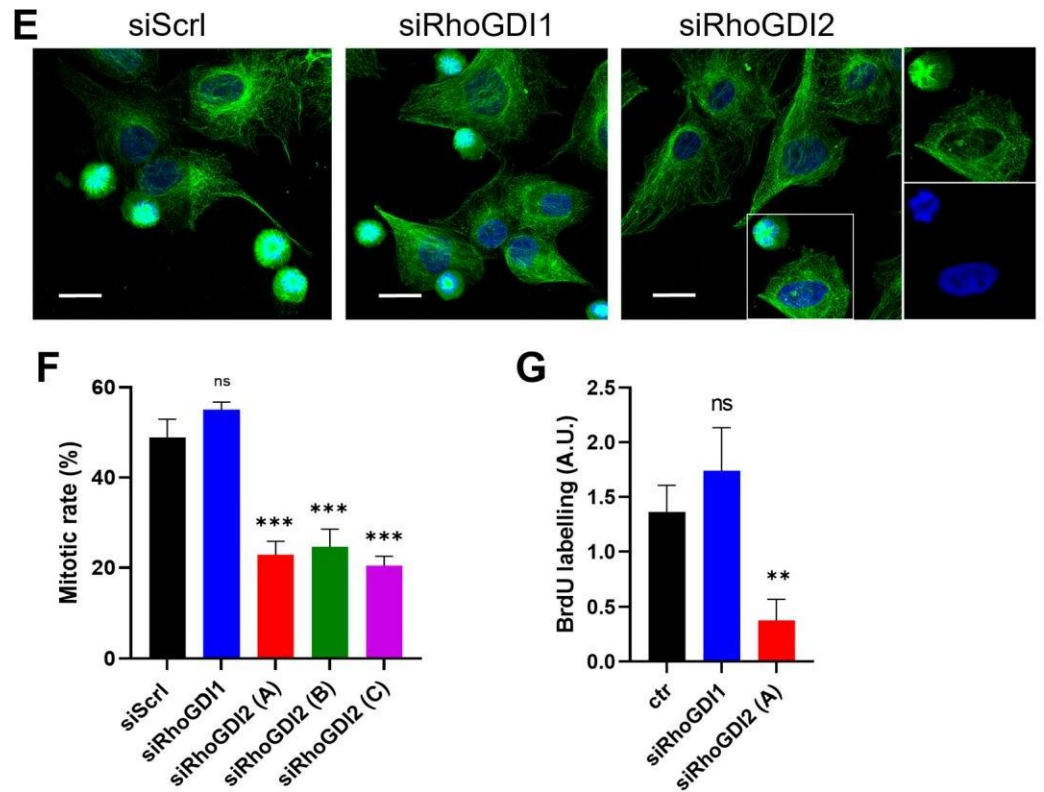


Figure 1. RhoGDI2 silencing represses the proliferation of MG-63 and U-2 OS osteosarcoma cells. Cells were transfected with a control siRNA (siScr), a siRNA targeting RhoGDI1 (siRhoGDI1), or three different siRNAs specific for RhoGDI2 (siRhoGDI2A, B, or C) and immediately seeded in 6-well plates. After 48 h, cells (U-2 OS in (A), MG-63 in (B)) were recovered for RT-qPCR analyses of RhoGDI1 and RhoGDI2 expression. A strong and specific silencing was observed, demonstrating the absence of cross-inhibition. Cell proliferation was also evaluated (U-2 OS in (C) and MG-63 in (D)). After transfection (as in (A,B)), cells were either collected immediately (0 h) or seeded into 24-well plates for further culture for 72 or 120 h. The DNA content of each well was then measured to assess cell proliferation. In parallel experiments, transfected cells were seeded on cover slips and treated with the CDK1 inhibitor Ro-3306 to arrest cells in the late G2 phase. After 24 h, cultures were extensively washed to remove the inhibitor, and mitosis was evaluated 40 min later by α -tubulin (green) and dapi (blue) staining. Representative images of siScr, siRhoGDI1, and siRhoGDI2-treated MG63 cells are provided in (E). The inserts on the right show separately the green and blue channels. The percentage of cells undergoing mitosis to total cells (mitotic rate) was obtained from 300–450 cells in each condition (F). In complementary experiments (G), transfected cells were seeded in 96-well plates. A total of 8 h after seeding, BrdU was incorporated for 16 h, and cells were processed following the manufacturer's protocol to assess BrdU incorporation. The graphs summarize the results of three independent experiments expressed as mean $\pm$ s.d. ns, non-significant; ** $p < 0.01$; and *** $p < 0.001$ as determined by ANOVA followed by Bonferroni analysis. Bar = 20 μ m.

To further determine how RhoGDI2 inhibits cell proliferation, siRNA-treated cells were first blocked for 24 h at the G2/M phase (in order to synchronize the cell cycle in the cultures) using the reversible Ro-3366 CDK1 inhibitor. Mitosis was then evaluated by nuclei staining 40 min after extensive washing to remove the inhibitor. We observed approximately 2.5-fold repression of mitosis upon RhoGDI2 inhibition. Once again, no inhibition was observed when RhoGDI1 was silenced, in line with our previous data (Figure 1E,F). This inhibition of proliferation was further confirmed through a complementary approach, by measuring the incorporation of BrdU, which was strongly repressed in RhoGDI2-silenced cells (Figure 1G).

3.2. RhoGDI2 Silencing Increases Supernumerary Centrosomes

A co-precipitation experimental model was developed to identify proteins potentially physically associated with RhoGDI2. Cells expressing RhoGDI2 tagged with FLAG® M2 were generated. This tag was placed at either the C- or N-terminus to reduce the probability of missing potential partners specific to the N- or C-terminal end domains of RhoGDI2. Tagged RhoGDI2 was immunoprecipitated with anti-FLAG® M2 antibody, and co-precipitated proteins were identified by mass spectrometry. Several dyneins and centrosome proteins were identified (Table 1), suggesting a role for RhoGDI2 in centrosome functions.

Table 1. Dyneins and centrosomal proteins (in blue) as potential RhoGDI2 interactants identified by immunoprecipitation followed by mass spec analysis. RhoGTPases (black) were also identified. RhoGDI2, the immunoprecipitated protein, was detected with the highest confidence and is indicated in red.

Unique Peptides	% Coverage	Score	Protein MW	Accession	Protein Name
22	87.5	276.2	23,044	P52566	Rho GDP-dissociation inhibitor 2
9	34.8	99.7	21,849	P63000	Ras-related C3 botulinum toxin substrate 1
4	18.2	46.2	21,827	P15153	Ras-related C3 botulinum toxin substrate 2
3	18.1	33.7	22,110	P61586	Transforming protein RhoA
4	5.3	28.7	235,152	Q8IZD9	Dedicator of cytokinesis protein 3
4	5.3	26.2	224,984	P11055	Myosin-3
4	2.2	24.3	384,118	A4UGR9	Xin actin-binding repeat-containing protein 2
4	2.4	24.1	524,524	Q96DT5	Dynein heavy chain 11; axonemal
4	7.0	23.8	215,310	Q8TEP8	Centrosomal protein of 192 kDa
4	2.4	23.1	515,918	Q9NYC9	Dynein heavy chain 9; axonemal
3	4.1	22.3	196,273	Q15811	Intersectin-1
3	1.7	21.6	404,166	Q0VDD8	Dynein heavy chain 14; axonemal
3	12.2	21.6	69,618	Q9GZS0	Dynein intermediate chain 2; axonemal
3	5.0	20.4	106,687	O43182	Rho GTPase-activating protein 6

During the cell cycle, centrosomes start duplicating between the G1 phase and the S phase, and this process is highly regulated. Therefore, we investigated whether RhoGDI2 knockdown induced centrosome abnormalities and observed that it led to a significant increase in the occurrence of supernumerary centrosomes (>2 centrosomes) (Figure 2). In some cases, these supernumerary centrosomes are observed in the close vicinity of an enlarged nucleus (compare the nucleus in the second and first examples of supernumerary centrosomes), suggesting aneuploidy. This effect was not observed upon RhoGDI1 knockdown (Figure 2), supporting our previous observations and further documenting that RhoGDI2-mediated regulations can be specific compared to RhoGDI1.

3.3. RhoGDI2 Silencing Reduces Ciliogenesis Through RhoA Activation

Centrosomes can form spindle poles during mitosis or lead to the formation of a primary cilium during cell cycle arrest (G0). To evaluate the role of RhoGDI2 on centrosome functions, we tested whether its silencing affects ciliogenesis.

The knockdown of RhoGDI2 reduced the number of cells with a primary cilium by approximately twofold compared to control, while repression of RhoGDI1 had only a small, albeit significant, effect (Figure 3). The next step was then to investigate the underlying molecular mechanisms responsible for the role of RhoGDI2 in centrosome functions. RhoGDI 1 and 2 interact with multiple RhoGTPases, sometimes with different affinities and effects, and can therefore influence multiple cellular phenotypes through RhoGTPase-regulated pathways. To assess whether RhoGDI2 regulates centrosome functions via the major RhoGTPases, we measured the active (GTP-bound) and total amounts of RhoA, Rac1, and Cdc42 under our different experimental conditions (Figure 4).

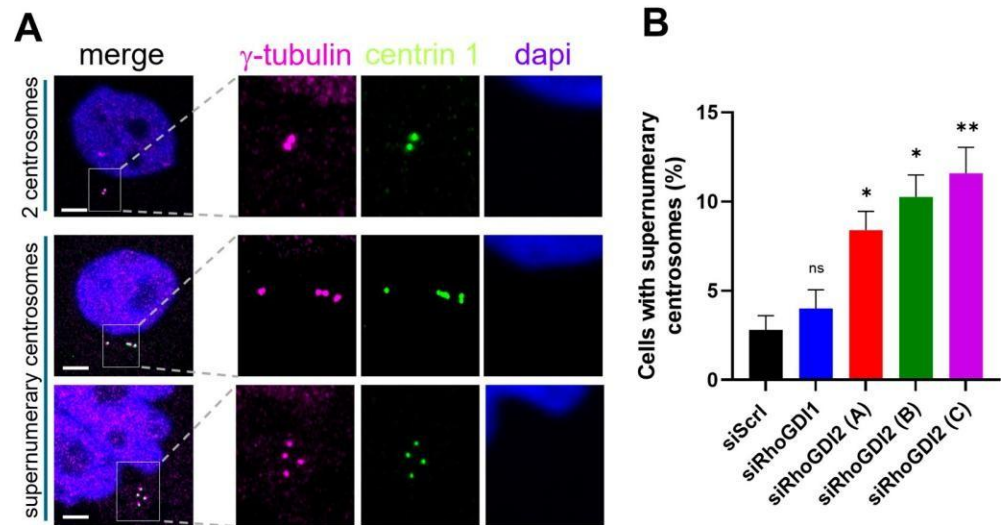


Figure 2. RhoGDI2 silencing increases supernumerary centrosomes. Immediately after transfection (as described in Figure 1), cells were seeded on cover slips and treated with the CDK1 inhibitor Ro-3306 to arrest cells in the late G2 phase. After 24 h, cells were stained for γ -tubulin (pink), centrin 1 (green), and dapi (blue). (A) Representative images of cells with 2 centrosomes and supernumerary centrosomes. The percentage of cells with supernumerary centrosomes (>2) in culture after transfection with the different siRNAs is indicated in (B). Data were obtained from 250 cells in each condition from three independent experiments, expressed as mean $\pm$ s.d. Bar = 5 μ m. ns: non-significant; * $p < 0.05$; and ** $p < 0.01$ as determined by ANOVA followed by Bonferroni analysis.

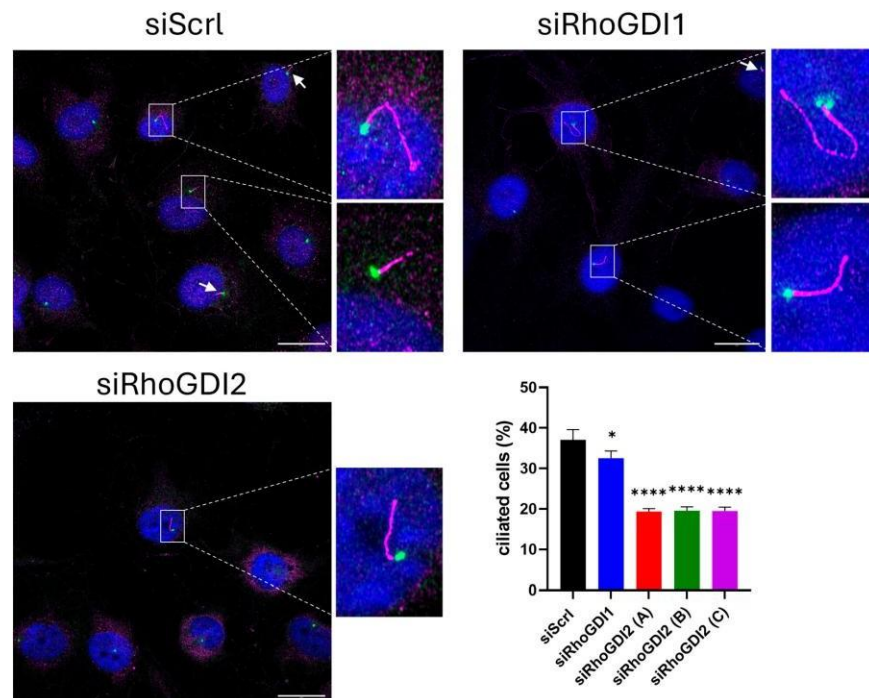


Figure 3. RhoGDI2 silencing reduces ciliogenesis. Immediately after transfection with a control siRNA (siScr1) or with a siRNA targeting RhoGDI1 (siRhoGDI1) or RhoGDI2 (siRhoGDI2A, B, or C), cells were seeded on cover slips and starved for serum for 48 h. They were then treated with the CDK1 inhibitor Ro-3306 for 24 h (to induce the accumulation of cells in the late G2 phase) and co-stained for γ -tubulin (green) and Arl13b (pink). The graphs summarize the results of 750 cells from three independent experiments, expressed as mean $\pm$ s.d. Bar = 20 μ m. * $p < 0.05$; **** $p < 0.0001$ as determined by ANOVA followed by Bonferroni analysis.

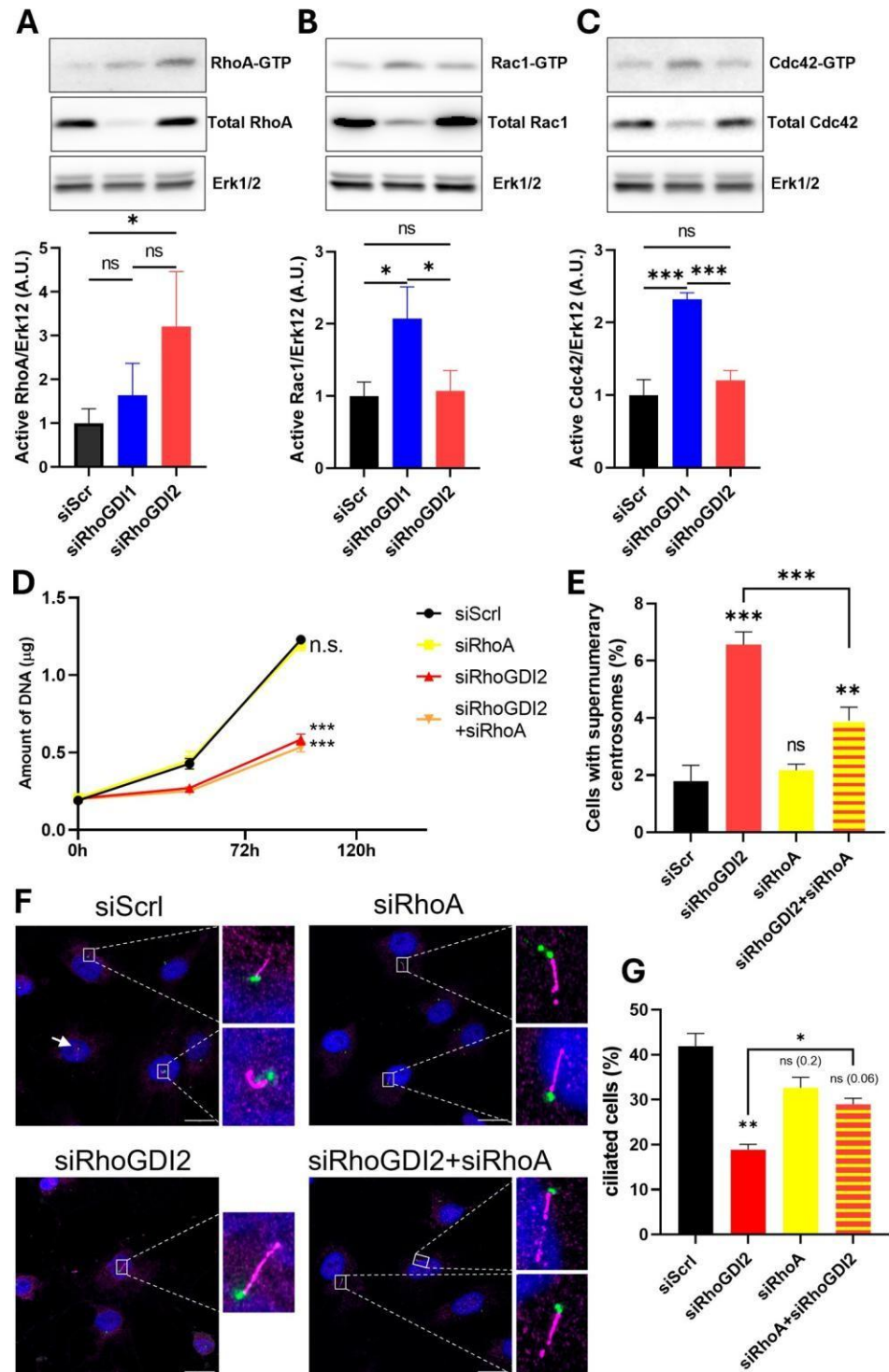


Figure 4. RhoGDI2 silencing increases the activity of RhoA, with no effect on Rac1 or Cdc42 (A–C). Immediately after transfection (as in Figure 1), cells were rinsed, seeded, and deprived of serum for 48 h. They were then collected and processed to measure, by Western blotting, the total amount and the active fraction (GTP-bound) of RhoA (A), Rac1 (B), and Cdc42 (C). The Erk1/2 abundance was also monitored as loading control in each condition. Quantifications are provided as histograms representing three independent experiments (mean $\pm$ s.d.). The potential role of RhoA in the RhoGDI2-mediated effects was also evaluated (D–G). Immediately after transfection (siScr, siRhoGDI2, siRhoA, siRhoGDI2 + siRhoA), cells were seeded in 24-well plates and collected at different time points for DNA measurements (D), or seeded on cover slips, deprived of serum for 48 h and treated with the CDK1 inhibitor Ro-3306 for 24 h to arrest cells in the late G2 phase, before co-staining for γ -tubulin (green) and Arl13b (pink) to label primary cilia (F,G). RhoA silencing did not rescue the inhibition of

proliferation upon RhoGDI2 silencing (D) but significantly mitigated the effect on supernumerary centrosomes (E) and ciliogenesis (F,G). The results of each graph are expressed as mean ($\pm$ s.d.) from three independent experiments. Data in (E,G) were obtained from 250–300 cells per experiment. Bar = 20 μ m. ns: non-significant; * $p < 0.05$; ** $p < 0.01$; and *** $p < 0.001$ as determined by ANOVA followed by Bonferroni analysis.

