

**The Investigation of RalGDS-Ral Signaling Pathway via
Molecular Dynamics Simulations**

by

Meltem Eda Ömür

A Dissertation Submitted to the
Graduate School of Sciences and Engineering
in Partial Fulfillment of the Requirements for
the Degree of
Master of Science

in

Molecular Biology and Genetics



July 31, 2019

The Investigation of RalGDS-Ral Signaling Pathway via Molecular Dynamics Simulations

Koç University

Graduate School of Sciences and Engineering

This is to certify that I have examined this copy of a master's thesis by

Meltem Eda Ömür

and have found that it is complete and satisfactory in all respects,
and that any and all revisions required by the final
examining committee have been made.

Committee Members:

Prof. Özlem Keskin Özkaya (Advisor)

Prof. Attila Gürsoy (Co-Advisor)

Prof. Halil Kavaklı

Asst. Prof. Mert Gür

Date:



To my lovely family

ABSTRACT

Ras family is composed of at least 35 proteins that are known as GTP-GDP mediated molecular switches that regulate most of the cellular processes through physical interactions with their wide variety of effector proteins. In response to the diverse extracellular stimuli, they become activated, and then recruit their effector proteins (Raf, PI3K, and RalGDS) to the plasma membrane and transmit the signal. Raf cascade and PI3K pathways are well-established effector pathways; RalGDS lately become the research scrutiny.

There are four prominent members of the family: H-Ras, N-Ras, K-Ras4A and K-Ras4B proteins that are found as the most frequently mutated proteins in human cancer types. The other well-known Ras members are Rap1 and Ral proteins. Whereas prominent members are well-characterized; the cellular functions of Rap1 and Ral are yet to be understood.

In this study, the main aim is to elaborate the RalGDS-Ral signalling pathway in complex with Ras family members (K-Ras, Rap1) and Ral effector proteins using molecular dynamics simulations. The five Ras systems and two Ral systems were generated to elaborate upstream and downstream of RalGDS signaling cascade. The five Ras systems are comprised of K-Ras4B^{wt}-RalGDS-RBD as a wild-type system, K-Ras4B^{G12D}-RalGDS-RBD and K-Ras4B^{G12V}-RalGDS-RBD as control systems, K-Ras4B^{G12V+E37G}-RalGDS-RBD as a mutational system, and Rap1^{wt}-RalGDS-RBD as a Rap1 system; the two Ral systems are composed of Ral-Sec5 and Ral-Exo84 effector interactions. The systems were designed to give insight into details of mechanism behind Ras-induced RalGDS-Ral pathway. The frequently involved residues that are responsible for stable complex formations and strong bond formations were investigated via MD simulations. We proposed the residues, Ser39 on Ras; Lys31 in Rap1; Tyr31, Asn54 and Asp56 on RalGDS as significant residues that amplify binding affinity between Ras family members and Ras Binding Domain of RalGDS. It is known that Sec5 and Exo84 have partially overlapping residues on RalA. We discovered most of the common involved residues on RalA in both interactions, which are Glu44, Ala48, Asp49, Ser50, Arg52, Tyr75.

The comparison of two Ral systems allowed us to identify important residues that amplify binding affinity of RalA to its effector proteins. These residues are Asp49, Glu73, Asp74, and Asn81. We further demonstrate the frequently involves important residues on Sec5 and Exo84.

Ras proteins have been scrutinizing for decades; however, they are still undruggable. The long-term purpose of this intense research is to develop small molecules that target Ras dependent RalGDS signaling particularly.



ÖZETÇE

En az 35 üyeden oluşmakta olan Ras ailesi, GTP-GTP aracılı moleküler anahtarlar olarak hücrede görev alırlar. Çeşitli hücre dışı uyaranlara karşılık olarak, aktif hale gelirler ve efektör proteinlerini (Raf, PI3K ve RalGDS) plazma zarına yakın bölgeye çağırarak onlarla fiziksel etkileşim kurarlar ve sinyal iletimini sağlamış olurlar. Raf kaskadı ve PI3K yolları uzun zamandır çalışılıyor olup, detaylandırılmış yollarlarken; RalGDS sinyal yolağı ise son zamanlarda araştırma konusu haline gelmiştir.

Ras ailesinin dört üyesi insan kanseri türlerinde en sık mutasyona uğramış proteinler olarak bilinirler: H-Ras, N-Ras, K-Ras4A and K-Ras4B. Ras ailesinin diğer iyi bilinen üyeleri, Rap1 ve Ral proteinleridir. Önde gelen dört üye iyi karakterize edilmişken; Rap1 ve Ral proteinlerinin hücresel fonksiyonları henüz tam olarak açıklığa kavuşmamıştır.

Bu çalışma, RalGDS-Ral sinyal yolunu moleküler dinamik (MD) simülasyon yöntemi kullanarak, Ras aile üyeleri (K-Ras, Rap1) ve Ral efektör interaksyonları doğrultusunda detaylı bir şekilde incelemeyi hedeflemiştir. Bu amaç doğrultusunda beş Ras sistemi ve iki Ral sistemi oluşturulmuştur. Beş Ras sistemi; K-Ras4B^{wt}-RalGDS-RBD kompleksini içeren yabancı tip system, K-Ras4B^{G12D}-RalGDS-RBD ve K-Ras4B^{G12V}-RalGDS-RBD komplekslerini içeren kontrol sistemleri, K-Ras4B^{G12V+E37G}-RalGDS-RBD kompleksini içeren mutasyonlu sistem ve Rap1^{wt}-RalGDS-RBD kompleksini içeren Rap1 sisteminden oluşurken; iki Ral sistemi, Ral-Sec5 ve Ral-Exo84 efektör interaksyonunu içeren sistemlerden oluşmaktadır. Totalde oluşturulmuş yedi sistem, Ras ilişkili RalGDS-Ral yolağının arkasındaki mekanizmayı ayrıntılı bir şekilde anlamak için tasarlanmıştır. Ral-efektör proteinlerinin kompleks oluşturmada önemli görevi olan Ral üzerindeki amino asitler, iki Ral sisteminin birbiriyle karşılaştırılmasıyla ortaya çıkartılmıştır.

Stabil kompleks ve güçlü bağ oluşturmada etkin role sahip olan amino asitler MD metodu ile incelenmiştir. Bu çalışmada, sistemleri oluşturan proteinler üzerindeki önemli amino asitleri ortaya çıkardık. Ras proteinindeki Ser39, Rap1'deki Lys31, RalGDS'deki Tyr31, Asn54, ve Asp56 amino asitleri, Ras aile üyeleri ile RalGDS arasındaki güçlü kompleks oluşumunu tetikleyici etkiye sahip olduğu düşünülen amino asitlerdir. Daha önceki çalışmalardan, Sec5 ve Exo84'ün RalA proteini üzerinde kısmi ortak bağlanma bölgeleri olduğu bilinmektedir. RalA proteinindeki bu bölgeye ait amino asitler bu çalışma sayesinde çoğunlukla keşfedilmiştir: Glu44, Ala48, Asp49, Ser50, Arg52, Tyr75. Ral-efektör proteinlerinin kompleks oluşturmada önemli görevi olan Ral üzerindeki amino asitler, iki Ral sisteminin birbiriyle karşılaştırılmasıyla ortaya çıkartılmıştır. Bu amino asitler, Asp49, Glu73, Asp74, ve Asn81 olarak belirtilmiştir. Aynı zamanda Sec5 ve Exo84 proteinleri üzerindeki kompleks oluşumunda önemli görevi olan amino asitler de ortaya çıkarılmıştır.

Ras proteinleri onlarca yıldır araştırma konusu olmuştur. Uzun süreli çalışmalara rağmen bu proteinlerin onkojenik etkisini hedef alan ilaç üretilmemiştir. Bu çalışmanın uzun vadedeki amacı özellikle Ras ilişkili RalGDS sinyal yolağını hedef alan küçük moleküller geliştirmektir.

AKNOWLEDGEMENTS

I would like to express my sincere gratitude to my advisors Prof. Özlem Keskin Özkaya and Prof. Attila Gürsoy for their guidance and support throughout my thesis project and graduate studies. I am appreciated that they allow me to be in COSBI family. I would also thank to our collaborators Prof. Ruth Nussinov and Dr. Hyunbum Jang for providing opportunity to be in Nussinov lab at NIH and contributing to my thesis study with their invaluable guidance.

I want to thank to all my thesis committee members Prof. İ.Halil Kavaklı and Dr. Mert Gür for their comments and feedbacks which improved in the progress of my study.

I would like to mention my dear friends and colleagues Kayra Kösoğlu, Asma Hakouz, Sıla Özdemir, Derya Soner Cavga, Jackson Weako, Damla Övek, Simge Şenyüz, Sukejna Valjevac and Zeynep Abalı who have always been by my side during these two years both in social and academic life.

I want to thank to my lovely friends Aslı Kürekçi, Buket Şengül, Fatma Büşra Işık, Sebile Arabacı, Beyza Kağan for their valuable friendships throughout my studies. I want to express my appreciation to my friends Saliha Sürme and Onur Taflan, who go through the same tough way to survive. I owe special thanks to Kayra Kösoğlu for her endless friendship and enjoyable coffee talks.

Words are powerless to express my deepest gratitude to my family, especially to my mother, my father, my sister and my brother. Without their unconditional love, support and encouragement, I would not accomplish this study successfully. Thank you for being always with me whenever I need.

