THE Cdc42 Cys81Tyr MUTATION: IMPACT ON GUANOSINE TRIPHOSPHATASE (GTPase) ACTIVATION STATUS AND ITS CELLULAR FUNCTIONS

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by

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k	Binding constant
Å	Angstrom
d	Distance from the charge
d_i	Distances from the atoms to the points of interest in the space
K	Kelvin
K_d	Dissociation constant
q_i	Atomic partial charges
ΔG	Binding affinity
ε	Dielectric constant
φ	Electrostatic potential

LIST OF ABBREVIATIONS

ACK	Activated Cdc42-associated kinase
Akt	Protein kinase B
ANOVA	Analysis of variance
APS	Ammonium persulfate
Arg	Arginine
Arp	Actin-related protein
C18A	Cysteine-to-alanine substitution at residue 18
C81F	Cysteine-to-phenylalanine substitution at residue 81
C81Y	Cysteine-to-tyrosine substitution at residue 81
CABS	C-α, β, and side chain
Cdc42	Cell division control protein 42 homolog
cDNA	Complementary DNA
CRIB	Cdc42/Rac-interactive binding
Cys	Cysteine
Dbl	Diffuse B-cell lymphoma
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
DOCK	Dedicator of cytokinesis
DTT	Dithiothreitol
ECL	Enhanced chemiluminescence solution
EGF	Epidermal growth factor
EMT	Epithelial-mesenchymal transition
ERK	Extracellular signal-regulated kinase
ESP	Electrostatic potential
EtBr	Ethidium bromide
FBS	Foetal bovine serum
G12V	Glycine-to-valine substitution at residue 12
GCP	Guanylyl β,γ-methylene-diphosphonate
G-domain	GTP-binding domain
GFP	Green fluorescent protein
G_p	Placental G-protein

Gp130	Glycoprotein 130
G-protein	GTP-binding protein
GST	Glutathione S-transferases
GTPase	Guanosine triphosphatase
H_2O_2	Hydrogen peroxide
HCC	Hepatocellular carcinoma
HLH	Haemophagocytic lymphohistiocytosis
HRP	Horseradish peroxidase
HVR	Hypervariable region
I21T	Isoleucine-to-threonine substitution at residue 21
IL	Interleukin
IL-10Rα	IL-10 receptor α
IL-10Rβ	IL-10 receptor β
IL-6Rα	IL-6 receptor α
IPTG	Isopropyl β-d-1-thiogalactopyranoside
JAK	Janus kinase
JNK	Jun N-terminal kinase
K16E	Lysine-to-glutamine substitution at residue 16
KO	Knockout
Lys	Lysine
MAPK	Mitogen-activated protein kinase
MD	Molecular dynamic
MMP-9	Matrix metalloproteinase 9
MSA	Multiple sequence alignment
MTT	Thiazolyl blue tetrazolium bromide
N132K	Asparagine-to-lysine substitution at residue 132
NF-κB	Nuclear factor kappa B
NOCARH	Neonatal-onset cytopoenia with dyshaematopoiesis, autoinflammation, rash, and HLH
PAK	p21-activated kinases
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDB	Protein data bank
PEI	Polyethyleneimine
Pen-Strep	Penicillin-streptomycin

P_i	Inorganic phosphate
PI3K	Phosphatidylinositol 3-kinase
PIDs	Primary immunodeficiencies
PKA	Protein kinase A
PKC-δ	Protein kinase C-δ
P-loop	Phosphate binding loop
PMSF	Phenylmethylsulfonyl fluoride
PTMs	Post-translational modifications
pTyr	Phosphorylated Tyr64
PVDF	Polyvinylidene difluoride
Q61L	Glutamine-to-leucine substitution at residue 61
R186C	Arginine-to-cysteine substation at residue 186
R66A	Arginine-to-alanine substitution at residue 66
R66E	Arginine-to-glutamine substitution at residue 66
R66G	Arginine-to-glycine substitution at residue 66
R68Q	Arginine-to-glutamine substitution at residue 68
Rac1	Ras-related C3 botulinum toxin substrate
Raf	Rapidly accelerated fibrosarcoma
Ras	Rat sarcoma
RCSB	Research Collaboratory for Structural Bioinformatics
Rho	Ras homolog
RhoA	Rho gene family member A
RhoGAPs	Rho GTPase-activating proteins
RhoGDIs	Rho guanine dissociation inhibitors
RhoGEFs	Rho guanine nucleotide exchange factors
RMSD	Root mean square deviation
RMSF	Root mean square fluctuation
RPMI	Roswell Park Memorial Institute
S. cerevisiae	*Saccharomyces cerevisiae*
S83P	Serine-to-proline substitution at residue 83
SD	Standard deviation
SDS	Sodium dodecyl-sulfate
Ser	Serine
SLE	Systemic lupus erythematosus

STAT	Signal transducer and activator of transcription
Thr	Threonine
T17N	Threonine-to-asparagine substitution at residue 17
TAE	Tris-acetate-EDTA
TCR	T-cell receptor
TEMED	Tetramethylethylenediamine
TME	Tumour microenvironment
Tyr	Tyrosine
WAS	Wiskott-Aldrich syndrome
WASp	Wiskott-Aldrich syndrome protein
Wnt-5a	Wingless-type MMTV integration site family member 5A
WT	Wild-type
Y32A	Tyrosine-to-alanine substitution at residue 32
Y64C	Tyrosine-to-cysteine substitution at residue 64
Y64E	Tyrosine-to-glutamine substitution at residue 64
Y64F	Tyrosine-to-phenylalanine substitution at residue 64

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MUTASI Cdc42 Cys81Tyr: KESAN TERHADAP PENGAKTIFAN GUANOSINA TRIFOSFATASE (GTPase) DAN FUNGSI SELULARNYA

ABSTRAK

Mutasi pada Rho guanina trifosfatase (GTPase) sentiasa dikaitkan dengan pelbagai penyakit. Salah satu ahlinya ialah Cdc42, yang memainkan peranan penting dalam pengisyaratan sel, organisasi sitoskeleton, dan tindak balas imun melalui kitaran antara keadaan terikat-GTP (aktif) dan keadaan terikat-GDP (tidak aktif). Penggantian sistein kepada tirosina pada posisi 81 (C81Y) dalam segmen β4 telah dikenal pasti dalam kalangan pesakit yang menghidap imunodefisiensi primer (PID) dan limfoma Hodgkin secara serentak. Bukti terkini menunjukkan bahawa mutasi ini mengganggu hubungan struktur-fungsi Cdc42, berpotensi menjejaskan pengisyaratan imun, dan mendorong onkogenesis. Meskipun begitu, peranan patogenik Cdc42 C81Y masih kurang difahami. Kajian ini bertujuan untuk menyiasat impak struktur dan biokimianya, dengan hipotesis bahawa ia mengganggu kitaran GDP/GTP, modifikasi pasca-translasi, dan pengisyaratan hiliran, seterusnya menyumbang kepada patogenesis penyakit. Untuk mencapai matlamat ini, pendekatan gabungan antara pengiraan dan eksperimen telah digunakan. Simulasi dinamik molekul (MD), analisis interaksi antara residu, dan kajian pengikatan molekul (docking) menunjukkan gangguan struktur yang signifikan dalam Rho insert domain, gangguan pada kawasan sensitif nukleotida, dan afiniti pengikatan GTP yang lemah, dan pengikatan GDP yang dipertingkatkan. Perubahan dalam profil elektrostatik turut mencadangkan gangguan dalam pengisyaratan efektor hiliran. Ujian menggunakan PAK1, WASp, dan N-WASp mengesahkan perubahan dalam dinamik pengaktifan, menyokong kesan dominan-negatif. Analisis biokimia selanjutnya menggunakan imunopresipitasi menunjukkan