The total amounts of RhoA, Rac1, and Cdc42 were greatly reduced upon removal of RhoGDI1, confirming its ability to protect them from degradation [10,19]. Such an effect was not found for RhoGDI2, illustrating once again the existence of different functions for these two proteins, even though they share many sequence similarities (Figure 4A–C). Moreover, we also observed that silencing RhoGDI2 or RhoGDI1 affects the activation of RhoGTPases differently: silencing RhoGDI1 resulted in an increase in GTP-bound Rac1 and Cdc42 but no modification of GTP-bound RhoA, while silencing RhoGDI2 induced an increase in active GTP-bound RhoA but no modification of GTP-bound Rac1 and Cdc42 (Figure 4A–C). This increased RhoA activity suggests its potential involvement in the phenotypical regulations observed following RhoGDI2 silencing. Hence, we then checked whether RhoA participates in the RhoGDI2-mediated regulations (Figure 4D–G).

The RhoA knockdown does not significantly modify the proliferation, supernumerary centrosomes, or the number of ciliated cells. In co-silencing conditions, it does not rescue the inhibition of proliferation induced by RhoGDI2 inhibition (Figure 4D), but it corrects the increased occurrence of supernumerary centrosomes (Figure 4E) and the repression of ciliogenesis induced by RhoGDI2 knockdown (Figure 4F,G), showing that RhoA is part of the RhoGDI2-mediated centrosome regulation. As expected from our data (Figure 4B), the repression of Rac1 in RhoGDI2-silenced cells did not rescue the ciliogenesis phenotype (Supplementary Figure S1).

3.4. RhoGDI2 Silencing Reduced Tumor Cell Killing Ability of NK-92 Cells

Having shown that RhoGDI2 repression affects centrosome functions in cancer cells, we wanted to extend these studies to immune cells, where a key function of the centrosome is the formation of the immune synapse (IS) [20] ISs are specialized cell–cell junctions that form between a T or natural killer cell and a target cell, such as a cancer cell, to initiate the killing process. As a functional approach, we tested whether reduced expression of RhoGDI2 affects the ability of NK cells (NK-92) to kill tumor cells, which would indicate changes in IS formation. NK-92 cells were first nucleofected with siRNAs, either control (siScr1) or targeting RhoGDI1 or RhoGDI2. They were then co-cultured with MG-63 osteosarcoma cells pre-loaded with a Caspase 3/7 substrate, which, when cleaved during cancer cell apoptosis, releases fluorescent DNA dye that stains the cell nucleus.

We observed that silencing of RhoGDI2 in NK-92 cells decreased their ability to kill tumor cells by ~30% as compared to control (Figure 5A–C). A smaller, albeit significant, decrease in the tumor-killing ability was also observed upon RhoGDI1 silencing. The efficacy of the gene silencing in NK-92 cells is shown in Figure 5D. These data show that RhoGDI2 controls centrosome function in multiple contexts and cell types.

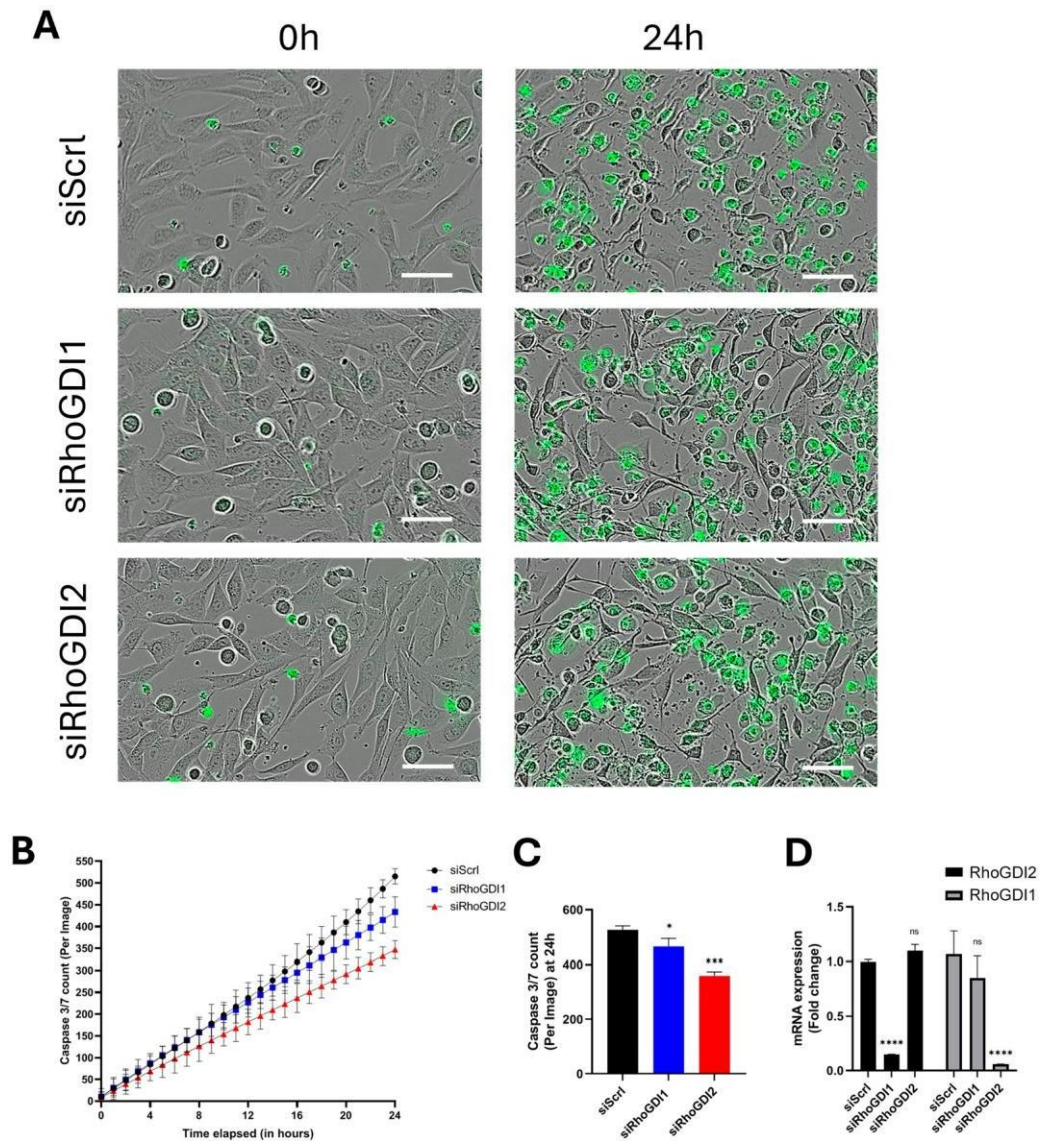


Figure 5. RhoGDI2 silencing reduces tumor cell-killing ability of NK-92 cells. NK-92 cells were nucleofected with a control siRNA (siScr) or with a siRNA targeting RhoGDI1 (siRhoGDI1) or RhoGDI2 (siRhoGDI2). After 24 h recovery, they were then co-cultured with MG-63 cancer cells loaded with Caspase 3/7 substrates to identify apoptotic cancer cells (in green). Imaging was performed over 24 h (Incucyte Live-Cell Analysis System). (A) Representative images of co-cultures at the first (0 h) and last (24 h) time point. (B) A 24 h time plot illustrating the killing ability of NK-92 cells (treated with either siScr, siRhoGDI1, or siRhoGDI2) on MG-63 cancer cells (expressed as green apoptotic cells per field). The killing activity of NK92 cells is strongly affected by RhoGDI2 silencing, even at the beginning of the co-culture. A slight decrease in the killing activity of siRhoGDI1-silenced cells was also observed, but mainly at longer co-culture time. (C) Quantification of killing activity at the final time point (24 h). (D) Evaluation of the efficiency of RhoGDI 1 and 2 gene silencing in NK-92 cells. The graphs summarize the results of three independent experiments expressed as mean $\pm$ s.d. Bar = 50 μ m. ns: non-significant; * $p < 0.05$; *** $p < 0.001$; and **** $p < 0.0001$ as determined by ANOVA followed by Bonferroni analysis.

4. Discussion

The Rho GDP dissociation inhibitors 1 (RhoGDI1) and 2 (RhoGDI2) are highly similar; however, they were reported to play differential roles in several types of cancer (review in [8,9]). Although they exert shared functions, notably the regulation of the RhoGTPase activation cycle, in vitro analyses revealed that they have some specific roles in cellular

physiology. For instance, in contrast to RhoGDI1, RhoGDI2 was reported to activate Rac1 via its binding to the guanosine exchange factor VAV1 [4]. Importantly, VAV1 could have a role in pancreatic cancer [21,22]. However, these observations can only partly explain the specific contribution of RhoGDI1 and RhoGDI2 to several types of cancers. Hence, our study has been designed to highlight cellular processes relevant to cancer biology that are differentially regulated by RhoGDI2 and RhoGDI1 and that could explain their specific role *in vivo*. First, we observed that knocking down RhoGDI2, but not RhoGDI1, regulates cell proliferation of various cancer cell types.

To characterize the mechanism involved, we set up an IP/MS (immunoprecipitation followed by identification of proteins by mass spectrometry) to identify proteins that interact with RhoGDI2 and could mediate the observed effects. These analyses revealed the presence of immunoprecipitated proteins related to the centrosome or its function. These proteins are not reported as common contaminants in IP/MS (The CRAPome: a Contaminant Repository for Affinity Purification Mass Spectrometry Data) and were identified with high confidence in terms of peptide identification and protein coverage. They are also consistent with a previous publication showing that RhoGDI2 can be found in association with centrosomes [23]. Surprisingly, Western blot analysis failed to identify these immunoprecipitated proteins. As these interactions between RhoGDI2 and its partners are expected to be highly transient, the efficiency of co-immunoprecipitation is expected to be low. Under these conditions, we speculate that MS analysis is sensitive enough to detect proteins present at low levels, whereas Western blotting is not. As an alternative approach, we then focused on studies that would indirectly confirm the role of RhoGDI2 on centrosome functions.

The centrosome is a non-membrane-bound organelle composed of a daughter and a mother centriole surrounded by a network of electron-dense material known as the pericentriolar matrix. It is the main microtubule-organizing center (MTOC) in the cell and, as such, regulates crucial cellular functions, such as cell shape, polarization, or division [24,25]. The mother centriole is responsible for organizing the cell's microtubule network, thereby driving the mitotic apparatus to allow the assembly of the mitotic spindle to ensure proper chromosome distribution [26]. It is also the basic element for the formation of primary cilia, a cellular organelle that plays the role of an antenna for various signal transduction, including growth factor signaling but also mechanotransduction or photoreception [27,28]. Alteration of centrosome function or supernumerary centrosomes can affect the faithful distribution of chromosomes between daughter cells and promote chromosome instability, aneuploidy, or mitotic catastrophe. While extra centrosomes are usually viewed as intrinsically pro-tumorigenic, recent investigations suggest that this belief must be adjusted: depending on the context, extra centrosomes can reduce cancer cell fitness and tumorigenesis [29].

We observed that knockdown of RhoGDI2, but not RhoGDI1, inhibited the formation of primary cilia, a microtubule-based organelle that is dependent on centrosomes [28,30]. The primary cilium acts as an 'antenna' that integrates various extracellular signals and therefore controls major cellular processes, including proliferation [28]. Ciliogenesis is a process that is strongly regulated by the degree of polymerization of the actin cytoskeleton. Inhibition of actin polymerization promotes ciliogenesis, whereas loss-of-function mutations in ciliopathy genes increase actin stress fibers [31].

The RhoGTPases Cdc42, Rac1, RhoA, and RhoC are involved in actin polymerization, leading to the formation of filopodia, lamellipodia, and stress fibers, respectively, and their activity can be regulated by RhoGDI2. RhoA and RhoC are also involved in centrosome amplification [32]. Therefore, through its association with the centrosome, RhoGDI2 could locally regulate its activity and thus contribute to correct centrosome duplication during

the cell cycle. This may explain why we observed that the inhibition of RhoGDI2, but not RhoGDI1, increased the number of cells with supernumerary centrosomes. The increased activation of RhoA, but not Rac1 and Cdc42, upon inhibition of RhoGDI2 suggested that RhoA may be involved in the inhibition of primary cilia formation observed in our model. This was confirmed by an experiment showing that RhoA knockdown significantly decreased the percentage of cells with supernumerary centrosomes and reversed the inhibition of ciliogenesis caused by RhoGDI2 inhibition. By contrast, proliferation inhibition upon RhoGDI2 silencing is not reversed by RhoA knockdown. Proliferation is a multi-step process that involves the coordination of several events in addition to centrosome duplication. RhoGDI2 could affect some of these events independently of RhoA, which would explain why RhoA knockdown is not sufficient to reverse the inhibition of proliferation following RhoGDI2 silencing.

Thus, RhoGDI2 is able to regulate centrosome-dependent cellular functions through local control of RhoA activity, which affects the organization of the actin cytoskeleton. Such local control of RhoGTPase signaling by RhoGDI2 has been suggested in other cellular models, notably in the nucleus of pancreatic b-cells [33]. Spatial regulation of RhoGTPase activity is not limited to RhoGDI2 and has also been described for RhoGDI1, whose local recruitment to cell–cell contacts has been reported to contribute to cell–cell contact maturation in epithelial cells through the regulation of both Rac1 and RhoA [34].

The elimination of target cells by immune cells requires the formation of the immune synapse, an organelle with striking similarities to the primary cilium, including the requirement of the centrosome for its formation [35]. Knowing that RhoGDI2 is highly expressed in immune cells, we then tested whether it regulates their function. We were able to show that inactivation of RhoGDI2 significantly altered the ability of NK cells to kill cancer cells, again highlighting its role in centrosome functions.

Taken together, our observations may explain the dual function of RhoGDI2 in cancer, where it is considered to be either a pro- or anti-cancer factor, as recently reviewed [9]. In cancer cells, it positively regulates centrosome functions and thus their efficient proliferation, while, in immune cells, it favors the formation of the immune synapse, which promotes the killing of cancer cells. Our data clearly indicate that RhoGDI1 and RhoGDI2, although very similar, can perform specific functions. This could be due to different affinities for binding partners [6] but also due to different subcellular locations resulting from differences in their amino acid composition. In particular, the N-terminal parts of their sequences are the most divergent [9], and the analysis of chimeric proteins composed of the N-terminal part of RhoGDI2 and the C-terminal part of RhoGDI1, and vice versa, could help to identify the contribution of these domains to the specific functions of RhoGDI1 and RhoGDI2.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/cells14221833/s1>, Figure S1: The repression of ciliogenesis following RhoGDI2 silencing is not rescued by co-silencing of Rac1.; Table S1: Primers used for RTqPCR measurements.; Table S2: Primers used to generate FLAG-RhoGDI2; Uncropped blots.

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3.3 Additional data

A few additional experiments were conducted during our characterization of the role of RhoGDI2 in cell biology, but they were not published because they seemed too preliminary and required further confirmation. However, they have been included in the manuscript for the sake of completeness and to provide an overview of what could be done in the future. We therefore ask readers to consider these data with caution and goodwill.

3.3.1. Suppression of RhoGDI2 causes defects in chromosome congression

Having established that reduced RhoGDI2 levels perturb centrosome-associated parameters, we next asked whether RhoGDI2 depletion compromises mitotic chromosome alignment. Accurate congression of chromosomes to the metaphase plate depends on the formation of a bipolar spindle, centrosome integrity, and coordinated microtubule–kinetochore interactions supported by centrosome- and motor-associated factors. To directly quantify chromosome alignment at metaphase, U-2 OS cells were transfected with a control siRNA (siScr1) or with siRNAs targeting RhoGDI1 or RhoGDI2, synchronized in late G2 using the CDK1 inhibitor RO-3306, and then released into mitosis to enrich for metaphase cells. Cells were fixed and co-stained for DNA (DAPI), α -tubulin to visualize spindle microtubules, and γ -tubulin to mark centrosomes (Figure 15A).

In control conditions, DAPI staining revealed a compact metaphase plate with chromosomes aligned along the spindle equator, consistent with efficient congression. A similar metaphase organization was observed following RhoGDI1 depletion, with no obvious broad disruption of chromosome alignment compared to control. In contrast, RhoGDI2-silenced cells frequently exhibited misaligned chromosomes and a broadened, less compact metaphase plate, indicative of impaired congression. Spindle microtubules were clearly detectable in all conditions by labeling of α -tubulin. γ -tubulin staining, a centrosome marker labeling the spindle poles, enabled us to verify that observed chromosome misalignment was not secondary to spindle abnormalities.

