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Computational Characterization of the Cancer-Associated GNB1
K57E Mutation: Structural and Dynamic Insights from Molecular
Dynamics Simulations

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Abstract

The GNB1 gene encodes the G β 1 subunit of heterotrimeric G proteins, which play a central role in G protein-coupled receptor (GPCR)-mediated signal transduction. Mutations in GNB1 have been implicated in a range of human diseases, including neurodevelopmental disorders such as GNB1 encephalopathy and various cancers. Despite increasing recognition of their clinical significance, the molecular mechanisms underlying the pathogenic effects of individual GNB1 mutations remain incompletely understood.

In this study, the cancer associated somatic K57E mutation was investigated within the G β 1 γ 2 system using molecular dynamics (MD) simulations and free energy calculations to characterize its structural and functional consequences. The thermodynamic impact of the lysine-to-glutamate substitution at position 57 was assessed by calculating the relative free energy difference ($\Delta\Delta G$) between the wild-type and mutant. Structural stability, residue flexibility, and residue–residue interactions were further analyzed using root mean square deviation (RMSD) and root mean square fluctuation (RMSF).

The K57E variant exhibited a positive $\Delta\Delta G$ of approximately +8.7 kJ/mol, indicating reduced thermodynamic stability compared with the wild type. Despite this energetic destabilization, RMSD analysis demonstrated preservation of the overall protein fold, while RMSF analysis revealed increased rigidity in several regions of the protein. Furthermore, the mutation disrupted local non-bonded interactions, suggesting altered electrostatic interactions at the protein interface. These computational findings are consistent with published experimental interaction data, which show preserved binding to G γ subunits but markedly reduced association with multiple G α subunits.

Overall, the results indicate that the K57E mutation does not compromise the global structure of G β 1 but instead alters its interaction interface, reducing its ability to engage G α proteins. These findings provide new insight into how the K57E variant may impair GPCR-mediated signal transduction and contribute to the molecular mechanisms underlying GNB1-associated cancers.

Zusammenfassung

Das GNB1-Gen kodiert für die Gβ1-Untereinheit heterotrimerer G-Proteine, die eine zentrale Rolle in der Signaltransduktion über G-Protein-gekoppelte Rezeptoren (GPCRs) spielen. Mutationen in GNB1 werden mit einer Vielzahl menschlicher Erkrankungen in Verbindung gebracht, darunter neurologische Entwicklungsstörungen wie die GNB1-Enzephalopathie sowie verschiedene Krebserkrankungen. Trotz zunehmender klinischer Erkenntnisse sind die molekularen Mechanismen, die den pathogenen Auswirkungen einzelner Mutationen zugrunde liegen, bislang nicht vollständig verstanden.

In dieser Arbeit wurde die somatische, krebsassoziierte K57E-Mutation im Gβ1γ2-System mithilfe von Molekulardynamik-(MD)-Simulationen und Freie-Energie-Berechnungen untersucht, um ihre strukturellen und funktionellen Auswirkungen zu charakterisieren. Der thermodynamische Einfluss der Substitution von Lysin durch Glutamat an Position 57 wurde durch die Berechnung der relativen freien Energiedifferenz ($\Delta\Delta G$) zwischen Wildtyp und Mutante bestimmt. Darüber hinaus wurden die strukturelle Stabilität, die Flexibilität einzelner Aminosäurereste sowie deren Interaktionen anhand der Root Mean Square Deviation (RMSD) und der Root Mean Square Fluctuation (RMSF) analysiert.

Die K57E-Variante zeigte eine positive $\Delta\Delta G$ von etwa +8,7 kJ/mol, was auf eine verringerte thermodynamische Stabilität im Vergleich zum Wildtyp hinweist. Trotz dieser energetischen Destabilisierung zeigte die RMSD-Analyse, dass die globale Proteinstruktur erhalten bleibt, während die RMSF-Analyse eine erhöhte strukturelle Starrheit in mehreren Proteinbereichen erkennen ließ. Darüber hinaus führte die Mutation zu einer Störung lokaler nichtkovalenter Wechselwirkungen, was auf veränderte elektrostatische Interaktionen an der Proteinoberfläche hindeutet. Diese computergestützten Ergebnisse stimmen mit veröffentlichten experimentellen Interaktionsdaten überein, die eine erhaltene Bindung an Gy-Untereinheiten, jedoch eine deutlich verminderte Assoziation mit mehreren Gα-Untereinheiten zeigen.

Insgesamt deuten die Ergebnisse darauf hin, dass die K57E-Mutation die globale Struktur von Gβ1 nicht beeinträchtigt, jedoch die Interaktionsoberfläche verändert und dadurch die Bindung an Gα-Proteine reduziert. Diese Erkenntnisse liefern neue Einblicke in die Auswirkungen der K57E-Mutation auf die GPCR-vermittelte Signaltransduktion und tragen zum Verständnis der Pathogenese GNB1-assoziiierter Krebserkrankungen bei.

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List of abbreviations

A	Alanine
AC	Adenylyl cyclase
aCML	Atypical chronic myelogenous leukaemia
ADHD	Attention-deficit/hyperactivity disorder
AML	Acute myeloid leukaemia
ASD	Autism spectrum disorder
ATP	Adenosine triphosphate
BAR	Bennet's Acceptance Ratio
CFT	Crooks Fluctuation Theorem
CHIP	Clonal haematopoiesis of indeterminate potential
CMA	Chromosomal microarray analysis
CMML	Chronic myelomonocytic leukaemia
CSF-1	Colony stimulating factor 1
DAG	Diacylglycerol
DBS	Deep brain stimulation
DEFA	Alpha-defensin
E	Glutamate
EEG	Electroencephalogram
EIEE	Early infantile epileptic encephalopathy
GABA	γ -aminobutyric acid
GDD	Global development delay
GDP	Guanosine diphosphate
GIRK	G-protein-gated inward rectifier K ⁺ channels
GNB1	G protein subunit beta 1
GNB1-E	GNB1-encephalopathy
GPCR	G protein-coupled receptor
GTP	Guanosine triphosphate

HSCT	Hematopoietic stem cell transplantation
ID	Intellectual disability
IP3	Inositol-1, 4, 5-trisphosphate
JAK	Janus kinase
K	Lysine
KPLBS	Keppen-Lubinsky-Syndrome
MD	Molecular dynamics
MDS	Myelodysplastic syndrome
MPN	Myeloproliferative neoplasm
MRI	Magnetic resonance imaging
NEMD	Non-equilibrium molecular dynamics
PDB	Protein Data Bank
PIP2	Phosphatidylinositol 4,5-bisphosphate
PLC β	Phospholipase C β
RMSD	Root mean square deviation
RMSF	Root mean square fluctuation
sAML	Secondary acute myeloid leukaemia
U	Potential energy
VAF	Variant allele frequency
WT	Wild-type
$\Delta\Delta G$	Change in Gibbs Free Energy

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1 Literature Overview

1.1 GNB1 Encephalopathy

GNB1 Encephalopathy (GNB1-E) is a rare neurological condition with solely 68 affected patients worldwide. It is caused predominantly by de-novo mutations in the GNB1 gene encoding for the G β 1 subunit of the heterotrimeric G protein. This affects essential signal pathways in GPCR signalling and leads to the typical clinical picture of GNB1-E (Nasvytis u. a. 2024).

GNB1 Encephalopathy, considered as a heterogenous neurodevelopment syndrome, is mostly marked by cluster of symptoms and differences in manifestations. First clinical signs usually appear in the first years of life, on average at 5 years old and are often hard to distinguish from other neurodevelopmental disorders. One unifying attribute shown in all patients of GNB1-E is global development delay (GDD). The clinical picture present in one in two patients includes typically cognitive or intellectual dysfunctions, differences in brain structure, epileptic seizures and/or muscle hypo- or hypertonia (Da Silva u. a. 2021; Revah-Politi u. a. 1993).

Due to the global distribution of the disease and its lack of restriction to a specific population, it is assumed to be most likely caused by de novo mutations in the GNB1 gene. Such mutations can arise in any population, regardless of ancestry (Lohmann und Masuho 2017).

1.1.1 Pathogenesis

G protein-coupled receptors (GPCRs) are a large group of transmembrane proteins that detect extracellular signals and activate G-proteins for intracellular response. Those cell regulating proteins consist of an alpha (G α), beta (G β) and gamma (G γ) subunit which form the heterotrimer (Revah-Politi u. a. 1993).

Before activation, the subunits are bound together as a heterotrimer with the α -subunit associated with a guanosine diphosphate (GDP) molecule. Through receptor triggering the GDP gets exchanged for a guanosine triphosphate (GTP) and the trimer dissociates into G α -GTP and G $\beta\gamma$. Both subunits initiate interaction with effector molecules causing the activation of diverse signalling pathways. With relevance to GNB1 Encephalopathy, the G β 1 γ subunit is known to interact directly with multiple targets including the inwardly rectifying K⁺ channels (GIRK1/2/3/4), SNAP-25, various adenylyl cyclases, N- and P/Q-type Ca²⁺ channels, PLC β (1-3), GPCR kinases and phosphoinositide 3 kinase γ (Smrcka 2008).

The GNB1-gene, coding for the β 1-subunit, displays a lot of different pathogenic mutations which cause a shift in receptor behaviour in GPCRs. These mutations lead to changes in the G β 1 subunit's structure and G β 1 γ function. The protein becomes either

unstable, leading to lose of protein function or is inhibited of forming a complex with the $G\alpha$, triggering a constant activation of $G\beta_1\gamma$. The binding of the heterotrimer $G\alpha\beta_1\gamma$ to the G-protein coupled receptor can be disrupted as well (Nasvytis u. a. 2024).

Next generation sequencing allowed the discovery of new disease-causing mutations of the GNB1 gene. Most of them, were confirmed to be developed spontaneously (de-novo mutation), meaning that it was not present in either parents DNA. Only in few cases autosomal dominant inheritance was proven (Lohmann und Masuho 2017). It was discovered that most of the pathogenic mutations are located in Exon 6 & 7 encoding for amino acids 76 – 112 in $G\beta_1$. This concludes the exon's central role in the function of the GNB1 gene (Da Silva u. a. 2021; Lohmann und Masuho 2017).