bahawa Cdc42 C81Y diekspresikan secara berlebihan, kemungkinan disebabkan oleh ubikuitinasi yang terjejas, seperti yang ditunjukkan oleh ramalan MD. Walaupun terdapat peningkatan ekspresi ini, tyrosin fosforilasi didapati berkurangan, menandakan kecacatan dalam pengisyaratan yang melibatkan RhoGDI. Ujian mendedahkan bahawa sel HEK293T yang mengekspresikan Cdc42 C81Y memaparkan perubahan dalam proliferatif dan migrasi. Ujian proliferasi menunjukkan pengurangan ringan. Sementara itu, ujian migrasi menunjukkan peralihan fenotip ke arah keadaan invasif, yang dicirikan oleh peningkatan pergerakan sel terarah yang signifikan, di mana tingkah laku invasif ini berkemungkinan melibatkan paksi MAPK/ERK. Akhir sekali, profil sitokin menunjukkan peningkatan IL-6 dan IL-10, menghubungkan mutasi ini dengan lata pengisyaratan keradangan. Kesimpulannya, kajian ini memberikan bukti yang kukuh bahawa mutasi Cdc42 C81Y mengganggu fungsi sel normal melalui ketidakstabilan struktur, pengikatan GTP yang terjejas, interaksi efektor yang berubah, dan pengisyaratan hiliran yang tidak terkawal. Kecacatan yang diperhatikan dalam proliferasi sel, migrasi, dan rembesan sitokin mencadangkan bahawa disregulasi imun dan limfoma yang berkaitan dengan mutasi Cdc42 C81Y berkemungkinan timbul secara serentak. Penemuan ini menekankan kepentingan Cdc42 sebagai perantara interaksi silang antara imun dan tumor serta memberikan pandangan baharu yang boleh memaklumkan kajian bertujuan untuk menyasarkan laluan yang diperantarai oleh Cdc42 bagi intervensi terapeutik.

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ABSTRACT

Mutations in small Rho guanine triphosphatase (GTPase) have been increasingly associated with various diseases. One of the members is Cdc42, a key regulator of cellular signalling, cytoskeletal organization, and immune responses by cycling between an active GTP-bound and an inactive GDP-bound states. A novel cysteine-to-tyrosine substitution at residue 81 (C81Y) within the β4 segment of Cdc42 has been identified in patients with concurrent primary immunodeficiency (PID) and Hodgkin's lymphoma. Notably, emerging evidence suggests that this mutation disrupts Cdc42 structure-function relationship, potentially impairing immune signalling, altering cytokine secretion profiles, and driving oncogenesis. Yet, the pathogenic roles of Cdc42 C81Y remains poorly understood. This study investigates its structural and biochemical impact, hypothesizing that it disrupts GDP/GTP cycling, post-translational modification, and downstream signalling, contributing to disease pathogenesis. To address this, a combined computational and experimental approach was employed. Molecular dynamics (MD) simulations, inter-residue interaction analysis, and docking studies revealed significant structural perturbations in the Rho insert domain, disruption of the nucleotide-sensitive region, weakened GTP binding affinity, and enhanced GDP binding. Alterations in the electrostatic profile further suggested disruptions in downstream effector signalling. Complementary pull-down assays with PAK1, WASp, and N-WASp confirmed a shift in activation dynamics, supporting a dominant-negative effect. Further biochemical analysis using

immunoprecipitation demonstrated that the Cdc42 C81Y mutant was overexpressed, potentially due to impaired ubiquitination, as MD predictions indicate altered ubiquitination sites. Despite this increased expression, phosphorylation at tyrosine residues was markedly reduced, suggesting defects in Cdc42-mediated signalling, particularly involving RhoGDI. Functional assays revealed that HEK293T cells expressing Cdc42 C81Y displayed altered proliferative and migratory capacities. Proliferation assay indicated a mild reduction in overall cell viability. Meanwhile, migration assay showed a significantly increased directed cell movement, in which this invasive behaviour likely involves the MAPK/ERK axis, initiated *via* pathways independent of typical GDP/GTP cycling. Lastly, cytokine profiling demonstrated elevated IL-6 and IL-10 secretion, linking the mutation to dysregulated inflammatory signalling cascades and potential immunomodulatory effects. In conclusion, this study provides compelling evidence that the Cdc42 C81Y mutation disrupts normal cellular function through structural destabilization, impaired GTP binding, altered effector interactions, and dysregulated downstream signalling. The observed defects in cell migration and cytokine secretion suggest that the immune dysregulation and lymphoma associated with the Cdc42 C81Y mutation appears to arise concurrently. These findings highlight the importance of Cdc42 as an immune-tumour crosstalk mediator and provide new insights that could inform future studies aimed at targeting Cdc42-mediated pathways for therapeutic intervention.

CHAPTER 1

INTRODUCTION

1.1 Background

Primary immunodeficiencies (PIDs), also known as inborn errors of immunity, are caused by mutations in over 485 genes (Tangye et al., 2022). A well-established consequence of PIDs is a significantly increased risk of malignancy, contributing to substantial morbidity and mortality (Barmettler et al., 2022). This heightened cancer susceptibility in patients with PID is attributed to several possible pathologies, including impaired immune surveillance due to immunosuppression (Maffeis et al., 2019; Salavoura et al., 2008). It is also recognized that certain immune disorders and malignancies share genetic predispositions arising from monogenic or polygenic factors that influence both immunity and cancer development (Dębniak and Lubiński, 2008; Fahed et al., 2020). Among the genes implicated in PIDs are those encoding actin-related monomeric small Rho guanosine triphosphatase (GTPase), such as Cdc42 (cell division control protein 42 homolog) (Hashim and Mokhtar, 2021; Tangye et al., 2022).

Cdc42, a member of the Rat sarcoma (Ras) homologous (Rho) superfamily GTPases, is a master regulator of the actin cytoskeleton (O'Neill et al., 2016; Wirth and Ponimaskin, 2023). Operating as a small GTP-binding protein (G-protein), it functions *via* a molecular switch cycling between two conformations — the active GTP-bound state and the inactive GDP-bound state (Brindani et al., 2023). This cycle is tightly controlled by three upstream regulator proteins, namely Rho guanine nucleotide exchange factors (RhoGEFs), Rho GTPase-activating proteins (RhoGAPs), and Rho guanine dissociation inhibitors (RhoGDIs) (Lam et al., 2019). Upon activation, Cdc42 undergoes a conformational change and transiently interacts with

diverse downstream effector proteins (e.g., PAK1, N-WASp, ACK), modulating multiple cellular pathways important for actin cytoskeleton reorganisation, cell-cycle progression, and motility (Hashim and Mokhtar, 2021). While a direct causal link between Cdc42 mutations and cancer remains to be definitively established (Arias-Romero and Chernoff, 2013; Murphy et al., 2021), a recent case study reported a novel heterozygous *de novo* missense variant of the monogenic *CDC42* gene (c.242G>A, p.Cys81Tyr [C81Y]) in a young boy with both severe immune dysregulation, PID, and Hodgkin lymphoma (Szczawinska-Poplonyk et al., 2020). This patient exhibited a range of immunological abnormalities, characterized by pancytopenia, elevated pro-inflammatory markers and a notable imbalanced T-cell population.