To quantify these differences, chromosome congression was measured from DAPI images using a chromosome congression index defined as the ratio between maximal chromosome plate width and length (as schematized by the red and white axes in Figure 15A). Consistent with the representative images, RhoGDI2 depletion significantly increased the chromosome congression index compared to control cells, indicating a wider and less focused metaphase plate (Figure 15B). This phenotype was reproduced with three independent siRNAs targeting RhoGDI2 (the efficacy of the silencing by the siRNAs was already checked as demonstrated in our manuscript), supporting that the congression defect is linked to reduced RhoGDI2 expression rather than an siRNA-specific off-target effect. In contrast, RhoGDI1 silencing did not significantly alter the congression index relative to control (Figure 15B). Together, these data show that RhoGDI2, but not RhoGDI1, is required for efficient chromosome alignment at metaphase in U-2 OS cells.

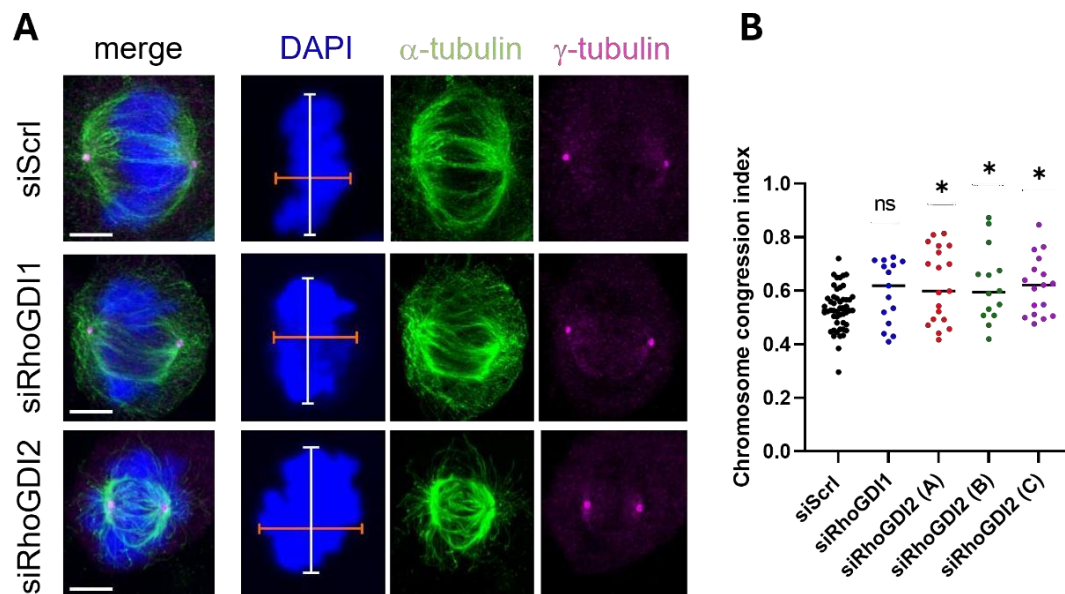


Figure 15. RhoGDI2 depletion impairs chromosome congression at metaphase

U-2 OS cells were transfected with a control siRNA (siScr1), with one siRNAs targeting RhoGDI1 (siRhoGDI1) or with three siRNA specific for RhoGDI2 (siRhoGDI2A, B, or C). Following transfection, cells were seeded onto coverslips and treated with the CDK1 inhibitor Ro- 3306 for 24 h to induce accumulation in late G2 phase, then released into mitosis for 40 min to allow progression into metaphase. Cells were subsequently fixed and co-stained for α -tubulin (green), γ -tubulin (magenta), and DNA (DAPI, blue). Representative images are shown in (A) where siRhoGDI2(A) was used to silence RhoGDI2. Chromosome congression was quantified based on DAPI staining as the ratio between the maximal chromosome plate width (red line) and length (white line). Quantification shown in (B) represents data from three independent experiments and is expressed as mean $\pm$ s.d. Scale bar = 5 μ m. Statistical significance was assessed by one-way ANOVA followed by Bonferroni post hoc analysis. ns, non-significant; *P < 0.05.

3.3.2 Creation of domain swapped chimeric RhoGDIs.

After determining that RhoGDI1 and RhoGDI2 had specific functions, we set out to characterize the molecular cause. As we know that their N-terminus constitute the most divergent domains between RhoGDI 1 and 2 (Figure 16A), we wondered if the functional differences between RhoGDI 1 and 2 could be mediated, at least partly, by these domains. We first generated inducible expression vectors coding for chimeric RhoGDI1/2 by swapping their respective coding sequences (Figure 16B). Vectors with these chimeric genes and recombinant genes for RhoGDI1 and RhoGDI2 were transduced into U-2 OS cells (Viral Vector Platform, GIGA, University of Liege) that can express these genes upon induction with doxycycline.

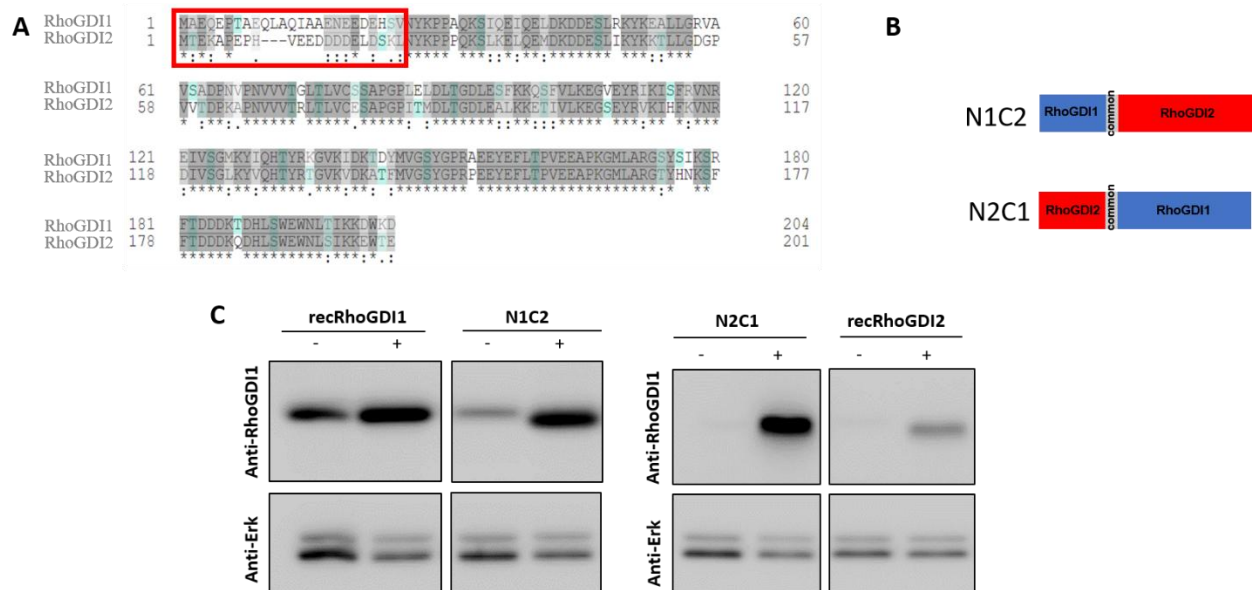


Figure 16. Generation of the RhoGDI1/RhoGDI2 chimeric constructs

A. Amino acid sequence alignment of RhoGDI1 (GDI1) and RhoGDI2 (GDI2). The two proteins are most divergent at their N-terminal extremity, as highlighted by the red box. Alignment symbols: '*' identical residues; '.' conserved substitution (similar physiochemical properties); ':' semi-conserved substitution; '-' gap introduced to optimise alignment.

B. Schematic representation of the domain-swapped chimeric constructs. The N1C2 construct contains the N-terminal sequence of RhoGDI1 (blue) joined to the C-terminal sequence of RhoGDI2 (red). The N2C1 construct contains the N-terminal sequence of RhoGDI2 (red) joined to the C-terminal sequence of RhoGDI1 (blue). The region of shared amino acids between the two proteins is indicated (grey, common aa).

C. Doxycycline-inducible lentiviral vectors containing the coding sequences of RhoGDI1, RhoGDI2, N1C2 and N2C1 were produced and transduced into U-2 OS cells (Viral Vector Platform, GIGA, University of Liège). Expression of recombinant proteins was induced with doxycycline (1 µg/ml) for 24 h. Samples with (+) or without (-) doxycycline were analysed by immunoblotting. Detection of recRhoGDI1 and N1C2 (left panel) was performed using an anti-RhoGDI1 antibody recognising the full-length protein. Detection of N2C1 and recRhoGDI2 (right panel) was performed using an antibody specifically directed against the C-terminal domain of RhoGDI1. Anti-Erk served as loading control. Data shown are representative of n=3 independent experiments.

To validate the expression of recombinant and chimeric RhoGDI constructs, Western blot analysis was performed. Cells were cultured in complete medium and expression of the recombinant/chimeric RhoGDIs was induced by doxycycline treatment for 24 h. Cell lysates were collected from all cell lines cultured in the presence (+) or absence (–) of doxycycline. Protein expression was assessed using an antibody directed against RhoGDI1 (recognizing the total protein and the C-terminal region).

As no antibody suitable for reliable detection of endogenous RhoGDI2 in U-2 OS cells is currently available, recombinant RhoGDI2 was visualized through cross-reactivity with the anti-RhoGDI1 antibody targeting the conserved C-terminal region. Consequently, the detected signal likely underestimates the actual expression level of recombinant RhoGDI2. As expected, doxycycline induction resulted in increased levels of RhoGDI1-containing constructs (Figure 16C), confirming successful expression of the recombinant and chimeric RhoGDI proteins. These results indicate that the experimental system for domain swapping between RhoGDI1 and RhoGDI2 was successfully established.

Next, we wanted to confirm that RhoGDI2 silencing had functional consequences on downstream RhoGTPase activity. For this, we tested the activity of Rac1 (a target of RhoGDI2) by GTPase activity pull down assays. This assay consists in extracting active (GTP-bound) Rac1 from complete cell lysates via its specific affinity for the p21-binding domain of PAK1 (PAK1-PBD), fused to GST and immobilized on glutathione-sepharose beads. Western blot analysis is then performed to compare the enrichment of the studied RhoGTPase (here Rac1) in the precipitate as compared to the total lysate.

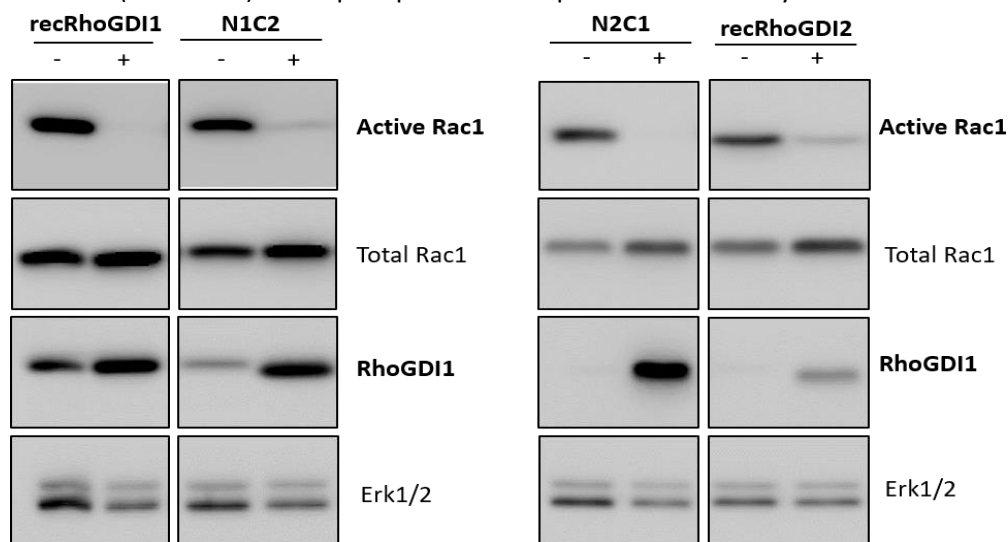


Figure 17: Characterization of the inhibitory capacity of the domain swapped chimeric on Rac1 activity
Chimeric/Recombinant (rec) RhoGDI expression was induced with doxycycline (1 µg/ ml) for 24 h. Cells with (+) or without (–) doxycycline were tested for active Rac1 by a pull down assay as previously described (Deroanne et al., 2003). Samples were analyzed by immunoblotting using antibodies specific for Rac1, RhoGDI1 and Erk1/2 (used for normalization purposes). Induction of recombinant RhoGDIs resulted in the reduction of the level of active Rac1.

The different cell lines were left untreated (-) or were incubated with doxycycline (24 h at 1µg/ml) (+) to induce the expression of the different types of recombinant RhoGDIs. Cells were then assessed for the presence of active Rac1 as previously described (Deroanne et al., 2003), followed by analysis via immunoblotting and compared with total Rac1 levels. The induction of the different RhoGDIs (RhoGDI1, RhoGDI2, N1C2, N2C1) resulted in a similar decreased active Rac1 levels (Figure 17).

3.3.3 Overexpression of chimeric RhoGDIs in RhoGDI2-silenced cells does not rescue the proliferative phenotype

As we observed that RhoGDI2 silencing represses the proliferation in multiple cell lines, we decided to verify whether this phenotype can be rescued by overexpressing the chimeric RhoGDIs in RhoGDI2-silenced cells. RhoGDI2 was silenced by RNAi and this inhibition lasted for at least 5 days. Cells were cultured in full medium, and chimeric/recombinant RhoGDIs were induced by doxycycline. Proliferation rate of these cell lines was assessed by DNA measurements at 0 h, 72 h and 120 h.

To determine the impact of RhoGDI expression on cell proliferation, we first assessed the effect of inducible recombinant and chimeric RhoGDIs in control cells (siScr1). In this condition, induction of recombinant RhoGDI expression resulted in a significant decrease in proliferation (Figure 18), consistent with excessive RhoGDI levels leading to reduced Rho GTPase activity, as supported by the immunoblot data.

As expected, silencing of RhoGDI2 alone also led to a reduction in cell proliferation. Importantly, induction of recombinant RhoGDIs in RhoGDI2-silenced cells did not restore proliferation to control levels. This indicates that increased RhoGDI expression, irrespective of RhoGDI2 knockdown, is sufficient to suppress proliferation, likely through global inhibition of Rho GTPase-dependent processes.

We next examined the effects of the N-terminal domain-swapped chimeric proteins N1C2 and N2C1. In control cells, induction of either chimera significantly reduced proliferation, mirroring the effect observed with recombinant RhoGDI1 and 2. Upon RhoGDI2 knockdown, proliferation was reduced in N2C1-expressing cells, whereas no additional decrease was observed in N1C2 cells. Notably, induction of either chimera failed to restore proliferation in RhoGDI2-silenced cells.

Collectively, these data indicate that overexpression of recombinant or chimeric RhoGDIs suppresses proliferation. Hence, it cannot rescue the inhibition of proliferation due to RhoGDI2 depletion.

Results

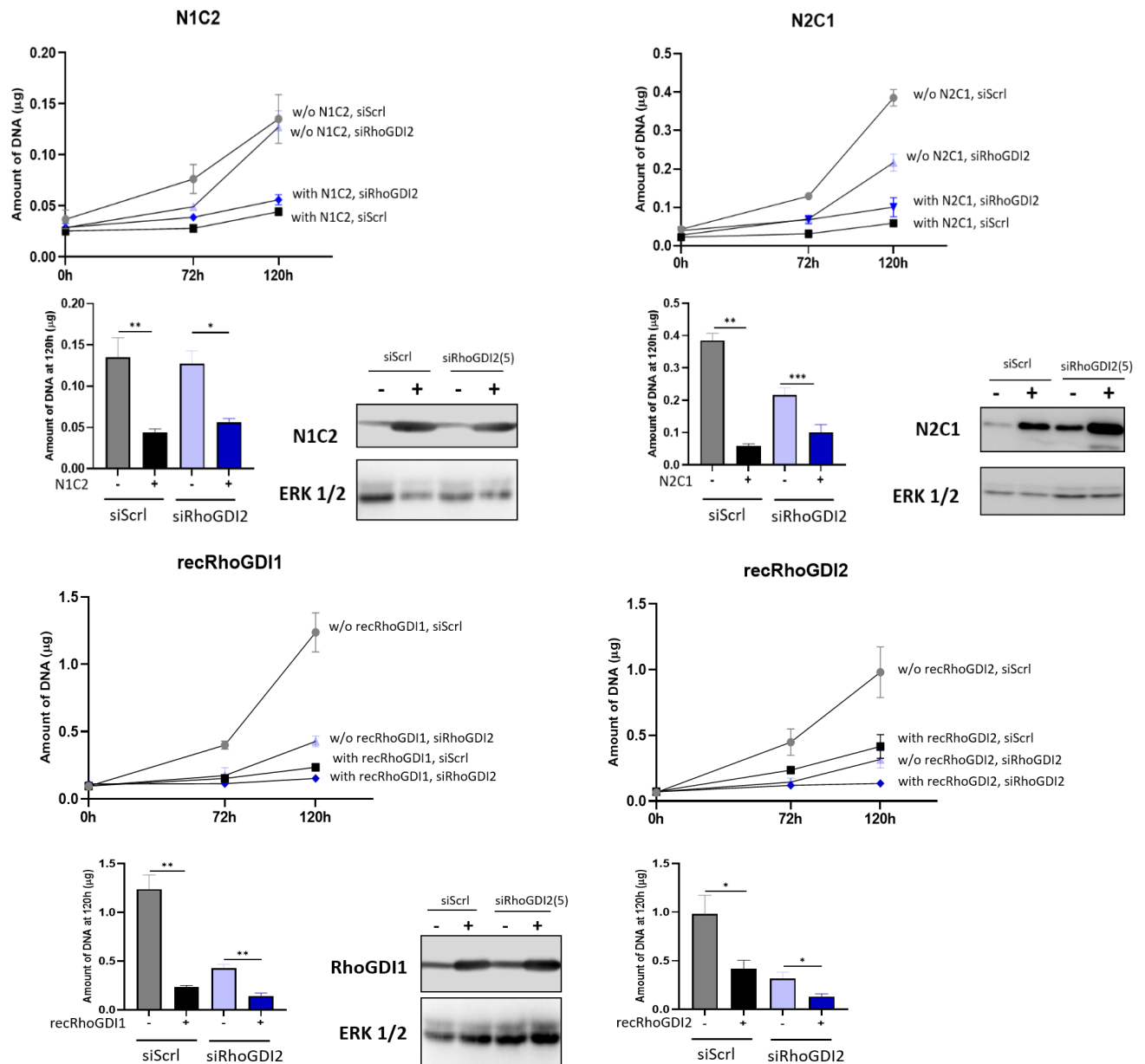


Figure 18: Induction of recombinant or chimeric RhoGDIs reduces cell proliferation independently of RhoGDI2 knockdown

Cells were transfected with a control siRNA (siScr1) or a siRNA targeting RhoGDI2 (siRhoGDI2) and seeded in 24-well plates. The expression of the different forms of RhoGDIs was induced with doxycycline (1 $\mu\text{g}/\text{mL}$) for 24 h. Cells were collected at the indicated time points, and proliferation was assessed by DNA content quantification as described in the Materials and Methods. The expression and effects of recombinant RhoGDI1 and 2 are shown in the upper panels, while data related to chimeric RhoGDIs (N1C2 and N2C1) are shown in the lower panels. recRhoGDI2 was not validated via WB as no specific anti-RhoGDI2 antibody is available. The C-terminal anti-RhoGDI1 antibody shows only partial cross-reactivity with RhoGDI2, precluding reliable confirmation of recRhoGDI2 expression by this approach. Graphs represent the mean $\pm$ s.d. of three independent experiments. DNA content at 120 h is summarized in the bar graphs. Immunoblots confirm induction of RhoGDI expression. Statistical significance was determined by one-way ANOVA followed by Bonferroni post hoc analysis. * $P < 0.1$; ** $P < 0.01$; *** $P < 0.001$.