Last but not least, I am grateful to Uğur Meriç Dikbaş for his eternal love and support throughout this process. Thank you for making my life more meaningful and easier to go through.

TABLE OF CONTENTS

ABSTRACT	iv
ÖZETÇE.....	vi
ACKNOWLEDGEMENTS	viii
TABLE OF CONTENTS	x
LIST OF TABLES.....	xi
LIST OF FIGURES.....	xii
ABBREVIATIONS.....	xiii
CHAPTER 1: INTRODUCTION	1
CHAPTER 2: LITERATURE REVIEW	4
2.1 Ras Subfamily.....	4
2.1.1 Ras Proteins	6
2.1.2 Rap1 proteins.....	8
2.1.3 Ral Proteins	9
2.2 RalGDS signaling pathway.....	11
2.2.1 Ral Activation.....	13
2.2.2 Ral effector proteins	14
2.2.3 Ral in cancer	16
CHAPTER 3: MATERIAL AND METHOD	19
3.1 MD Simulations.....	19
3.1.1 Generating initial configurations	20
3.1.2 Atomistic MD Simulations.....	23
3.1.3 Binding free energy calculation for the Ras dimers	24
3.1.4 Stability and interaction analysis.....	26

CHAPTER 4: RESULTS AND DISCUSSION	27
4.1 Binding free energy analysis.....	44
4.2 Discussion.....	48
CHAPTER 5: CONCLUSION	53
APPENDIX A.....	55
APPENDIX B.....	61
BIBLIOGRAPHY	79



LIST OF TABLES

Table 1. The simulated systems in this study.	19
Table 2. Hydrogen bond and Salt bridge analysis of RalA-Sec5 complex.....	41
Table 3. Hydrogen bond and Salt bridge analysis of RalA-Exo84 complex	42
Table 4. Gibbs binding free energy values of five Ras systems.	45
Table 5. Gibbs binding free energy values of two Ral systems.....	45



LIST OF FIGURES

Figure 1. Diagram of Ras superfamily members.	4
Figure 2. Active GTP-bound Ras and inactive GDP-bound Ras cycle.	5
Figure 3. Multiple Sequence Alignment of K-Ras, Rap1 and Ral proteins.	7
Figure 4. Ras-effector interactions..	10
Figure 5. The RalGEF family members.	12
Figure 6. The effector proteins of active Ral-GTP	14
Figure 7. The illustration of Ras-RalGDS-Ral interactions.....	18
Figure 8. The initial structures of the simulated seven systems.	22
Figure 9. Total internal energies of five Ras systems.....	29
Figure 10. Total internal energies of Ral systems.	29
Figure 11. The electrostatic and van der Waals energy distributions as a function of time for five Ras systems.....	30
Figure 12. The electrostatic and van der Waals energy distributions as a function of time for Ral systems.	31
Figure 13. RMSD plots of five Ras–RalGDS-RBD systems.	32
Figure 14. RMSD plots of two Ral systems.	32
Figure 15. RMSF plots for five Ras systems.....	34
Figure 16. RMSF plots for Ral systems.....	35
Figure 17. Hydrogen bond analysis of five simulated Ras systems.	37
Figure 18. Salt-bridge analysis of five simulated Ras systems.	37
Figure 19. Representations of hydrogen bond formations for Ras systems.	38
Figure 20. 3D representations of hydrogen bond formations in the interface region of Ral systems.....	43
Figure 21. The binding energy contributions of five Ras-RalGDS systems.	46
Figure 22. The binding energy contributions of two Ral systems.....	47

ABBREVIATIONS

GAP	GTPase-Activating Proteins
GEF	Guanine Nucleotide Exchange Factor
GDP	Guanosine diphosphate
GTP	Guanosine-5'-triphosphate
PIP2	Phosphoinositide-3,4-diphosphate
PIP3	Phosphatidylinositol 3,4,5-trisphosphate
Ras	Rat sarcoma
Ral	Ras-like protein
RalGDS	Ral Guanine Dissociation Stimulator
Rap	Ras proximate protein
RBD	Ras Binding Domain
RMSD	Root Mean Squared Deviations
RMSF	Root Mean Squared Fluctuations



Chapter 1: INTRODUCTION

Ras proteins are small GTPases that cycle between GDP-bound inactive state to GTP-bound active state. They are known with their significance on regulating most of the cellular processes. They function through physical interactions with the effector proteins in response to the various type of extracellular stimuli at the plasma membrane. They were first characterized as retroviral oncogenes. Then, the constitutively active Ras proteins have been discovered in most of the human cancers [1]. Ever since, the investigation of Ras proteins has become the main subject of the researches. Despite numerous research and pharmaceutical efforts, Ras family members are still known as non-druggable proteins [2]. In the long-term, development of Ras-targeted antagonists has been aimed to achieve for cancer treatments.

As the researches on Ras mechanism have been scrutinized for decades, the accumulative achievements of the studies have enlightened the Ras functions and underlying mechanism behind its functionality [3]. Throughout the investigation process, new questions have arisen. The focus of some studies has shifted to the different aspects to investigate the mechanism of Ras effector signaling pathways. Ras proteins have mainly three fundamental downstream pathways: PI3K, Raf-ERK-MAPK, RalGDS [4]. The Raf and PI3K cascades are well-characterized due to their known contributions in tumor growth, whereas RalGDS is relatively less studied one.

RalGDS was reported as a substantial component in Ras-induced carcinogenesis. The absence of RalGDS was shown to reduce tumor progression in mice. In previous experimental studies, it was shown that RalGDS regulate cell survival via activation of the JNK cascade [5]. There are accumulating studies that show the contributions of RalGDS-Ral signaling pathway in oncogenesis.

This pathway has also taken attention with the crosstalk function of RalGDS in Ras family members. RalGDS is an effector protein of Ras and exchange factor of Ral proteins. This is a unique case which one of the Ras effectors is an activator of another Ras family member [6].

Since the importance of RalGDS signaling pathway has been discovered in tumor growth and metastasis, the downstream mechanism of RalGDS and Ras-dependent Ral activation have been in the limelight.

Three Ras family members; K-Ras, Rap1 and Ral were selected for this study. The reasons behind our interest on these three proteins are in the following: K-Ras4B is the most frequently mutated isoform in Ras-driven cancers [7]; Rap1 can either inhibit transforming function of Ras or function in similar way with Ras to induce Raf-MEK-ERK pathway [8]; and the activators of Ral proteins are regulated by direct Ras binding [9].

This study provides comprehensive investigation of RalGDS pathway. We aim to characterize the interactions between Ras family members (K-Ras4B and Rap1) and Ras Binding Domain of RalGDS, and consequent Ral interactions with its effectors Sec5 and Exo84 to enlighten important residues in these interactions. As RalGDS pathway is yet to be understood, the involved residues on Ras Binding Domain of RalGDS (RBD) in interaction with Ras family members are still uncharted. Substantially, the frequently involved residues in the activation of Ral via RalGDS-RBD needs to be elaborated.

In total, seven systems were simulated; five Ras systems and two Ral systems. The five Ras systems consist of a wild type system, K-Ras4B^{wt}-RalGDS-RBD; two control systems, K-Ras4B^{G12D}-RalGDS-RBD and K-Ras4B^{G12V}-RalGDS-RBD; a mutational system, K-Ras4B^{G12V+E37G}-RalGDS-RBD; and Rap1-RalGDS-RBD system. Rap1 binding to RalGDS-RBD is the strongest interaction among other Ras family members. The control systems contain G12D and G12V mutations to keep Ras proteins in their constitutively active state. The mutational system contains G12V with E37G mutations on K-Ras to target RalGDS pathway not the other effectors [10]. In this way, the comparison of mutational system with control systems and Rap1 system allows us to reveal the significantly involved residues coupling with RalGDS-RBD. Ral systems, RalA-Sec5 and RalA-Exo84 were generated to discover critical residues on Ral and its effector proteins, which are yet to discovered completely. We identified the significant residues amplifying the binding affinity between Ras members and RalGDS-RBD as Ser39 on Ras, Lys31 on Rap1, Tyr31, Asn54 and Asp56 on RalGDS,

We showed some conserved residues on Ras (Glu37, Asp33, Asp38, Ser39, Arg41) and RalGDS (Arg20, Gly28, Met30, Lys52), that maintain complex formation between these proteins. We further discovered some conserved residues on RalA in complex with Sec5 and Exo84 as Glu44, Ala48, Asp49, Ser50, Arg52, Tyr82; and some residues on RalA amplifying affinity to its effectors as Glu73, Asp74, Asn81.

Chapter 2 presents an elaborated review on RalGDS signaling pathway and Ras-dependent Ral signaling pathway. The RalGDS pathway is scrutinized interaction with Ras proteins, and additionally Ral signaling pathway is detailed in a Ras-dependent manner.

Chapter 3 gives the extensive explanation of materials and method used in this study. The Molecular Dynamics (MD) simulation and the analysis methods are described in detail.

Chapter 4 provides the results of MD simulations and the discussion of the results in the aspect of integration of novel findings with known information.

Chapter 5 presents the conclusion and future aspects.

Chapter 2: LITERATURE REVIEW

2.1 Ras Subfamily

Small GTPases (G-proteins) are also known as Ras superfamily, form a superfamily with more than 100 known proteins [11]. This superfamily is composed of five major members: Ras family, Rab family, Rho family, Sar1/Arf family, and Ran family based on their sequence similarity and functions (Figure 1). They are accepted as ancestors of molecular switches that mediate various essential cellular processes through cycling between GTP-bound state to GDP-bound state [1].

