

# Potential Mechanism of CDC42 Promoting HCC Metastasis

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## Abstract

Hepatocellular carcinoma (HCC) is an aggressive malignancy with increasing morbidity and mortality worldwide. The migration and motility of HCC tumor cells are enhanced by the formation of invadopodia, which comprise membrane protrusions at the leading edge. Previous studies have showed that cell division cycle 42 (CDC42) plays an essential role in remodeling the cytoskeleton, which is associated with invadopodia formation and thus mediates cellular movement. Therefore, aberrant expression of CDC42 is hypothesized to promote tumor cell migration. In this review, we discuss the important role of CDC42 activation induced by guanine nucleotide-exchange factors (GEFs), which function as upstream regulators to activate CDC42, thereby mediating HCC invasion and metastasis by facilitating invadopodia formation. Furthermore, inhibitors targeting the CDC42-GEF interaction might be developed as an intervention against HCC metastasis.

**Keywords:** CDC42, GEFs, HCC, Cell motility, Invadopodia, Metastasis, Inhibitors

## Introduction

Hepatocellular carcinoma (HCC) is the sixth most common cancer worldwide, as well as the third leading cause of cancer-related deaths [1], with aggressive malignancy and high invasiveness. The extravasation of HCC cells plays an important role in distant metastasis; however, its precise mechanism remains unclear. Invadopodia, which are associated with invasive tumor cells, are crucial for cellular invasion and metastasis [2]. During the process of HCC invasion and metastasis, invadopodia formation occurs in tumor cells, thus enhancing the cellular motility.

Cell division cycle 42 (CDC42) is a 21.3 kDa Rho GTPase protein, which is activated when bound to GTP and inactivated when bound to GDP [3]. The release of GDP requires the participation of a guanine nucleotide-exchange factor (GEF). GEFs bind to CDC42-GDP and induce the exchange of GDP for GTP to activate CDC42 [4]. Subsequently, active CDC42-GTP interacts with its specific effectors and triggers biological effects, including cell migration. CDC42 is aberrantly activated

in many human cancers, and multiple biological functions mediated by CDC42 are associated with tumor metastasis [5].

In this review, we summarize the role of active CDC42 induced by GEFs in HCC metastasis. Certain inhibitors targeting the Rho GEFs that are involved in aberrant CDC42 activation (thus mediating HCC invasion and metastasis), which might provide new insights into the prevention and treatment of HCC metastasis.