1.1.2 Clinical manifestations

The neurodevelopmental disability GNB1-E is characterized by a collection of distinct symptoms. Even though the disease is primarily neurological in origin, there are several secondary systemic manifestations observed in patients (Petrovski 2016; Reddy u. a. 2021).

1.1.2.1 Global Development Delay (GDD)

A shared clinical presentation in all patients diagnosed with GNB1-E, is global development delay. Even though there is no data available regarding pregnancy and childbirth in relation to GNB1-E, infants show first symptoms solely through feeding difficulties resulting in growth delay (Nasvytis u. a. 2024).

As the condition progresses GDD gets more prominent. Most affected children show motor difficulties, unable to walk without assistance or achieve independent walking at a later age than typical. Those patients who start walking develop usually gait disorders, coordination issues and a history of recurrent falls. In some cases, paralysis on one side of the body (*hemiplegia*), uncontrolled movements affecting legs and hands (*dyskinetic quadriplegia*) and stiffness in both legs (*spastic diplegia*) occurred (Nasvytis u. a. 2024; Vitrikas u. a. 2020).

In addition, a lot of individuals are reported to be non-verbal or display difficulties particularly in their speaking ability as well as dysarthric speech (Nasvytis u. a. 2024).

1.1.2.2 Intellectual disability (ID)

Around 74% of the GNB1-E patients exhibit an intellectual disability varying from mild to severe. Yet, it should be taken into consideration that some patients were too young for a meaningful assessment of cognitive function and that adequate documentation was lacking for certain older patients (Revah-Politi u. a. 1993).

1.1.2.3 Abnormal muscle tone

The most common early sign in infants, diagnosed with GNB1-E is hypotonia, showing a spectrum of clinical severity. In severe cases, patients have trouble keeping controlled

movement of both legs and arms and coordinating muscles for swallowing. Usually, children's muscle tone shifts with growth and evolves over time from low tone to spastic hypertonia, resulting in stiffness and higher resistance to movement. Moreover, studies have emphasized the pattern of low tone in neck, trunk and spine combined with higher tone in limb muscles (Nasvytis u. a. 2024).

1.1.2.4 Dystonia

Dystonia is a disorder associated with constant or sustained episodic muscle contraction causing twisting and patterned movement or/and abnormal posture (Grütz und Klein 2021). It represents the most frequently reported movement disorder in patients with GNB1-E. A fraction of patients is reported to suffer from unpredictable muscle jerks (*myoclonus*) as well as intermittent status dystonicus. Tics, chorea, and ataxia are among the less commonly observed clinical manifestations (Nasvytis u. a. 2024; Reyes u. a. 2023).

1.1.2.5 Epilepsy

A subset of GNB1-E patients appears to have either focal or generalized epileptic seizures. There is a big variation in observed seizure types including tonic, atonic, myoclonic and tonic-clonic seizures. A small number of cases were associated with West syndrome, an early infantile epileptic encephalopathy (EIEE) (Peng u. a. 2018; Nasvytis u. a. 2024).

1.1.2.6 Visional and hearing impairment

The most common sensory impairments in GNB1-Encephalopathy patients are nystagmus, with roughly 40% reported cases. Other eye movement disorders include strabismus, ophthalmoplegia or gaze deviation. One out of ten patients suffer from cortical visual impairment or optic nerve atrophy, including one blind individual. None of the patients showed differences in genetic variation that could allow a connection to their clinical picture. The observed hearing dysfunction were either bilateral sensorineural or unilateral sensorineural hearing loss (Revah-Politi u. a. 1993; Hemati u. a. 2017).

1.1.2.7 Other symptoms

Other reported symptoms include cardiovascular defects, gastrointestinal issues, genitourinary anomalies, cutaneous mastocytosis, behavioural issues like ASD or ADHD, hypothyroidism and malignancy (Hemati u. a. 2017; Revah-Politi u. a. 1993).

1.1.3 Prognosis

Due to the lack of long-time data, it is difficult to predict a patient's life expectancy. While GNB1-E predominantly is observed in children and young adults, some cases extending into adulthood have been documented. Typically, the condition is not associated with any congenital anomalies that could reduce longevity, indicating a favourable prognosis with appropriate medical care (Nasvytis u. a. 2024).

1.1.4 Diagnostics

Even though, identifying different mutations in the GNB1 gene improved with next generation sequencing, the complexity of genetic interpretation remains challenging. Patients often exhibit more than one potential pathogenic variant which makes it difficult to draw conclusions regarding disease aetiology. Even if identified mutations are proven to change protein structure, it is possible that those variants have no pathogenic effect (Lohmann und Masuho 2017).

Due to the overlap of symptoms with other neurological conditions, GNB1-Encephalopathy cannot be diagnosed based on clinical features alone. Establishing a diagnosis is only possible with genetic analysis by identifying a pathogenic or likely pathogenic variation, primarily through chromosomal microarray analysis (CMA) (Nasvytis u. a. 2024; Revah-Politi u. a. 1993).

There is no specific laboratory marker that provides reliable detection of GNB1-E, making diagnosis more challenging. It is recommended for patient to undergo an EEG to rule out asymptomatic brain seizures as well as an MRI to detect potential brain abnormalities (Nasvytis u. a. 2024).

1.1.5 Treatment

At present, there is no cause-targeted therapy for GNB1-Encephalopathy available, therefore management is limited to supportive care and symptomatic treatment. To maximize the patient`s benefit, a suitable therapy should be initiated at an early stage. Feeding therapy, physiotherapy and occupational therapy are considered as core treatments for infants as well as individualized education plans and mobility aids upon reaching school age (Nasvytis u. a. 2024).

Patients affected by epileptic seizures receive a medication with standard anti-epileptic drugs. Even though no drug of choice has been established, ethosuximide has demonstrated promising results in preclinical studies (Nasvytis u. a. 2024).

The current therapy for movement disorders includes the use of anticholinergics, dopamine agonists and antagonists, dopamine depleting agents, benzodiazepines and baclofen. However, these drugs display limited and inconsistent benefits. In severe cases, deep brain stimulation (DBS) has been shown to improve myoclonus and dystonia (Nasvytis u. a. 2024).

Physical and occupational therapy are considered crucial in GNB1-E treatment, since they help prevent secondary complications like contractures, falls, stiffness or muscle tightness. Most importantly, the patients care must be multidisciplinary and individualized because GNB1-Encephalopathy affects typically more than just one organ system (Nasvytis u. a. 2024).

1.2 Guanine nucleotide-binding proteins (G-Proteins)

G-proteins (guanine nucleotide-binding proteins) are central mediators in several mammal signal pathways and key factor for regulation of signal transduction. Multiple extracellular stimuli (e.g. hormones, photons, neurotransmitters, drugs) are known to activate GCPR and leading to promotion of GDP to GTP exchange and dissociation of the heterotrimeric G-protein into its subunits (Tennakoon u. a. 2021).

The human genome includes 5 $G\beta$ (GNB1 – GNB5) and 12 $G\gamma$ (GNG1 – GNG12) genes. This demonstrates how versatile cell signalling can be and how large GPCRs functional diversity is (Tennakoon u. a. 2021).

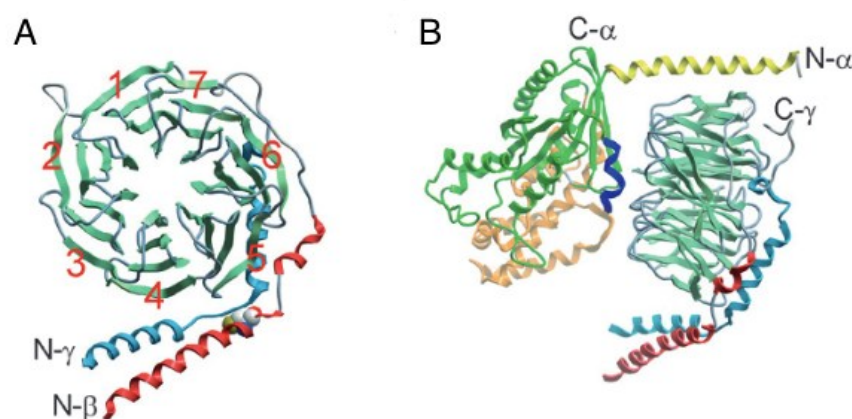


Figure 1: G-protein structure.

(A) Crystal structure of the $G\beta\gamma$ subunits with numbered blades 1-7. Red illustrated $G\beta$ subunit is interacting with the blue $G\gamma$ helix. (B) G-protein heterotrimer depicted with the two main interaction regions between $G\beta\gamma$ and $G\alpha$. The N-terminus of $G\alpha$ is shown in yellow and the switch II region in dark blue (Smrcka 2008).

The $G\beta$ subunits are arranged in a round structure (β -propeller) consisted of seven repeated units (“blades”). Each part of the propeller blade is made of a four-stranded β -sheet. At the N-terminus (first 57-70 amino acids) $G\beta$ contains a α -helical domain which displays a tight coiled-coil interaction with the γ subunit. This implies that $G\beta$ and $G\gamma$ form a tightly associated, stable complex. The $G\alpha$ subunit engages with the $G\beta\gamma$ complex at two main regions. First interaction includes the N-terminus of $G\alpha$ binding to the side of the first blade of the propeller. The second interaction is between the switch II region of $G\alpha$, which changes conformation upon GTP binding, and the top surface of the β -propeller (Smrcka 2008).

1.2.1 G protein-coupled receptor signalling

The primary mechanism regulating biological function across all levels, from individual cells to the whole organism, is chemical signalling. The recognition of these signals largely depends on receptors, therefore playing a key role in signal transduction (Rang 2006).

G protein-coupled receptors (GPCRs) have been a major focus of pharmacological research, due to their wide range of cellular function and their role as the predominant class of drug targets. Most of the signal transmission in mammals are controlled by neurotransmitters and hormones acting through GPCRs. They form the largest family of membrane bound proteins and can be classified into subfamilies A, B1, B2, C, F and taste 2 sensory receptor subfamily, highlighting their high diversity (Latorraca u. a. 2016; Wootten u. a. 2018).