Critically, the C81Y mutation is located within the β4 segment, a structurally important region adjacent to the nucleotide-binding loop, which is essential for efficient GTPase action. Because this segment contributes to protein conformational stability (Murphy et al., 2021), amino acid substitutions at this site are presumed to significantly impact protein dynamics and potentially its function. A prior study demonstrated that a cysteine-to-phenylalanine substitution at residue 81 (C81F) obstructs the Cdc42's intrinsic GTP hydrolysis, resulting in a gain-of-function effect (Martinelli et al., 2018), highlighting the sensitivity of this region to perturbation. Meanwhile, the Cdc42 C81Y variant unique clinical context, characterized by impaired B-cells function and thrombocytopenia, suggesting molecular and functional consequences that diverge from those observed with the Cdc42 C81F, which, unlike Cdc42 C81Y, has not been associated with severe immune dysregulation and cancer (Hashim and Mokhtar, 2021). Although the importance of this region is clear, the precise mechanisms by which different substitution at position 81 (particularly the

C81Y variant) influence Cdc42 functional state remain to be elucidated. These observations, supported by findings from similar knockout (KO) studies (Burbage et al., 2015; Heib et al., 2021; Pleines et al., 2010), lead us to hypothesize that the C81Y mutation may trigger a loss-of-function effect by potentially altering Cdc42 structure, thereby hindering Cdc42 GDP/GTP cycling and subsequent signalling.

Beyond the typical regulation of Rho family members activity by GDP/GTP cycling, post-translational modifications (PTMs), such as phosphorylation, provide another crucial layer of control by modulating their stability, localization, and interactions (Chang et al., 2011; Forget et al., 2002; Hodge and Ridley, 2016). Among these, the dynamic and reversible process of phosphorylation is particularly relevant in Cdc42 signalling, particularly at tyrosine (Tyr) residues (Olson, 2016). Specifically, several Tyr residues on Cdc42 located within or near the Switch I/II domain, including Tyr23, Tyr32, Tyr40, Tyr51, and notably Tyr64, are known phosphorylation sites that influence interactions with RhoGDI (Moritz et al., 2010; Trost et al., 2012; Tu et al., 2003; Yin et al., 2023). For instance, reduced Tyr64 phosphorylation of Cdc42 diminishes RhoGDI interaction and influence GDI-mediated cell transformation (Tu et al., 2003). Studies of Takenouchi-Kosaki syndrome, which features a Cdc42 tyrosine-to-cysteine substitution at residue 64 (Y64C), similarly demonstrated critical role of Tyr64 in Cdc42-RhoGDI interaction, as this variant is accompanied by both thrombocytopenia and impaired RhoGDI binding (Daimon et al., 2021). Given the established role of Tyr phosphorylation in Cdc42 regulation and its impact on GDI-mediated cellular transformation, the C81Y substitution, resulted in altered phosphorylation might contribute to the haematological malignancy observed (Szczawinska-Poplonyk et al., 2020), is of particular interest. Consequently, it is crucial to determine whether this mutation would indirectly affects Cdc42

phosphorylation at other sites by potentially altering kinase binding sites or proper positioning for phosphorylation to occur by specific kinases (Songyang et al., 1995). Either scenario could have distinct implications for Cdc42-RhoGDI interactions and their role in the observed pathology, making further investigation key to pinpointing the relevant signalling pathways and establishing a mechanistic link.

In addition to its role in cancer, Cdc42 is also important for immune system regulation, controlling innate and adaptive immunity (Gerasimcik et al., 2015; Guo, 2021; Guo et al., 2011; Kalim et al., 2022; Smits et al., 2010), including the modulation of cytokine and T-cell receptor (TCR) signalling (Assing et al., 2023; Chemin et al., 2012). Interestingly, besides haematological malignancy, the patient also exhibited immune dysregulation, including a reduced absolute lymphocyte (T-cells) count, severe CD4+ and regulatory T-cell deficiency, and haemophagocytic lymphohistiocytosis (HLH). HLH, a severe hyperinflammatory syndrome, is characterized by uncontrolled cellular activation leading to excessive cytokine production and tissue damage (Gioia et al., 2024). This prompts a further question: could the Cdc42 C81Y variant contribute to these observed immune abnormalities by disrupting Cdc42's normal function in immune cells? This also raises the possibility that the variant may impair immune regulatory mechanisms, promote a sustained inflammatory state, and obstruct anti-tumour immunity. Interleukin (IL)-6 or IL-10 are two such cytokines that may mediate these effects (Hao et al., 2023; Hirano, 2020; Shouib and Eitzen, 2022), hinting at a crosstalk between Cdc42 C81Y in immune-cancer interactions.

1.2 Problem statement

PIDs resulting from monogenic mutations such as CDC42 C81Y can impair immune surveillance and are increasingly linked to malignancy. The identification of this mutation in a clinical case involving both immune dysregulation and Hodgkin lymphoma raises important questions regarding its pathogenic potential. Despite this association, the molecular and functional consequences of the Cdc42 C81Y variant remain poorly characterised. Specifically, it is unclear how this mutation affects Cdc42 structural integrity, activation state, and post-translational regulation, including phosphorylation. The β4 segment of Cdc42, where the C81Y substitution is located, plays a critical role in maintaining proper GTPase function and is therefore of particular interest for structural and functional investigation.

Current gaps in knowledge also extend to whether this mutation promotes oncogenic features through altered cell behaviour or immune signalling pathways. In particular, the role of Cdc42 C81Y in regulating cytokine production, such as IL-6 and IL-10, has not been elucidated. Addressing these gaps requires an integrative approach that combines computational predictions with functional assays to clarify the mutation's mechanistic impact. This study seeks to define the potential contribution of Cdc42 C81Y to immune dysregulation and malignancy in PID by characterising its effects on protein conformation, activation, phosphorylation, cell behaviour, and cytokine secretion. All the data obtained should help in providing a foundation for future therapeutic advancements in PID patient care and, importantly, contributing to strategies aimed at reducing cancer risk in this population.

1.3 Research objectives

This study aims to elucidate the structural and functional consequences of the Cdc42 C81Y mutation in cellular processes.

1.3.1 Specific objectives

To achieve this, the study is designed to:

1) To investigate the effect of C81Y mutation on the Cdc42 structure and activation status through computational studies and effector pull-down assay.
2) To investigate the effect of Cdc42 C81Y on the protein expression levels and phosphorylation status using immunoprecipitation and Western blotting.
3) To investigate the effect of Cdc42 C81Y on cellular proliferation, migration, and cytokine profiles using MTT, wound healing assays, and ELISA.