## CDC42 Plays an Essential Role in Cell Motility

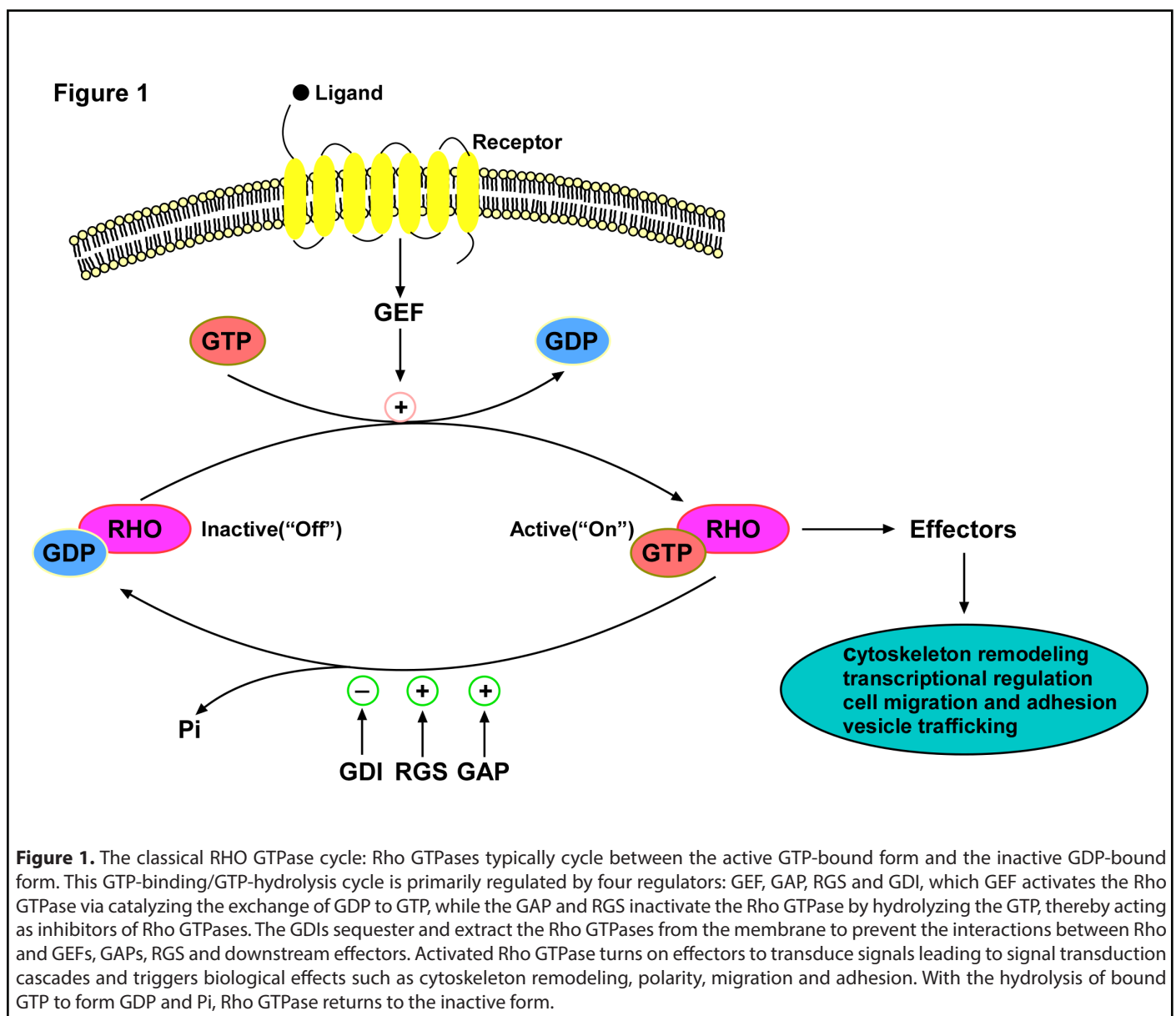
The Rho GTPases form a subfamily of the RAS superfamily of small GTP-binding proteins with a size of 21 to 25 kDa [6]. These proteins function as molecular switches that trigger signal transduction cascades. Rho GTPases have recently been expanded to over 20 members [7]. Among them, CDC42 has been extensively studied.

CDC42 is a key signal transduction protein that plays a central role in intracellular signaling networks, mediating a variety

of signal transduction pathways initiated by growth factors, cytokines, and cell adhesion proteins [8]. CDC42 is mainly regulated by four classes of regulatory proteins: guanine nucleotide exchange factor (GEF), GTPase-accelerating protein (GAP), regulator of G protein-signaling (RGS), and guanine nucleotide dissociation inhibitor (GDI) (**Figure 1**). As a molecular switch, CDC42 shows an active "on" form when bound to GTP and an inactive "off" form when combined with GDP. The conversion of the inactive GDP-bound form to the active GTP-bound form is mediated by GEFs [9]. In addition, the hydrolysis rate of GTP is promoted by GAP and RGS, but inhibited by GDI. In response to external signals, GEFs engage CDC42 and form macromolecular complexes with scaffolding proteins and/or kinases, resulting in CDC42 activation [3]. Activated CDC42 then mediates a signaling cascade leading to the activation of various downstream effectors that influence

many cellular processes, such as cell adhesion, motility, polarity, cytokinesis, and growth [7-12].