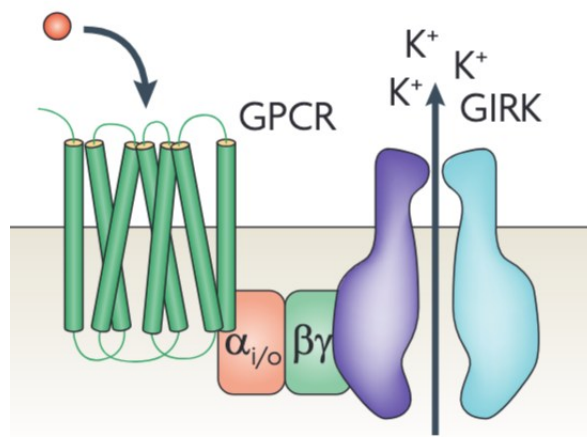


Figure 2: GPCR signalling illustrated by GIRK activation.

Upon ligand binding GPCR catalyses GDP-GTP exchange and promotes dissociation of the subunits. The $\beta\gamma$ subunit interacts directly with the GIRK channel and activates it through conformation change. K^+ can leave the cell resulting in the hyperpolarization of the cell membrane (Lüscher und Slesinger 2010).

GPCRs contain a transmembrane domain that is connected to an extra- and intracellular end, that can vary in length and sequence. Most of the receptors possess an additional extracellular binding side, attached to the N-terminus for ligand-binding (Latorraca u. a. 2016). Upon binding of a neurotransmitter or other ligand, the receptor stimulates the exchange of GDP for GTP, which leads to the dissociation of the heterotrimeric protein into α and $\beta\gamma$ subunits. These subunits are mediating further downstream signals by targeting other proteins or channels. The $G\beta\gamma$ subunit interacts directly with ion channels, adenylyl cyclases, PLC β and the GIRK channel, stimulating there activation primarily through allosteric regulation (Weis und Kobilka 2018).

1.3 G-protein-gated inward rectifier (GIRK/Kir3.x)

G protein-gated inwardly rectifying K^+ channels belong to the Kir family (Kir1–Kir7) and are found predominantly in heart and pancreatic tissue as well as the nervous system. Neurotransmitter like acetylcholine, dopamine, serotonin or GABA stimulate several different GPCRs resulting in the release of its subunits and $G\beta\gamma$ directly binding and opening the GIRK channel. When activated, K^+ can enter the cell, leading to hyperpolarization of the membrane and a reduced neural and cellular activity (Nguyen u. a. 2024).

Another mandatory co-factor for activation of the GIRK channel is phosphatidylinositol 4,5-bisphosphate (PIP2), a lipid signalling molecule located in the plasma membrane. PIP2 facilitates binding between the G protein and the GIRK channel (Nguyen u. a. 2024).

Several Studies identified GIRK activity as a key factor in pain perception, learning and addiction. Abnormal activity of the channel is associated with a variety of neurological conditions like epilepsy, Down-syndrome, Keppen-Lubinsky Syndrome (KPLBS) and drug addiction. Due to the involvement of GIRK channels in the cardiac and endocrine system, dysregulation can also lead to heart failure, Long QT syndrome or type II diabetes mellitus (Nguyen u. a. 2024).

1.3.1 Structure

GIRK channels are tetrameric complexes meaning that one functional channel consists of 4 GIRK protein subunits. The genes encoding for these proteins are KCNJ3/GIRK1/Kir3.1, KCNJ6/ GIRK2/Kir3.2, KCNJ9/GIRK3/Kir3.3, and KCNJ5/GIRK4/Kir3.4. Each subunit has two transmembrane helices, a pore loop functioning as a K⁺ selective filter, and a N-terminal and C-terminal cytoplasmic domain. The cytoplasmic tails show the highest degree of variation among the four GIRK subunits. These differences effect binding, channel trafficking and gating (Luo u. a. 2022).

Kir3.x channels can assemble as either homo- or hetero-tetramers (**Figure 3**), but they predominantly form heterotetrameric complexes, such as GIRK1/3 and GIRK2/3. In contrast, only GIRK2 is able to form a functional channel consisting of the same subunits, the GIRK2/2 (Lüscher und Slesinger 2010; Luo u. a. 2022).

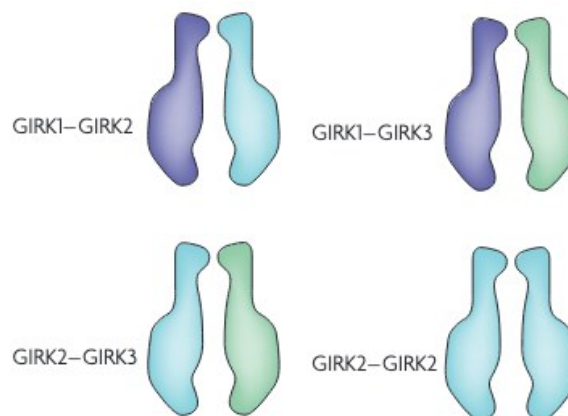


Figure 3: GIRK channel complexes.

The core subunits GIRK1-3 form heterotetrameric complexes. Solely GIRK2 is able to form a homotetramer (Lüscher und Slesinger 2010).

Neuronal GIRK channels predominantly consist of the GIRK1 and the core GIRK2 subunit. GIRK2 contains important trafficking signals, contributing to the ER export of proteins and its final delivery to the cell surface. Several mouse knock-out studies proved that by deleting the GIRK2 gene, channels loose normal function, allowing less ions passing

through the membrane resulting in reduced hyperpolarization. Additionally, studies have observed a alteration in GIRK1 stability and assembly, indicating a dependence on the GIRK2 subunit (Luo u. a. 2022).

1.4 K57E Mutation

This study focuses on the somatic, cancer associated K57E mutation located on the G β 1 subunit. In this case, the positively charged amino acid lysine at position 57 is replaced by the negatively charged glutamic acid, suggesting a potential alteration in protein structure and function (Yoda, Weigert, u. a. 2015).

Lysine is often found in specific locations within protein structure. It possesses a hydrophilic and hydrophobic side chain making the amino acid somewhat amphipathic. The hydrophilic side chain contains an amino group, which gives the amino acid its positive charge, whereas the longer hydrophobic side part consists mostly of carbon and hydrogen. Additionally, lysine often forms salt bridges with opposite charges and helps to stabilize protein structure (Betts und Russell 2003).

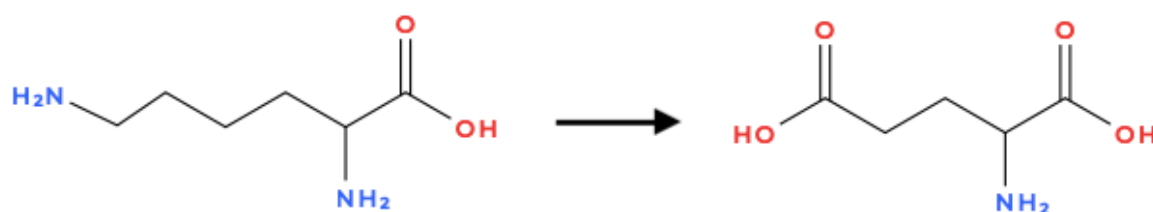


Figure 4: Substitution of lysine to glutamic acid in position 57

Glutamate carries a negative charge due to his carboxylate group. This amino acid primarily favours polar or hydrophilic environment and is often located on the protein surface. Like lysine, glutamate is often involved in salt bridges (Betts und Russell 2003).

1.4.1 Patients reported with K57E mutation

At the moment there are eleven confirmed cases of patients carrying one K57 variant. These mutations are emphasized to display a lineage specificity, almost exclusively causing myeloid cancer. Out of the 11 cases, 6 patients have been identified with myelodysplastic syndrome (MDS), 2 patients with acute myeloid leukaemia (AML) as well as 2 patients with atypical chronic myelogenous leukaemia (aCML) and one with polycythaemia vera (PV) (Yoda, Adelmant, u. a. 2015).

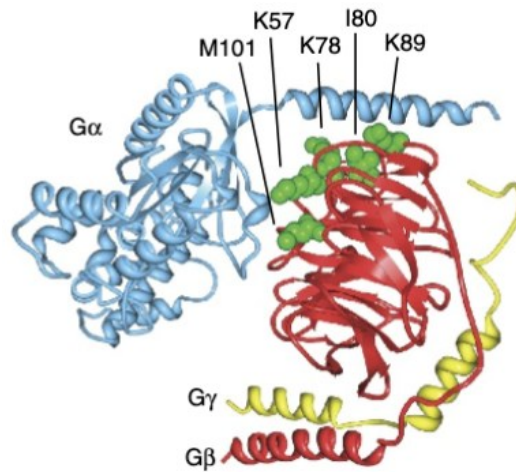


Figure 5: G-protein crystal structure.

The location of multiple recurrent oncogenic mutation is illustrated in green. K57E mutation is located in the β -propeller region. α subunit depicted in blue, β subunit in red and γ subunit in yellow (Yoda, Adelmant, u. a. 2015).

This work focuses on the K57E missense mutation, which was observed in at least three cases with myelodysplastic syndrome (MDS). One previously case of K57E mutation was reported in a patient with sAML (Walter u. a. 2013; Yoda, Weigert, u. a. 2015).

It should also be noted that GNB1 mutations are among the most frequent genes affected in clonal haematopoiesis of indeterminate potential (CHIP), suggesting their ability to promote and initiate myeloid diseases (Vedula u. a. 2018).

Myelodysplastic syndrome (MDS)

Myelodysplastic syndrome is a group of clonal bone marrow diseases characterized by ineffective haematopoiesis. The medical consequences of MDS are ineffective blood cell production, cytopenia and abnormally developed blood cells. Affected patients typically display a reduced blood cell number in the peripheral blood. The treatment varies and is subtype-dependent (Hasserjian 2018).

Secondary Acute Myeloid Leukaemia (sAML)

Secondary acute myeloid leukaemia is a type of AML that develops due to a previous condition, like MDS or myeloproliferative neoplasm (MPN) or due to exposure to cytotoxic chemotherapy and radiation therapy. Because of changed terminology and diagnostic criteria over time, diagnosing sAML can be difficult. An affected patient must undergo a detailed evaluation including medical history, physical examination and several bone marrow analyses. sAML has an overall poor prognosis and achieves the best results through chemotherapy prior to hematopoietic stem cell transplantation (HSCT) (Green und Wang 2025).