CHAPTER 2

LITERATURE REVIEW

2.1 Primary immunodeficiencies (PIDs)

The highly orchestrated immune system network comprised of immunocompetent cells and inflammatory mediators that functions adeptly to combat disease-causing antigens, including pathogens and cancer cells. An effective immune function is typically marked by the coordinated interaction between immune components, achieved through cascades and feedback loops that sustain robust immune surveillance. However, this hallmark is compromised in primary immunodeficiencies (PIDs), or often recognised as inborn errors of immunities (Yamashita et al., 2021). Unlike secondary immunodeficiencies, which occur after extrinsic factors such as immunosuppressive medications or infections (e.g., HIV), PIDs mostly adhere to Mendelian inheritance patterns or, in small cases, manifest as acquired forms (McCusker et al., 2018; Mortaz et al., 2016). On global scale, the occurrence of PIDs exceeds the prevalence rate observed in Malaysia which is 0.37 cases per 100,000 population, differing by ~11 to 55-fold (Abd Hamid et al., 2020). This seemingly low cases in Malaysia suggests possible underreporting and underdiagnosis that can be linked to a lack of awareness among the public and healthcare professionals (Abd Hamid et al., 2020).

Interestingly, a wide array of genetic mutations underlies the spectrum of PIDs, a group of clinically and genetically distinct disorders currently numbering over 485 types (Tangye et al., 2022). This enumeration of identified PID-related genes has experienced more than a twofold surge in comparison to the previous decade, mainly because of the advancements in upfront molecular diagnosis tools (Yamashita et al.,

2021). Each disorder is distinguished by poor or dysfunction critical immunological cells or effector molecules, affecting processes such as receptor signalling, activation, maturation, and chemokine-induced motility (Tangye et al., 2022). Certain manifestations of PIDs can be so subtle that they might remain unnoticed for extended periods till reaching adulthood (Condino-Neto and Espinosa-Rosales, 2018; Meyts et al., 2020). Conversely, more severe forms become apparent almost immediately after the birth of an affected baby with severe clinical presentations (Dvorak et al., 2023). Although the clinical presentations of PIDs differ markedly, most affected individuals are physically vulnerable and predisposed to infections, uncontrolled inflammation, autoimmunity, and malignancy (McCusker et al., 2018; Yamashita et al., 2021).

2.1.1 PID malignancies

Examining data collected across different countries, it becomes evident that patients with PIDs face a substantial mortality rate of ~8.7 to 15.8% (Aghamohammadi et al., 2021). Apart from infections, the occurrence of malignancies proves to be another critical life-threatening event for this patient population (Barmettler et al., 2022; Haron et al., 2023). Indeed, alarming statistics demonstrate that children diagnosed with PIDs bear an overall risk up to 25% for malignancy development (Mortaz et al., 2016; Verhoeven et al., 2018). Within the spectrum of cancers observed, malignancies of haematologic origin, particularly lymphoma are diagnosed at a higher rate (Mayor et al., 2018; Mortaz et al., 2016; Resnick et al., 2012), with Hodgkin lymphoma comprising for 10% and non-Hodgkin lymphoma for 48.6% of cases (Mortaz et al., 2016). Similarly, secondary immunodeficiency, observed in individuals such as those with HIV/AIDS (Berhan et al., 2022), those on immunosuppressive medications (Wang et al., 2023), the elderly (Soverini and Tucci, 2022), and individuals undergoing

chemotherapy (Swerdlow et al., 2011), is often reported with an elevated incidence of lymphoma.

As covered in several comprehensive reviews, the understanding of cancer aetiology in PID patients involves a nuanced consideration of both intrinsic and extrinsic factors (Abolhassani et al., 2021; Baris and Kolukisa, 2021; Haas, 2018; Hauck et al., 2018). Undeniably, the common thread among these factors is the inadequacy of immune mechanisms, ultimately impeding effective immunosurveillance. This concept, initially proposed by Burnet in the early 1960s (Burnet, 1963), has evolved into the immunoediting theory as shown in Figure 2.1 (Dunn et al., 2002), where the immune system are capable of distinguish between normal cells and pre- or cancerous cells (Haas, 2018). Such immunosurveillance in patients with PIDs fail to establish a good fitness barrier for neoplastic cells, rendering it insufficient to block the acquisition and progression of these cells into cancer (Verhoeven et al., 2018). Certain malignancies may also originate from monogenic or polygenic defects linked to immune and/or cancer-related signalling, thereby promote the risk for malignant transformation (Derpoorter et al., 2018).

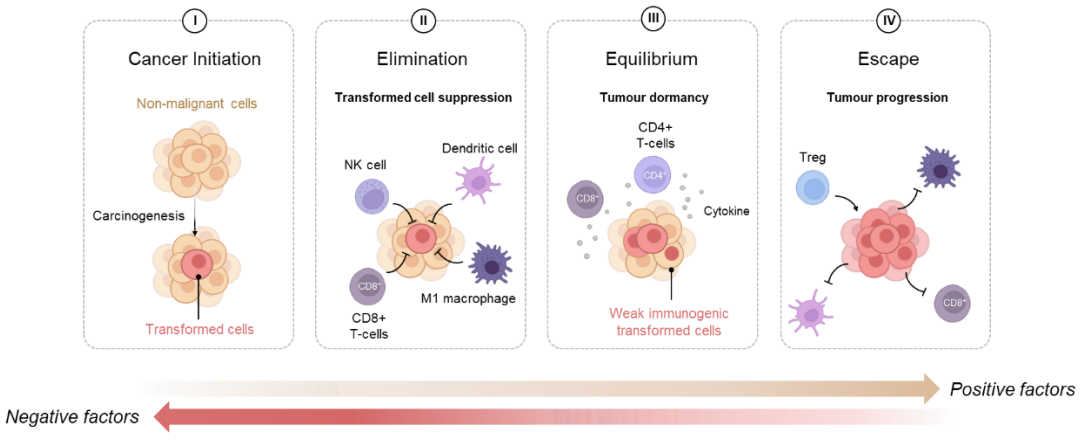

Figure 2.1 A schematic representation of the cancer immunoediting theory. Cancer immunoediting involves four stages: (1) initiation, where normal cells transform into cancerous cells; (2) elimination, where the immune system attacks and eliminates those cells; (3) equilibrium, a period of balanced tumour dormancy; and (4) escape, where tumour cells evade immune surveillance and progress. The gradient bar indicates the shift from factors hindering tumour growth (red arrow) to those promoting it (yellow arrow).

2.1.2 A case report of Cys81Tyr (C81Y) mutation in Cdc42 with PID and malignancy

Szczawinska-Poplonyk et al. (2020) reported a compelling case of syndromic immunodeficiency associated with a novel monogenic *CDC42* variant: a nucleotide change c.242G>A results in a C81Y amino acid substitution (Szczawinska-Poplonyk et al., 2020). The patient, an eleven-year-old boy diagnosed with a complex set of cellular and physiological abnormalities, including pancytopenia (comprising thrombocytopenia and hypoimmunoglobulinaemia), reduced absolute peripheral lymphocyte counts (affecting both T- and B-cells), imbalanced T-cell subsets (with severely lowered CD4+ and regulatory T-cells), and facial abnormalities, features reminiscent of Takenouchi-Kosaki syndrome (Szczawińska-Popłonyk et al., 2023). This patient presented with an unusual combination of clinical manifestations, notably the development of cancer, which has not been reported in other PID cases associated with mutations in the *CDC42* gene (Hashim and Mokhtar, 2021).