Cell motility is a multistep process that includes a migration process involving the formation of protrusions at the leading edge [13]. The initial step of cell migration is extension of the cytoplasm, which is induced by actin polymerization at the leading edge. Finger-like structures called filopodia (the actin-based structure of membrane protrusions at the cell periphery) are induced by CDC42 [14,15]. During the process of membrane protrusion, new actin polymerization is involved and actin nucleators, such as Wiskott–Aldrich syndrome protein (WASP), neuronal-WASP (N-WASP) and the actin-related protein-2/3 (Arp2/3) complex, are required [16-18]. WASP family proteins function as scaffold proteins that link upstream signals to activate the Arp2/3 complex, the initiation



**Figure 1.** The classical RHO GTPase cycle: Rho GTPases typically cycle between the active GTP-bound form and the inactive GDP-bound form. This GTP-binding/GTP-hydrolysis cycle is primarily regulated by four regulators: GEF, GAP, RGS and GDI, which GEF activates the Rho GTPase via catalyzing the exchange of GDP to GTP, while the GAP and RGS inactivate the Rho GTPase by hydrolyzing the GTP, thereby acting as inhibitors of Rho GTPases. The GDIs sequester and extract the Rho GTPases from the membrane to prevent the interactions between Rho and GEFs, GAPs, RGS and downstream effectors. Activated Rho GTPase turns on effectors to transduce signals leading to signal transduction cascades and triggers biological effects such as cytoskeleton remodeling, polarity, migration and adhesion. With the hydrolysis of bound GTP to form GDP and Pi, Rho GTPase returns to the inactive form.

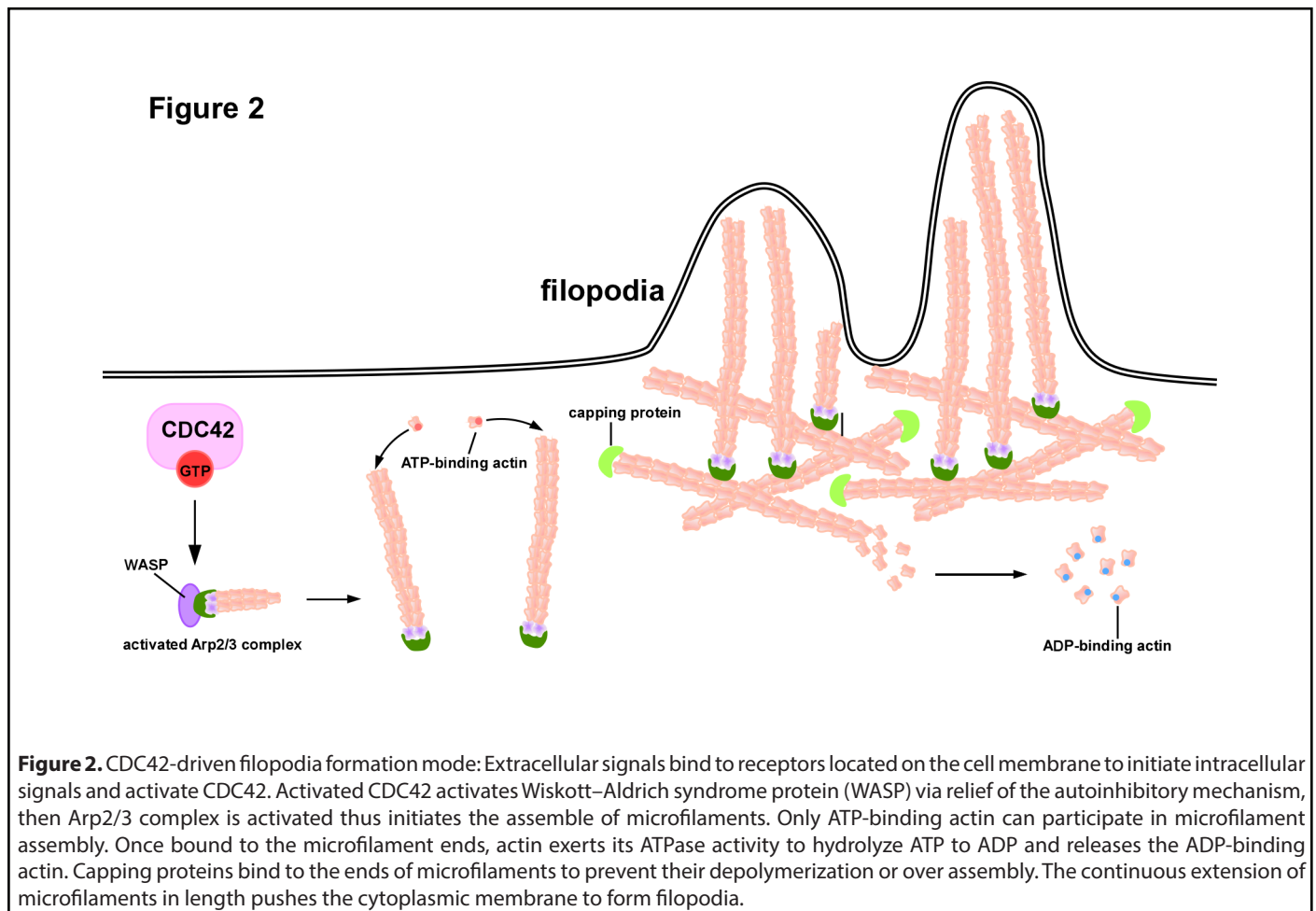
complex for microfilament assembly, which leads to a burst of actin polymerization. The first identified member of the Wiskott–Aldrich syndrome protein (WASP) family was WASP itself, which was initially found as the causative gene of WAS, an X-linked recessive disease that was described as a clinical trial of immunodeficiency, thrombocytopenia, and eczema [19]; and N-WASP, the second member of the WASP subfamily, was identified in the brain but widely expressed in different tissues, which shares several functional motifs with WASP. Under resting conditions, WASP and N-WASP are inactive forms due to the interaction between the GTPase-binding domain and the carboxy-terminal region, thereby inhibiting the binding of the Arp2/3 complex [20]. Once activated by various external stimuli, CDC42-GTP activates WASP and N-WASP via relief of the autoinhibitory mechanism. Thus, CDC42 contributes to the indirect activation of the Arp2/3 complex to initiate peripheral actin polymerization, thereby leading to filopodia formation [21] (**Figure 2**).