1.4.2 Affected molecular targets

The somatic mutation K57E is located in the β -propeller of the GNB1 protein. It is suggested that this region is important for providing stability for protein-protein interaction, concluding that mutations alter GNB1 communication with other signal molecules. Especially, mutations at position K57 are known to interact with the α subunit. Through immunoprecipitation, the K57E mutation has shown a significant decrease in association with almost all G α subunits (e.g. GNAI1, GNAI2, GNA11) (Yoda u. a. 2014; Walter u. a. 2013).

Additionally, mutations on the codon K57 affect downstream mitogenic signalling similar to RAS and tyrosine kinase mutations. They promote cytokine-independent growth of human TF1 myeloid cells through activation of the MEK/ERK and PI3K/AKT/mTOR pathway while also stimulating the RAS/MAPK signalling cascade (Vedula u. a. 2018; Walter u. a. 2013; Yoda u. a. 2014).

A Western blot analysis of TF-1 cells expressing GNB1 variants performed by Yoda demonstrated that the K57E mutation increased the phosphorylation of key downstream signalling proteins that serve as effectors of the PI3K, mTOR and MAPK pathways, including AKT, MEK and ERK. Increased cell proliferation resulting from the release of G α was excluded, by inhibition of G α signalling with pertussis toxin (Yoda, Adelmant, u. a. 2015).

Research described the substitution of alanine on position K57 and discovered an increased binding avidity of GNB1 to the G-protein receptor kinase GSK3, leading to a stabilization of β -catenin and its enhanced down signalling. Moreover, K57A exhibits an increased association of GNB1 with the β -adrenergic receptor kinase (GRK) as well as a decrease in adenylate cyclase 2 (AC2) activation, which disrupts normal cellular regulation further. This raises the question of whether a substitution with glutamic acid (K57E) would result in similar effects. (Walter u. a. 2013).

1.4.2.1 Effects on Phospholipase C β 3 (PLCB3)

Phospholipase C (PLC) are enzymes that stimulate phosphatidylinositol-4, 5-bisphosphate (PIP2) to produce the two important second messenger diacylglycerol (DAG) and inositol-1, 4, 5-trisphosphate (IP3). These molecules then activate protein kinase C (PKC) and initiate intracellular Ca^{2+} release (**Figure 6**) which are essential for cell proliferation and differentiation (Ratti u. a. 2020; Xiao u. a. 2010).

PLC β isoforms are primarily activated by G-protein coupled receptors through its α - and $\beta\gamma$ subunits. Aside of its catalytic role, PLC β 3 also serves as a scaffold for protein interactions and plays a key role in controlled cell growth. It forms, together with the protein phosphatase SHP-1 and the transcription factor Stat5, the SPS-complex. As a result, SHP-1 dephosphorylates the activated Stat5 and stops its induced gene activation and cell growth signals. Phospholipase C β 3 also supports the interaction

between SHP-1 and Lyn, a tyrosine kinase. Lyn phosphorylates and activates the SHP-1 and enables Stat5's deactivation in the first place (Xiao u. a. 2010; Park u. a. 1993).

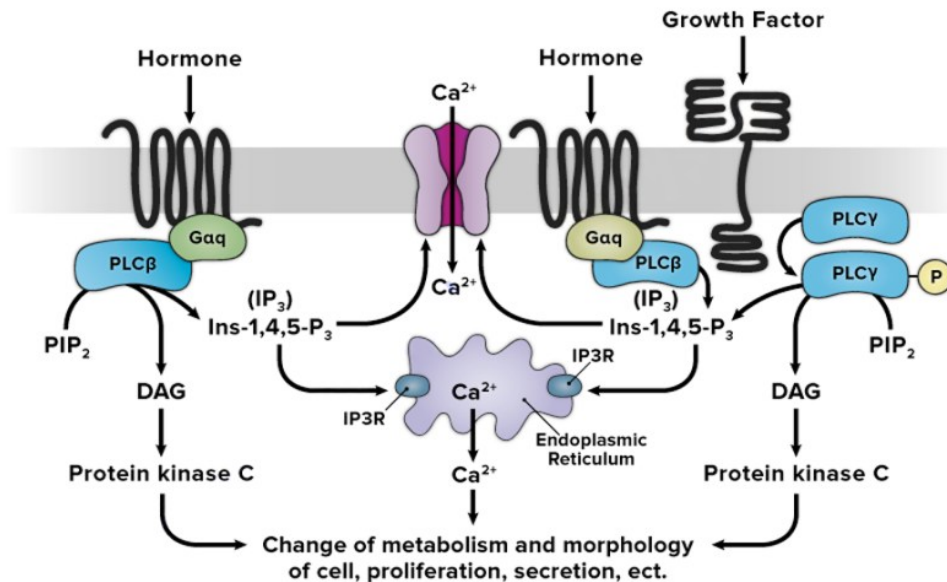


Figure 6: G-protein receptor-mediated activation of PLCβ (mking 2022)

Moreover, PLCβ3 acts as a tumour suppressor in bone marrow by controlling the proliferation of hematopoietic stem cells, its downregulation or dysfunction is therefore linked to the development of cancers like MDS or AML (Ratti u. a. 2020). In AML, PLCβ is a main regulator of the non-canonical Wnt/ Ca^{2+} signalling pathway. If this pathway is disrupted, by e.g. K57E mutation's reduced $G\alpha$ stimulation, myeloblasts are prevented to differentiate into mature blood cells, leading to AML outbreak (Gruszka u. a. 2019).

Besides GPCR signalling, the PLCβ subtype 3 can be also activated by P2RY6 which is stimulated by the colony stimulating factor 1 (CSF-1), leading to autophagy and monocytic differentiation. If this pathway is altered chronic myelomonocytic leukaemia (CMML) can occur. In CMML, the production of P2RY6 antagonists, alpha-defensins DEFA1 and DEFA3, is elevated. They disrupt the CSF1-P2RY6-PLCβ3 signalling and impair induced autophagy and monocytic differentiation (Ratti u. a. 2020).

1.4.3 Effects on the PAM signalling pathway

The PI3K/AKT/mTOR (PAM) signalling pathway acts as a critical regulator of essential cellular functions, like cell survival, growth, proliferation and metabolism in higher eukaryotic cells. External growth factors or hormones stimulate the PAM pathway through binding to receptor tyrosine kinases (RTKs) or GPCRs, resulting in PI3K recruitment. This major functional protein converts PIP2 into PIP3, which serves as a docking station for AKT. Through phosphorylation by PDK1 and mTORC2, AKT migrates

into the cytoplasm and nucleus to regulate various downstream substrates like GSK3 or TSC2 (**Figure 7**). The tumour suppressor protein PTEN, is tightly regulating the PAM pathway by dephosphorylating PIP3 back into PIP2 and switching the signalling cascade off (Glaviano und Foo 2023).

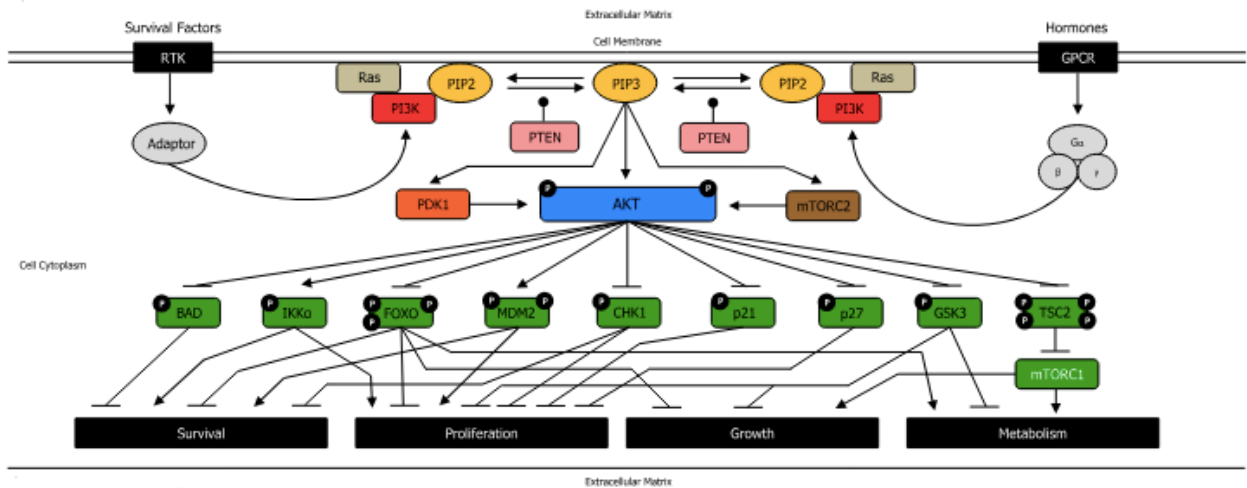


Figure 7: PAM pathway activation including signalling targets and cellular substrates (Glaviano und Foo 2023).

Aberrant activation of the PI3K/AKT/mTOR pathway is one of the most common drivers of human cancer, occurring in approximately 50% of all tumours. Oncogenic mutations in PI3K or alterations in G-proteins (e.g. K57E) enhance proliferative signalling by increasing PIP3 production, resulting in hyperactivation of the signal transduction pathway and uncontrolled cell proliferation. Sustained activation of the PAM pathway suppresses apoptosis by inhibiting the activity of pro-apoptotic factors such as FOXO transcription factors and BAD, thereby promoting cell survival (Glaviano und Foo 2023).

1.4.4 Therapeutic strategies

Currently, there are no mutation-specific treatments for K57 variation-driven cancers. Therapeutic options for AML patients are allogeneic stem cell transplantation (HSCT) followed by chemotherapy, increasing the life expectancy on average for 2 years (Vedula u. a. 2018).

There have been some therapeutic strategies, like the use of JAK inhibitors, however it lacks formal evaluation through clinical studies. Since patients with oncogenic GNB1 mutations often display kinase alterations as well, the clinical intervention remains challenging. In vitro studies have shown that mutations in GNB1 along with JAK mutations (JAK2 V617F) can promote a resistance in kinase inhibitors, like ruxolitinib, without therapeutic selection pressure as their origin. Although PI3K/mTOR signalling inhibitors may represent a promising therapeutic approach, further studies are required to determine their effectiveness in this disease context (Vedula u. a. 2018; Yoda u. a. 2014).