4 Discussion

Aberrant signaling of Rho GTPases and their regulators, including RhoGDI2, have been found to be implicated in cancer^{2,12,17,79,97,184}. The overarching theme of this research is directly or indirectly related to deciphering the dual role of RhoGDI2 in cancer biology, with special emphasis at the functional differences between RhoGDI1 and 2. RhoGDI2 was chosen as the subject of research because we still do not have a clear understanding of its mechanistic functions beyond the conventional Rho GTPase regulation and of its complex role in cancer.

4.1 RhoGDI2 potentially interacts with centrosomal and dynein proteins

RhoGDI1 and RhoGDI2 are differentially expressed across multiple cancer types and have been associated with distinct cellular phenotypes. Previous studies have shown that these two RhoGDIs share limited overlap in their non-Rho GTPase interactomes. For example, RhoGDI2 has been reported to interact with the RhoGEF Vav1, whereas RhoGDI1 does not, highlighting functional divergence between the two proteins.

These findings suggest that RhoGDI2 regulates specific signaling pathways through mechanisms that are not shared with RhoGDI1. Given the comparatively limited characterization of RhoGDI2 relative to RhoGDI1, it is likely that additional pathways and molecular mechanisms involving RhoGDI2 remain unidentified. This underscores the importance of identifying novel RhoGDI2-interacting proteins to better understand its unique functional roles.

To this end we perform IP/MS analyses aimed at discovering potential novel interactors of RhoGDI2. In this IP/MS, we identified RhoG for the first time as a potential interactor of RhoGDI2 that we confirmed by Co-IP. Moreover, we also found multiple centrosomal and dynein proteins in the same IP/MS. These findings suggest that RhoGDI2 may play a significant role in centrosome dynamics and microtubule organization. However, we were unable to validate by complementary techniques the direct interactions between RhoGDI2 and centrosomal proteins, due to a dearth of high-quality antibodies available for centrosomal proteins detected in our IP/MS.

Interestingly, some studies show that RhoG plays a role in T-cell receptor internalization from the immunological synapse (IS) by phagocytosis in human Jurkat T cells and Raji cells and in mice models^{185–187}. As noted before in this thesis, centrosomes are also involved in the formation of an IS by reorganizing the microtubule cytoskeleton. In addition, RhoGDI2 has been observed to localize in the centrosomes of human cervical cancer HeLa cells, where it plays a role in centrosomal amplification, prolonged mitosis, and aberrant cytokinesis^{188,189}.

4.2 Reduced RhoGDI2 expression disrupts centrosomal functions and promotes mitotic errors

It is important to note that centrosomal and dynein proteins, along with Rho GTPases, play key roles in microtubule organization within cells. Centrosomal proteins are crucial for the nucleation and anchoring of microtubules, ensuring proper cell division and intracellular transport. Dynein motor proteins, encoded by at multiple genes, interact with microtubules to facilitate the movement of cellular components and the organization of the mitotic spindle during cell division. Rho GTPases regulate various aspects of the cytoskeleton, including microtubule dynamics and stability. Through their coordinated actions, these proteins ensure the accurate spatial and temporal organization of microtubules, which is essential for maintaining cellular structure and function ^{22,129,136,143,144,153,156,190}.

4.2.1 RhoGDI2 silencing repressed cell proliferation and mitosis in cancer cells

Investigating additional functions of RhoGDI2 in the cell division cycle is highly relevant to study for enhancing our understanding of its dual role in cancer. Our results show a decrease in cell proliferation upon RhoGDI2 silencing. In the same experiment, we also observe that silencing of RhoGDI1 does not affect this phenotype, highlighting a key functional difference between the two RhoGDIs. It was important to show these functional differences to emphasize the influence of RhoGDI2 on cancer cell phenotypes.

The decrease in cell proliferation upon RhoGDI2 silencing observed in our research is consistent with literature, where RhoGDI2 has been shown to be positively correlated with the proliferation in pancreatic (PDAC), colorectal (CRC), and hepatocellular (HCC) cancer cell lines, thereby suggesting that RhoGDI2 promotes proliferation of cancer cells either through regulation of key Rho GTPases or by participating in other signaling pathways ^{50,52,86,93,96}. However, RhoGDI2 expression has also been observed to be inversely correlated in proliferation of lung adenocarcinoma (LUAD) cells ^{55,56,111}, thus underscoring its dual role in cancer.

The term "proliferation" specifically refers to an increase in the number of cells, typically measured as the cell count over time. Cells achieve proliferation by undergoing a series of steps known collectively as the mitotic cycle, or mitosis ¹⁹¹. Our results also demonstrated that RhoGDI2 silencing caused a repression in mitotic rate. As expected, we did not see any effect upon RhoGDI1 silencing.

Depletion of RhoGDI2 expression suppressed both the cell proliferation and mitotic rate *in vitro*, establishing that RhoGDI2, and not RhoGDI1, functions as a positive regulator of cell cycle progression in cancer cells.

During the cell cycle, centrosomes begin duplicating immediately after the G1 phase and at the onset of the S phase. This process is tightly regulated by cell cycle control and is a crucial event for cell cycle progression and proliferation ¹²⁴. Due to our combined observations of IP/MS results and mitosis repression upon RhoGDI2 silencing, and previous work by Jiang *et al* wherein RhoGDI2 was observed to be localized in centrosomes ¹⁸⁸, we hypothesized that RhoGDI2 plays a role in centrosome dynamics and microtubule regulation.

4.2.2 RhoGDI2 silencing causes centrosomal defects in cancer cells

Because we were unable to functionally validate direct interactions between RhoGDI2 and centrosomal proteins, we next assessed whether depletion of RhoGDI2 resulted in measurable centrosomal defects, thereby providing functional insight into its potential role in centrosome regulation.

Analysis of centrosome integrity revealed that silencing of RhoGDI2, but not RhoGDI1, led to pronounced centrosomal amplification in osteosarcoma cells. Specifically, RhoGDI2 knockdown resulted in a significant increase in the number of cells exhibiting centrosome amplification, indicating a role for RhoGDI2 in maintaining centrosome number homeostasis. Importantly, this phenotype was not observed upon RhoGDI1 silencing, underscoring the specificity of this defect to RhoGDI2 depletion rather than a general consequence of RhoGDI loss.

These findings are consistent with previous work by Jiang *et al.*, who reported an increase in supernumerary centrosomes following RhoGDI2 repression in HeLa cells, suggesting that the role of RhoGDI2 in centrosome regulation is conserved across cancer cell types ¹⁸⁷. Centrosome amplification is now widely recognized as a hallmark of cancer and is frequently associated with additional centrosomal abnormalities that can promote chromosomal instability, aberrant mitoses, and tumor progression. Consequently, therapeutic strategies targeting cells harboring supernumerary centrosomes are being actively explored. In this context, our data identify RhoGDI2 as a regulator of centrosome number homeostasis, whose loss contributes to centrosome amplification in cancer cells ^{171,183,188}. This suggests that RhoGDI2 may be crucial for microtubule organization and anchoring during the cell cycle, a function that is subsequently utilized to organize and stabilize the spindle pole during mitosis, regulate ciliogenesis during G1/G0 phase and form IS in immune cells ^{137,143,153,158,162}. Without RhoGDI2, the microtubule network perhaps cannot maintain its normal association with the centrosome, resulting in overall disorganization and destabilization of the microtubule arrays. As the N-terminal domains of RhoGDI 1 and 2 are completely divergent, whilst the C-terminal domains share

a 73.6% identity²³, it is possible that the divergent N-terminal domains of RhoGDI1 and RhoGDI2 are responsible for their distinct effects on centrosomal regulation.

Despite observing role of RhoGDI2 in centrosome duplication and chromosome congression, we were unable to confirm its localization in the centrosomes or at the spindle poles because of the absence of commercially available and specific RhoGDI2 antibody. As mentioned before, the 3 key Rho GTPases, i.e. RhoA, Rac1 and Cdc42, are involved in cell cycle progression and in centrosome dynamics¹⁶⁵.

Moreover, Kim *et al* show that, in gastric cancer cells, RhoGDI2 acts as a scaffold protein to promote Rac1 interactions with other actin-binding proteins which promotes their invasive ability⁴⁹. Whether RhoGDI2 facilitates interactions between Rho GTPases and centrosomal proteins to maintain microtubule organization remains to be established, but would be consistent with its known role as a scaffold for Rho GTPase complexes.

When preparing for cell division, the mitotic spindle begins to define two poles and an equator. When mitosis starts, chromosomes condense and disperse towards the spindle equator. This movement is known as chromosome congression and it ensures that the chromosomes are properly aligned during metaphase before being segregated¹⁹². Defects in spindle pole formation often cause impaired chromosome congression. Aneuploidy, the presence of abnormal number of chromosomes in a cell, is also being considered a hallmark of cancer and is often accompanied by chromosomal instability (CIN)¹⁹³. There are numerous reports of CIN contributing to tumorigenesis and is being considered as a potential anti-cancer therapy^{194,195}. Chromosomal congression defects is one of the pathways that contributes to CIN¹⁹⁶.

Our data show a significant decrease in chromosome congression upon RhoGDI2 silencing, while RhoGDI1 silencing has no significant effect. This suggests that RhoGDI2 is essential for proper chromosomal congression, potentially by regulating microtubule dynamics or interacting with dynein proteins that transport chromosomes towards the spindle equator. Given that chromosomal misalignment can impair accurate chromosome segregation, RhoGDI2's role in congression may contribute to genomic stability, which could, in turn, support cancer cell proliferation. Alternatively, its influence may be mediated through mitotic signaling pathways that facilitate efficient cell division.

One key observation to highlight is that we notice that RhoGDI1 silencing does not induce defects in chromosome congression during metaphase. This could be due to the distinct GTPase client specificities of RhoGDI1 and RhoGDI2. RhoGDI1 preferentially regulates RhoA and Rac1 in the context of actin cytoskeleton remodeling, whereas RhoGDI2 appears to have a more specific role in

centrosome homeostasis, potentially through its interactions with centrosomal and dynein-associated proteins identified in our IP/MS data. The absence of a congression defect upon RhoGDI1 silencing therefore suggests that the centrosomal function identified here is specific to RhoGDI2 and is not a general consequence of RhoGDI family member loss ^{143,155,192,197}.

We further investigated whether RhoGDI2 depletion affects specific centrosome-dependent functions beyond mitosis. Our data show that RhoGDI2 knockdown markedly impairs ciliogenesis, indicating a role for RhoGDI2 in centrosome-associated processes required for primary cilium formation. Notably, although RhoGDI1 silencing also resulted in a statistically significant reduction in ciliogenesis, this effect was substantially less pronounced than that observed upon RhoGDI2 depletion, highlighting a predominant role for RhoGDI2 in this process.

Primary cilia are crucial for signaling and sensory functions where they play roles in signal transduction and maintaining cellular homeostasis ¹⁶². Reduced ciliogenesis can also have other significant consequences on the cell where it can lead to many development abnormalities such as ciliopathies like polycystic kidney disease, Joubert syndrome, etc ^{159,160}. Moreover, primary cilia also play a role in regulating the cell cycle. During cell division, the primary cilium is resorbed, and its components are recycled for mitotic spindle formation.

Reduced ciliogenesis can disrupt centrosome-dependent signaling pathways involved in cell cycle regulation. In many contexts, impaired ciliary function has been associated with defective cell cycle checkpoint control and tumour progression.

We further find that RhoGDI2 suppression increases the activity of RhoA, but not that of Rac1 or Cdc42. To investigate the impact of RhoA on ciliogenesis, we performed a double knockdown of RhoGDI2 and RhoA. We observed that the loss-of-phenotype of ciliogenesis resulting from RhoGDI2 silencing was partially reversed when RhoA was concomitantly silenced. RhoA is known to cause excessive actin polymerization so this reduction of RhoA can mitigate the negative effects on actin polymerization and cytoskeletal organization, thereby allowing ciliogenesis to occur more normally ¹⁴⁵. Moreover, silencing RhoA alone did not significantly affect ciliogenesis, ruling out non-specific effects and indicating that the partial rescue observed in RhoGDI2-silenced cells is not due to a basal increase of ciliogenesis via RhoA. However, repression of Rac1 in RhoGDI2-silenced cells did not mitigate the ciliogenesis phenotype.

This suggests that RhoGDI2 promotes ciliogenesis through regulation of RhoA. This is in line with literature where many studies have highlighted the role of RhoA in ciliogenesis. Increased RhoA activity and excessive actin polymerization at the basal body in mice has resulted in primary cilia defects ¹⁹⁸. In polycystic kidney disease (PKD1), cystic cells showed disorganized actin cytoskeleton with elevated total and centrosomal active RhoA and ROCK signaling. By examining cilia length as a marker for centrosomal RhoA activity, ARHGAP5, -29, and -35 were noted as key ciliogenesis regulators in normal human renal tubular cells ¹⁴⁷. Moreover, in a study focusing on a ciliary disease, the RhoA/ROCK1 pathway was reported to mediate cell migration and wound-healing defects suggesting that RhoA is integral to ciliary function ¹⁹⁹.

Although our data indicate that RhoGDI2 promotes ciliogenesis through regulation of RhoA activity, this does not imply that all centrosome-associated functions are regulated through the same mechanism. Importantly, the experimental conditions used to study cell proliferation, mitosis, and centrosome amplification differed from those used to assess ciliogenesis, as these assays were performed in full-serum conditions rather than under serum starvation.

Serum availability is known to differentially modulate Rho GTPase activity, with serum deprivation predominantly reducing RhoA-driven actin polymerization, while Rac1 and Cdc42 activities may be maintained or transiently activated as part of an adaptive stress response ²⁰⁰. Therefore, the relative contribution of individual Rho GTPases downstream of RhoGDI2 is likely context-dependent. This may explain why RhoGDI2 strongly affects ciliogenesis, an event closely linked to RhoA-mediated cytoskeletal regulation, while its impact on other centrosomal functions, such as mitotic progression or centrosome amplification, may involve distinct or additional signaling pathways ²⁰¹. With that, we propose the following model of postulated RhoGDI2 functions at the primary cilia (Figure 19):

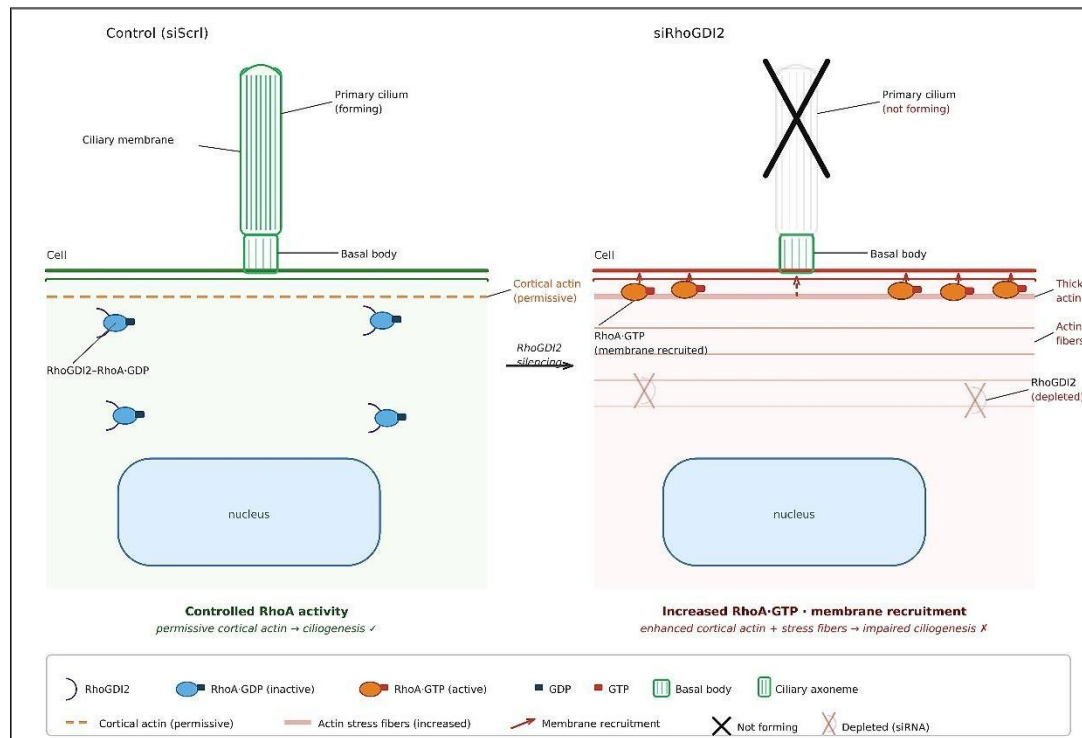


Figure 19: Working model linking RhoGDI2 depletion to impaired ciliogenesis via increased RhoA activity.