Furthermore, a variety of other molecules are required to direct the CDC42-dependent formation of filopodia. The interaction of CDC42 with IRSp53 (an SH3 domain-containing scaffold protein) allows the recruitment of Mena to the IRSp53 SH3 domain, which initiates actin filament assembly

into filopodia [22]. In addition, CDC42 phosphorylates and activates LIM motif-containing protein kinase (LIMK) via P21 activated kinase 1 (PAK1), which in turn efficiently induces the phosphorylation and inactivation of cofilin, resulting in decreased depolymerization of F-actin, ultimately leading to filopodia formation [23]. Many effectors of CDC42 also function downstream of RAC [14]; consequently, CDC42 is considered a potential regulator that drives the activity of RAC-dependent lamellipodia, suggesting a molecular model for the coordinated control of cell motility. In conclusion, the basic mechanism of CDC42 in cell motility is that CDC42 is activated by GEFs and then induces the formation of a protrusion at the leading edge to facilitate cell migration.

### CDC42 in HCC Invasion and Metastasis

HCC is a common cause of cancer-related mortality and morbidity worldwide. Despite the substantial improvement in novel diagnostic methods and therapeutic strategies, the prognosis of patients with HCC is still unsatisfactory [1], with median 5 years survival rate of only 18% [24]. Moreover, for patients with advanced HCC, the median survival is only 6-8 months [25], and the agent that has been shown to improve survival, sorafenib, can only extend the median



overall survival to 10.7-14.7 months [26]. The poor prognosis of HCC is due to its aggressive invasiveness and high metastasis. The lung is the most common site of extrahepatic metastasis of HCC [27]. In the past, it was considered that the pulmonary metastases of HCC were mostly multiple lesions, which did not have surgical indications, and comprehensive non-surgical treatment was often adopted [28]. However, non-surgical therapies including systemic chemotherapy for pulmonary metastasis of HCC have limited efficacy. Furthermore, tumor cell extravasation is a key step during cancer metastasis, yet the precise mechanisms are unclear, and there is no consensus on the treatment strategy for pulmonary metastasis of HCC. Therefore, there is an urgent need to determine the underlying mechanism of extravasation in HCC pulmonary metastasis.