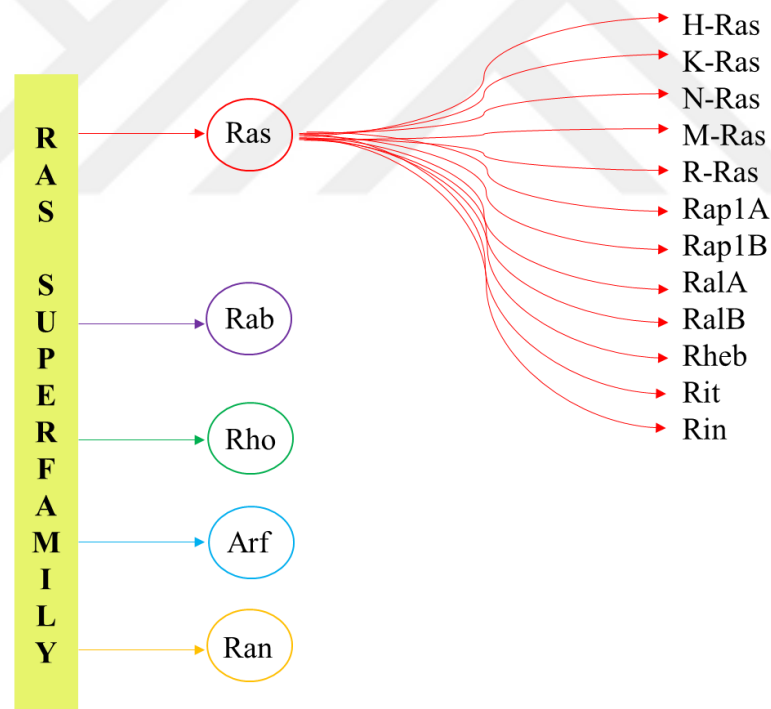


Figure 1. Diagram of Ras superfamily members. This figure is adapted from van Dam et.al. study [12].

Rat sarcoma (RAS) genes were first characterized as oncogenes. Ras subfamily consists of at least 35 members, which are four Ras proteins (H-Ras, N-Ras, K-Ras4B, K-Ras4A), RalA, RalB, Rheb, R-Ras, the Raps proteins and lately involved Rit and the Rin proteins (Figure 1). All Ras family members contain evolutionary conserved catalytic G domain [13]. Ras proteins are small GTPases that cycle between GDP-bound inactive and GTP-bound active forms to mediate a wide range of cellular processes, including cell cycle progression, cell differentiation and survival, vesicular and nuclear transportation [14].

The transition between GTP to GDP and vice versa is regulated by guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs), respectively (Figure 2). GTPases have weak intrinsic GTPase activity, which is catalyzed by GAPs. This intrinsic activity hydrolyzes GTP to GDP. Once the extracellular stimuli activate GEFs, they induce the release of guanosine diphosphate (GDP) from Ras and lead to the binding of guanosine triphosphate (GTP) to Ras [6]-[8]. Upon GTP binding, conformational change occurs in the effector binding region of Ras to couple with its effector proteins such as Raf kinase, PI3K, and Ral guanine nucleotide dissociation stimulator (RalGDS) [18].

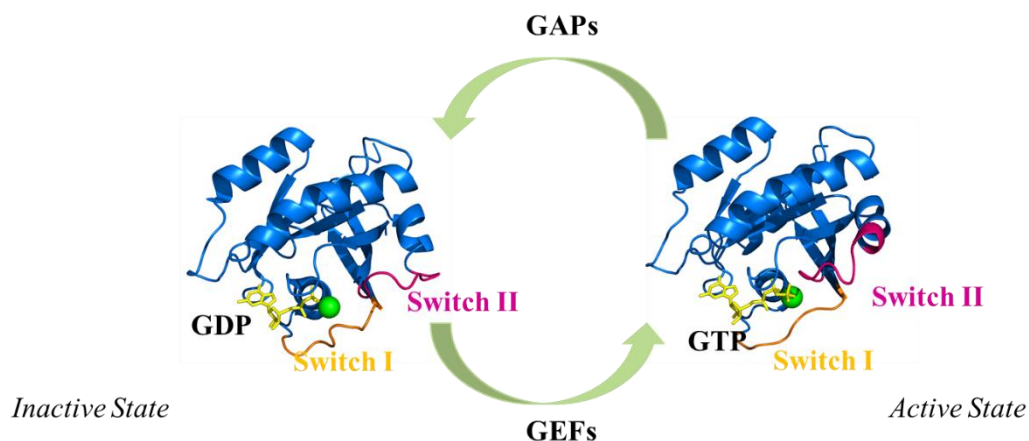


Figure 2. Active GTP-bound Ras and inactive GDP-bound Ras cycle. GEF and GAP proteins are responsible for the switch between Ras GDP to GTP.

2.1.1 Ras Proteins

The H-Ras, N-Ras, K-Ras4A and K-Ras4B proteins are oncogenic Ras proteins encoded by *H-ras*, *N-ras*, *K-ras* genes. The sequences of the four Ras isoforms have ~85% identity. Even their catalytic G domains are very similar, they differ in their hypervariable regions that contain a CAAX motif (Figure 3) [19].

The catalytic domain consists of the functional P-loop (residues 10-17), Switch I (residues 30-38), and Switch II (residues 59-67) regions that are responsible for GTP hydrolysis and effector interactions [20], [21]. In response to GTP binding, conformational change occurs in Switch I and Switch II regions. Switch I (SI) is the region for effector-protein interactions. Switch II (SII) is important in GEF binding. [6].

Although they are highly homologous, they are distinct in their binding partners and physiological functions. Whereas K-Ras is confirmed as the most frequently mutated isoform in Ras-driven cancers (86%); N-Ras (11%) and H-Ras (3%) are relatively less mutated isoforms among Ras-driven cancers according to the Catalog of Somatic Mutations in Cancer (COSMIC) records [4],[7].

Most of the oncogenic Ras proteins contain mutations at G12, G13 and Q61 residues. These mutations on Ras might cause conformational change and preclude GAP binding to Ras. As a result, Ras proteins stay in their constitutively active state and amplify tumorigenic effects (Figure 2) [22] .

The frequency of these residues to be mutated varies among them. The order of frequencies of mutated residues on K-Ras are in the following; G12 (89%), G13 (9%) and Q61 (1%). The residue G12 most frequently mutates to aspartate G12D (36%) among three frequent mutations G12C (14%), and G12V (23%) [23],[24].

The combinations of the substitutions on Ras proteins are specific to the effector such as G12V and E37G double mutations on Ras activate RalGEFs; G12V and T35S, and G12V and D38E activate Raf; and double mutations G12V and Y40C activate PI3K, in particular [10] ,[25].

The other prominent Ras family members are Ral proteins (RalA, RalB) and Rap proteins. These members are functionally and sequentially similar to the four Ras proteins (H-Ras, N-Ras, K-Ras4A, K-Ras4B). Upon GTP binding, they cycle between GTP-bound state to GDP-bound state. Once they turn into GTP-bound state, they further interacts with their effectors [26].

The Rap1 proteins have been known with their antagonistic effects on transforming activity of Ras. Now, it has been suspected that Rap1 proteins may have more complicated functions beside their antagonistic functions [27].

The Ras-like proteins RalA and RalB are highly homologous small GTPases which have ~80% sequence identity. Even they are highly similar, they differ in their functionality. Most of the GEFs of Ral proteins contain Ras Binding Domain (RBD) [28]. It is an unique case which one Ras family member is activating the another member of Ras family [6]. Lately, the significance of Ral proteins has taken attention in tumorigenicity. Ral can be activated either through Ras-dependent or Ras-independent pathway via Rap1 activation through increased Ca^{+2} in the environment. The underlying mechanism of these interactions is not yet understood [27].

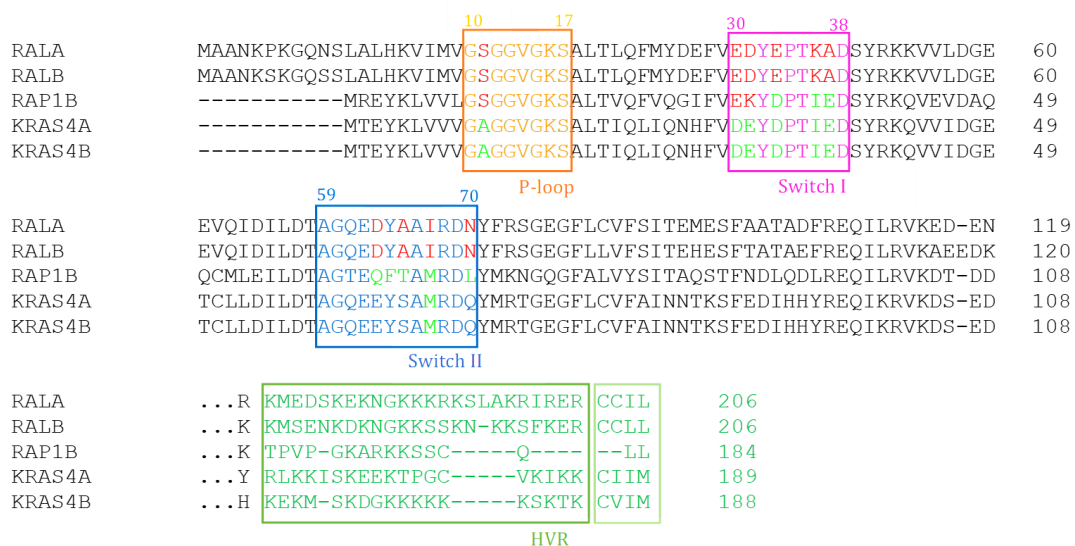


Figure 3. Multiple Sequence Alignment of K-Ras, Rap1 and Ral proteins.