In control cells (siScr1), RhoGDI2 maintains RhoA in its inactive GDP-bound state in the cytoplasm, limiting its recruitment to the plasma membrane. Actin remodelling is kept at permissive levels, allowing the basal body to nucleate and extend the primary cilium axoneme into the extracellular space. Upon RhoGDI2 silencing (siRhoGDI2), RhoA is released from its inhibitory complex and accumulates in its active GTP-bound form. RhoA-GTP is recruited to the plasma membrane, where it drives enhanced actin assembly and the formation of actin stress fibers. The resulting dysregulation of the actin cytoskeleton interferes with basal body function and impairs primary cilium formation. The plasma membrane is represented as a double leaflet. Symbols are defined in the legend. Arrows indicate membrane recruitment of RhoA-GTP. The crossed-out cilium indicates absence of ciliogenesis. Data are representative of findings from U-2 OS cells. (Created with Biorender)

4.2.3 RhoGDI2 silencing decreases immune cell killing potential, a function also regulated by the centrosome

As we have previously seen that RhoGDI2 promotes ciliogenesis, we also investigated if RhoGDI2 promotes other centrosomal functions. In immune cells, a key function of the centrosome is the formation of the immune synapse (IS). IS is the interface between a T cell or a natural killer cell and a target cell, and is a key structure in the killing process^{163,202}. We observe a significant decrease in the ability of NK-92 cells to kill tumour cells after RhoGDI2 silencing, highlighting that RhoGDI2 plays a role in this centrosomal function in immune cells. RhoGDI1 repression was also able to induce this effect, albeit less dramatically.

The reduced tumour-killing capacity of NK-92 cells upon RhoGDI2 suppression may reflect impaired formation or functional maturation of the IS. Notably, the IS and the primary cilium share strong architectural and mechanistic parallels, including centrosome docking, polarized microtubule organization, and tightly regulated actin remodeling. Given our findings that RhoGDI2 promotes primary ciliogenesis through regulation of RhoA-dependent actin dynamics, it is plausible that RhoGDI2 similarly contributes to IS formation by modulating actin organization at the immune synapse. This hypothesis is further supported by the high expression of RhoGDI2 in immune cells, where it is considered the predominant RhoGDI, and by previous reports implicating Rho GTPases in IS assembly and function. Together, these observations suggest that RhoGDI2 may regulate IS formation through mechanisms analogous to those governing ciliogenesis, potentially involving the same Rho GTPase-dependent cytoskeletal pathways^{37,76,77,109}. In addition, given our identification of RhoG as a RhoGDI2 interactor and prior reports implicating RhoG in IS dynamics, RhoG may represent an alternative or complementary Rho GTPase through which RhoGDI2 influences immune synapse function^{185,186}.

Immune cells infiltrate the tumour microenvironment (TME) and play a pivotal role in the body's defense against cancer. The TME is a complex network composed of various cell types, including cancer cells, endothelial cells, and a diverse array of immune cells such as T cells, natural killer (NK) cells, macrophages, and dendritic cells. The TME can also be immunosuppressive, with cancer cells and associated cells often producing cytokines and other factors that inhibit immune cell function and promote tumour growth. The balance between immune activation and suppression within the TME is critical for determining the outcome of the immune response to cancer^{72,109,165}. Therefore, understanding the dynamics of immune cells is essential for developing new therapeutic strategies to enhance anti-tumour immunity and improve cancer treatment outcomes. The role we propose for RhoGDI2 in centrosomal functions is summarized in Figure 20:

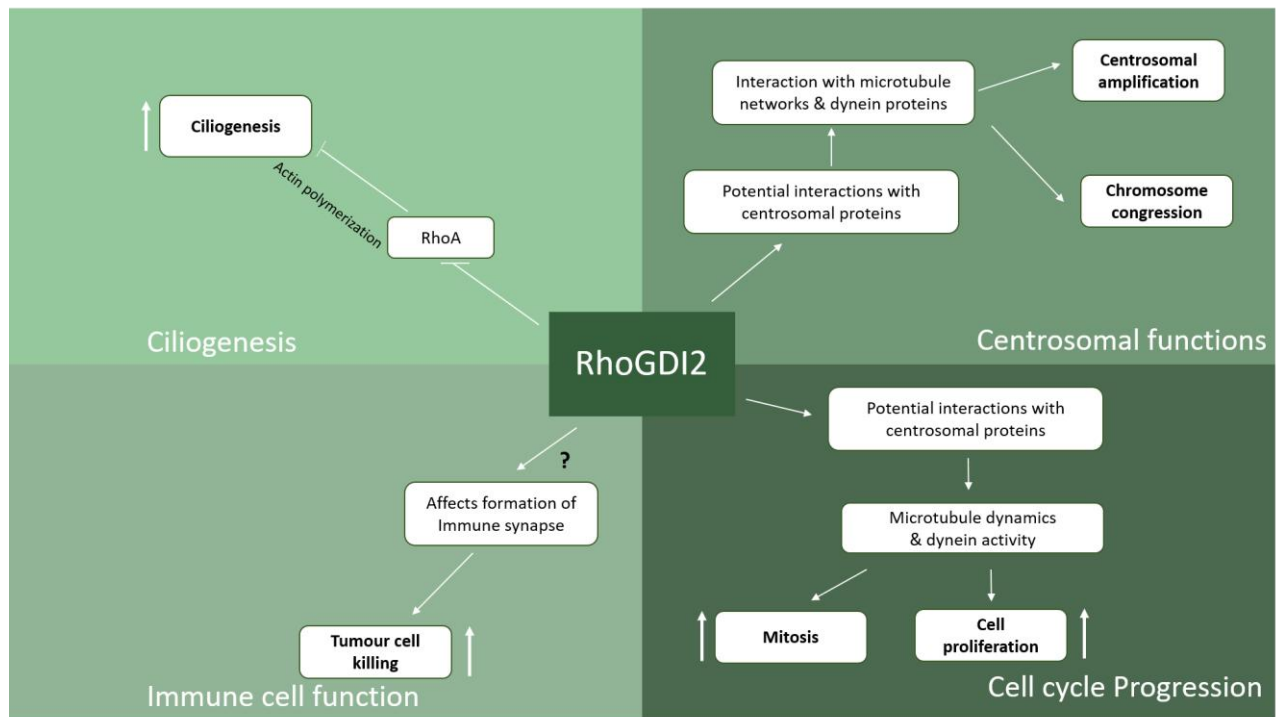


Figure 20: Role of RhoGDI2 in centrosomal functions

Functions of RhoGDI2 in cell cycle progression, centrosomal amplification & chromosome congression, ciliogenesis, and immune cell functions. RhoGDI2 regulates key Rho GTPase to promote cell proliferation, it also promotes mitosis. RhoGDI2 may interact with centrosomal proteins, or actors related to tubulin dynamics, to influence centrosomal amplification and spindle pole formation. RhoGDI2 negatively regulates RhoA to promote ciliogenesis. Lastly, RhoGDI2 promotes the formation of normal immune synapse, consequentially promoting tumour cell killing, however the mechanism is unknown.

4.3 The functional role(s) of the extreme N-terminal domain in RhoGDIs

Despite their structural similarities, RhoGDI1 and RhoGDI2 display significant divergence in functions. These differences may be due to varying affinities for Rho GTPases or their co-regulators, leading to distinct regulatory impacts on Rho GTPase-dependent biological processes. Moreover, both RhoGDI 1 and 2 might be differentially localized within the cytoplasm or membranes across various cell types, further contributing to their distinct roles. The most obvious difference lies in their extreme N-terminal domains. RhoGDI1 and RhoGDI2 fold differently at this region, with the N-terminal domain of RhoGDI2 being acidic and negatively charged. This unique feature allows RhoGDI2 to form long-range electrostatic interactions with co-regulators, a capability that RhoGDI1 lacks. This structural distinction may quite possibly underscore the unique functional attributes of RhoGDI2, and explain why it can interact with specific binding partners.

To investigate further, we created inducible expression vectors encoding chimeric RhoGDI1/2 proteins by swapping their respective extreme N-terminal domains. We confirm that RhoGDI2 silencing had functional consequences on downstream RhoGTPase activity, and noticed that both RhoGDI1 and RhoGDI2 decrease Rac 1 activity. However, in literature many studies have reported that RhoGDI2 regulates numerous biological processes by promoting Rac1 activity, as observed in Jurkat T cells, myofibroblasts, and various human cancers, possibly by directly or indirectly interacting with Rho GEFs Vav1 and Rac1-specific Trio ^{46,49,54,76,109}.

Our initial hypothesis was that re-expression of recombinant RhoGDI2 in RhoGDI2-silenced cells would reverse the proliferation defect, and that comparison with N-terminally swapped chimeric RhoGDIs would help delineate the contribution of the extreme N-terminal domains of RhoGDI1 and RhoGDI2 to this phenotype. However, neither recombinant RhoGDI2 nor the chimeric RhoGDIs restored cell proliferation in RhoGDI2-depleted osteosarcoma cells. A likely explanation for this lack of rescue is that inducible expression of recombinant and chimeric RhoGDIs results in aberrantly high protein levels, leading to excessive inhibition of Rho GTPase activity and, consequently, impaired cell proliferation. This limitation suggests that precise regulation of RhoGDI expression levels is critical for maintaining normal cellular function, and that overexpression systems may not be suitable for accurately dissecting domain-specific functions of RhoGDIs in proliferation control.

5 Conclusion

This research set out to understand the dual and context-dependent role of RhoGDI2 in cancer biology and to define its functional divergence from the closely related RhoGDI1. Together, our findings reveal previously unrecognized functions of RhoGDI2 that extend well beyond its classical role as a passive regulator of RhoGTPase cycling.

Using osteosarcoma cells as a model, we demonstrate that RhoGDI2 regulates centrosomal integrity, ciliogenesis, and cell proliferation, functions not shared by RhoGDI1 despite their high structural similarity. Mechanistically, these effects are mediated at least in part through local regulation of RhoA activity, whose silencing partially rescues the centrosomal and ciliary defects induced by RhoGDI2 loss. Extending this to the immune context, we show that RhoGDI2 is also required for efficient NK cell-mediated tumour killing, consistent with a role in immune synapse organization, a structure with striking parallels to the primary cilium.

Taken together, these data provide a mechanistic explanation for the reported dual role of RhoGDI2 in cancer: its pro-tumorigenic or anti-tumorigenic behavior is not contradictory but context-dependent, reflecting its distinct functions in cancer cell proliferation on one hand and anti-tumour immunity on the other. Critically, our results establish that RhoGDI1 and RhoGDI2 are not functionally interchangeable, particularly in centrosome-related pathways, underscoring the importance of isoform-specific analysis when investigating RhoGTPase regulation in disease.

6 Perspectives

Our research has identified many novel properties of RhoGDI2 that might explain its dual function in cancer through its roles in centrosomes. However, many questions still remain about how, or why, RhoGDI2 plays a role in these different functions.

In our IP/MS, we see multiple centrosomal and dynein proteins that might be potentially interacting with RhoGDI2, and we hypothesize that through these interactions RhoGDI2 might exert its role in centrosomal functions. It is imperative to validate these interactions to further understand the proteomic landscape of RhoGDI2.

In addition, we see that RhoGDI2 plays a role in all centrosomal functions studied. However, the role of RhoGDI1 in these centrosomal functions was not consistent. In the future, data to study this inconsistency can be gathered. As we observe that RhoGDI2 plays a role in ciliogenesis through regulation of RhoA activity, we still do not know if this regulation is direct or indirect. Further studies that elucidate how this regulation plays through would be most effective in understanding the mechanisms behind this function.

In terms of immune cells, it would be highly relevant to see if RhoGDI2 causes reduced or abnormal immune synapse formation. Moreover, we still do not know if RhoGDI2 affects centrosomal functions in these cells through its interactions with centrosomal proteins or through regulation of Rho GTPases.

Lastly, the strategy used to swap the extreme N-terminal domains of RhoGDI1 and RhoGDI2 represents a first step toward dissecting isoform-specific functions of RhoGDIs. However, the use of inducible overexpression systems limits precise control of protein levels, which likely confounds functional rescue experiments. Future studies could address this limitation by inserting recombinant or chimeric RhoGDI cDNAs into the endogenous RhoGDI1 or RhoGDI2 loci using CRISPR/Cas9-mediated homologous recombination, thereby ensuring physiological expression levels. Such an approach would allow a more accurate assessment of whether the N-terminal domains are sufficient to rescue centrosome-related phenotypes in cancer and immune cells. If a functional contribution of the extreme N-terminal domain is confirmed, targeted point mutations at putative regulatory sites, including phosphorylation sites (e.g. T2 or S20) or within the acidic domain, could further elucidate the molecular mechanisms governing RhoGDI2 function *in vitro* and *in vivo*.

Overall, understanding RhoGDI2's multifaceted roles could provide critical insights into cancer biology and identify new avenues for targeted cancer therapies, particularly in the realm of cancer immunotherapy. The potential of RhoGDI2 to significantly impact cancer immunotherapy highlights the necessity of exploring its roles not only in cancer cells but also in immune cells, paving the way for promising advances in novel cancer therapeutics.

7 Materials and Methods

The published paper contains its own materials and methods section. This section represents the methods and materials used for the additional experiments in the thesis which are outside the paper, notably of the subsection 3.3.

7.1 Measurement of chromosomal congression

To measure metaphase chromosome congression cells were treated with Ro-3306 for 24 h and a mitotic release of 40 mins. Image processing and analysis followed that of Green and Kaplan²⁰².

7.2 Preparing RBD and CRIB bacteria

An aliquot of CRIB/RBD bacteria in 50% glycerol stock was streaked onto an LB plate followed by incubation at 37°C overnight. A single colony was inoculated in 2.5 ml of LB broth (10 g/L Tryptone, 5 g/L Yeast Extract, 5 g/L NaCl) (with 100 µg/mL ampicillin), which was subjected to shaking at 37°C for 4h. 50 µl of this bacterial culture was further inoculated in 80 ml of fresh LB broth (supplemented with 100 µg/mL ampicillin) and shaken at 37°C overnight. 20 ml of overnight culture was then inoculated in 3 x 400 ml of fresh LB broth (supplemented with 100 µg/mL ampicillin) and incubated at 37°C for 1h 40min. 0.1 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to each 400 ml culture and left shaking at 37°C (CRIB) or 30 °C (RBD) for 3h. The bacterial cells were harvested by centrifugation at 5000 rpm for 20 mins. The bacterial pellets were frozen in liquid nitrogen and stored at -80°C in 2 ml aliquots.

RBD bacteria express GST fused to the Rho-Binding Domain of Rhotekin which is a downstream effector of RhoA that binds selectively to the active (GTP-bound) form. It was used to pull down active RhoA from cell lysates. CRIB bacteria express GST fused to the Cdc42/Rac Interactive Binding domain of PAK1 (also called PAK1-PBD), which selectively binds active (GTP-bound) Rac1 and Cdc42. It was used to pull down active Rac1 (and Cdc42) from lysates.

7.3 PCR amplification of RhoGDI 1 & 2 N-terminus domains to generate domain swapped constructs

Primers were designed to amplify upstream and downstream of the N- and C- terminal of RhoGDI1 and RhoGDI2, the domain swapped constructs would be called N1/C2 and N2/C1. Plasmids pcDNA4/TO/Rhogdibeta and pcDNA3/TO/mRhoGDI1 were used for RhoGDI1 and RhoGDI2, respectively. The polymerase-chain reaction (PCR) contained phuHF buffer 1x (ThermoFisher Scientific F518L 5x), 0.3 µM forward primer, 0.3 µM reverse primer, 0.2 mM dNTPs, 1U Phu enzyme mix

(ThermoFisher F531S) and 30 ng of the respective genomic DNA. The reactions were labelled **1** (RhoGDI1 N-terminal), **2** (RhoGDI2 C-terminal), **3** (RhoGDI2 N-terminal) and **4** (RhoGDI1 C-terminal). The reactions 1, 2 and 4 were then incubated for 30 seconds at 98°C, followed by 13, 16 and 19 cycles of 10 s at 98°C, 20 s at 66°C and 30 s at 72°C. For reaction 3, incubation was for 30 seconds at 98°C, followed by 13, 16 and 19 cycles of 10 s at 98°C, 20 s at 62°C and 30 s at 72°C. The products were visualized in 1% agarose gel supplemented with 2.5 µl of EtBr. Samples were treated with 4 µl of Xylene Cyanol and SmartLadder gene Ladder (Eurogentec MW-1700-10) was used as marker. Samples were run in an electrophoresis cell coupled to 30 mins at 100 V and the gel visualized using a VersaDoc™ 4000 MP system (Bio-Rad).

For the second step, DNA was migrated on agarose gel and extracted by centrifugation and then precipitated with ethanol. All 4 products were resolubilised using 25 µl of 1x PhuBuffer. The reactions were prepared 5 (with product 1 and 2 for N1/C2) and 6 (with product 3 and 4 for N2/C1). The polymerase-chain reaction (PCR) contained phuHF buffer 1x (ThermoFisher Scientific F518L 5x), 0.2 mM dNTPs, 1U Phu enzyme mix (ThermoFisher F531S) and 30 ng of the 2 products. The reactions were then incubated for 30 seconds at 98°C, followed by 5 cycles of 10 s at 98°C, 20 s at 66°C and 30 s at 72°C. After this, 0.5 µl of forward and reverse primers for the respective RhoGDI were added to each mix. The reactions were then incubated for 30 seconds at 98°C, followed by 9, 12, 15 and 18 cycles of 10 s at 98°C, 20 s at 66°C and 30 s at 72°C. The products were visualized as before on agarose gel.

7.4 Molecular cloning into vectors

DNA was extracted from the agarose gel and precipitated using ethanol. A restriction digestion was performed with NotI and KpnI, from which the insert was extracted using 25 µl of restriction digest buffer, 50 µl of phenol and 120 µl of chloroform. The mix was centrifuged at 20000 rpm for 15 seconds at 4°C. Phenol was removed and it was centrifuged 4x at same conditions. To the final DNA, 1.5 µl of glycerol, 1/10th of the final flow-through (FT) volume of ligation buffer (100 mM Tris/HCl pH 7.5, 5 mM MgCl₂) and 2x the final FT volume of 100% ethanol was added. The mix was frozen for a few minutes and centrifuged at 20000 rpm, 4°C for 30 minutes. After centrifugation, all supernatant was removed, and the ethanol was air-dried. 6 µl of 1x TBE (0.13 M Tris pH 7.6, 45 mM boric acid, 2.5 mM EDTA) was added to the final pellet of insert and it was visualized on an agarose gel to ensure correct size.