Cell motility changes markedly during tumor cell invasion and metastasis, and is always deregulated in metastatic cancer. Given that RHO GTPases participate in various cellular functions, it is believed that they are connected with almost every stage of cancer development and progression, including tissue invasion and metastasis [29]. Previous studies have demonstrated frequent dysregulation of CDC42 in numerous human cancers, which is mainly caused by dysregulation of its upstream regulators, especially GEFs [30]. CDC42 is presumed to facilitate the aggressive biological behaviors of tumor cells via remodeling the cytoskeleton, which affects cell motility. Recent studies indicated that CDC42 participates in the tumorigenesis and progression of HCC [6]. Here, we discuss the molecular mechanism by which CDC42 plays an essential role in migration and metastasis by affecting the cell motility.

CDC42-induced modulation of cell motility is involved in the underlying molecular mechanism of HCC invasion and metastasis. Strikingly, CDC42 was found to enhance the ability of HCC cells to invade surrounding tissues by inducing filopodia formation [31]. In our latest published study, we found that the upregulated expression of Rho guanine nucleotide exchange factor 37 (ARHGEF37) correlated positively with high risk of HCC pulmonary metastasis [32]. Furthermore, we demonstrated that overexpression of ARHGEF37 increased the CDC42-GTP level in HCC cells, and enhanced their extravasation and lung metastatic capability by promoting the formation of invadopodium, a special F-actin-rich matrix-degrading structure, arises on the ventral surface of the cell membrane and found in a variety of carcinomatous cells whereas filopodia appears on the leading edges of migrating cells and function to command the direction of migration [33], consequently disrupting the interaction between endothelial cells and pericytes [32]. Meanwhile, overexpression of the GEFs faciogenital dysplasia 1 (FGD1) in HCC leads to CDC42 activation, thus facilitating invadopodia formation and extracellular matrix (ECM) degradation, and subsequent tumor cell invasion [34]. Moreover, RICH2, (Rho GTPase activating protein 44, also known as ARHGAP44), regulates the formation of filopodia in HCC in a CDC42 dependent

manner, and stable overexpression of RICH2 significantly inhibited the invasion of HCC cells *in vitro* [35]. In addition to upstream regulators of CDC42, other cellular components are also involved in HCC invasion and metastasis by activating the CDC42 axis. For example, CDC42 interacting protein 4 (CIP4) is a CDC42 effector that assists membrane deformation and actin polymerization. Specifically, phosphorylation at T225 on CIP4 facilitates the interaction with CDC42, thus promoting the formation of functional invadopodia, which is involved in regulating cancer cell invasiveness and metastasis [36].

In brief, the molecular mechanism by which CDC42 mediates HCC invasion and metastasis is mainly regulated by the overexpression of upstream regulators, especially GEFs, thus facilitating invadopodia formation, ECM degradation, and subsequent tumor cell invasion.

### Treatment Strategies Targeting the CDC42-GEF Interaction

Until recently, Rho GTPases themselves were deemed to be “undruggable” considering their inaccessible structure and multifaceted function [37]. However, the upstream regulators of Rho GTPases play a crucial role in tumor invasion and metastasis, thus making them potential targets to develop anti-tumor agents. Although no therapeutically effective drugs targeting CDC42 signaling for cancer treatment are available, small molecule inhibitors targeting the CDC42 axis combined with other anti-cancer agents could be promising and of potent according to recent studies [30,38-42].

Unlike protein kinases, which can be targeted by their nucleotide analogs, it is difficult to target Rho GTPases by such small molecule modulations because of the micromolar GTP concentration in cells and the sub-nanomolar binding affinity of Rho GTPases for GTP or GDP [37]. Given that Rho GTPases are activated by different RhoGEFs, which respond to different signals in a temporally and spatially manner, targeting specific RhoGEFs provides feasible selectivity. Several small molecule inhibitors targeting specific RhoGEFs and preventing their binding to Rho GTPases have been developed. For example, compound CID2950007 and its analog CID44216842 were synthesized, both of which inhibited GTP binding to CDC42 in a dose-dependent manner [40]. Inhibition of CDC42 activation induced by CID2950007 effectively inhibited filopodia formation and led to ineffective cell migration in human ovarian cancer cells. In addition, intersectin 1 (ITSN1) has been identified as a CDC42-specific GEF using *in vitro* studies [43] and the small molecule inhibitor ZCL278 can dock into the ITSN1 binding groove of CDC42 [39]. ZCL278, synthesized and purified based on the CDC42-ITSN1 interaction interface, is able to fit into the surface groove of CDC42, which is critical for GEF binding, with low micromolar affinity ( $K_d = 6.4 \mu\text{M}$ ) [39]. And it suppresses CDC42-mediated actin-based motility [32] and migration [44] without disrupting cell viability, thus