2.1.2 Rap1 proteins

Rap1 proteins (Rap1A and Rap1B) are, also named as *Krev-1*, Ras-like small GTPases that are activated by a wide variety of receptors such as EGF, GPCRs, and PDGF [29]. They are found to couple with most of the Ras effectors including RalGEFs and Raf kinases (B-Raf and c-Raf) through the same effector binding domain that other Ras proteins have. Even Ras proteins and Rap1 proteins share structural similarities, they are distinct in the aspect of GEF proteins, intrinsic GTPase activities, and their placement inside the cells.

Rap1 proteins are mostly found at the intracellular vesicular membranes as endocytic vesicle. The localization of the Rap1 proteins in the intracellular environment provide them accessibility via second messengers. At least three second messengers (DAG, cAMP, Ca^{+2}) have been reported as an activator of Rap1 proteins providing transition between Rap1-GDP state to Rap1-GTP state [8].

Rap1 can be activated by cAMP which regulates various cellular processes either through protein kinase A (PKA)-dependent or PKA-independent manner [30], [31]. In PKA dependent manner, Protein kinase A becomes activated by cAMP in response to extracellular stimuli, and then activates Rap1 [32]. Rooji and colleagues discovered that activation of Rap1 proteins occurs through interaction between new identified Rap1 specific GEF, Epac, and cAMP [33]. Kawasaki and colleagues identified that Rap1 GEFs have diacylglycerol and calcium binding sites that provide secretion of calcium [34]. The activation of Rap1 proteins was observed as a result of the increase in intracellular Ca^{+2} in response to the extracellular stimuli [35]. Studies showed that Rap1 proteins might have crucial role in Ca^{+2} dependent Ral activation, but also, Rap1 proteins has been shown to be involved in RalGDS dependent Ral activation. Kikuchi and colleagues discovered that Rap1 proteins have more affinity to RalGDS rather than Ras proteins. The interaction of Rap1 with RBD of RalGDS results in recruiting RalGDS to the plasma membrane and induce Ral activation in Ras-dependent manner [36]. In some in vitro studies, Ral proteins are shown to be induced via Rap1 coupling with RalGDS-RBD more efficiently than other Ras protein interactions with RalGDS-RBD [37].

2.1.3 Ral Proteins

The Ras-like (Ral) guanyl nucleotide-binding proteins are small GTPases that regulate wide variety of cellular processes through acting as a molecular switch between GDP-bound inactive state to GTP-bound active state. Ral proteins consist of two isoforms: RalA and RalB, that are encoded by *RalA* and *RalB* genes, which were first discovered by screening of B-lymphocyte cDNAs library. In their catalytic domain, RalA and RalB isoforms have ~85% sequential similarity and ~50% similarity in their HVR region. The differences in the membrane targeting region include different phosphorylation sites for RalA and RalB, which makes them unique in functionality in the aspect of effector binding. Ral proteins have at least 50% sequence similarity with Ras proteins. While Ras and Ral proteins share similar catalytic G domain, Ral proteins have 11 more residues at the N-terminus and 6 more residues at the C-terminus (Figure 3). These sequential differences affect their effector interactions, and subsequently functionalities at the cellular level [28].

Ras-Effector Interactions

All reported Ras effectors contain a conserved structural Ras Binding Domain (RBD) or Ras Association (RA) domain that allows them to bind Ras [38]. Once Ras turns into GTP-bound active state, it then interacts with its effector proteins as Raf protein kinase, phosphatidylinositol 3-kinase (PI3K) and RalGDS, members of the Ral guanine nucleotide exchange factor (RalGEF) family (Figure 4). While Raf and PI3K pathways are well-known effectors in cancers, RalGDS is accordingly less studied. However, there are compelling studies that are suggesting RalGDS pathway has important role in tumorigenesis and metastasis [4], [39], [40].

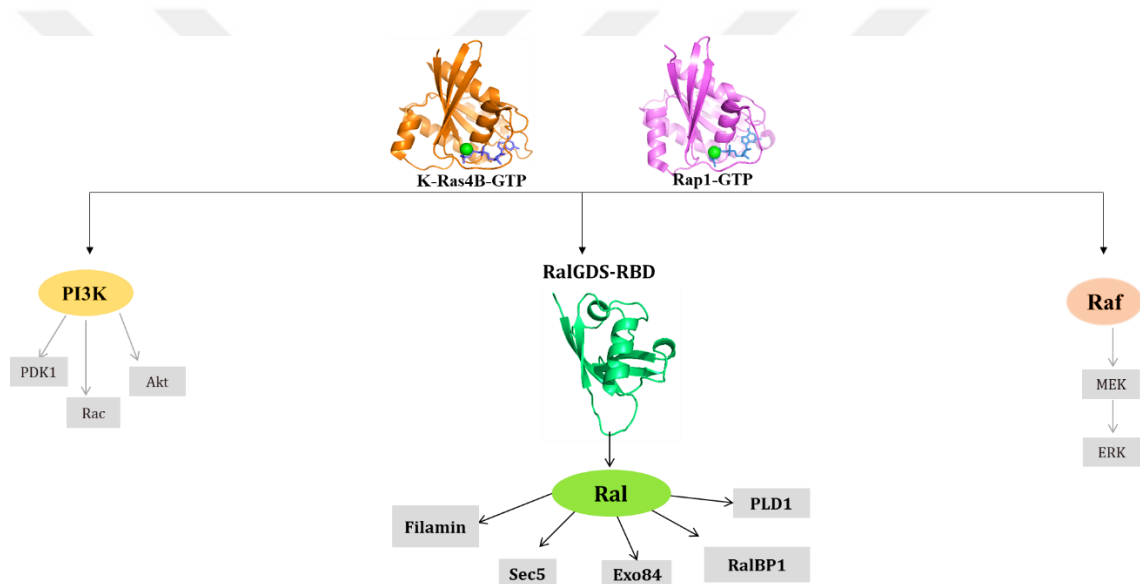


Figure 4. Ras-effector interactions. Ras proteins regulate various cellular functions through physical interactions with their effector proteins. It is the illustration of three Ras effector proteins with two representative members (K-Ras and Rap1) of Ras subfamily.

2.2 RalGDS signaling pathway

The guanine nucleotide exchange factors are mediators of GDP-GTP cycle. There are seven RalGEF members that are grouped into two subgroups as (RalGDS, Rgl, Rgl3 [41], Rlf [42]) and (RalGPS1, RalGPS2 and Rgl4) depend on activation mechanisms.

The first subgroup is activated in a Ras-dependent manner, whereas the second subgroup is activated in a Ras-independent manner [28]. The first subgroup members of RalGEFs share high structural and sequential similarities. They contain REM (Ras Exchange Motif) at the N-terminal end, CDC25 catalytic domain in the center and Ras Binding Domain (RBD) at the C-terminal end (Figure 5).

The second subgroup members have CDC25 region in common with the first subgroup members. They contain PH domain and SH3-binding motif, instead REM and RBD. RalGEFs perform their intrinsic exchange factor activity through CDC25 catalytic domain in the center of the structure. The catalytic domain is not sufficient to proceed GDP-GTP exchange cycle [43]. It requires REM domain to function properly. REM is also known as pivotal region for Sos activity that is Ras-specific exchange factor [18], [28].

RalGDS is the first identified and characterized RalGEF protein. RalGDS have been taken attention due to the Ral-specific GEF activity [44]. Activated Ras proteins bind to RBD of RalGDS and induce Ral activation through recruiting RalGDS to the plasma membrane where Ral proteins are located.

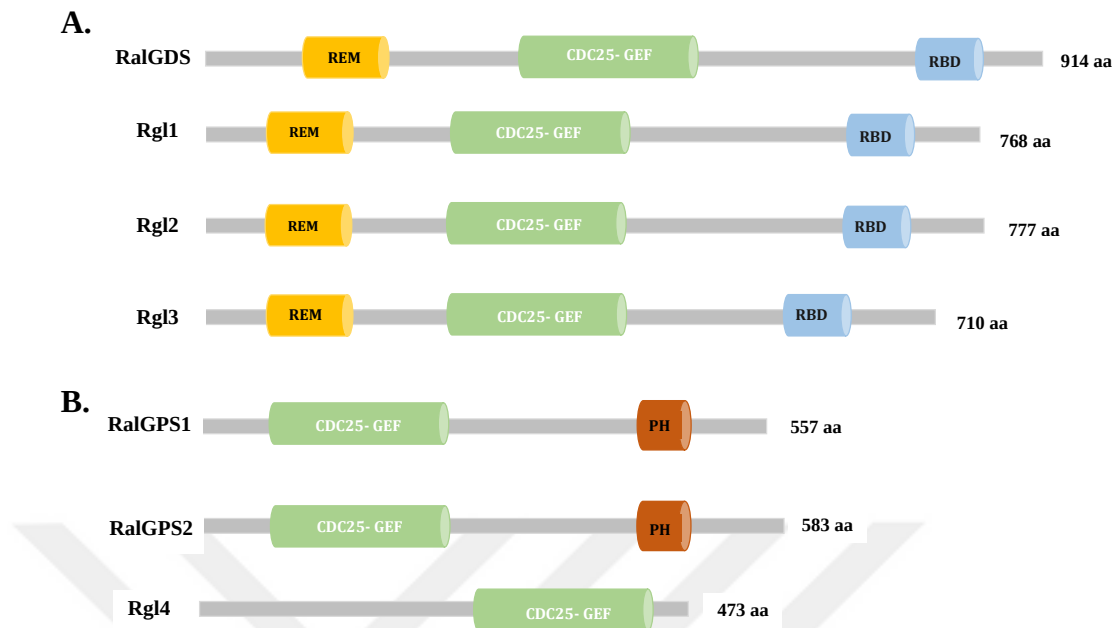


Figure 5. The RalGEF family members. RalGEF family members can be divided into two subgroups depend on containing Pleckstrin homology (PH) domain or RBD domain. **A.** These are the first subgroup members of human RalGEFs. They all contain RBD domain for Ras association, REM (Ras Exchange Motif) and CDC25 domains which are regulators of catalytic activity. **B.** These are the second subgroup members of human RalGEFs. They have PH domain instead RBD. This figure is adapted from Channing Der review [28].