Concurrently, he met diagnostic criteria for HLH, alongside elevated chronic inflammatory markers like fibrinogen and C-reactive protein. This hyperinflammation state, likely attributable to a dysregulated pro-inflammatory cytokine profile (Zinter and

Hermiston, 2019), consistent with a contribution of the Cdc42 in regulating various immune cell functions (Assing et al., 2023; Chemin et al., 2012; Gerasimcik et al., 2015; Guo, 2021; Guo et al., 2011; Kalim et al., 2022; Smits et al., 2010). The subsequent histopathological diagnosis confirmed the presence of stage IV nodular sclerosis Hodgkin lymphoma, supported by immunophenotype with strong positivity for key biomarkers CD30 and CD15. This specific *CDC42* mutation is of particular interest given its potential implications for its role in both PID and haematological malignancy. The temporal relationship between the PID and Hodgkin lymphoma is therefore a key factor to understand the underlying mechanisms at play, including whether the malignancy observed precedes or coincides with the immune deficiency.

2.2 Cell division control protein 42 homolog (Cdc42)

Cell division control protein 42 homolog (Cdc42; gene name *CDC42* [MIN: 116952]), is a monomeric small GTPase categorised within the Cdc42 subfamily of the classical Ras (rat sarcoma) homology (Rho) family, which itself belongs to the Ras superfamily (Figure 2.2). This small GTP-binding protein (G-protein), with molecular weight of ~21 kDa, was first isolated from the human placental membranes and originally termed placental G-protein (G_p) (Evans et al., 1986). Significant breakthroughs in understanding Cdc42 functions came through genetic screening focused on cell division cycle protein mutants in the budding yeast *Saccharomyces cerevisiae* (*S. cerevisiae*). This investigation pinpointed *CDC42* as the gene pivotal in mediating the interplay between cell cycle events and cellular processes involving both cytosolic/membrane-associated components (Adams et al., 1990; Johnson and Pringle, 1990).

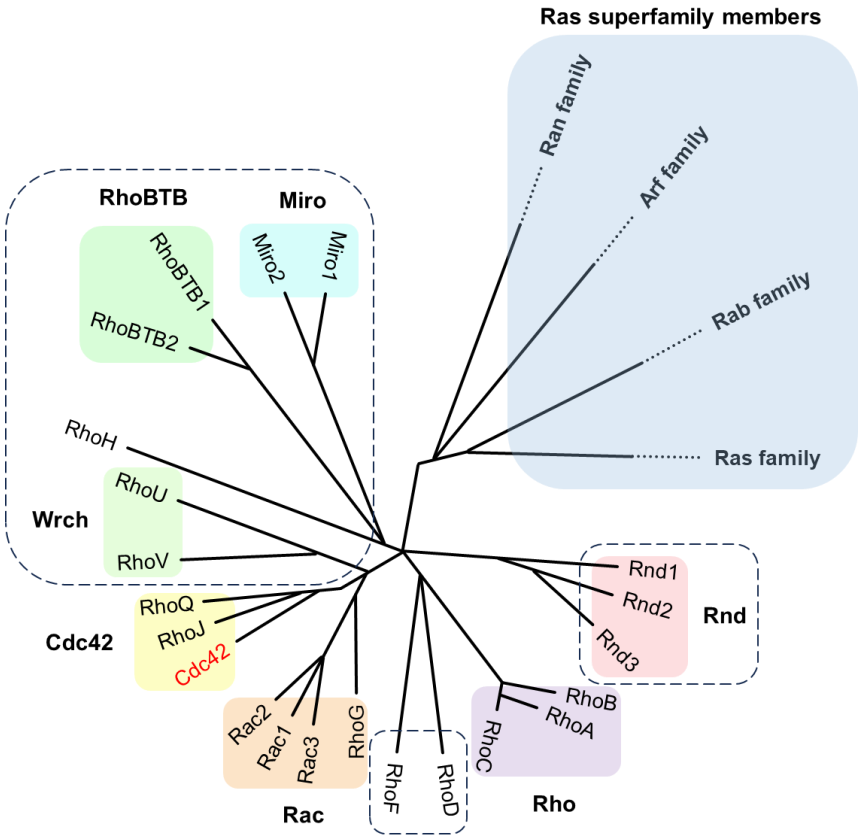

Figure 2.2 Phylogenetic relationships of within the Ras superfamily. A phylogenetic tree was constructed using Clustal Omega (Sievers et al., 2011) based on amino acid sequence alignment of small Rho GTPases, with the Ras, Rab, Ran, and Arf families included for broader superfamily context. The Rho family is resolved into eight major subfamilies: Rho, Rac, and Cdc42 (classified as typical), and Wrch, Rnd, Miro, RhoBTB, and others (classified as atypical), which are encircled by dotted squares.

In an interesting turn of events, Shinjo and collaborators managed to purify a complementary DNA (cDNA) clone corresponding to G_p, referred to as G25K. It closely resembling yeast Cdc42p and exhibiting >80% peptide sequence identity (Polakis et al., 1989; Shinjo et al., 1990). Further insight arose as two distinct cDNA isolates from humans unveiled the presence of two closely associated proteins, each

sharing 95% identity, but differing in residues 163 to 191 (Johnson, 1999). These entities were identified as the human brain isoform G25K (Munemitsu et al., 1990) and the ubiquitously expressed Cdc42Hs (Kozma et al., 1995). Notably, Cdc42 is evident across diverse eukaryotic species, from *Drosophila melanogaster* to *Caenorhabditis elegans* to *Homo sapiens*, with homologues displaying 80-95% amino acid sequence similarity in their predicted amino acid sequences (Johnson, 1999; Thompson, 2013). This conservation is underscored by the complementarity of most eukaryotic Cdc42 homologues with the Cdc42-1 mutant originating from *S. cerevisiae*, indicative of an ancient evolutionary origin and conserved functionalities (Johnson, 1999).

Presently, our understanding indicates that mammals possess a broad array of Rho GTPase family members (Figure 2.2), encompassing approximately 22 distinct types (Chong et al., 2023). Among these, Cdc42 stands alongside other extensively characterised Rho GTPases such as Ras-related C3 botulinum toxin substrate (Rac1) and Rho gene family member A (RhoA). They have traditionally been recognised as master regulators governing actin cytoskeleton architecture, whereby Cdc42 particularly associated with filopodia induction (Nobes and Hall, 1995). Continual investigation throughout the years has progressively uncovered involvement of Cdc42 in myriad mammalian cellular processes, including but not confined to intracellular vesicle trafficking, cell division, cell-to-cell adhesion, chemotaxis, and cell polarisation (Wirth and Ponimaskin, 2023).

2.2.1 Key structural features

X-ray crystallography and nuclear magnetic resonance spectroscopy have proven to be indispensable tools since 1990s to decipher the shared fundamental structural

attributes among various small GTPases within the Ras superfamily (De Vos et al., 1988; Hirshberg et al., 1997; Ihara et al., 1998; Owen et al., 2000; Rudolph et al., 1999). Cdc42, like other small GTPases, conforms the canonical Rossmann fold, featuring a core with six stranded mixed β-sheet (labelled β1 through β6) flanked by five α-helices (labelled α1 through α5) that interconnected by a series of loops (Yin et al., 2023). Typically, the primary structure of Cdc42 can be dissected into two distinct sections as summarized in Figure 2.3: (1) the G-domain (GTP-binding domain), responsible for nucleotide interaction, and (2) the C-terminal HVR (hypervariable region), crucial for membrane association.