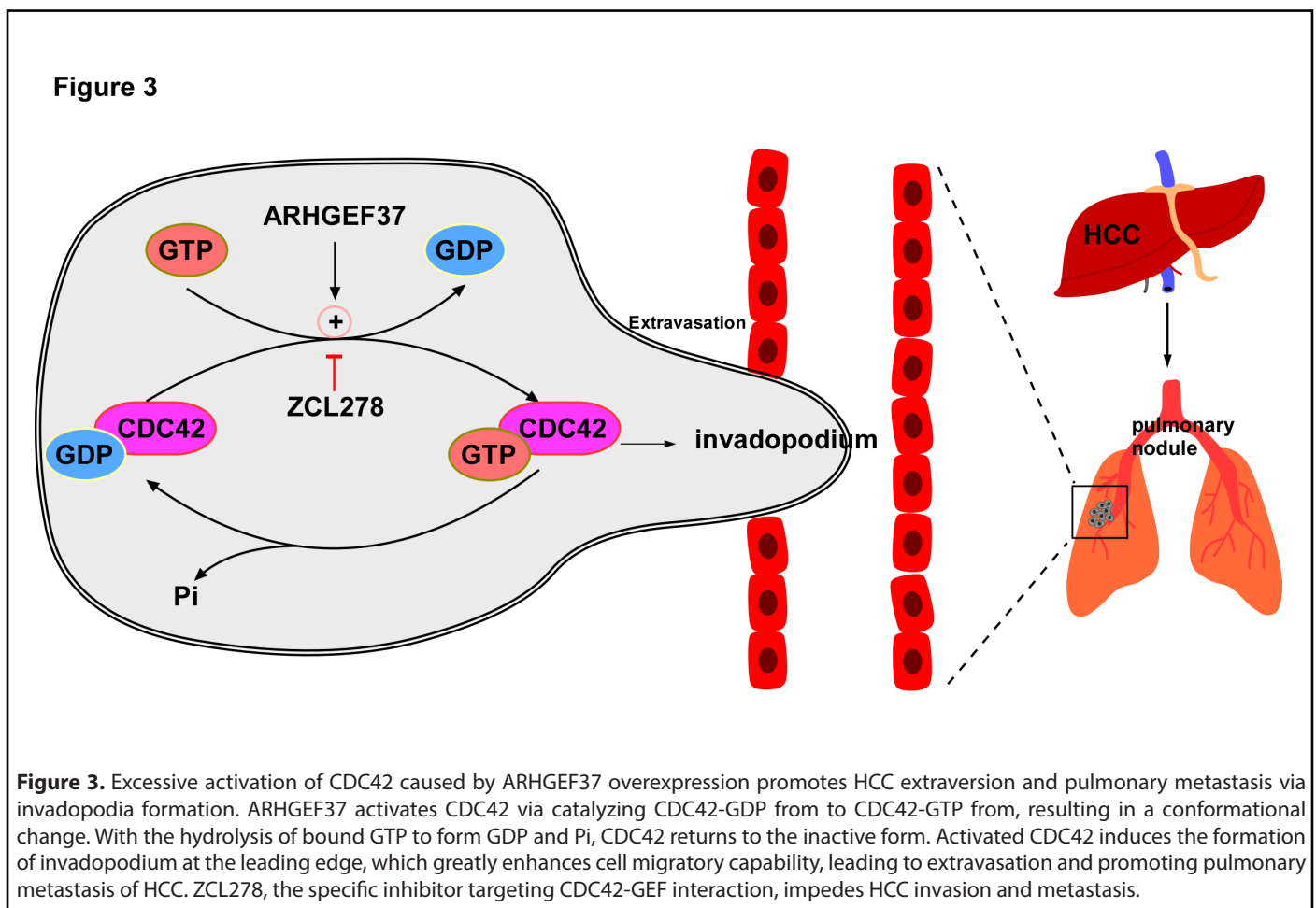
providing a powerful tool for researching the interaction of CDC42-GEF in human pathogenesis, such as those of cancer. In our latest published study, we demonstrated that ARHGEF37, the most salient molecules screened through integrated two public datasets of pulmonary metastasis in HCC, The Cancer Genome Atlas (TCGA) HCC dataset and online public Kaplan-Meier Plotter database, while ITSN1 was not in the candidate list, catalyzed the release of GDP from CDC42 to bind with GTP, activating CDC42 to induce the formation of invadopodium at the leading edge, which greatly enhanced cell migration, leading to pulmonary metastasis of HCC. Importantly, treatment with ZCL278 dramatically inhibited that active CDC42 induced by ARHGEF37 mediated HCC invasion and metastasis (**Figure 3**). Our findings further confirmed that the inhibition of the CDC42-GEF interaction by the small molecule ZCL278 impedes HCC invasion and metastasis. Taken together, inhibitors targeting the CDC42-GEF interaction to inhibit tumor invasion and metastasis will be an excellent candidate for lead compound optimization in further anticancer studies.