2.2.1 Ral Activation

The Ral proteins, RalA and RalB, can be activated either through Ras-dependent manner or Ras-independent manner. In Ras-dependent manner, Ral activation can be mediated through Ras interactions with RalGEFs (RalGDS, Rgl, Rfl) and PI3K.

Ras can interact with Ras-binding region of RalGDS (RalGDS-RBD) in response to extracellular stimuli and induce Ral activation through targeting RalGEF family member to the plasma membrane where Ral proteins are located.

The Ral proteins can also be induced via Ras-dependent PI3K activation. Once PI3K becomes activated, it produces phosphoinositide-3,4-diphosphate (PIP₂) and phosphatidylinositol 3,4,5-trisphosphate (PIP₃) [45] that enable to bind and induce PH (Pleckstrin homology) domain of PDK-1 (PI3K-dependent kinase 1) and consequent Akt phosphorylation [46].

The pivotal role of RalGEFs is to induce Ral activation through relieving the autoinhibition of its catalytic activity. However, the downstream mechanism of RalGEF activation is poorly understood. PDK1 interaction with N-terminus of RalGDS relieves autoinhibition of RalGDS catalytic activity, and subsequently accelerates its GEF activity [43]. PH domain is not required for PDK1 dependent RalGDS activation[47]. PDK1 can activate RalGDS at their N-termini in response to EGF receptor activation [45], [48]

Ral proteins were discovered as Calmodulin binding proteins in a Ca⁺² dependent manner [47]. In Ras-independent manner, Ral proteins can be induced in response to the increased level of intracellular Calcium (Ca⁺²) due to the PLC activation and Ca⁺²/calmodulin binding [49].

RalGDS have Ca⁺²/CaM binding region, IQ-like CaM-binding motif, at its N-terminus. Ca⁺²/CaM binding to RalGDS via IQ-motif is known to relieve autoinhibition of RalGDS GEF activity [50]. Once RalGDS becomes activated, it is translocated to the plasma membrane and activate RalA at the carboxy-terminal. Moreover, the activation of RalA protein by Calmodulin binding was observed in CaM-binding domain in the C-terminus of RalA [47].

Ral proteins can be induced by one of the other Ras family members, Rap1, in a Ras-independent manner. Rap1 and Ral proteins were shown to be coregulated due to the increase in Calcium level in intracellular environment [51]. Rap1 protein is thought to involve in Ca^{+2} dependent Ral activation [51].

2.2.2 Ral effector proteins

Activated GTP-bound Ral proteins mediate a wide variety of downstream pathways including endocytosis, exocytosis, cytokinesis, regulation of second messengers through interactions with its effectors as RalBP1, filamin, ZONAB, Sec5/Exo84 [9],[52]. RalBP1-RLIP76 and the Sec5-Exo84 are well-characterized Ral effectors (Figure 6) [28].

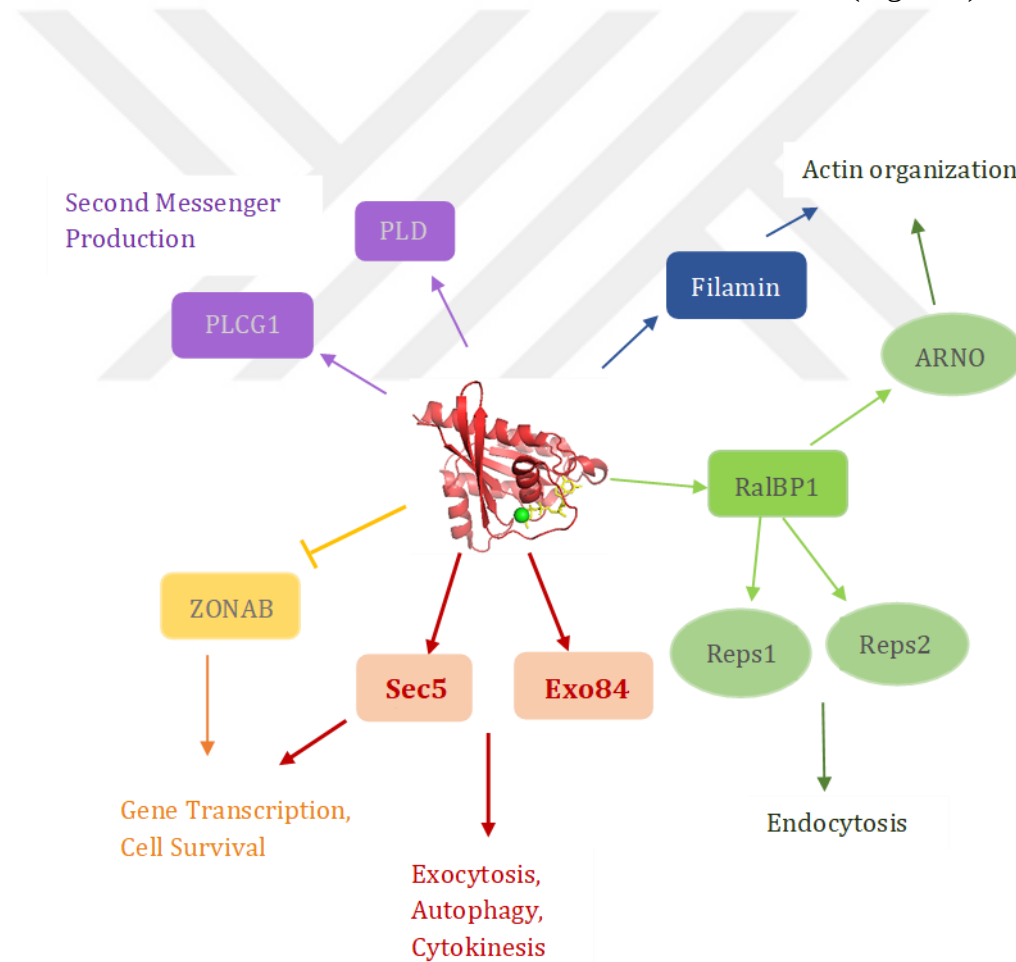


Figure 6. The effector proteins of active Ral-GTP. This figure is adapted from Channing Der review [28].

The exocyst complex

The octameric protein complex, also named as the exocyst complex, is comprised of Sec3, Sec5, Sec6, Sec8, Sec10, Sec15, Exo70 and Exo84 proteins [53][54]. The exocyst complex is responsible for targeting secretory vesicles to the plasma membrane before vesicle fusion. Sec5 and Exo84 proteins are the main mediators of exocyst function through direct binding to Ral proteins [55],[56]. The subcellular localizations of these subunits of exocyst complex are distinct; Sec5 is localized at the plasma membrane, whereas Exo84 is localized in vesicular membrane. Sec5 is a Ral-specific effector which serves as a tag at the plasma membrane, where Exo84-containing vesicles can recognize and interact [57]. GTP-bound Ral proteins bind to Sec5, and subsequently Sec5 couples with TBK1 (TANK-binding kinase 1) that is a crucial kinase in the regulation of NF- κ B signaling and nuclear localization [58],[59]. This activation is important in cell protection against apoptosis under oncogenic stress. In conclusion, the interactions of Exo84 and Sec5 exocyst subunits with Ral proteins regulate a wide variety of mechanisms that might contribute to oncogenesis [60]. In vivo studies showed Ral-exocyst complex formation is important in tumor initiation and growth [28].

Other effectors

RalBP1 (RLIP76) proteins are the other well-characterized Ral effector proteins that are regulating actin organization, cell differentiation, and cell cycle progression through coupling with CDK1, Epsin, Repp1 and Repp2 proteins [61],[62]. The role of RalBP1 in endocytosis is interacting with wide variety of proteins to function as a molecular scaffold and mediator [30],[56]. It has been showed that the over-expression of RalBP1 causes the amplification of the cellular invasion and migration [64], while the suppression of RalBP1 diminishes the tumor growth [65].

There are less-characterized effectors that Ral binds to such as PLD1, Filamin, ZO-1-associated nucleic acid binding protein (ZONAB) [28]. RalA and RalB can couple with phospholipase D (PLD) which is found in the Golgi apparatus, and endoplasmic reticulum [66]. The main role of PLD1 is to produce phosphatidic acid and choline [67]. The activity of PLD is increasing in response to oncogenic Ras activation [68]. Activation of RalA can be promoted by calmodulin in a calcium dependent manner [61],[62].