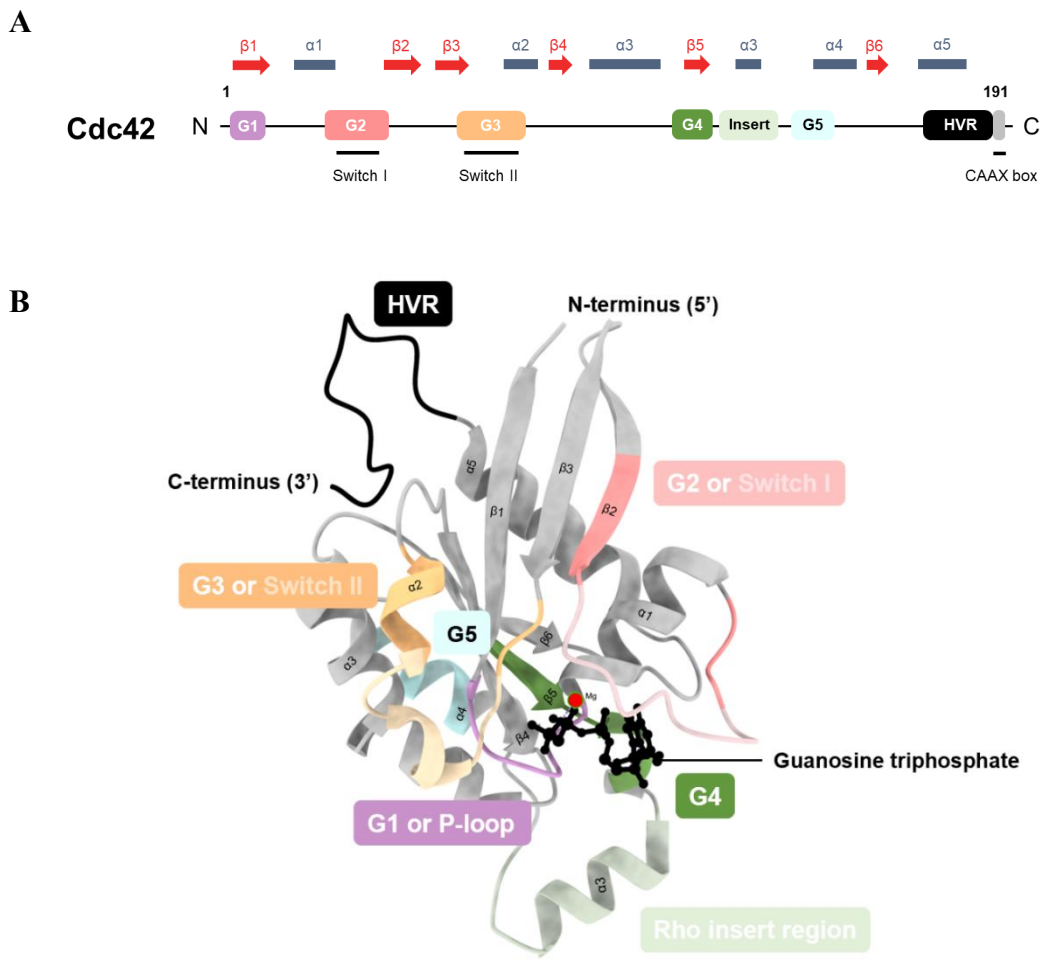

Figure 2.3 Domain structure of Cdc42 (PDB code 2QRZ). (A) Cdc42 shared several conserved features of typical small Rho GTPase, including the G-domain, HVR, CAAX box, and uniquely the Rho insert region. **(B)** The motifs are colour-coded as follows: G1 (residues 10-15, denoted in purple), G2 (residues 27-42, in red), G3 (residues 57-72, in orange), G4 (residues 112-119, in green), Rho insert (residues 122-135, in light green), G5 (residues 141-148, in blue), and HVR (residues 178-189, in black). Within the G2 and G3 motifs are the Switch I (in pink) and Switch II (in light orange), respectively. The stick models depict ligand (guanine nucleotide; in black) and a red sphere representing the magnesium ion. ***Note***: HVR, hypervariable region.

In the G-domain, five motifs (G1-G5) assumes critical roles in nucleotide binding and coordinating the magnesium ion (Schaefer et al., 2014). G1 (residues 10-15) firmly establishes a grip on the α- and β-phosphates of nucleotide (Murphy et al., 2021), earning its name as the 'phosphate binding (P)-loop', and this loop contains mutational hotspot, a glycine-to-valine substitution at residue 12 (G12V) that renders the Cdc42 constitutively active (Stengel and Zheng, 2011). Two other motifs, G2 (residues 27-42) and G3 (residues 57-72), encompass the majority of regions, named Switch I and Switch II, respectively. They serve a dual purpose, involving magnesium ion coordination and engagement with γ-phosphate of nucleotide (Zhang et al., 2023). As such, their inherent structural plasticity is intimately linked to nucleotide binding, capable of undergoing ample conformational changes depending on the nucleotide state (GDP/GTP) (Mosaddeghzadeh and Ahmadian, 2021). Notably, G3 houses another mutational hotspot, a glutamine-to-leucine substitution at residue 61 (Q61L), where the sidechain of Gln61 implicate in GTP-hydrolysis; this mutation also leads to constitutive activation and contributes to oncogenic signalling (Stengel and Zheng, 2011).

Moving beyond these intramolecular interactions, two structural regions, Switch I (residues 30-38) and Switch II (residues 59-67), underlie Cdc42's role as a molecular switch (Yin et al., 2023). These regions, adopting distinct conformations, dictate the specificity of interactions with a diverse array of regulatory and downstream effectors proteins. The G4 motif (residues 112-119), widely recognised as the 'guanine-binding loop', and the G5 motif (residue 141-148) are responsible for engaging with the ribose and guanine base to ensure proper nucleotide binding, while governing the selective binding for guanosine over adenosine (Yin et al., 2023). Interestingly, what sets small Rho GTPases apart from other Ras isoforms is the Rho insert domain (residues 122-135 in Cdc42), nestled between the G4 and G5 motifs (Haspel et al., 2021). An early study

identified this region as having a distinct role in cell transformation (Wu et al., 1998). Despite not exerting any profound structural influence in nucleotide-bound forms (Hakoshima et al., 2003), this unique feature remain central to perpetuating cellular signalling by interacting with RhoGEFs, RhoGAPs and enabling the regulation of effector proteins activation (Haspel et al., 2021; Schaefer et al., 2014).

Besides G-domain, Cdc42 comprises a flexible C-terminal HVR, with an ending consensus tetrapeptide motif of C-A-A-X (C, conserved Cys188; A, aliphatic amino acid; and X, any amino acid) (Mosaddeghzadeh and Ahmadian, 2021; Smithers and Overduin, 2016). Within this HVR lies a densely packed cluster of positively charged residues (primarily lysine [Lys] and arginine [Arg]), forming what is known as the polybasic patch. The CAAX box represents an important site for the sequential PTMs. Initiated at the conserved Cys residue, this process involves a series of isoprenylation, such as geranylgeranylation, where a 20-carbon isoprenoid lipid is attached (Michaelson et al., 2005). The prenylated protein then undergoes further modification through endoproteolysis and carboxyl methylation (Michaelson et al., 2005). Consequently, the C-terminal region acquires hydrophobic properties, drastically enhancing its affinity for anchoring and localisation to the plasma membrane, which is further regulated by RhoGDI that can extract Cdc42 from membrane and sequester it in the cytosol. This critical step, combined with the action of the polybasic patch, which interacts with the negatively charged headgroups of phospholipids, serves to stabilise membrane anchoring (Mosaddeghzadeh and Ahmadian, 2021).