The clonal status of the mutation plays a big role in the treatment as well. Meaning, wherever it is present in the founding clone, disease originated cancer cells, or in the subclone, which evolves out of the founding clone and contains new additional mutations. GNB1 mutation show a wide range of variant allele frequency (VAF), which is used to determinate clonal status. Put differently, GNB1 mutations can be found in both founding and subclone (Walter u. a. 2013).

1.5 Molecular dynamics simulation

Molecular dynamics (MD) simulation is a computational approach used for prediction of atom's movement in a protein or biomolecular system over time. This technique has been around since the late 1970s but has gained a lot of importance in the last years due to its use in drug discovery. Key factors for MD's increased popularity were new insights into complex structures, like GPCRs, through advanced X-ray crystallography and cryo-EM. Subsequently, the new discovered targets serve as refined starting points for the simulations. Other important factors are the increased computing power, enhanced accuracy and greater access to MD simulations (Hollingsworth und Dror 2018; Karplus und McCammon 2002).

MD simulations are commonly used for prediction of protein folding or ligand binding and captures protein movements with its atom's positions in short intervals, typically lasting for a few femtoseconds. This method allows observation of biomolecular processes in action that are normally difficult to obtain with solely experimental techniques (Hollingsworth und Dror 2018).

1.5.1 Principle

The core idea behind a MD simulation is to predict how every atom in a system, e.g. protein surrounded by water and lipids, moves over time. Prior to simulation, we must know the positions of each atom in the biomolecular system. During each step, the system calculates the forces acting on each atom based on its interaction with the rest atoms in the system and uses those forces to update the position and velocity of every atom. The principle for the specific prediction of the atoms positions as a function of time is based on Newton's laws of motions. The final output is a trajectory normally visualized in a 3D-movie, demonstrating the atomic-level configuration at every simulated step (Hollingsworth und Dror 2018).

During MD simulation, forces are calculated using a mathematical model, a molecular mechanics force field. Typically force fields include information about coulombic interactions, preferred length of covalent bonds and other interactions like torsing, Van der Waals forces or angle bending. Put together, these informations allow prediction of molecules behaviour.

Molecular interactions can be described with the empirical potential energy function:

$$\vec{F} = -\nabla U(\vec{r})$$

A force field is a mathematical model that combines a group of empirical energy functions, and the parameters utilized for calculating the potential energy (U) of a molecular system. This model is defined as a potential energy function based on the molecular coordinates of the atoms and includes only pairwise bonded and non-bonded interactions:

$$U(\vec{r}) = \Sigma U_{bonded}(\vec{r}) + \Sigma U_{non-bonded}(\vec{r})$$

Non-bonded interactions include non-electrostatic and electrostatic engagements between pairs of atoms. The Lennard-Jones potential is mostly used to describe the non-electrostatic potential energy:

$$V_{LJ}(r) = \frac{c_{12}}{r^{12}} - \frac{c_6}{r^6}$$

The term r^{-12} stands for the strong Pauli repulsion derived from the overlapping of the electron orbitals. The r^{-6} represents the weaker attractive forces between the dipoles located in the valence orbitals.

An alternative way to express LJ potential is by including the well depth (ε) and the van der Waals radius (σ):

$$V_{LJ}(r) = 4\varepsilon \left[\left(\frac{\sigma}{r} \right)^{12} - \left(\frac{\sigma}{r} \right)^6 \right], \quad C_{12} = 4\varepsilon\sigma^{12}, \quad C_6 = 4\varepsilon\sigma^6$$

The Coulomb's law can be used to describe electrostatic potential around a molecule:

$$V_{Elec} = \frac{q_i q_j}{4\pi\epsilon_0\epsilon_r r_{ij}}$$

q_i – charge of particle i , q_j – charge of particle j , r_{ij} – distance between particles i and j

The different bonded terms, like the bond potential (V_{Bond}), the angle potential (V_{Angle}) and the torsion (dihedral) angle potential (V_{Dihed}) are described as followed ([www.1](#)):

$$V_{Bond} = k_b(r_{ij} - r_0)^2, \quad r_0 - \text{bond length}, k_b - \text{bond constant}$$

$$V_{Angle} = k_\theta(\theta_{ijk} - \theta_0)^2, \quad \theta_0 - \text{equilibrium angle}, k_\theta - \text{force constant}$$

$$V_{Dihed} = k_\phi(1 + \cos(n\phi - \delta)) + \dots, \quad n - \text{periodicity}, \delta - \text{phase shift angle}$$

Once the force is determined, Newton's second law is used to calculate the atom's acceleration, which is then used to update the positions and velocities over the short time steps (Scheraga u. a. 2007):

$$f_i(t) = m_i a_i(t) = - \frac{\partial V(x(t))}{\partial x_i(t)}$$

$f_i(t)$ – net force on atom i at time t , $a_i(t)$ – related acceleration, m_i – atom mass

1.5.2 Benefits and Limitations

MD simulations show a broad range of benefits for molecular biology and drug discovery by providing a dynamic, atomic-level insight of biomolecules and complex structures that experimental data often cannot capture alone. It helps understand functional mechanisms like conformational changes or ligand binding. Unlike lab experiments, conditions in the simulation can be controlled and precisely known. This allows testing of the molecule's response to mutations, phosphorylation, protonation or environmental changes in membrane composition. In our case the MD simulation was used to predict changes in functional effects of a mutated protein in comparison to its wild-type structure. (Hollingsworth und Dror 2018).

Even though, MD simulations have improved during the last years, it is important to note that there are still limitations that should be considered while analysing the obtained results. Force fields are empirical models with a built-in uncertainty meaning that they are only necessarily approximate. Additionally, during molecular dynamics simulations the covalent bonds do not break or bend therefore do not accurately reflect reality. To ensure numerical stability, the simulations use short time steps for its calculations. However, biological processes mostly occur on nano- or microseconds or even longer. Reaching these biological timescales requires a huge amount of steps which makes simulations extremely computationally demanding (Hollingsworth und Dror 2018).

Before simulations one requires a starting point, typically an experimental protein structure. The systems accuracy heavily depends on the starting structures quality or resolution to avoid errors or deficient results, as well as the amount of simulation runs. Due to the nature of molecular systems small changes in initial conditions, like the velocity of a molecule, can lead to different trajectories, therefore making multiple runs under the same conditions inevitable (Hollingsworth und Dror 2018).

Lastly, for the effective use of MD simulations a significant expertise is needed. Making sure the simulations offer high quality work one must figure out if one's problem can be addressed by MD. If so, appropriate simulations must be set up and a correct interpretation of the outcome must be conducted (Hollingsworth und Dror 2018).

1.6 Free Energy Changes Upon Amino Acid Mutation

The primary objective in simulation-based molecular design is the accurate prediction of free energy changes upon amino acid mutation. Free energy calculations are crucial for designing proteins with desired thermodynamic properties, like improved stability or stronger binding, due to its key role in thermodynamics and kinetics (Aldeghi u. a. 2019).

Free energy refers to two different thermodynamic parameters: the Helmholtz energy (A) and the Gibbs energy (G). Both represent the part of a system's energy that performs work instead of being contained as thermal motion. In biochemistry and chemistry, free energy is mostly referring to the Gibbs energy, defined as $G = H - TS$, because specific

changes occurring at constant pressure are more common in biological processes than at constant volume. In this term H stands for the enthalpy of the system, T for the temperature and S for the entropy (Atkins u. a. 2018).

The Gibbs energy serves as a fundamental tool for predicting the direction of spontaneous change and the maximum work a system can perform. Important in this context is the change in Gibbs energy (ΔG). A decrease in free energy (negative ΔG value), at constant temperature and pressure, indicates that a reaction is spontaneous, meaning the process converts reactants into products without the need of induced energy. If ΔG is positive, the process is happening non-spontaneously, driving the system to seek the spontaneous reverse reaction. If both forward and backward reaction occur at equal rates, the system is in the state of equilibrium and the $\Delta G = 0$ (Atkins u. a. 2018).

There are multiple methods used to estimate free energy changes in relation to different binding affinities or stabilities of mutant and wild-type proteins. One such method is the alchemical free energy calculation based on all-atom simulations that properly capture the Boltzmann distribution. The transfer of one amino acid to another can be performed reversibly and irreversibly, labelled as equilibrium and non-equilibrium free energy calculations (Aldeghi u. a. 2019).

The free energy profile of a system defines the thermodynamic and kinetic parameters and provides insight into biophysical processes, like protein-folding, protein-protein interaction and ligand binding. Two sets of conformation can be defined: State A referring to an unfolded system (disordered) and state B a folded system (ordered). The free energy difference between state A and state B provides the relative equilibrium probability of the protein being rather in its unfolded than folded state (Aldeghi u. a. 2019):

$$\frac{p_A}{p_B} = \frac{e^{-\beta G_A}}{e^{-\beta G_B}} = e^{-\beta(G_A - G_B)} \quad \Delta G = G_A - G_B = -k_B T \ln \frac{p_A}{p_B}$$

G_A – free energy of the unfolded state, G_B – free energy of the folded state, k_B – Boltzmann constant, T – absolute temperature, $\beta = \frac{1}{k_B T}$

Further, the free energy difference also defines the maximum work obtained from the closed system, which is only reached in an ideal reversible process. The system must be in constant thermodynamic equilibrium, where changes are almost zero and very slow. If a process happens in a real, limited amount of time the energy is lost e.g. as heat and the process is irreversible. The second law of thermodynamics states that the work that is performed during a process is equal or larger than the free energy difference between initial and final state:

$$\langle W(\tau) \rangle \geq \Delta G$$

The equality applies only if the process is reversible with $\tau \rightarrow \infty$, while an irreversible process loses energy in heat dissipation and causes the two values to differ. The control parameter λ is used to drive the non-equilibrium process from his initial ($\lambda = 0$) to its

final state ($\lambda = 1$). λ changes continuously at each time step and thereby modifies the system's energy landscape. The performed work can be calculated by integrating the energetic cost needed for the systems modification:

$$W(\tau) = \int_{\lambda=0}^{\lambda=1} \frac{\partial \mathcal{H}(x, v, \lambda)}{\partial \lambda} d\lambda$$

$\mathcal{H}$ – Hamiltonian of the system, x – phase space coordinates, v – velocities