Activated RalA can interact with the enzyme phospholipase C- δ 1(PLCD1) in the presence of calcium which is responsible for regulation of nuclear phosphoinositide cycle [63],[64].

Active RalA can form direct interaction with ZONAB as a transcription factor, which then mediates gene expression [72]. Active GTP-bound RalA can also bind to filamin protein which is significant in actin crosslinking and filopodia formation [73].

2.2.3 Ral in cancer

Ras proteins regulate three major pathways: Raf-MEK-ERK, PI3K and RalGDS. Whereas the Raf pathway is the most well-characterized pathway among them [52], RalGDS pathway has been overlooked until Counter and colleagues showed that RalGEF-Ral pathway is significant for Ras-induced growth transformation of immortalized cells [74]. Once the immortalized human cell lines were developed in the latest 1990s, studies on Ral became accelerated [75]. Further studies showed that RalA and RalB have antagonistic roles in cancer cell migration, even their sequence are highly similar [76],[77]. Later, White and colleagues showed that while RalA has an important role in tumor growth in anchorage-independent manner, RalB is necessary for the survival of only cancerous cells [28],[78]. There are wide range of compelling studies that support the roles of Ral proteins in tumor growth through RNA inhibition (RNAi) of Ral expression [79],[80]. Both RalA and RalB proteins serve significant roles in human tumor growth and invasion through activation of its effector proteins or activation of signaling pathways as PLD1, Cyclin D, NK-kB or phosphorylation of Ral proteins [75], [77],[81],[82]. Consequently, the activation of Ral proteins has been observed in most of the cancer types including prostate [83], colorectal [84], melanoma [85], carcinoma [86], pancreatic [87].

H-Ras, K-Ras, N-Ras and M-Ras proteins are well-known members of Ras family, whilst Rap1 and Ral proteins are relatively less-validated ones. Ral proteins are important, because their GEF proteins are regulated by direct Ras binding; Rap1 is important because of its suppressive effect on Ras signaling and activator effect on Ral signaling [26].

White and colleagues showed the importance of RalGDS- Ral signaling pathway in tumor growth [88]. For the first time, Marshall et al. discovered that RalGDS induces Ras-dependent tumor growth through encouraging oncogenic cell survival [5]. Moreover, there are pathogenic mutations on RalGDS itself reported in TCGA [89], ICGC [90] and COSMIC [7] databases. These findings have shifted the focus on RalGDS, which might be a potential Ras effector in the development of anti-oncogenic Ras suppressors for cancer treatment.

The aim of this study is to investigate the downstream mechanism of RalGDS signaling pathway in detail. For this purpose, RalGDS-Ral signaling pathway was scrutinized in the aspect of its interactions with Ras family members (K-Ras and Rap1) and Ral effectors through identifying significant residues involving in the strong stable complex formation using Molecular Dynamics simulation method. Seven systems were generated; five Ras system and two Ral systems. The five Ras systems include a wild type system, two control systems containing oncogenic mutations, mutational system, and Rap1 system. The control systems, K-Ras4B^{G12D}-RalGDS-RBD and K-Ras4B^{G12V}-RalGDS-RBD, are aimed to observe the oncogenicity of Ras proteins [91]. G12D and G12V substitutions are the most likely found mutations in Ras related cancer types. These two control systems, System 2 and System 3, are expected to yield more affinity to RalGDS-RBD than wild-type system. The mutational system, K-Ras4B^{G12V+E37G}-RalGDS-RBD, contain G12V and E37G mutations which are known as the specific RalGDS-RBD directed mutations for K-Ras4B. System 4 is expected to yield the best binding energy value among the other K-Ras including systems (System1, System2, System3, and System 4). System 5 contains Rap1 complex with RalGDS-RBD, which was proposed as the strongest interaction seen in Ras-RalGDS-RBD complex formations [92]. Therefore, the best binding affinities to the RBD are expected to belong System 4 and System 5. System 5 will yield the most favorable complex formation. The comparison of five Ras systems with each other will allow us to identify frequently involved residues in the

complex formation with RBD, whilst the comparison of System 4 and System 5 will provide the distinct residues that encourage the binding affinity to RBD.

RalGDS becomes activated in response to activate Ras binding, then it induces Ral signaling as a GEF of Ral proteins (Figure 7). To scrutinize the Ral signaling as the continuation process of RalGDS activation, System 6 (RalA-Sec5) and System 7 (RalA-Exo84) were generated. These systems contain RalA interactions with Ral effectors, Sec5 and Exo84, which are known as the most prominent associating proteins with oncogenic Ral activity [93].

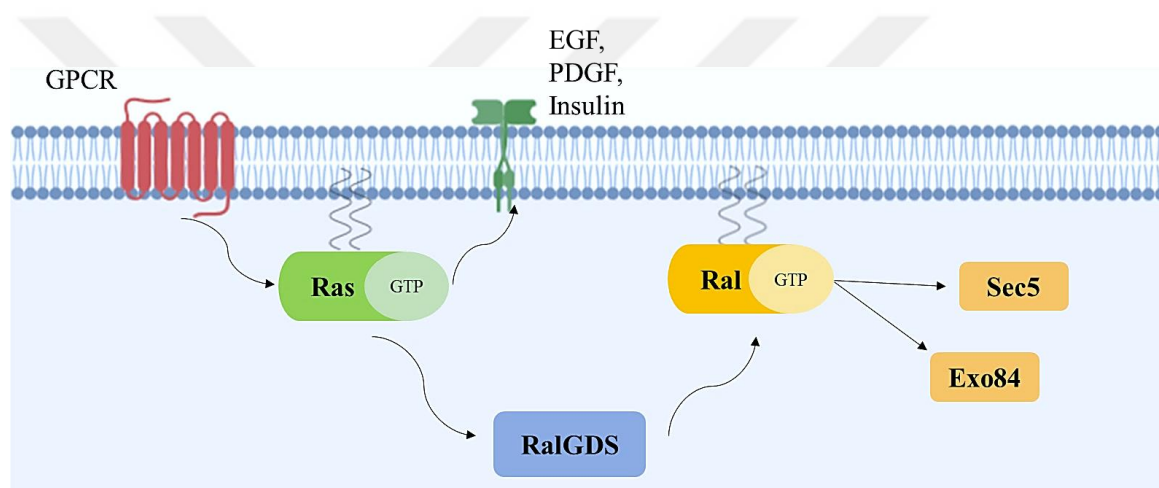


Figure 7. The illustration of Ras-RalGDS-Ral interactions. This figure is adapted from J.L Bos study [27].

The main contribution of this dissertation is to investigate the most frequently involved important residues on each monomer in seven systems; Ras proteins, Ras Binding domain of RalGDS, and interface regions of RalA and its effectors, Sec5 and Exo84. The Ras systems confirmed that Rap1 has the tightest interaction with RBD, and G12D+E37G mutations increase the binding affinity to RBD. The comparison of System 4 and System 5 identified the significant residues for Ras and RBD for the stable complex formation, addition to frequently involved residues in all Ras systems. The Ras systems confirmed that Sec5 and Exo84 are competitor proteins to bind RalA and increase the probability of locating in distinct subcellular complexes. Furthermore, the comparison of System 6 and System 7 demonstrate the residues on RalA that increase the binding affinity of effectors.

Chapter 3:

MATERIAL AND METHOD**3.1 MD Simulations**

To elicit RalGDS-Ral signaling pathway, the five Ras systems and two Ral systems were designed (Table 1). System 1 was used as a wild type system; System 2 and System 3 were used as control systems; System 4 denotes the mutational system, which was aimed to intensify Ras–RalGDS-RBD interaction; System 5 was used to compare with other Ras systems, as Rap1 competes with K-Ras in the binding of RalGDS-RBD. System 1 to 5 contain 167 residues for K-Ras4B and 87 residues for RalGDS-RBD; System 6 and System 7 contain 173 residues for RalA; and 92 residues for Sec5, 109 residues for Exo84.

Table 1. The simulated systems in this study.