2.2.1(a) Cysteine (Cys) in position 81: a residue within β4 segment

The interested mutation (see Section 2.1.2) occurs at position 81 within the β4 segment, a structurally unique region of the protein. While not part of the five canonical GTPase motifs, this segment is buried within the core of Cdc42 (Figure 2.4). Although its precise structural-functional relationship remains incompletely understood, the extreme evolutionary conservation patterns of Cys residues suggests biological significance (Marino and Gladyshev, 2010). While some Cys are located on protein surfaces and exhibit more typical polar characteristics with lower conservation, those burial type displays high conservation. This distinction is crucial since the fact that C81 is conserved in Cdc42 across diverse species (Martinelli et al., 2018), implying that C81 is not merely a structural component but a residue which plays a key role in Cdc42 function. As supported by experimental evidence, C81F abolishes Cdc42's intrinsic GTPase activity and promotes a higher degree of activation (Martinelli et al., 2018). The gain-of-function effect can be attributed to the β4 segment's proximity to the nucleotide-binding region. However, different substitutions at this critical residue can have distinct functional consequences. In contrast to previous C81F studies, the present investigation of the C81Y mutation reveals a distinct clinical context and suggests a loss-of-function effect, based on the observed impaired B-cell function and thrombocytopenia (Hashim and Mokhtar, 2021), justifying for further work to clarify the molecular basis of this C81Y-associated pathology.

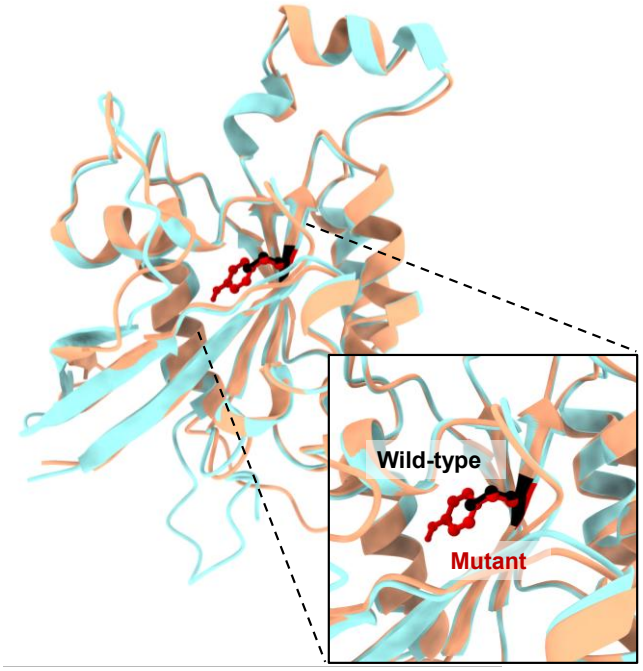

Figure 2.4 Structural localization of Cys81 within the β4 segment of the Cdc42. This visualization illustrates the spatial positioning of the C81Y substitution within the β4 strand, a structurally significant region adjacent to the nucleotide-binding pocket. The side chain of the wild-type cysteine at position 81 (Cys81) is shown in black, while the mutant tyrosine (Tyr81) is shown in red.

2.2.2 Regulatory proteins

Being a classical Rho GTPase, Cdc42 functions as a binary switch within the intracellular signal transduction networks. This protein dynamically shifts between two molecular conformations: the "off" state (GDP-bound or inactivation), and the "on" state (GTP-bound or activation). The regulatory machinery controlling this involves three set of proteins (Figure 2.5): (1) RhoGEFs that facilitate the GDP/GTP exchange reaction; (2) RhoGAPs that accelerate the intrinsically slow GTP-hydrolytic reaction; (3) RhoGDIs, which inhibit nucleotide exchange and maintain large pool of inactive

small G-protein in the cytosol. Importantly, this status dictates the cyclic nature of GTP binding and hydrolysis, subsequently influencing downstream biological effects.

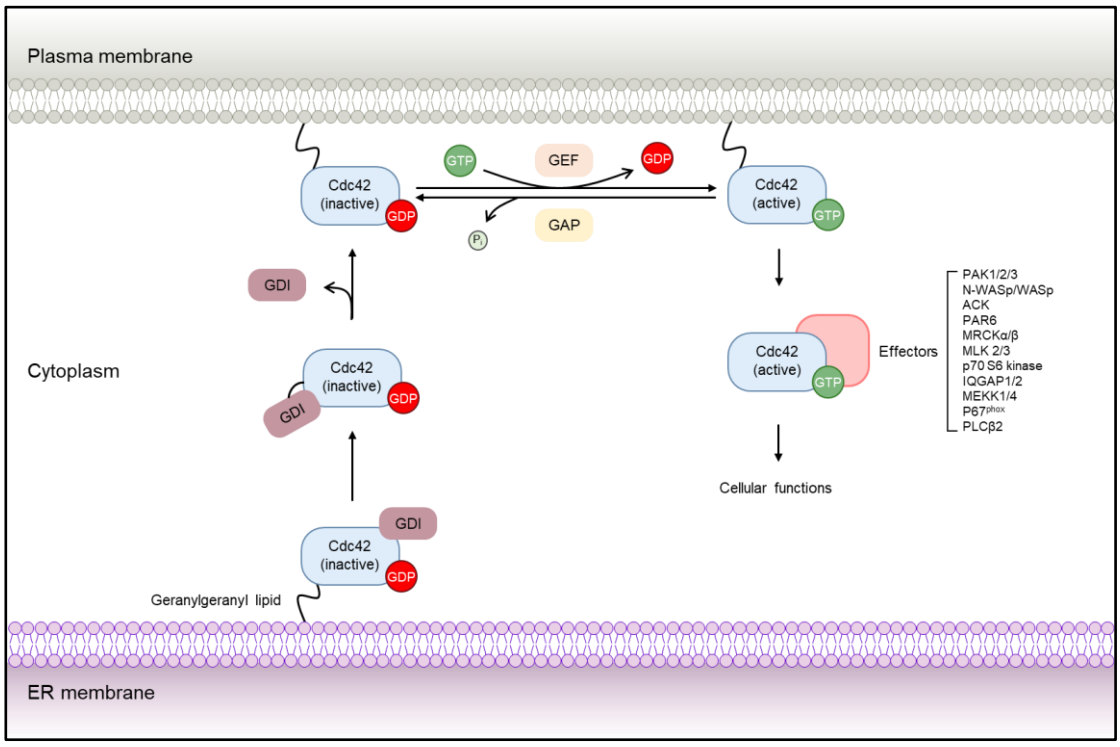

Figure 2.5 Key regulatory mechanisms for Cdc42. The prenylated Cdc42 acquires its geranylgeranyl lipid attachment at Cys188 within the CAAX box region after processing on the ER membrane. Negative regulatory proteins, known as GDIs, can bind to the Cdc42-GDP and extract it from the lipid bilayers, with the geranylgeranyl moiety embedded in hydrophobic pocket of GDI. This interaction facilitates the solubilization of Cdc42 into the cytosol while preventing the dissociation of guanine nucleotide. As such, the small G-protein stays inactive. Upon stimulation, GDI detaches from Cdc42-GDP, allowing it to anchor to the plasma membrane. This transition enables signal-activated GEFs to activate Cdc42 by exchanging GDP for GTP. Conversely, GAPs enhance the intrinsic GTPase function of Cdc42, which accelerates the conversion of GTP to GDP. When Cdc42 is in its active GTP-bound form, it undergoes a conformational change that promote effector binding and activation, as

well as driving various cellular activities. ***Notes***: ER, endoplasmic reticulum; GAP, GTPase-activating protein; GDI, guanine nucleotide-dissociation inhibitor; GEF, guanine nucleotide exchange factors; GTPase, guanine triphosphatase.