To ligate the inserts into vector, 2 µl of 7 ng/µl of vector cut with KpnI and NotI, 0.4 µl of inserts and 2.4 µl of ligation solution was mixed and incubated at RT for 15 minutes.

7.5 Co-immunoprecipitation assay

U-2 OS (WT, tagged with anti-FLAG M2 at N- or C- terminal of RhoGDI2) were washed 2 times with ice cold PBS and harvested in Co-IP lysis buffer (50 mM Tris/HCl pH 7.4, 150 mM NaCl, 1 mM MgCl₂, 0.5% Triton, 50 mM NaF) supplemented with inhibitors 0.1 mM AEBSF and 4 µg/ml aprotinin just before use. Lysates were centrifuged at 16000 g for 10 minutes and 4°C. Anti-FLAG® M2 Magnetic beads (Merck, #A2220) were washed first in MQ, then in Co-IP buffer and added to 90% of supernatants for 2h at 4°C with end over end rotation. At the same time, 10% of supernatant is added to Laemli for total protein concentration. After incubation, samples are centrifuged for 30 seconds at 4°C and 5000g. Supernatant is discarded and the beads were washed 4 times afterwards with Co-IP buffer and taken up in 1x Laemli buffer. Samples are denatured for 5 minutes at 95°C before western blotting.

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The Dual Function of RhoGDI2 in Immunity and Cancer

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Abstract: RhoGDI2 is a guanine nucleotide dissociation inhibitor (GDI) specific for the Rho family of small GTPases. It is highly expressed in hematopoietic cells but is also present in a large array of other cell types. RhoGDI2 has been implicated in multiple human cancers and immunity regulation, where it can display a dual role. Despite its involvement in various biological processes, we still do not have a clear understanding of its mechanistic functions. This review sheds a light on the dual opposite role of RhoGDI2 in cancer, highlights its underappreciated role in immunity and proposes ways to explain its intricate regulatory functions.

Keywords: RhoGDI2 1; RhoGDI1 2; Rho GTPases 3; cancer 4; immunity 5

1. Introduction

Rho GTPases are highly conserved members of the Ras superfamily, which are best known to organize the actin and microtubule cytoskeleton thereby defining the cell shape and migration. They also control a wide variety of signaling pathways that regulate crucial biological processes such as vesicle transport, cell division and gene transcription [1–3]. Rho GTPases cycle between an active GTP-bound form and an inactive GDP-bound form. This activity is regulated by three classes of proteins: guanine nucleotide exchange factors (GEFs) catalyze the exchange of GDP for GTP to activate the GTPase; whereas GTPase-activating proteins (GAPs) increase the intrinsic GTP hydrolysis rate of the GTPase and inactivate it; and guanine nucleotide dissociation inhibitors (GDIs) sequester the GDP-bound form of GTPases in the cytosol to prevent their activation by GEFs or ubiquitin-mediated degradation (Figure 1) [4]. Aberrant signaling of Rho GTPases and their regulators is commonly found in many human cancers and has been attributed to several mechanisms [5–10].

To this date, nearly 85 RhoGEFs and 66 RhoGAPs have been identified for nearly 20 Rho GTPase family members, wherein, in stark contrast, only three human RhoGDIs have been identified so far: RhoGDI1 (or RhoGDI α), RhoGDI2 (or RhoGDI β or D4-GDI or Ly-GDI) and RhoGDI3 (or RhoGDI γ) [8]. All three reside exclusively in the cytoplasm wherein RhoGDI1 is ubiquitously expressed [11,12]. RhoGDI2 was initially believed to be expressed specifically in hematopoietic cells [13,14] but subsequently has also been found in various other cell types and tissues, including cancer cells [8]. RhoGDI3 is primarily expressed in the brain, lung, kidney, testis and pancreas where it targets the Golgi, and shows specificity towards RhoB and RhoG [15]. There is not much known about RhoGDI3 in cancer and immunity, therefore it will not be discussed further in this review.

RhoGDI1 and RhoGDI2 have been implicated in multiple human cancers through their involvement in cancer cell migration, invasion and metastasis and, thus, are regarded as attractive targets for cancer biology [8]. RhoGDI2 has largely remained in RhoGDI1's shadow because of its lower abundance and more restrained distribution. It is, however, starting to garner more attention due to discoveries hinting that RhoGDI2 may play more complex roles in multiple human cancers and many key cellular processes. This review highlights the similarities and differences between RhoGDI1 and RhoGDI2, whilst also encapsulating the multiple roles the latter has shown or has been proposed to play. Finally,



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we also suggest possible novel functions for RhoGDI2 and tie everything together in the context of cancer.

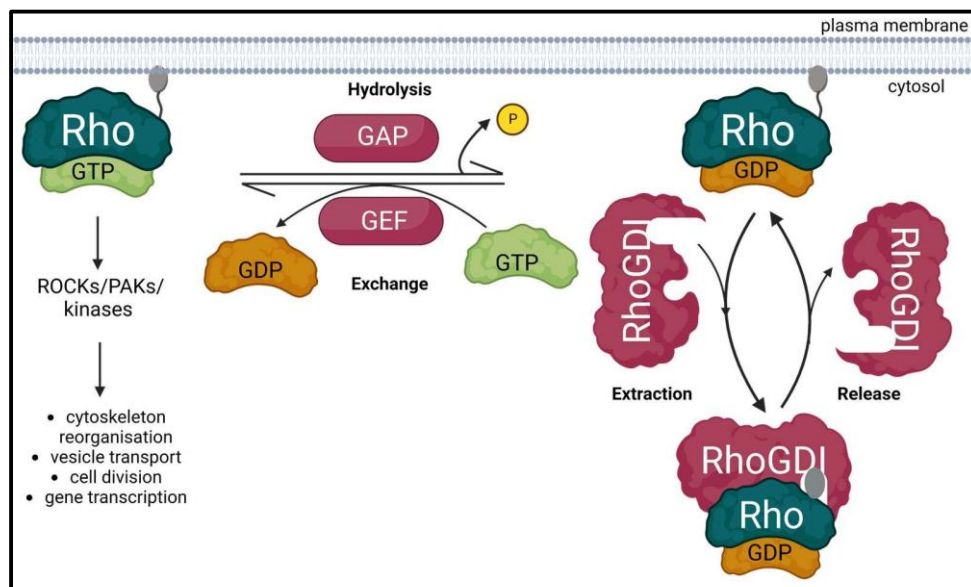


Figure 1. Schematic diagram of the Rho GTPase regulatory cycle. Inactive Rho GTPase is dissociated from its GDP and uptakes GTP to get activated through a process promoted by the Rho guanine exchange factors (RhoGEFs). Active GTP-bound Rho GTPase can then interact with its effectors such as Rho-associated coiled-coil containing kinases (ROCKs), PAK family of serine/threonine kinases (PAKs) and other kinases to participate in various biological processes. This interaction ceases when Rho GTPase-activating proteins (RhoGAPs) stimulate the hydrolysis of the bound GTP to GDP, thereby inactivating the Rho GTPase. The inactive GDP-bound form of Rho GTPase is free to bind to and be sequestered by Rho guanine nucleotide dissociation inhibitors (RhoGDIs). This induces their relocation to the cytoplasm, prevents their ubiquitin-mediated degradation and regulates the activation of Rho GTPases by GEFs. Created with [BioRender.com](https://www.biorender.com) (accessed on 28 January 2023).

2. RhoGDI1 and RhoGDI2: Similarities and Differences

RhoGDI1 was the first RhoGDI to be discovered in rabbit intestine and bovine brain cytosol in 1989 and is widely considered to be the prototype of RhoGDIs. Subsequently, corresponding human cDNA was isolated and a RhoGDI protein was also identified in yeast [11]. Leffers et al. characterized RhoGDI2 and found that it was largely expressed in hematopoietic cells [16]. RhoGDIs interact with the GDP-bound Rho GTPases and extract Rho GTPases from the membrane to regulate them from undergoing the GDP/GTP exchange cycle. [17]. The N-terminal domain of the RhoGDIs interacts with the switch 1 and switch 2 regions of GDP-bound Rho GTPases which prevents the exchange of GDP for GTP and therefore keeps them in their inactive form [18,19], whereas the C-terminal domain also contributes towards their inhibition by extracting Rho GTPases from the membrane [17,20].

RhoGDIs may also shuttle inactive Rho GTPases towards membranes leading to their activation [17,21]. Moreover, RhoGDIs can protect its interacting Rho GTPases from proteasomal degradation [22], demonstrating that RhoGDIs are not merely inhibitors for Rho GTPases but also have a key role in their regulation and signaling. Quite expectedly in view of these functions, both the RhoGDIs are involved in the regulation of multiple biological processes such as actin cytoskeletal organization, cell migration and immune response [23–26]. As mentioned previously, they are also implicated in many human cancers where they can either be upregulated or downregulated (Table 1). The correlation between RhoGDIs and prognoses in multiple human cancer will be discussed in a later section.

Table 1. Expression of RhoGDI1 and RhoGDI2 in human cancers. Biphasic, up and then down.

Cancer	RhoGDI	Regulation	Reference(s)
Colorectal cancer	RhoGDI1	Up	[27]
	RhoGDI2	Up	[28]
Breast cancer	RhoGDI1	Up	[29]
	RhoGDI2	Up Biphasic	[30,31] [32,33]
Hepatocellular carcinoma	RhoGDI1	Down	[34]
	RhoGDI2	Up	[35]
Bladder cancer	RhoGDI1	Down	[36]
	RhoGDI2	Down	[37]
Ovarian cancer	RhoGDI2	Down	[38,39]
Hodgkin's lymphoma	RhoGDI2	Down	[40,41]
Gastric cancer	RhoGDI2	Up	[42,43]
Pancreatic cancer	RhoGDI2	Up	[44–46]
Melanoma	RhoGDI2	Up	[47]
Lung cancer	RhoGDI2	Down	[48,49]
Osteosarcoma	RhoGDI2	Down	[50,51]
Leukemias	RhoGDI2	Down	[52,53]

Although their extreme N-terminal domain (25 and 22 amino acids for RhoGDI 1 and 2, respectively) are completely divergent, RhoGDI 1 and 2 show 73.6% identity for the remaining C-terminal sequence (Figure 2). RhoGDI1 and RhoGDI2 interact with and form complexes with the classical Rho GTPases, i.e., RhoA, RhoC, Rac1, Rac2, Rac3, RhoG and Cdc42 [19,54–56]. However, the interaction potency of RhoGDI2 with Cdc42 is 10–20 folds lower than that of RhoGDI1. Platko et al. observed that a single residue (Ile 177 in RhoGDI1/Asn 174 in RhoGDI2) is responsible for this difference in their affinity for Cdc42 [57].

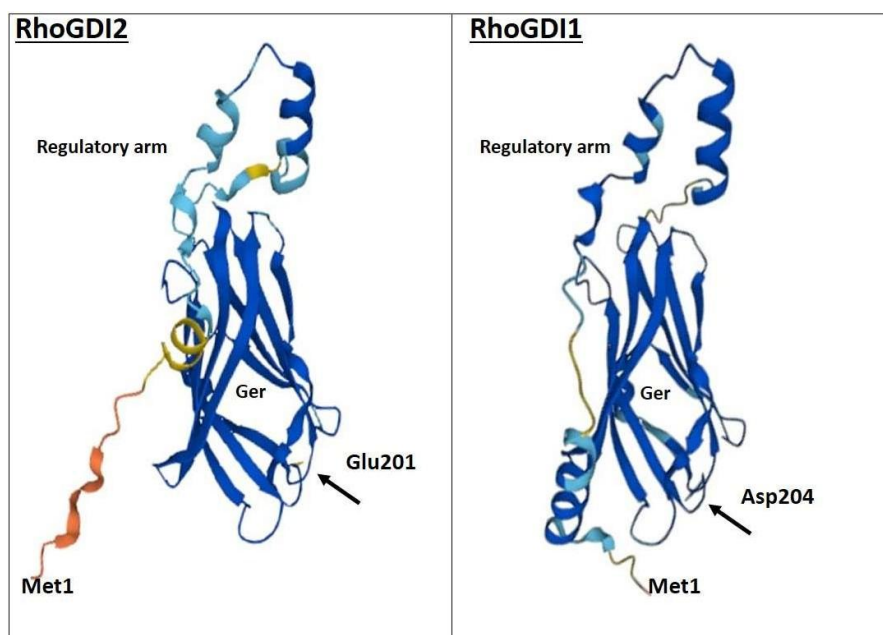
Several other proteins that are not part of the Rho GTPase family have been found to interact with RhoGDI 1 or 2 or both, mainly through high throughput experiments. Upon examining Uniprot (<https://www.uniprot.org> (accessed on 23 January 2023)) and Biogrid (<https://thebiogrid.org> (accessed on 23 January 2023)) databases, the interactors of both RhoGDIs can be extrapolated. Both RhoGDI 1 and 2 have been found to interact with ubiquitin-fold modifier 1 (UFM1), small ubiquitin-like modifier 4 (SUMO4), U2 small nuclear RNA auxiliary factor 2 (U2AF2) and DEAD (Asp-Glu-Ala-Asp) box polypeptide 58 (DDX58). However, RhoGDI1 interacts with Cullin3, whereas RhoGDI2 does not. RhoGDI1 also interacts with EWS RNA-binding protein 1, ezrin, moesin and radixin. On the other hand, RhoGDI2 interacts with RhoGEF Vav1, whereas RhoGDI1 is unable to do so. RhoGDI2 also interacts with acyl-CoA thioesterase 7, B cell CLL/lymphoma 6 and cadherin1. Perhaps the differences in functions of both RhoGDIs may be attributed to their interactions with different proteins that do not belong to the Rho GTPase family.

Mouse models were used to identify the respective functions of RhoGDI 1 and 2. Yin et al. generated RhoGDI2-null mice to explore its functions in lymphocytes. They observed that there were no abnormalities in lymphoid development and immune responses. However, in vitro cultivation of B and T cells from these mice showed de-regulated interactions and other impaired phenotypes. They inferred that RhoGDI2 regulates Rho GTPases in lymphocyte survival and responsiveness, wherein the absence of RhoGDI2 can be compensated in vivo by other Rho GTPase regulatory proteins [26]. It was later shown that RhoGDI1-null mice display abnormalities in the kidneys and reproductive system in

adulthood and that levels of RhoGDI2 expression did not change in WT and RhoGDI1-null mice [58]. Double knockouts of RhoGDI 1 and 2 were then generated in order to get a better insight into their specific and shared functions. These mice are characterized by aberrant homeostasis of lymphocytes and an increased eosinophil population. T cells derived from the mice display defective in vitro proliferation and development and lower levels of CD3 expression. These results show that RhoGDI 1 and 2 share similar functions and can partly substitute for each other in lymphocytic migration and development [59].

P52565	MAEQEPTAEQLAQIAAENEEDEHSHV	NYKPPAQKSIQEIQLDKDDSLRKYKEALLGRVA	60
P52566	MTEKAPEPH--VEEDDDDELDSKL	NYKPPPKSLKELQEMDKDDSLIKYKLTLLGDGP	57
	* : * : : * : *	***** : * : * : * : * : * : * : *	
P52565	VSADPNPNVVVTGLTLVCSAPGPLELDTGDLE	ESFKQSFVLKEGVEYRIKISFRVNR	120
P52566	VTDTPKPNVVVTRLTLVCSAPGPTIMDLTG	LEALKKETIVLKEGSEYRKIHKFNVR	117
	* : * : * : * : * : * : * : * : *	* : * : * : * : * : * : * : * : *	
P52565	EIVSGMKYIQHTYRKGVKIDKTYMVG	SYGPRAEYEFLLTPVEEAPKGMLARGSYSIKSR	180
P52566	DIVSGLKYVQHTYRTGVKVDKATFMVG	SYGPREEYEFLLTPVEEAPKGMLARGTYHNKSF	177
	* : * : * : * : * : * : * : * : *	* : * : * : * : * : * : * : * : *	
P52565	FTDDDKTDHLSWEWNLTIKDKWDK	204	
P52566	FTDDDKQDHLSEWNLSIKKEWE	201	
	***** : * : * : * : * : * : * : *		

(A)



(B)

Figure 2. Comparison of primary and tertiary structures of human RhoGDI1 and RhoGDI2. (A) Protein sequences were compared using EMBL-EBI's Clustal Omega tool. The accession numbers are as follows: human RhoGDI1, P52565 and human RhoGDI2, P52566. Identical residues are indicated by asterisks; substitutions for amino acids possessing highly similar or somehow similar characteristics are indicated by double and single dots, respectively. The highly divergent extreme N-terminal domains are enclosed in the red box. RhoGDI2 is phosphorylated at Y24, S31 and Y153 by $\beta 2$ integrin-related kinases Src, c-Abl and Syk in response to PSGL-1 antibody ligation. On the contrary, RhoGDI1 is phosphorylated at S45, S48 and T52 by calcium-dependent protein kinase CPK3.

(B) Predictions of the 3D structure of RhoGDI1 and RhoGDI2 are from the AlphaFold project (AF-P52565-F1 and AF-P52566-F1, respectively). Confidence regarding the 3D structure corresponding to different parts of the proteins is provided by color code, with dark blue representing the highest confidence and orange the lowest confidence. Ger: pocket accommodating the geranylgeranyl moiety of the Rho GTPases.

3. Regulatory Functions of RhoGDI2

RhoGDI2 is implicated in multiple biological processes in the human body either due to their regulation of Rho GTPases or independently (Figure 3).