Systems		
Ras-RalGDS Systems	System 1	K-Ras4B ^{wt} -GTP – RalGDS-RBD
	System 2	K-Ras4B ^{G12D} -GTP – RalGDS-RBD
	System 3	K-Ras4B ^{G12V} -GTP – RalGDS-RBD
	System 4	K-Ras4B ^{G12V+E37G} -GTP – RalGDS-RBD
	System 5	Rap1 – RalGDS-RBD
Ral Systems	System 6	RalA-Sec5
	System 7	RalA-Exo84

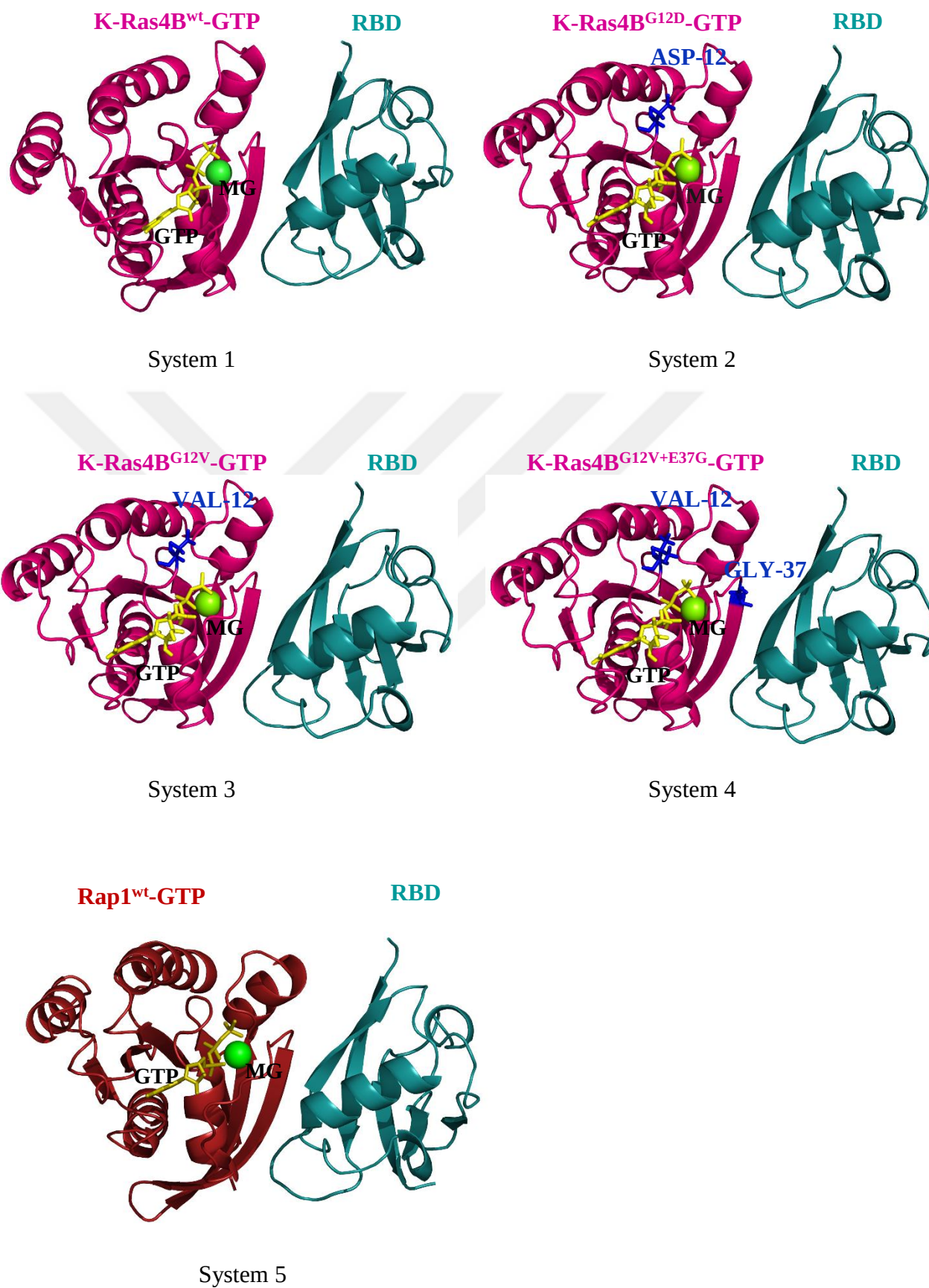
3.1.1 Generating initial configurations

In the simulation system of K-Ras4B-GTP-RalGDS-RBD in aqueous solution, K-Ras4B^{Q61H} (PDB ID: 3GFT) was obtained from Protein Data Bank (PDB). 3GFT has Q61H mutation, H61 was mutated back into Q61 native structure to construct structure for K-Ras4B^{wt}-GTP-RalGDS-RBD. G12V and G12D mutations were applied to 3GFT separately to construct the control systems of K-Ras4B-GTP-RalGDS-RBD. In PDB, there is no protein structure for K-Ras4B^{wt}-GTP-RalGDS-RBD. The protein structure for this system was constructed with superimposition of K-Ras4B-GTP (PDB ID: 3GFT) into GTP-bound H-Ras with RalGDS-RBD (PDB ID:1LFD).

In the simulation system of Rap1-RalGDS-RBD, GSP bound Rap1 (PDB ID: 4DXA) was selected [94]. In 4DXA, the residue V12 was mutated back to G12 for Rap1^{wt}-GTP. The complex structure for Rap1^{wt}-GTP-RalGDS-RBD was constructed with superimposition of Rap1^{wt}-GTP into GTP-bound H-Ras with RalGDS-RBD (PDB ID: 1LFD). The Ral systems were constructed as in the following: the complex structure (PDB ID: 1ZC3) is obtained from PDB [56] for RalA-Exo84; 1UAD is obtained from PDB [55] for RalA-Sec5.

Some of those PDB structures contain GppNHp and GSP, a nonhydrolyzable GTP analogues. They were modified to physiological GTP. The parameters for GTP and GDP were taken from CHARMM parameter database [95].

A.



B.

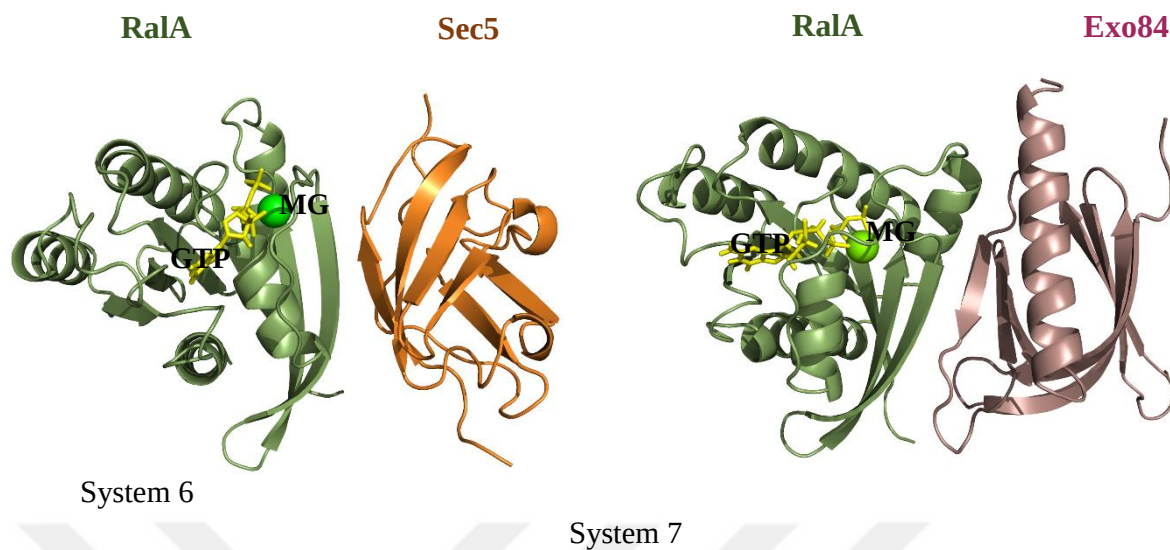


Figure 8. The initial structures of the simulated seven systems. **A.** These are the structures of RalGDS complexes with K-Ras and Rap1. System 1 is a wild type system, System 2 and System 3 address the control systems. System 4 is the mutated system. System 5 represents Rap1–RalGDS-RBD simulated complex. **B.** These are the simulated systems of Ral systems; System 6, RalA-Sec5; and System 7, RalB-Exo84.

3.1.2 Atomistic MD Simulations

The crystal structures for the systems subjected to Molecular Dynamics (MD) simulations in an aqueous environment. A total of seven systems were performed. Initial configuration and protein structure files were prepared by using all-atom additive CHARMM36 force field [96]. For Ras systems, 300 ns of five simulations were performed, and for Ral-effector systems, 1000 ns of two simulations were performed.

The initial configurations were solvated by the modified TIP3P water model [97]. The modified TIP3P water model is used to construct isomeric unit cell box in the dimensions of $90 \text{ \AA} \times 90 \text{ \AA} \times 90 \text{ \AA}$. Each unit cell box contains 73,858 atoms including 40 Cl^- , 53 Na^+ , 1 Mg^{+2} for K-Ras^{wt}-RalGDS-RBD; 73,840 atoms including 40 Cl^- , 54 Na^+ , 1 Mg^{+2} for K-Ras^{G12D}-RalGDS-RBD; 73,855 atoms including 40 Cl^- , 54 Na^+ , 1 Mg^{+2} for K-Ras^{G12V}-RalGDS-RBD; 73,846 atoms including 40 Cl^- , 52 Na^+ , 1 Mg^{+2} for K-Ras^{G12V+E37G}-RalGDS-RBD; 73,780 atoms including 40 Cl^- , 55 Na^+ , 1 Mg^{+2} for Ral^{wt}-Sec5; and 73,702 atoms including 40 Cl^- , 52 Na^+ , 1 Mg^{+2} for Ral^{wt}-Exo84 system. The counterions and required number of ions were added to neutralize the systems up to reaching $\sim 100 \text{ nM}$ concentration.

In the pre-equilibrium stages, several minimizations (10,000 steps) were applied to the all initial systems and then, 50,000 steps dynamics cycles were performed for each system with the harmonically restrained proteins in the systems. At the final pre-equilibrium stage, the harmonic restraints (from $k = 5 \text{ kcal per mol per \AA}^2 \text{ per atom}$ to $k=0$) removed to relax and adapt the protein to the surrounding heat bath. To *calculate* long range *electrostatic* interaction potentials, particle mesh ewald (PME) method was used [98]. The Langevin temperature control and Nosé-Hoover Langevin piston pressure control were used to maintain the constant temperature at 310 K, and the pressure at 1 atm, respectively.