2.2.2(a) Rho guanine nucleotide exchange factors (RhoGEFs)

The rate of nucleotide exchange of Rho GTPases (i.e., Cdc42) is inherently slow (binding constant, k of 0.0168 min^{-1}), attributed to their high affinity for GDP/GTP in sub-nanomolar to picomolar range (Bos et al., 2007; Rossman et al., 2002). For this reason, the presence of RhoGEFs as positive regulators are important, with roughly 35 of the 85 identified RhoGEFs associated with Cdc42 thus far (Bagci et al., 2019; Müller et al., 2020). These multidomain proteins can be divided into two main groups: diffuse B-cell lymphoma (Dbl)-related proteins and the dedicator of cytokinesis (DOCK) family proteins. Under certain triggers, including the activation of cell surface receptors, they accelerate nucleotide exchange by modifying the phosphate-binding site (Bos et al., 2007; Vetter and Wittinghofer, 2001), and hence reducing Rho GTPase nucleotide affinity to facilitate subsequent replacements. Initially, a RhoGEF binds to the Rho GTPase-GDP in the presence of Mg^{2+}, forming a transient quaternary complex characterized by low affinity. This complex rapidly becomes destabilised when the orientation of specific amino acids (Lys16, Thr17, Cys18, Phe28) changes, leading to the release of GDP-Mg^{2+} (Kang et al., 2019; Rossman et al., 2002). With intracellular GTP typically present in twenty-fold molar excess than that of GDP in the cytoplasm (Kang et al., 2019), this promotes the formation of another unstable quaternary complex involving RhoGEF-Rho GTPase and GTP-Mg^{2+}. Eventually, the dissociation of RhoGEF results in stable assembly of the Rho GTPase-GTP complex (Rossman et al., 2002).

Studies using mutagenesis approaches have revealed Thr17, a residue near the phosphate binding loop (P-loop) that, whose substitution with asparagine (Asn) results in a Cdc42 mutant (T17N) that is defective in guanine nucleotide exchange (Vanni et al., 2005) and hence confers a dominant-negative phenotype (Table 2.1). This mutant alters Cdc42 signalling *in vivo* by competitively binding to RhoGEF, forming a more stable Cdc42 T17N-RhoGEF complex than its WT counterpart, thereby hindering RhoGEF to catalyse GDP release of endogenous Cdc42 (Kang et al., 2019; Melendez et al., 2011). Thr17 typically forms a hydrogen bond with GDP (Kang et al., 2019) and coordinates with Mg^{2+} to stabilise Cdc42 in GDP-bound state (Acuner et al., 2021). As a consequence, the Asn substitution simultaneously weakens affinity for GDP and block effective GTP binding, causing the protein to predominantly adopt a nucleotide-free or GDP-bound conformation. Another less appreciated residue in Cdc42, Phe28, interacts with the guanine ring of GDP *via* π-π stacking, This interaction occurs only when RhoGEF is not bound, which explains why the Cdc42 F28L mutant shows impaired GDP binding and a significantly increased GDP/GTP exchange rate – roughly fifty times the rate of Cdc42 WT – without requiring RhoGEF (Fidyk et al., 2006; Kang et al., 2019). Despite this rapid exchange, the associated effects of the fast-cycling phenotype, such as an abnormally high pool of activated Cdc42, can be mitigated by promoting GTP hydrolysis through increased RhoGAP expression (Fidyk et al., 2006).

2.2.2(b) Rho GTPase-activating proteins (RhoGAPs)

As a Rho GTPase, Cdc42 is capable of weakly hydrolyse GTP, which cleaves the third (γ)-phosphate bond to produce GDP and inorganic phosphate (P_i). However, compared to the timescale of Cdc42's cellular functions, this hydrolysis reaction occurs at a low rate (k = 0.05 min^{-1}) (Self and Hall, 1995). Thus, negative regulators (RhoGAPs) intervene to accelerate the hydrolytic process by several factors, and return Cdc42 to its basal inactive state (Csépányi-Kömi et al., 2013) with ~25 of the 70 available RhoGAPs are known to regulate Cdc42 such as p50RhoGAP, CdGAP, and IQGAP1(Müller et al., 2020). Structurally, RhoGAPs are marked by a GAP domain (approximately 150 amino acids) that suffices to enable protein binding and catalytic stimulation (Bidaud-Meynard et al., 2017; Moon and Zheng, 2003; Peck et al., 2002). Within this domain, an invariant Arg residing in a loop commonly referred to as the "arginine finger" plays a catalytic function, following stable interactions with GTP-binding core (P-loop and Switch I/II) of Cdc42. It appears that the GAP domain and Cdc42's GTP-binding core are oriented at a 20° angle relative to each other, from the initial state (Rittinger, Walker, Eccleston, Nurmahomed, et al., 1997) to the transition state (Rittinger, Walker, Eccleston, Smerdon, et al., 1997), a configuration believed to protrude the "arginine finger" into the active region of Cdc42. Here, the Tyr32 residue of Cdc42 contributes to stabilizing interaction with the "arginine finger" (Fidyk et al., 2006). Along with that, the stabilization of negative charges generated during the state shift, the close contact between catalytic Gln61 and water molecules, as well as a lowering in the transition state energy barrier collectively enhance the efficacy of the GTP hydrolysis reaction (Bidaud-Meynard et al., 2017; Moon and Zheng, 2003; Scheffzek and Ahmadian, 2005).

The well-known mutations, Cdc42 G12V and Cdc42 Q61L (Table 2.1), represent RhoGAP-insensitive mutants that remain constitutively hyperactivated due to weakening of interactions between "arginine finger" and Cdc42's active pocket. Specifically, Cdc42 G12V block the catalysis-favoured orientation of Gln61 *via* steric hindrance (Chen et al., 2022; Haspel et al., 2021), while Cdc42 Q61L prevents the necessary structural conformation required for effective nucleophilic attack (Mosaddeghzadeh et al., 2022). Unlike these mutants that are completely defective in GTP hydrolysis, Cdc42 Y32A (a tyrosine-to-alanine substitution at residue 32) retains partial intrinsic GTPase activity (slow-cycling) while being insensitive to RhoGAP. As a result, it is not permanently locked in activated state (Fidyk et al., 2006). Additionally, certain sites, such as the Rho insert domain, has been shown to influence the binding affinity of Cdc42 for the IQGAP1. For example, a asparagine-to-lysine substitution at residue 132 (N132K) in Cdc42 Q61L cause a slight reduction in interaction with IQGAP1 (Mosaddeghzadeh et al., 2022). Nevertheless, complete deletion of the insert loop preserves a RhoGAP binding affinity similar to that of Cdc42 WT. This suggests that while the insert domain is near the GAP interface, it is not essential for RhoGAP binding in the resting state. Instead, it is only relevant in the transition state of GTP hydrolysis reaction, perhaps by influencing the conformation of the complex (Owen et al., 2008). This is further supported by the observation that replacing the bulky Lys132 to a smaller Ala132 alleviates potential steric hindrance and stabilizes the α-helix at C-terminal region.