The Jarzynski equation relates the average non-equilibrium work to the equilibrium free energy difference and is expressed as:

$$\langle e^{-\beta W(\tau)} \rangle = e^{-\beta \Delta G}$$

Each non-equilibrium transition is required to begin from an equilibrium state, whereas the final state of the system is not. While the Jarzynski equation considers only transitions in one direction, from $\lambda = 0$ to $\lambda = 1$, the Crooks Fluctuation Theorem (CFT) includes forward ($\lambda: 0 \rightarrow 1$) and reverse directions ($\lambda: 1 \rightarrow 0$) (Aldeghi u. a. 2019). The relation is described as followed (Aldeghi u. a. 2019):

$$\frac{P_f(W)}{P_r(-W)} = e^{\beta(W-\Delta G)}$$

$P_f(W)$ and $P_r(-W)$ represent the normalized probability distributions of work values received from both transformation paths. By overlapping the forward and reverse work distributions, the free energy difference can be assessed:

$$\widehat{\Delta G} = W + k_B T \ln \frac{P_f(W)}{P_r(-W)}$$

$\widehat{\Delta G} = W$ stands for the intersection of the work distributions. Nevertheless, this approach is limited and can be diminished by applying ΔG estimators, e.g. the BAR (Bennet's Acceptance Ratio). The BAR combines both forward and backward distributions and allows an optimal estimation of ΔG :

$$\sum_{i=1}^{N_f} \frac{1}{1 + \frac{N_f}{N_r} e^{\beta(W_i - \widehat{\Delta G})}} = \sum_{j=1}^{N_r} \frac{1}{1 + \frac{N_r}{N_f} e^{-\beta(W_j - \widehat{\Delta G})}}$$

N_f, N_r – number of trajectories in forward and reverse direction

This equation must be solved numerically by applying the Newton-Raphson or Nelder-Mead solver (Aldeghi u. a. 2019).

To calculate the free energy difference, the initial and final states of interest as well as the path connecting both, must be clarified first. To illustrate the protein folding, the initial state is determined as the unfolded protein and the final state as the folded

protein. Theoretically, the transformation from one state to another can be done by pulling the protein's N- and C- termini apart and afterwards measuring the work needed to unfold the protein. Yet, this principle demands extensive calculation in order to achieve convergence. The more efficient strategy is to calculate the folding free energy changes ($\Delta\Delta G_{\text{folding}}$) by establishing a thermodynamic cycle, allowing the use of alchemical (non-physical) pathways. They introduce smaller and more easily converging perturbations within the system. Since ΔG is a state variable and depends only on the initial and final states of the system and therefore path independent, it is more efficient mutating a wild type protein into its variant and simulating the folding and unfolding process (Aldeghi u. a. 2019).

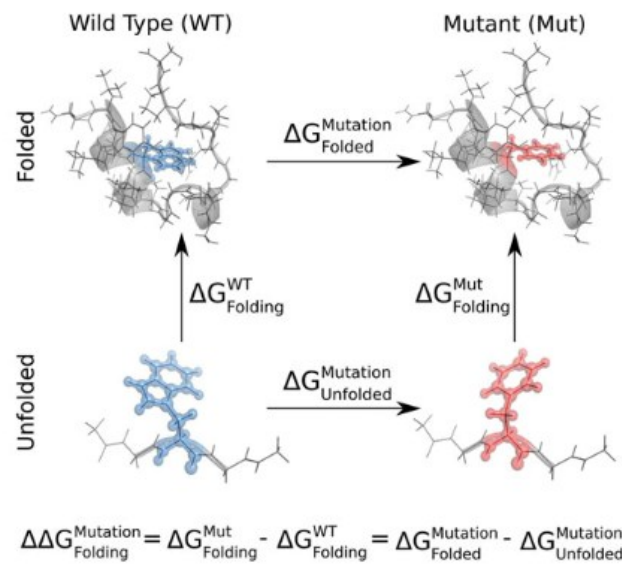


Figure 8: Thermodynamic cycle for calculation of free energy changes in protein folding upon mutation
($\Delta\Delta G_{\text{Folding}}^{\text{Mutation}}$)

The left side represents the folding process of the WT-protein ($\Delta G_{\text{Folding}}^{\text{WT}}$), the right side shows the process for the mutant ($\Delta G_{\text{Folding}}^{\text{Mut}}$). The horizontal transformations ($\Delta G_{\text{Folded}}^{\text{Mutation}}$ and $\Delta G_{\text{Unfolded}}^{\text{Mutation}}$) depict the non-physical pathway. (Aldeghi u. a. 2019).

A wild-type protein, in its folded and unfolded stage, can be transformed into a mutant, through morphing the amino acids, via the alchemical path (**Figure 8**). The free energy difference of protein folding upon amino acid mutation ($\Delta\Delta G_{\text{Folding}}^{\text{Mutation}}$) can be obtained, either through this non-physical pathway ($\Delta G_{\text{Folded}}^{\text{Mutation}} - \Delta G_{\text{Unfolded}}^{\text{Mutation}}$) or the more demanding physical path, by folding the wild-type and mutant protein ($\Delta G_{\text{Folding}}^{\text{Mut}} - \Delta G_{\text{Unfolding}}^{\text{Mutation}}$).

In pursuit of $\Delta\Delta G_{\text{Folding}}^{\text{Mutation}}$ calculation, the protein's unfolded state must be suitable for simulation. Since the full length of the protein is rather challenging to simulate, only short fragments like independent tripeptides, are commonly used (Aldeghi u. a. 2019).

2 Objective

Mutations in the G β 1 subunit induces dysregulation of the normal signalling pathway of G-proteins. Since only limited information is available on the impact of the pathogen variant K57E on G β 1 γ 2 complex, molecular dynamics simulations were performed to identify potential structural and functional changes.

This study aims to provide insight into the effects of the cancer related K57E mutation on the G β 1 γ 2 dimer on a molecular scale. For identification and visualization of changes in protein structure, the variant K57E was implemented into the G-protein. In addition, this work seeks to improve understanding of the GNB1 pathophysiology and to uncover new potential treatment approaches.

In order to examine the mutation's impact on the G β 1 γ 2 subunit and accomplish the objective of this work, several steps were performed:

- Constructing a simulation system including K57E mutation in the G β 1 γ 2 system based on PDB 4KFM
- Performing non-equilibrium molecular dynamics simulations in a double system/single box setup for the tripeptide and protein model of wild type and mutant G β 1 γ 2.
- Calculation of $\Delta\Delta G$ upon the K57E mutation for new insights into potentially altered protein-protein interaction and protein complex stability.
- Extension of run 5 to 100 ns and further analysis of trajectories, focusing on RMSD and RMSF parameters.

These procedures contribute to a deeper understanding of structural and functional impacts of the K57E mutation in the G β 1 γ 2 complex.

3 Materials and Methods

3.1 Simulation Software and Preparation

The molecular dynamics simulations were performed with GROMACS version 2022.3 running on the operating system LINUX Ubuntu version 20.04.6 (Abraham u. a. 2015; Van Der Spoel u. a. 2005).

The protein structure for the G β 1 γ 2 system is based on PDB 4KFM. For simplifying this complex molecular system, preparations were carried out by using CHARMM-GUI. The mutation-containing tripeptide was obtained through the pmx-webserver (<http://pmx.mpibpc.mpg.de>) (Aldeghi u. a. 2019; Jo u. a. 2008).

The applied force field in this study was Amber99SB*ILDN and the simulation was performed in a protein-in-water system (TIP3P) with a double system/single box setup.

3.2 Protocols

Before calculation of the free energy for a charge-changing mutation, like K57E, a system with the tripeptide and the protein was created. This approach secures a persistent simulation charge and avoids systematic errors. After preparation of the hybrid topologies via GROMACS, that include both the wild-type (State A) and mutant (State B) parameters, the two systems, starting from opposing states, were combined into the same simulation box. The distance between protein and tripeptide amounted to 50 Å, to ensure no interaction through short-range electrostatics (Aldeghi u. a. 2019).

Due to the double system/single box set up, additional steps were conducted before starting the simulation process. The two systems were configured in opposing states, one as the folded protein in its wild-type state whereas the other one was set as the unfolded tripeptide in its mutated state. During the alchemical transition, the two systems flip:

- Forward (0 $\rightarrow$ 1): Folded (WT) $\rightarrow$ Folded (Mut) & Unfolded (Mut) $\rightarrow$ Unfolded (WT)
- Reverse (1 $\rightarrow$ 0): Folded (Mut) $\rightarrow$ Folded (WT) & Unfolded (WT) $\rightarrow$ Unfolded (Mut)

For calculation of the free energy the first step in the pipeline was ensuring the system's stable, low energy starting point, which was achieved through energy minimisation. This step verifies that our system exhibits no steric clashes or unfavourable geometry. To prevent our system from collapsing the solvent must be properly adjusted for temperature and pressure and must achieve proper orientation around the protein (the solute). The next procedures were conducted in two phases: NVT (constant number of particles, volume, temperature), also known as "isothermal-isochoric", and NPT (constant number of particles, pressure, temperature), "isothermal-isobaric", equilibration. Once these two phases have been completed, 10-ns MD simulations

followed by non-equilibrium MD simulations (10ns) were carried out. Two independent equilibrium samples of 10ns for the end states, one for $\lambda = 0$ and $\lambda = 1$, were conducted, which is sufficient for capturing conformational changes in the WT and mutant systems. From each equilibrium trajectory retrieved from the last 8 ns, 100 frames every 80 ps are extracted and serve as the starting conformation for the non-equilibrium transitions. Based on that, the work values $\partial H / \partial \lambda$ for both directions were obtained. The free energy difference between the two states was calculated and estimated by applying the BAR. These steps were repeated for 5 runs („Lysozyme in Water“, o. J.; Aldeghi u. a. 2019).

Run 5 was extended to 100 ns after which RMSD and RMSF calculations were carried out with GROMACS. The root-mean-square deviation (RMSD) is a method used to analyse the similarity of two structures and provide information about the simulation's quality, meaning how well a simulation maintains a stable structure. Additionally, it is used to define clusters of related conformations. The root-mean-square fluctuation (RMSF) is a standard measure used to estimate the average deviation of an atomic position from a reference position and evaluate the molecules' structural flexibility. It serves as an important dynamic index that indicates which parts of a molecule structure fluctuate the most or least (Pitera 2014; Song u. a. 2024).