### Conclusion

The Rho family GTPase CDC42 regulates cytoskeletal organization in physiological processes, including cell

proliferation, motility, and polarity. The activation of CDC42 in response to upstream signals is mediated by GEFs, which convert the GDP-bound inactive form to the GTP-bound active form of CDC42. Activated CDC42 transduces signals to downstream effectors, the autoinhibitory mechanism of WASP/N-WASP was relieved and thus effectual, then Arp2/3 complex is activated thus initiates the assemble of microfilaments, the continuous extension of microfilaments in length pushes the cytoplasmic membrane to form invadopodia; and generates cellular effects, such as cytoskeleton remodeling, polarity, migration and adhesion. Aberrant activation of CDC42 results in pathogenesis, such as tumor invasion and metastasis. Tumor invasion requires orchestration of actin-based protrusions capable of ECM degradation and cell locomotion. In numerous human tumors, including HCC, the ability of cells to form invadopodia and their invasive potential are directly correlated.

Recent studies indicate that CDC42 plays an essential role in hepatocellular carcinoma invasion and metastasis [6]. Mechanistically, CDC42-mediated HCC invasion and metastasis are mainly regulated by the overexpression of upstream regulators, particularly GEFs, which thus facilitates invadopodia formation, ECM degradation and subsequent





tumor cell invasion. Based on the molecular mechanism, novel strategies targeting CDC42-GEF interactions might lead to drugs to prevent and treat HCC invasion and metastasis. Of particular interest, in our latest published study, we identified ARHGEF37, a specific CDC42 guanine nucleotide exchange factor, catalyzes the release of GDP from CDC42 to bind with GTP, which activates CDC42 by triggering a conformational change, resulting in the formation of invadopodia, which greatly enhances cell migration, leading to extravasation and promoting pulmonary metastasis of HCC. ZCL278, the specific inhibitor targeting CDC42-GEF interaction, impedes HCC invasion and metastasis [32]. Therefore, our study provides a new insight into the underlying mechanisms on the ARHGEF37 overexpression-mediated extravasation and pulmonary metastasis of HCC, indicating that agents targeting CDC42-GEF interaction should be of potency in therapeutic interventions of HCC metastasis.

Several inhibitors that interfere with CDC42's role on cellular motility have been identified, such as compound CID2950007 and its analog CID44216842, and ZCL278, although none have been applied to clinical therapeutics. The regulatory mechanism of the RHO GTPase family should be further clarified to permit the design of additional therapeutic interventions and more attention should be paid to the design of inhibitors targeting RHO GTPase effectors and regulators. Moreover, further studies are needed to understand the detailed roles of RHO GTPases in the development of HCC.

## Conflicts of Interest

The authors declare no conflicts of interest.

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## Author Contributions Statement

M.T. and R.F. wrote the manuscript text. R.F. created the figure and edited the manuscript for important intellectual content. M.T. and J.L. supervised the study. All authors read and approved the final manuscript.

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