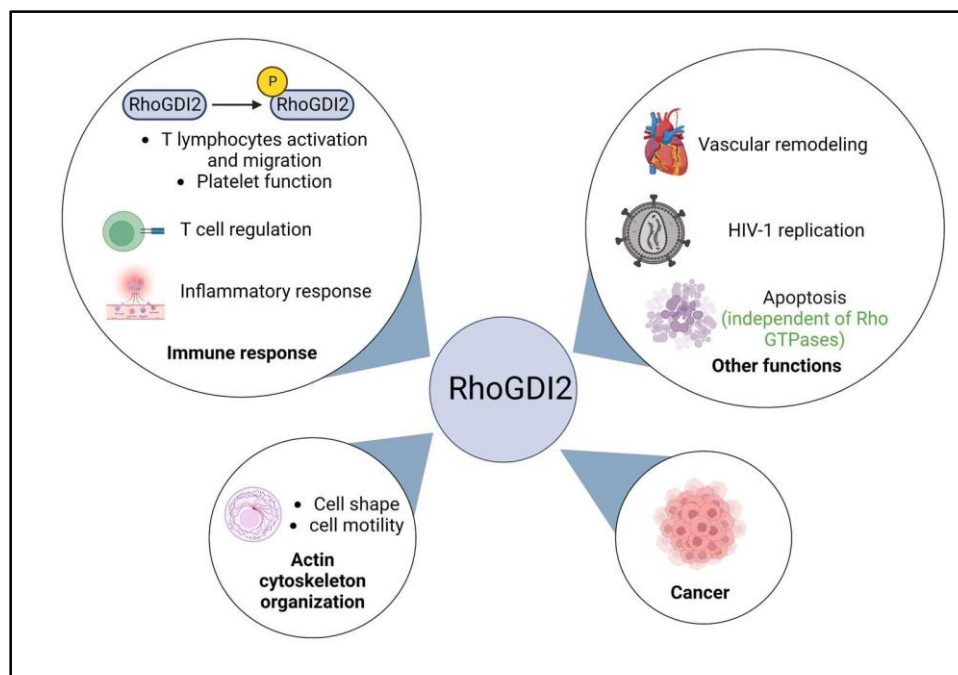


Figure 3. Schematic representation of the main functions of RhoGDI2 in the human body. RhoGDI2 is known to play a major role in multiple biological processes. It is involved in actin cytoskeleton organization, where it affects cell shape and movement. It is also involved in immune response wherein it is phosphorylated by kinases (Src, c-Abl and Syk) at Y24, S31 and Y153. Phosphorylated RhoGDI2 plays a role in T cell lymphocyte activation and migration and platelet function. RhoGDI2 is also involved in T cell regulation and inflammatory response. It regulates vascular remodeling by helping the migration of smooth muscle cells and is also implicated in HIV-1 replication and apoptosis. Lastly, RhoGDI2 is also involved in multiple cancers. Except for apoptosis, RhoGDI2 plays a Rho GTPase-dependent role in all other biological processes mentioned here. Created with [BioRender.com](https://www.biorender.com) (accessed on 28 January 2023).

3.1. Actin Cytoskeletal Organization

The three best characterized Rho GTPases are RhoA, Rac1 and Cdc42. They transduce signals in response to a chemical or mechanical stimuli to regulate different signaling pathways [60]. These Rho GTPases have been shown to modulate actin cytoskeleton organization, thereby defining cell shape and movement. RhoGDI2 sequesters Rho GTPases in the cytosol and may cause a rounding up of cells in various cell lines upon overexpression [16]. CRIF-1 (or CR6-interacting factor-1) is involved in mitochondrial functions and the regulation of cell growth. In human umbilical vein endothelial cells (HUVECs), RhoGDI2 is upregulated upon CRIF-1 silencing, which results in reduced cell migration possibly through the regulation of the activity of Rho GTPases [25]. In addition, RhoGDI2 has been shown to negatively regulate trophoblast migration via the inhibition of Rac1 activity. Trophoblasts need to proliferate and migrate to ensure a successful pregnancy [61].

3.2. Immune Response

3.2.1. Innate Immune Response

The innate immune system is the body's first line of a quick and non-specific mechanism of defending itself against foreign organisms entering the body [62].

RhoGDI2 negatively regulates Fcγ receptor (FcγR)-mediated phagocytosis in Jurkat T cells by preventing the localization of Rac1 to the membrane and thereby inactivating it.

Phagocytosis, or the process of engulfing a foreign particle, is driven by a finely controlled rearrangement of the actin cytoskeleton. It is essential to regulate the actin cytoskeleton as phagosome and multicomponent signaling pathways transduce signals from phagocytic receptors to the cytoskeleton. FcγR is one such receptor that clusters on monocytes and macrophages to activate multiple signaling cascades to initiate phagocytosis; its function has also been implicated in the pathophysiology of autoimmune diseases and in mediating cytotoxic effects of monoclonal anti-tumour antibodies [63,64].

Though not related to its function, it is worth mentioning that RhoGDI2 was recently identified as an extremely sensitive fecal biomarker to quantify gut inflammation in patients with inflammatory bowel disease (IBD) to estimate IBD severity [65]. Severe IBD can lead to gastrointestinal cancers as well as cardiovascular diseases; immunological disorders and inflammatory biomarkers can be exploited for information about the immune system of an individual, even if they aren't directly involved in inflammation [66].

Using mRNA microarray analysis, RhoGDI2 was found to be differentially expressed in chronic chagasic cardiomyopathy (CCC), a myocardial disease characterized by severe cardiac inflammation leading to heart failure. RhoGDI2 was identified as one of the top genes, along with Rac2, from a module that is enriched in the pathway of natural killer (NK) cell-mediated cytotoxicity [67]. This suggests that RhoGDI2 may be involved in CCC pathogenesis via NK cell-mediated cytotoxicity, possibly via Rac2 regulation [68]. Despite the fact that NK cells belong to the family of T and B cell lymphocytes, the level of RhoGDI2 protein expression in NK cells has not yet been documented [69].

The association of RhoGDI2 and Rac2 was also reported in a signaling pathway that is implicated in the pathogenesis of oral lichen planus (OLP), a chronic inflammatory and immune-mediated disease affecting skin, nail, hair and mucous membranes. Caspase-1, an inflammatory caspase, is upregulated in OLP tissues wherein Rac2 and RhoGDI2 were identified as its potential interactors. Moreover, it was also observed that the gene and protein expression levels of Rac2 and RhoGDI2 are upregulated in OLP tissues, where they positively correlate with Caspase-1 leading to immune deregulation and inflammation in OLP [70].

3.2.2. Adaptive Immune Response

The highly specific and relatively slow adaptive immune system, made up of B and T cell lymphocytes and antibodies, takes over when the innate immune system is unable to destroy the germs [62].

Though also not a function of RhoGDI2, it was recently reported through a large nationwide cohort study that antibodies against RhoGDI2 can be used as a diagnostic biomarker for long-term kidney graft loss and fibrosis in patients with kidney transplants from deceased donors. Further studies showed that RhoGDI2 autoantibodies were associated with chronic antibody-mediated rejection and inferior graft survival [20,71–74]. It would be interesting to explore if the autoantibodies against RhoGDI2 can be used as a diagnostic biomarker in other organ transplants.

3.2.3. Phosphorylated RhoGDI2

RhoGDI2 phosphorylated at unspecific site(s) was immunoprecipitated from Jurkat T cells stimulated by phorbol myristyl acetate (PMA). However, it could not be immunoprecipitated from resting T cells, suggesting that phosphorylation of RhoGDI2 could have potential consequences for lymphocyte activation [14]. Phosphorylated RhoGDI2 was also immunoprecipitated from monocyte U937 immune cells after PMA treatment. Further experiments showed that RhoGDI2 was phosphorylated at unspecified tyrosine residue(s) in anti-CD3 monoclonal antibody (mAb)-activated Jurkat and Raji T cells. It was also observed that RhoGDI1 remained unphosphorylated in PMA-induced U937 cells and anti-CD3mAb-activated T cells. This is of interest, as PMA induces the differentiation of U937 cells from non-adherent myelomonocytic cells to adherent macrophage-like cells and anti-CD3 mAbs are a well-recognized protocol for T cell activation.

It was also reported that upon T cell activation by anti-CD3 mAbs, RhoGDI2, but not RhoGDI1, interacts with Vav1, a RhoGEF that is also expressed abundantly in immune cells. Both Vav1 and RhoGDI2 then co-localize at the periphery of the immune synapse, the interface between the T cell and an antigen-presenting cell [75,76]. Theoretically, RhoGDI2 and Vav1 have opposing functions, so their interaction is intriguing. This could suggest that RhoGDI2 might be involved in the regulation of hematopoietic-specific Rho GTPases via different mechanisms leading to their inhibition. In Jurkat T cells, knocking down RhoGDI2 reduces their adhesive and migratory ability by decreasing the activities of Rac1 and Cdc42. It has also been recently discovered that RhoGDI2 can be phosphorylated at Tyr24, Ser31 and Tyr153 (by Src, c-Abl and Syk), which would play a role in Rac1 activation by RhoGDI2. Phosphorylation of RhoGDI2 at Y24/153 increased its ability to bind to Vav1, which could explain the promoting role of RhoGDI2 in T cell adhesion and migration, while the phosphorylation at S31 is required for the opening of RhoGDI2 and the subsequent release of its interacting Rho GTPases [24,76]. This is in contrast with the anti-migratory effect of RhoGDI2 in multiple other cell types [62], bolstering the fact that perhaps RhoGDI2 participates in different regulatory pathways in T lymphocytes. This is of additional importance as RhoGDI2 was initially observed in B and T lymphocytes where it is abundantly expressed [16], suggesting that RhoGDI2 is the predominant RhoGDI in hematopoietic cells and that it plays a dual role in the immune response.

In human platelets, RhoGDI1 has a homogenous distribution whereas RhoGDI2 is distributed unevenly or in a polarized manner within the same cell. Downregulating RhoGDI2 in human platelets inhibited their spreading, which was not observed upon RhoGDI1 silencing. It was also noticed that RhoGDI2, and not RhoGDI1, was phosphorylated at PKC substrate motifs upon platelet activation and co-localized with PKC in adherent platelets. This study suggests that RhoGDI2 may also play a role in platelet function by regulating Rho GTPases activity and affect the hemostasis [77]. This could mean that despite RhoGDI1 being the preferred binding partner for Rac1 and Cdc42 in most cell types, these key Rho GTPases preferentially bind to RhoGDI2 in human platelets.

3.3. Apoptosis

Apoptosis driven by caspase-3 can be induced by anti-Fas antibody, etoposide, taxol, daunorubicin, overexpression of PMA and staurosporine [78–80]. RhoGDI2, but not RhoGDI1, is cleaved at Asp19 by caspase-3 during apoptosis in Jurkat T cells, ML-1 and HL-60 human leukemic cell lines and macrophages. The cleaved form of RhoGDI2 (deprived of its N-terminal extremity) is then translocated into the nucleus suggesting a pro-apoptotic role of RhoGDI2. An identical caspase-3-dependent cleavage is also observed in spontaneous and TNF- α induced apoptosis in polymorphonuclear neutrophils (PMN) and in camptothecin-induced apoptotic human promyelocytic cells. This is of particular importance as apoptosis is a critical step when PMN migrates into affected tissues and interacts with extracellular matrix (ECM) proteins to ameliorate inflammation [81]. Cleavage of RhoGDI2 by caspase-3 is now considered a hallmark of the apoptosis [20,80].

3.4. HIV-1 Replication

Rho GTPases have been reported to regulate the replication of human immunodeficiency virus type 1 (HIV-1), the causative agent of acquired immunodeficiency syndrome (AIDS), by promoting its entry and its infection into T cells [82]. It was noted, however, that RhoGDI2 negatively regulated the rate of HIV-1 replication in infected MT-4 T cells by reducing the F-actin content and the activity of both Rac1 and RhoA at the early phase of the viral life cycle. Env are glycoproteins anchored in the viral membrane essential for the viral entry into cells, and the activation of RhoA mediates the reorganization of the cytoskeleton to facilitate this entry [83,84]. Upon further analysis, it was observed that expression of recombinant RhoGDI2 affected Env-mediated processes, possibly via receptor clustering and virus-cell membrane fusion.

3.5. Vascular Remodeling

Vascular remodeling is involved in the pathogenesis of many life-threatening cardiovascular diseases, transplantation and chronic rejection [85,86]. Vascular smooth muscle cells (VSMCs) undergo a transition from contractile to proliferative phenotype for successful vascular development and remodeling. Upon binding of angiotensin (Ang) II, a peptide hormone, to its receptor angiotensin II receptor type I, RhoA becomes activated and further mediates migration of VSMCs. A recent study also reported that Ang II induces proteasomal degradation of RhoGDI 1 and 2 by upregulating ubiquitination, which leads to reduced proliferation in VSMCs [87].

Consistent to this, the expressions of RhoGDI1 and RhoGDI2 are significantly increased by TGF β 1 in human aortic adventitial fibroblasts, wherein TGF β 1 drives the differentiation of vascular myofibroblasts from fibroblasts, and positively correlates with vascular remodeling [88]. Upon further investigation, it was discovered that TGF β 1 promotes the interactions between RhoGDI2 and Rac1 or Cdc42 via the Smad signaling pathway, with no effect on the interactions between RhoGDI1 and Rac1/Cdc42. This suggested that RhoGDI2, and not RhoGDI1, participates in TGF β 1 induced myofibroblast differentiation [89].

4. Regulatory Functions of RhoGDI2 in Cancer

RhoGDI2 is either upregulated or downregulated in many cancers depending on its type and stage. More interestingly, its level of expression can be either meaningless or strongly correlated with a good or poor prognosis. Figure 4 reports different cancer types for which studies identified RhoGDI2 as having pro- or anti-cancer tumour effects. This section reviews recent findings on RhoGDI2 in cancer progression and highlights its dual functions.

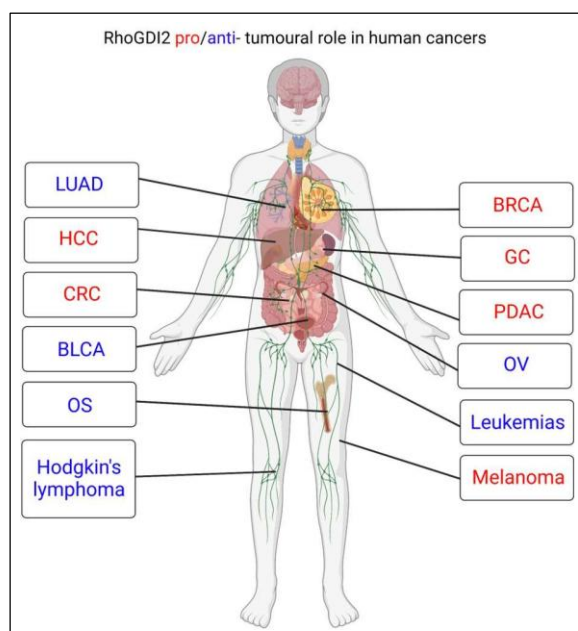


Figure 4. RhoGDI2 in cancer. Based on in vitro experiments and on correlation studies using human tumour samples, RhoGDI2 has been described as possessing either pro- or anti-tumour functions, depending on cancer types. It plays a tumour promoting role in hepatocellular carcinoma (HCC), colorectal carcinoma (CRC), breast cancer (BRCA), gastric cancer (GC), pancreatic ductal adenocarcinoma (PDAC) and skin cutaneous melanoma (SKCM). It plays a tumour suppressive role in lung adenocarcinoma (LUAD), bladder cancer (BLCA), osteosarcoma (OS), Hodgkin's lymphoma, ovarian (OV) cancer and in multiple leukemias. Created with [Biorender.com](https://www.biorender.com) (accessed on 28 January 2023).

4.1. Ovarian Cancer

While RhoGDI1 is uniformly expressed in multiple ovarian (OV) cancer cells, RhoGDI2 protein expression is either upregulated or downregulated depending on the OV cancer cell line. RhoGDI2 has also been shown to act as a tumour and metastasis suppressor in OV cancer by enhancing Rac1 activity to activate p38 and JNK/MAPK cascades [38]. This was corroborated by a recent study that observed that Adenosine (Ado), a purine nucleotide exerting anti-tumour activity in multiple human cancers, enhances RhoGDI2 expression in A2780 OV cancer cells and A2780 subcutaneous xenografts in nude mice. They also observed that Ado inhibited tumour growth in the mice in a RhoGDI2-dependent manner [39].

4.2. Breast Cancer

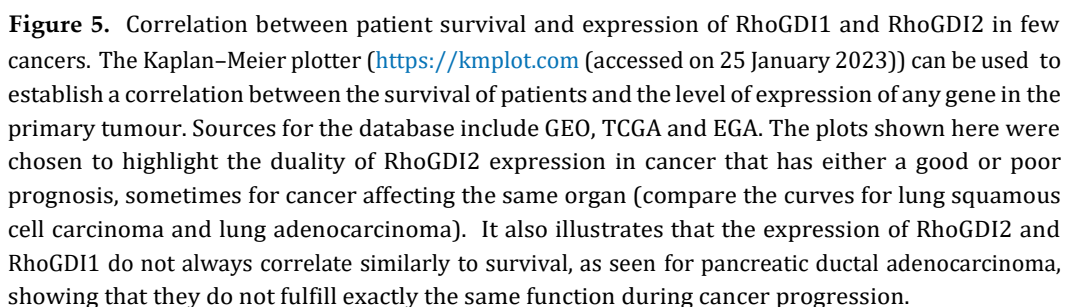
Similar to OV cancer, RhoGDI2 protein expression was shown to be either upregulated or downregulated depending on the breast cancer (BRCA) cell line, while RhoGDI1 is similarly upregulated in all BRCA cell lines [30,31]. Eventually, a stage-dependent biphasic pattern of RhoGDI2 expression has been evidenced in breast cancer tissues with a marked increase from normal to hyperplasia, followed by a decrease from in situ to invasive lesions. An inverse correlation between RhoGDI2 expression and lymph node metastasis was observed, implying that RhoGDI2 might act as a tumour promoter but metastasis suppressor in BRCA [32]. However, it is important to remember that RhoGDI2 is not only expressed by cancer cells but also by cells of the tumour micro-environment, such as immune cells. Therefore, modifications in RhoGDI2 expression measured in tumour samples can be influenced also by differences related to immune cell populations infiltrating the tissue.