4 Results

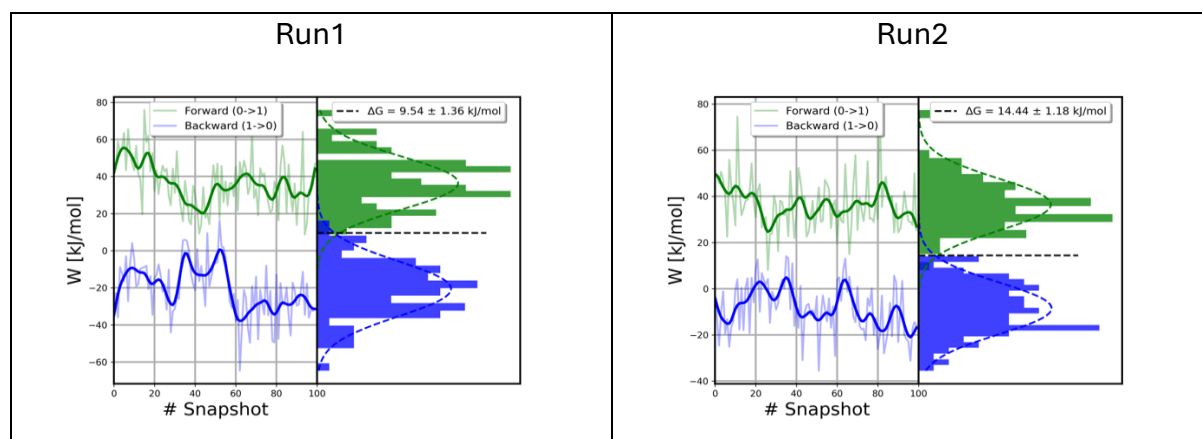
This study aimed to investigate potential structural and functional impacts of the K57E mutation on the G β 1 γ 2 system using molecular dynamics simulations. The required structural data for G β 1 γ 2 was taken from the Protein Data Bank (4KFM). This chapter summarizes the findings concerning the effect of the K57E variant on the G β 1 γ 2 system associated with GNB1 encephalopathy, which will be addressed in greater detail in the discussion section.

Free energy changes upon amino acid mutation were calculated, and after an extended MD simulation run (Run 5), RMSD and RMSF analyses were performed additionally.

The table above summarizes the $\Delta\Delta G$ values obtained from each run:

Run	$\Delta\Delta G$ (kJ/mol)
1	$+9,54 \pm 1,36$
2	$+14,44 \pm 1,18$
3	$+0,71 \pm 3,01$
4	$+10,32 \pm 0,98$
5	$+8,53 \pm 1,52$

Table 1: Overview of the calculated $\Delta\Delta G$ values from each run.



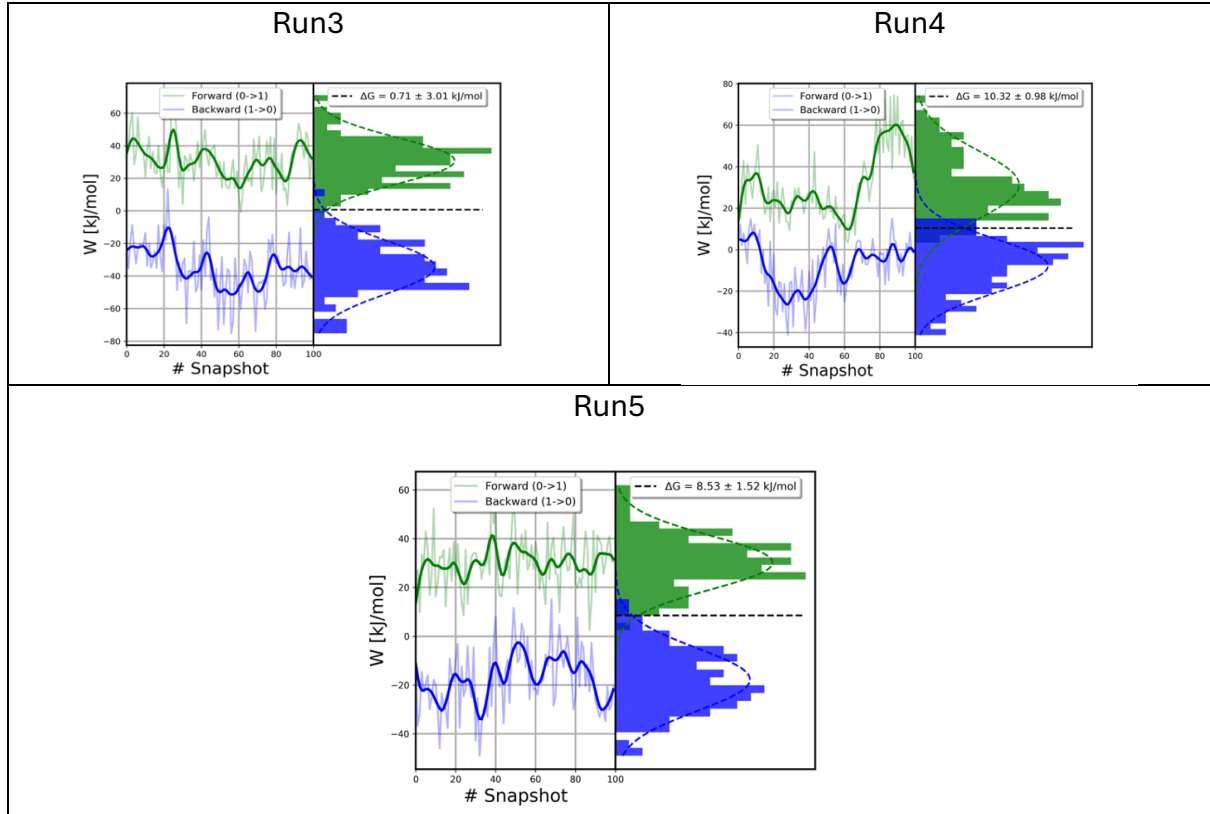


Table 2: Generated histograms for each run illustrating the transition of WT to K57E mutation.

Left: the work values for the forward and backward transition. Right: obtained distribution curves for the work values with the intersection, demonstrating the free energy change.

4.1 $\Delta\Delta G$ calculations

The calculated free energy difference, derived from the plots above, is directly equal to the $\Delta\Delta G$ of the mutation, due to the double-system setup. Using the obtained free energy values, one can calculate the average $\Delta\Delta G$ value for the K57E mutation:

$$\Delta\Delta G_{folding\ avg}^{mutation} = \frac{9,54 + 14,44 + 0,71 + 10,32 + 8,53}{5} = + 8,71 \text{ kJ/mol}$$

The average $\Delta\Delta G$ value for the mutation K to E on position 57 is + 8,71 kJ/mol. These findings suggest that the K57E mutation could slightly destabilize the G β 1 γ 2 structure.

4.2 Root mean square deviation (RMSD)

After the extension of run 5 to 100 ns, the G β -backbone of wild-type and K57E variant was observed through RMSD analysis. The values obtained from the analysis are shown above (**Figure 9**).

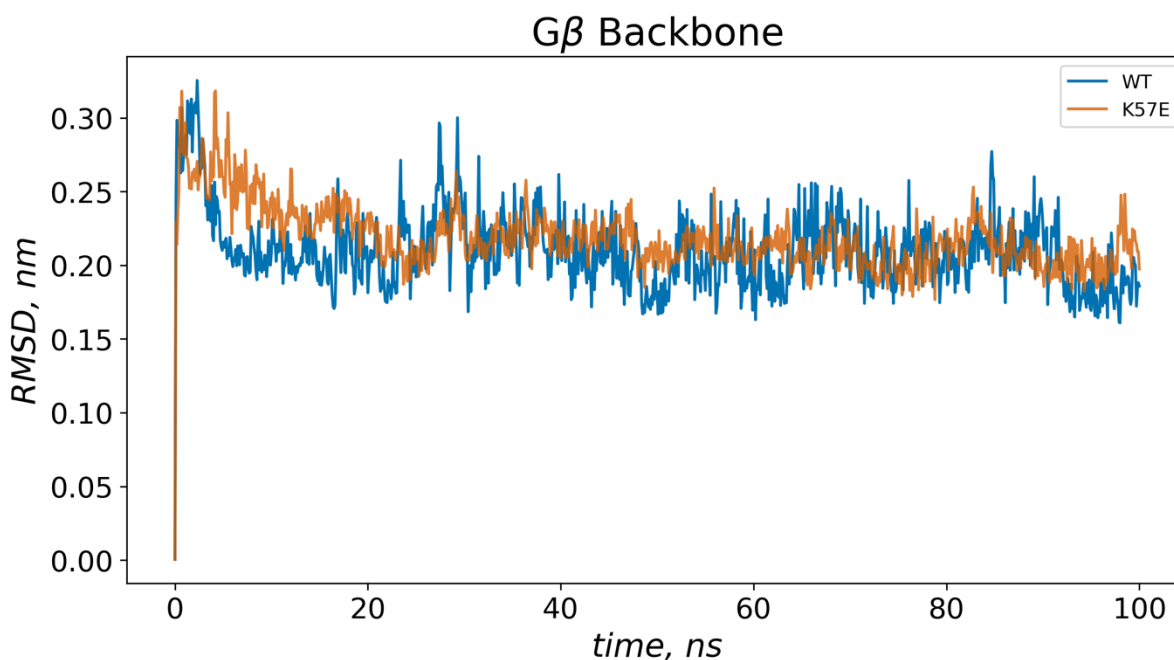


Figure 9: Root-mean-square deviation (RMSD) values for the wild-type (blue) and K57E (orange) G β subunits within the G $\beta\gamma$ 2 complex.

RMSD measures the extent to which the G β 1 backbone deviates from its starting conformation over time. Both WT and K57E show a rapid increase in RMSD up to 0,25 – 0,32 nm, which represents the typical initial relaxation phase. The mutation shows slightly higher RMSD in the early stage, suggesting a higher structural adjustment. After the initial rise, both systems settle into a stable range around 0,18 – 0,25 nm and show overall similar trajectories.

The results indicate that both WT and Mut are structurally stable throughout the simulation and that the variant does not significantly affect the global G β 1 backbone stability. Therefore, K57E mutation likely induces local or dynamic effects rather than global destabilization of the protein structure.