Another study further demonstrated that Rictor/mTOR pathway, which is upstream of Rho GTPases signaling, downregulates RhoGDI2 expression and promotes Rac1 activity, thereby stimulating cell invasion and metastasis in BRCA cells. They confirmed that lower RhoGDI2 levels were associated with poor prognosis in the human epidermal growth factor receptor 2 (HER2)⁺ BRCA [33]. However, the data obtained from public databases including all BRCA subtypes and analyzed with the “Kaplan-Meier Plotter” (an online tool available for exploring the correlation between the expression of any gene and survival of patients with cancer (<https://kmplot.com> (accessed on 25 January 2023)) [90], show that a high RNA expression level of RhoGDI2 is associated with a lower probability of overall survival (Figure 5).

4.3. Bladder Cancer

RhoGDI2 has largely been reported to be associated with a good prognosis in bladder cancer (BLCA) patients, where it represses metastasis by inhibiting Rac1 activity. However, it does not affect the primary tumour growth [37].

Despite being accepted as a metastasis suppressor, recent reports from Huang lab unanticipatedly find RhoGDI2 levels to be consistently elevated in most human and mouse BLCA tissues. They have identified three different pathways—RhoGDI2/miR-200c/JNK2/Sp1/MMP2 pathway, XIAP/Erk/nucleolin/RhoGDI2 and, lastly, miR-145/Sp1/USP8/AUF1/RhoGDI2 pathway—through which they show that RhoGDI2 promotes BLCA invasion in vitro and lung metastasis in vivo. They observe that RhoGDI2 acts as a tumour and metastasis promoter, despite being widely demonstrated in the past as a metastasis suppressor, with no effect on the tumour [91–93]. Further investigations are required to better define the exact function of RhoGDI2 in BLCA.



4.4. Osteosarcoma

WSB1 (hypoxia-driven-WD repeat and SOCS box containing 1) is upregulated in many human cancers and acts as a suppressor of cytokine signaling. In osteosarcoma (OS), RhoGDI2 (but not RhoGDI1) is degraded by WSB1. In U2OS and MG63 human osteosarcoma cell lines, it results in the activation of Rac1 which increases the amount of F-actin, promotes the formation of lamellipodia and leads to enhanced cell motility and migration ability. WSB1 was also shown to drive the metastatic potential of OS in vitro and in the BALB/c (nu/nu) mouse model. Of most interest in this context is that RhoGDI2 overexpression in OS cells and mice is able to reverse the spreading of the WSB1-induced metastasis [50,51], demonstrating its direct participation in OS progression.

4.5. Leukemias

RhoGDI2 was found to be mutated at two positions (V68L and V69A) in KM3 and Reh human acute myeloid leukemia (AML) cell lines. Neither wild-type (wt) nor mutated (mt) RhoGDI2 overexpression altered cell proliferation. However, overexpressing mtRhoGDI2 promoted cell adhesiveness and invasiveness in vitro. These induced phenotypes were reversed by the overexpressing of recombinant wtRhoGDI2, which probably illustrates a competition between wtRhoGDI2 and mtRhoGDI2 for a common interacting partner that regulates AML progression [52].

CXCL12 is a chemokine that binds primarily to CXCR4 (CXCR4) to induce several signaling pathways related to chemotaxis, cell survival and proliferation and gene transcription. RhoGDI2 is expressed quite highly in all acute lymphoblastic leukemia (ALL) cell lines, including Jurkat T-ALL cells. It was found that downregulation of RhoGDI2 increases CXCL12-driven T-ALL migration. RhoGDI2 mutants were created to mimic phosphorylation induced by PMA on Y24 and Y153 (discussed in previous section). These phosphomimetic mutants were seen to rescue the inhibiting phenotype induced by wtRhoGDI2 on CXCL12-mediated ALL migration. Additionally, phosphorylation of RhoGDI2 on Y24 or Y153 reduce its affinity for RhoA or RhoC, leading to their increased activity. Further investigations showed that the phosphorylation of RhoGDI2 was due to non-receptor protein tyrosine kinases Src and ABL1 in response to CXCR4 stimulation by CXCL12 in T-ALL [24,53,76,94].

4.6. Hodgkin's Lymphoma

RhoGDI2 gene expression is selectively downregulated in Hodgkin's lymphoma when compared with B cell non-Hodgkin's lymphoma (B-NHL) cells [40]. Due to RhoGDI2's abundance in hematopoietic cells and its possible role in apoptosis (explained in the previous section), a study attempted to evaluate the functional relevance of RhoGDI2 in apoptosis. Hodgkin L428 cells, which do not express endogenous RhoGDI2, were modified to conditionally express recombinant RhoGDI2. Its induction led to only moderate levels of apoptosis, questioning its importance in this model [41].

4.7. Pancreatic Cancer

RhoGDI2 was found to be significantly upregulated in tumour samples from patients with pancreatic adenocarcinoma (PDAC) wherein its expression positively correlated with tumour size, differentiation, clinical stage, lymph node metastasis, vascular invasion and reduced life expectancy (see Figure 5). Silencing of RhoGDI2 was shown to decrease the invasiveness of PDAC cells in vitro, and to reduce the expression of matrix metalloproteinase (MMP) 2, possibly through regulation of Rac1 activity [44]. It suggests that RhoGDI2, or the pathway it regulates, could be an interesting target in that particular highly aggressive cancer for which the current therapeutic options are of limited long-term efficacy. IFN- γ is a cytokine predominantly produced by natural killer cells and has anti-tumorigenic effects [45]. It was observed that IFN- γ suppresses the expression of RhoGDI2, which reduces Rac1 activity and CXCL8 expression. Moreover, overexpression of RhoGDI2 in PDAC cells in vitro enhanced their proliferation, migration, invasiveness and apoptotic

resistance to Gemcitabine (a chemotherapeutic drug). It does so by increasing the expression of vimentin and Snail, a master regulator of epithelial-to-mesenchymal transition (EMT), and by decreasing the expression of the epithelial marker E-cadherin. As an additional validation of the involved mechanisms, the downregulation of Snail expression inverted the phenotypes seen by overexpressing RhoGDI2 [46].

4.8. Colorectal Cancer

RhoGDI2 gene and protein expressions are upregulated in the highly invasive and metastatic colorectal cancer (CRC) cell lines as compared to less invasive cell lines. It is also upregulated in CRC tissues wherein its expression negatively correlates with patient survival. Overexpression of RhoGDI2 enhances the proliferation, migration and invasive capacities of CRC cells partly via the activation of the PI3K/Akt pathway [28]. Although not explored further, the activation of PI3K/Akt pathway by RhoGDI2 overexpression in CRC could occur via its regulation of Rho GTPases.

4.9. Hepatocellular Carcinoma

RhoGDI2 has been shown to be upregulated in hepatocellular carcinoma (HCC) cells where its overexpression increased their rates of proliferation and invasion, via activating the PI3K/Akt pathway and increasing the levels of MMP2 and MMP9 (consistent to PDAC). MMPs are ECM-degrading enzymes involved in tumour invasion and metastasis. They are also intimately involved in the regulation of the activities of cytokines and cytokine receptors [95]. No further research was conducted to delve into the relation between RhoGDI2 and PI3K/Akt pathway in HCC [35].

A recent report claims that synergistic effects of traditional Chinese medicines *Euphorbia Pekinesis* and *Glycyrrhiza glabra* against HCC ascites in mice models is due to the downregulation of the expression of RhoGDI2 and Frk (Fyn-related kinase), a member of the Src non-receptor tyrosine kinase family. Frk and RhoGDI2 are both involved in the pathway of vasopressin-regulated water re-absorption, which has been implicated in fluid retention in cirrhosis and seems to be a common therapeutic target pathway of various anti-ascites drugs [96].

4.10. Gastric Cancer

RhoGDI2 shows significantly elevated expression levels in gastric cancer (GC) samples as compared to normal and para-cancerous tissues. In addition, knocking down RhoGDI2 expression decreases the migration and invasion of GC cells, enhances their sensitivity towards chemotherapeutic drugs and reduces tumour growth in mice models. RhoGDI2 acts as a scaffold protein to promote the binding of Rac1 to FilaminA, an actin binding protein. FilaminA further promotes Rac1 activation via binding to Trio, a Rac1 specific RhoGEF, and thereby enhances the invasive abilities of GC cells. Both RhoGDI2 and FilaminA are indicated to be associated with poor prognosis of GC patients. Whether RhoGDI1 can also associate with FilaminA is not known. In addition to promote Rac1 binding to FilaminA, the RhoGDI2-Rac1 axis is also involved in another signaling pathway in GC since suppressing the expression of RhoGDI2 repressed the Rac1/Pak1/LIMK1 axis, which is involved in EMT, cell migration and invasion in multiple human cancers [42,43].

4.11. Melanoma

Myosin VIIA (Myo7a) is an unconventional myosin serving in intracellular movements. It was shown to promote proliferation, migration, invasion and tumour growth in B16 melanoma cells (a mouse model of skin cutaneous melanoma). Knocking down Myo7a decreases the expression of RhoGDI2 and Rac1 activity. Most interestingly, restoring the expression of RhoGDI2 in these cells is sufficient to also restore Rac1 activity, showing its direct implication in the process. Due to the involvement of Rac1 in multiple human cancers, it was inferred that Myo7a could exert tumour-promoting phenotypes by promoting RhoGDI2 expression levels and consequently Rac1 activity in B16 melanoma [47].

4.12. Lung Cancer

Knocking down RhoGDI2 significantly increased the proliferation, migration and invasion of A549 lung adenocarcinoma (LUAD) cells via increased Rac1 activity. Rac1 promotes migratory and invasive abilities in LUAD along with cytoskeleton rearrangements. When overexpressed, RhoGDI2 scatters F-actin filaments within the cytoplasm and induces loss of cell-cell adhesion and stress fibers formation. Contrary to what was observed in PDAC, overexpression of RhoGDI2 decreased the expression of Snail and increased the expression of E-cadherin, which might account for the anti-tumoral role of RhoGDI2 in LUAD which is suggested by correlation, showing an increased survival for patients having high tumour RhoGDI2 expression (Figure 5). Surprisingly, however, the reverse was observed for lung squamous cell carcinoma (Figure 5), again illustrating the critical importance of defining the different regulatory pathways involving RhoGDI2 in different cancer types.

As opposed to what was observed in HCC, downregulating the RhoGDI2 expression in LUAD increased the expression and activity of MMP9, which resulted in increased migratory and invasive abilities. Further studies showed that, in stark contrast to HCC, suppressing the expression of RhoGDI2 also increased the phosphorylation of Akt and PI3K, hinting that RhoGDI2 may exert anti-tumoral activities in LUAD by decreasing Rac1 activity and deactivating the PI3K/Akt pathway [48,49].

FHL1 (Four and a half LIM protein 1) is downregulated in multiple human cancers, including LUAD, where it exerts anti-tumoral activity [97]. As a potential underlying explanation, it was shown that FHL1 stimulates the expression of RhoGDI2, but not of RhoGDI1. However, the precise pathway is not yet elucidated.

5. Discussion and Future Directives

Although the role of RhoGDI2 in regulating the activity of Rho GTPases is well established, recent findings have highlighted complex molecular mechanisms of this regulation. In this review, we have presented the similarities and differences between RhoGDI1 and RhoGDI2 in regulating Rho GTPases and subsequently many biological processes. We also talked about the regulatory functions of RhoGDI2 in multiple biological processes such as immune response, HIV-1 replication and vascular remodeling and paid attention to its dual role in cancer.

It is important to note that despite sharing a high level of similarity in their structures, RhoGDI1 and RhoGDI2 have proven to be quite divergent in some functions. Both RhoGDIs may have different affinities for Rho GTPases or their co-regulators, which may cause them to regulate differently to Rho GTPases-dependent biological processes. In addition to their specific affinities towards Rho GTPases, the RhoGDIs may also be differentially distributed in the cytoplasm and/or membranes in different cell types, contributing to their diverging roles. However, the most crucial aspect to highlight is the completely divergent extreme N-terminal domains of RhoGDI 1 and 2. Both the RhoGDIs fold differently at their N-terminal domain (as highlighted in Figure 2B) and the N-terminal domain of RhoGDI2 is acidic in comparison to the N-terminal domain of RhoGDI1, thereby possessing a negative charge that may enable RhoGDI2 to establish long-range electrostatic interactions with co-regulators, whilst RhoGDI1 would be unable to do so.

As illustrated by their names, RhoGDIs were first considered only as inhibitors of the activity of Rho GTPases. However, as we have described, RhoGDI2 regulates many biological processes by not only inhibiting, but also promoting the activity of Rac1, as noticed in Jurkat T cell and myofibroblasts, and multiple human cancers [38,47,89]. To the best of our knowledge, RhoGDI1 does not promote the activity of key Rho GTPases. Further research should aim at explaining why RhoGDI2 is able to promote Rac1 activity in some cell types and not others by focusing on three main points: (i) the search for direct or indirect interactions between RhoGDI2 and third partners, such as the Rho GEFs Vav1 and Rac1-specific Trio [24,43,76]; (ii) investigation of the potential role of RhoGDI2 as a scaffold protein that can enhance the binding between Rac1 and actin-binding proteins, which would regulate its functions [42]; and (iii) analysis of dynamics of the subcellular

localization of RhoGDI2, which can vary within different cell types, and also contribute to its dual role in regulating Rac1 activity.

As shown in Figure 6, RhoGDI2 is involved in several hallmarks of cancer, its expression correlates to either high or low aggressiveness in different human cancers. There are multiple drugs being developed to target other regulators and effectors of Rho GTPases [4,6,21]. Although no drugs specifically targeting RhoGDIs are in development, some drugs and traditional medicine involved in ameliorating cancer phenotypes have been shown to be implicated in different signaling pathways involving RhoGDI2. Therefore, inhibiting RhoGDI2 activity has therapeutic potential for some cancers and other diseases, but it is essential to tread carefully due to its complicated role in the human body. As an alternative, a better understanding of the implications of RhoGDI2 in pro- and anti-cancer pathways could lead to the identification of novel therapeutic targets. Further experiments aiming to decipher the intracellular molecular pathways affected by RhoGDI2 are required to appreciate its role in cancer biology.

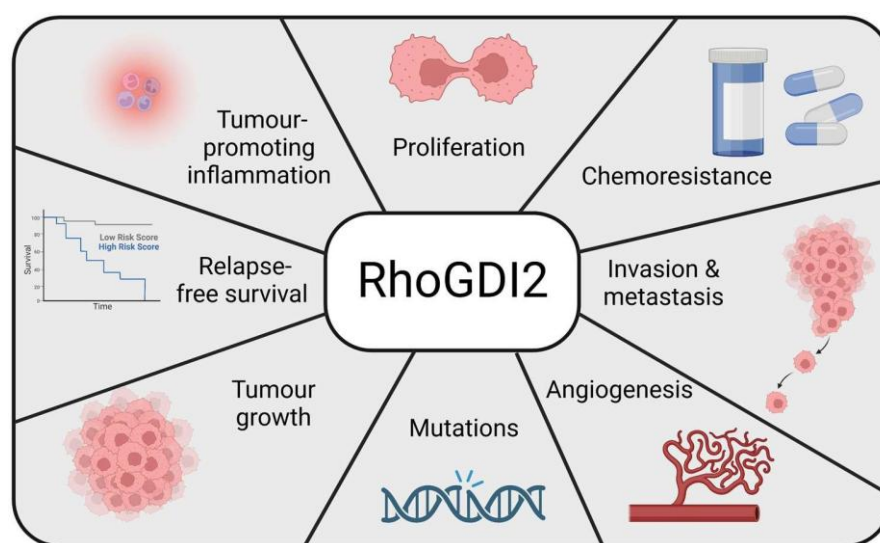


Figure 6. RhoGDI2 is involved in many hallmarks of cancer. RhoGDI2 is involved in many aggressive phenotypes of cancers via its regulation of Rho GTPases either directly or indirectly by interacting with other regulators of Rho GTPases. It modulates cell proliferation, tumour growth, cell migration, invasion and metastasis. It also provides chemoresistance and is shown to be mutated in some human cancers. It also contributes to tumour-promoting inflammation, angiogenesis and relapse-free survival in multiple human cancers. Created with [BioRender.com](https://www.biorender.com) (accessed on 28 January 2023).

A critical role of Rho GTPases, and therefore of RhoGDIs, is the organization of the actin cytoskeleton and all the downstream processes. Changes in the actin cytoskeleton affect other cellular responses such as changes in gene transcription, the microtubule cytoskeleton or vesicular transport. Besides transport, mobility and mechanical support, the microtubule cytoskeleton is also involved in chromosome segregation, centriole duplication and formation of primary cilia [98–100]. Rho GTPases have been extensively probed for their roles in processes involved in centriole duplication and cell division [101–103]. Despite the extensive research done to scrutinize RhoGDI2 for its involvement in multiple human cancers, no work has been done to investigate if and how RhoGDI2 is involved in the different steps regulating cell division.

RhoGDI2 has been shown to localize in centrosomes of human cervical cancer HeLa cells, wherein it is involved in centrosomal amplification, prolonged mitosis and aberrant cytokinesis. Mitosis is part of the cell cycle where the replicated chromosomes are separated into two new nuclei, and perturbations in the centrosomal function are a hallmark of cancer [104,105]. It will be interesting to investigate more functions of RhoGDI2 in the cell

division cycle to increase our understanding of its involvement in cancer and other diseases to ensure appropriate targeting of RhoGDI2 for cancer therapeutics.

Besides its role in cancer cells, RhoGDI2 is highly expressed in immune cells where it regulates some of their key functions. Therefore, when considering studies correlating patient prognosis and RhoGDI2 expression, we must take into account the immune cells infiltrating the tumour microenvironment. This aspect of the role of RhoGDI2 in cancer progression has so far received little attention, while it could, however, have significant implications in cancer immunotherapy which is considered to be one of the most promising approaches for treating cancers.

6. Conclusions

Due to its dual role, it is crucial to study RhoGDI2 in the context of cancer. For this purpose, an enhanced characterization of its functions in cellular processes related to cancer, of its potential partners and of the dynamics of its subcellular localization are mandatory. However, one must remember to carry out these investigations not only in cancer cells but also in immune cells. Following this, RhoGDI2 can prove to be significant in cancer immunotherapy, which has made promising advances toward novel cancer therapeutics.

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