4.3 Root mean square fluctuation (RMSF)

Additional to RMSD, the backbone root mean square fluctuation profile comparing wild-type and K57E mutation was conducted (**Figure 10**). The RMSF measures how much each residue fluctuates during the MD simulation.

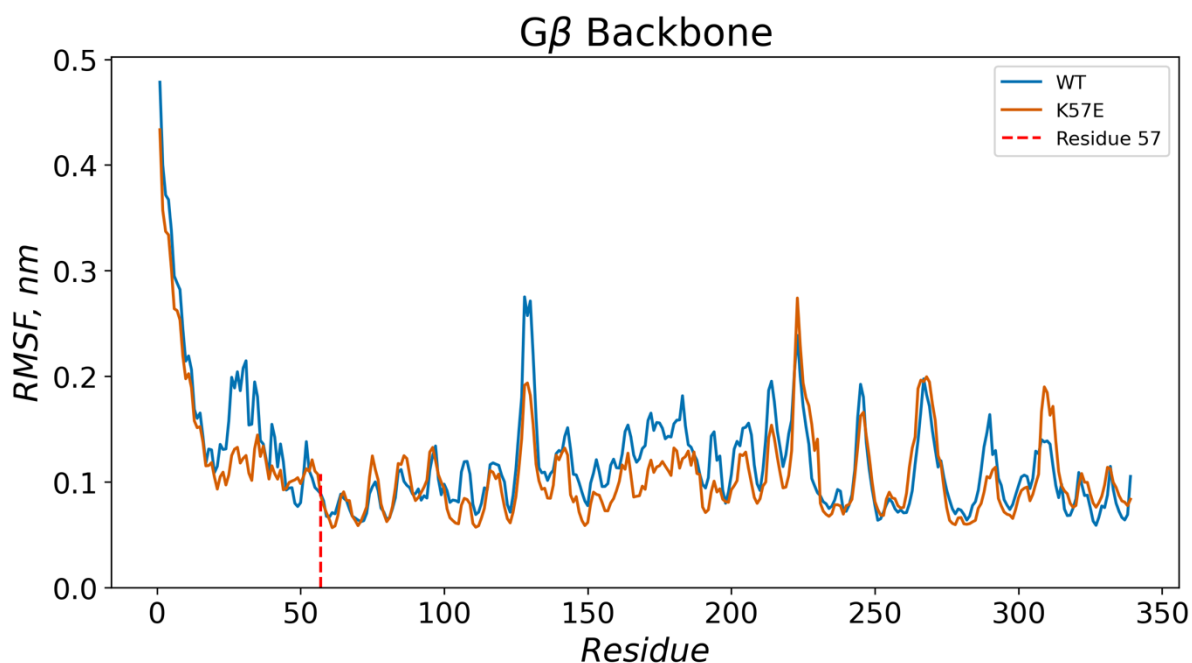


Figure 10: Root-mean-square fluctuation (RMSF) profile for wild-type (blue) and K57E mutation (orange) with the highlighted residue 57

Overall, the WT and K57E profiles show similarity, suggesting that the variant does not alter the Gβ1 backbone dynamics substantially and that the protein's fold remains largely stable. Looking at the residue 57, RMSF is low meaning that this region is structurally stable, and the fluctuation profile of the mutant is not drastically changed compared to the wild type. However, the mutant exhibits lower fluctuations in specific regions (residue 25-50, 125-140 and 160-200), implying that these parts of the Gβ1 backbone are more rigid compared to the wild type.

Both the RMSD and RMSF results indicate a preserved protein structural stability.

4.4 Non-bonded residue interaction

In addition, nonbonded interaction energies between residue 57 (wild type and the K57E variant) and the surrounding residues within the G β 1 subunit were calculated across five independent simulation runs. The histogram displays the median interaction energies across all runs (bar heights), while the individual dots represent the median value obtained from each simulation run (**Figure 11**).

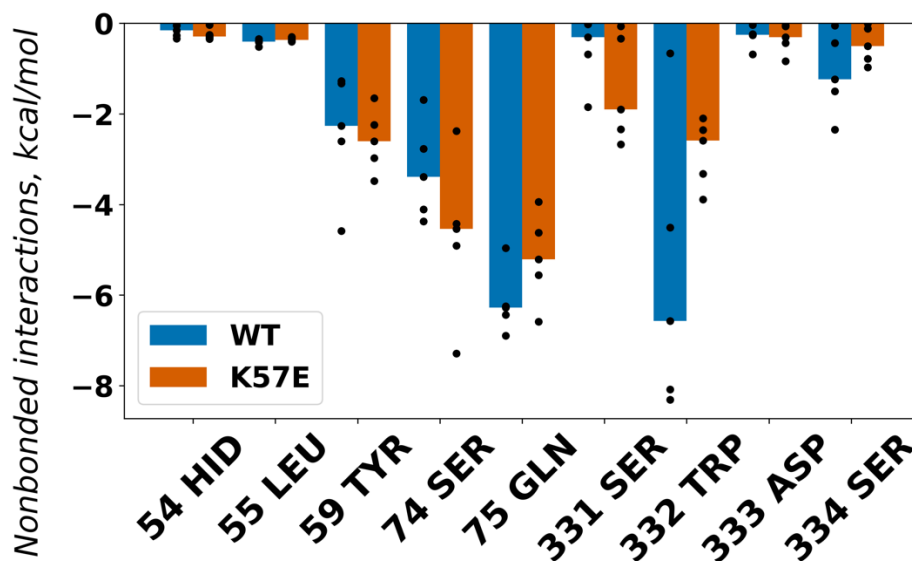


Figure 11: Non-bonded interactions with surrounding residues for wild-type (blue) and K57E (orange)
vertical axis contains the listed neighbouring residues for position 57, black dots represent obtained median value from each simulation run

The analysis indicated significant changes in the mutant's nonbounded interaction energies regarding the residues 332 TRP, 75 GLN, 331 SER and 74 SER. The K57E mutation demonstrates the largest difference at TRP332 compared to the WT, which may indicate loss of protein stabilization at this residue. Another noticeable weaker interaction of the variant can be observed at residue GLN75. On the other hand, the mutant interaction with SER331 and SER74 becomes more favourable, possibly compensating for losses in the other residues.

Collectively, the results suggest that the K57E mutation redistributes the interaction energies rather than profoundly affecting overall conformational stability.

5 Discussion

The purpose of this study was to investigate the influence of the somatic, cancer associated K57E mutation on potential structural alterations and changes in residue–residue interactions within the G β 1 γ 2 system. As important downstream effectors in GPCR signalling, these subunits are critical for cellular signal transduction and regulated cell proliferation.

The thermodynamic consequences of the substitution of lysine with glutamate at position 57 were investigated using non-equilibrium free energy calculations. Thereby, the MD simulations provide an insight into overall protein stability, local interactions and structural flexibility. The calculated relative free energy difference between the wild-type and mutant yielded an average $\Delta\Delta G$ of +8,71 kJ/mol. Comparable values were obtained in four out of the five simulation runs, demonstrating the reproducibility of the observed trend. This positive value suggests that the K57E mutation is energetically less favourable than the wild type, indicating a decrease in overall thermodynamic stability.

While the RMSD values of the K57E variant initially exhibit slightly higher deviations during the first 20 ns, it settles into a profile very similar to the wild type, suggesting that the core fold of the protein is not disrupted by the mutation and is therefore unlikely to impair Gy binding. This structural stability is further supported by the relative abundance of proteins interacting with GNB1 wild-type and mutant measured by mass spectrometry, which demonstrated maintained or increased association of the GNB1 K57E mutant with several Gy subunits (GNG10, GNG5, GNG2), as well as the chaperone PDCL (Yoda, Adelmant, u. a. 2015).

Similarly, the overall RMSF profile for both wild type and mutant were largely comparable, including at residue 57. However, the K57E mutation exhibits increased structural rigidity in residues 25-50, 125-140 and 160-200. This increased stiffness may limit the induced-fit conformational changes required for G β 1 γ binding to its signalling partners like G α , which is in line with prior observations regarding the K57E mutation (1.4.2).

Moreover, the substitution of the positively charged lysine with the negatively charged glutamate in position 57 exhibits a disruption in the local nonbonded interaction profile, with K57E exhibiting a substantial loss of interaction with TRP332 compared to the wild type. Although both lysine and glutamate are polar, the shift from positive to negative charge likely causes the loss of critical electrostatic interactions necessary for efficient G α recruitment. This is supported by the experimental data generated by Yoda for nearly all detected G α subunits (including GNAQ, GNAI1, GNAI2, GNAI3, GNA11, and GNA13), indicating a marked reduction in binding affinity compared to the wild-type (Yoda, Adelmant, u. a. 2015).

Although the overall structure of the protein remains intact and Gy binding is preserved, the combination of energetic destabilization ($\Delta\Delta G \approx +8.7$ kJ/mol), reduced local flexibility, and disruption of key residue–residue interactions appear to impair interactions with G α subunits. The experimental data presented by Yoda provide strong support for this functional distinction, demonstrating preserved interactions with Gy subunits while simultaneously revealing substantially reduced association with G α proteins. Together, these findings suggest that the K57E mutation primarily alters the interaction interface rather than the global fold of G $\beta 1$, thereby impairing G-protein signalling without compromising overall structural integrity.

6 Conclusion

This thesis aimed to provide a molecular analysis of the cancer-associated GNB1 K57E mutation using molecular dynamics simulations and free energy calculations for G β 1 γ 2 complex. The results represent an initial step toward elucidating the structural and functional consequences of this mutation.

The findings of this study indicate that the K57E mutation induces subtle structural changes that increase G β 1 backbone rigidity and alter its interaction surface without compromising overall protein stability or fold integrity. Rather than causing protein unfolding, the mutation appears to disrupt critical contacts at the G β 1 - G α interface and reduce conformational flexibility. These local alterations are likely to impair binding and communication between the G β 1 γ complex and its signalling partners. In particular, G α and phospholipase C β 3 appear to be affected, both of which have been implicated in cancer development and progression.

Collectively, these findings provide a molecular-level explanation for the pathogenicity of the K57E mutation in GNB1 and support a model in which the protein retains its structural scaffold but loses the conformational flexibility required for proper signalling.

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