The simulations were applied to NPT ensemble that indicates the constant number of atoms, pressure, and temperature. The NAMD parallel computing code was used for the production runs on a HPC Biowulf cluster at the NIH (Bethesda, MD) [99]. Simulation trajectories were analyzed with the updated CHARMM. The salt bridges and H-bonds were analyzed with VMD [100].

3.1.3 Binding free energy calculation for the Ras dimers

To investigate the strength of the interactions within the systems, the binding free energies, van der Waals energy and internal energies were analyzed using the molecular mechanics energies combined with the Poisson–Boltzmann surface area continuum solvation (MM-PBSA) method [87],[88].

The average binding free energy is formulated as a sum of the gas phase contribution, the solvation energy contribution and the entropic contribution which is shown as:

$$\langle \Delta G_b \rangle = \langle \Delta G_{gas} \rangle + \langle \Delta G_{sol} \rangle - T \Delta S$$

The average of gas-phase and solvation free energy values were taken throughout MD trajectories. $\langle \Delta G_{gas} \rangle$ defines the gas phase contributions of binding free energy which is the summation of the internal energy, van der Waals interaction and the electrostatic energy.

$$\Delta G_{gas} = \Delta H_{intra} + \Delta H_{vdW} + \Delta H_{elec}$$

$\langle \Delta G_{sol} \rangle$ denotes the free energy of solvation, which is thermodynamic description of a solution. It is the sum of the electrostatic contribution and the non-polar contribution.

$$\Delta G_{sol} = \Delta G_{sol}^{elec} + \Delta G_{sol}^{nonpolar}$$

ΔG_{sol}^{elec} denotes the electrostatic contribution of solvation that is the free energy turns on the solute partial charges and calculated using the linear Poisson Boltzmann equation within the CHARMM program [95]. $\Delta G_{sol}^{nonpolar}$ indicates the nonpolar contribution of solvation that is derived from linear relation to the solvent accessible surface area (SASA).

$$\Delta G_{sol}^{nonpolar} = \gamma SASA + \beta$$

γ ($\gamma = 0.00542$) and β ($\beta = 0.92$) [103] are constants of the solvation free energy per unit surface area and the free energy of hydration for a point solute [104]. The entropy term is decomposed into three terms: rotational, translational and vibrational components. The configurational entropy is obtained from normal modes via quasi-harmonic analysis of the MD trajectories in the VIBRAN module of the CHARMM program. Then, these results are summed up with translational and rotational contributions that are derived from calculation of principal moment of inertia [95].

$$T\Delta S = T\Delta S_{trans} + T\Delta S_{rot} + T\Delta S_{vib}$$

The change in binding energy was calculated with the following formula for KRas4B–RalGDS-RBD systems:

$$\Delta G_b = G_b^{System} - (G_b^{K-Ras4B} + G_b^{RalGDS-RBD})$$

3.1.4 Stability and interaction analysis

To investigate the stability of the systems, the fluctuations of the system and the residues were calculated. Root Mean Square Deviation (RMSD) and Root Mean Square Fluctuation (RMSF) measurements were performed. RMSD measures the square root of average squared distance between corresponding atoms; RMSF calculates the deviations in the position of each residue throughout the simulations [105].

RMSD is measured as followed:

$$RMSD = \sqrt{\frac{1}{N} \sum_{i=1}^N \delta_i^2}$$

N indicates the total number of atoms, δ_i is the distance between atoms from different structures.

RMSF is computed as followed:

$$RMSF = \sqrt{\frac{1}{T} \sum_{t_j=1}^T (x_i(t_j) - \bar{x}_i)^2}$$

T denotes the time of the simulation in time steps, $x_i(t_j)$ indicates the coordinates of corresponding residue at time t_j and $\bar{x}_i$ is the time-averaged coordinates of i^{th} residue.

To explore the strengthen of the bond formation, salt-bridges and hydrogen bond formations were investigated using VMD. If the frequency of bond formations more than 10% throughout the simulation, then these salt-bridges and h-bonds were counted as strong stable interactions.

Chapter 4:

RESULTS AND DISCUSSION

To scrutinize the underlying mechanism of RalGDS-Ral signaling pathway, five systems for Ras-RalGDS interactions and two systems for Ral-effector interactions were generated. System 1 addresses KRas^{wt}-RalGDS-RBD complex; System 2, KRas^{G12D}-RalGDS-RBD complex; System3, KRas^{G12V}-RalGDS-RBD complex; System 4, KRas^{G12V+E37G}-RalGDS-RBD complex; System 5, Rap1-RalGDS complex. System 6 and System 7 represent Ral-effector complexes; RalA-Sec5 and RalB-Exo84. The systems were generated to enlighten the RalGDS-Ral pathway in detail. System 1 is the wild-type system; System 2 and System 3 are control systems having G12D and G12V mutations to keep KRas in its constitutively active state; System 4 is a mutational system that is mutated to target RalGDS particularly; System 5 contain Rap1 protein hat is expected to result in the strongest coupling with RalGDS.

The five Ras systems were simulated for 300 ns; System 6 and System 7 were simulated for 1000 ns explicit MD simulations. The equilibration of the systems was controlled via internal energy calculations (Figure 9, Figure 10, Figure 11, Figure 12) and RMSD analysis (Figure 13 and Figure 14). The RMSF analysis were performed to determine the fluctuations of each residue throughout the trajectories (Figure 15 and Figure 16). To discover the significant residues and interactions within complexes, salt-bridges and hydrogen bond analysis were performed in VMD (Figure 17 and Figure 18). The binding free energies of the stabilized systems were then calculated throughout MD trajectories using MM-PBSA method (Table 4 and Table 5).

The stability of the systems was analyzed calculating RMSD values and internal systematic energies. System 5 yields more stable conformation throughout 300 ns simulations, followed by System 3 and System 4 (Figure 9 and Figure 13). The fluctuation of the complexes ranges from 1 Å to 2 Å for System 5; from 1 Å to 2.5 Å for System 3; and from 1 Å to 4 Å for System 4. System 1 and System 2 are relatively fewer

stable complexes within the Ras systems. These are compelling with the distance calculations between proteins in the systems (Figure A. 1 A-E).

The stability of the Ras systems were checked calculating RMSD values of the complexes and internal energy of the systems throughout 1000 ns trajectories. The RMSD values for System 6, RasA-Sec5 and System 7, RasA-Exo84 are synchronized up to ~500 ns simulation trajectories (Figure 14). Throughout 1000 ns MD simulations, System 6 and System 7 preserve their own stabilities to form stable complex formations (Figure 10). Their stability can be observed in distance measurements between proteins in the systems throughout trajectories (Figure A. 2 F,G).



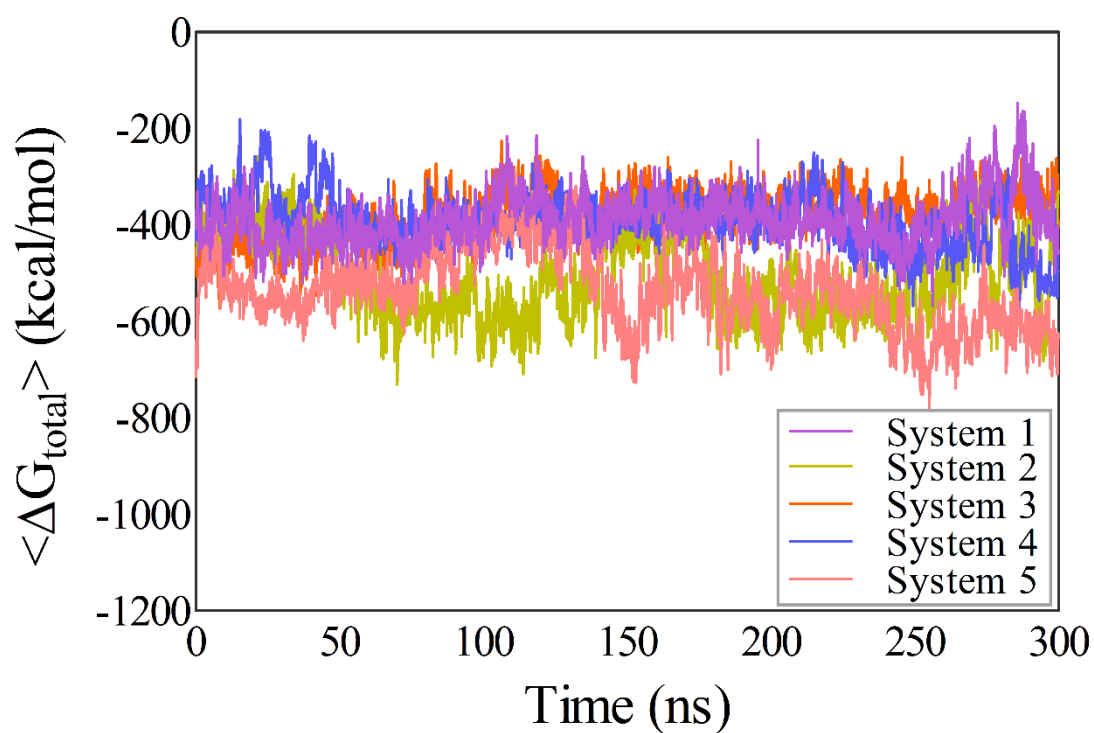


Figure 9. Total internal energies of five Ras systems.

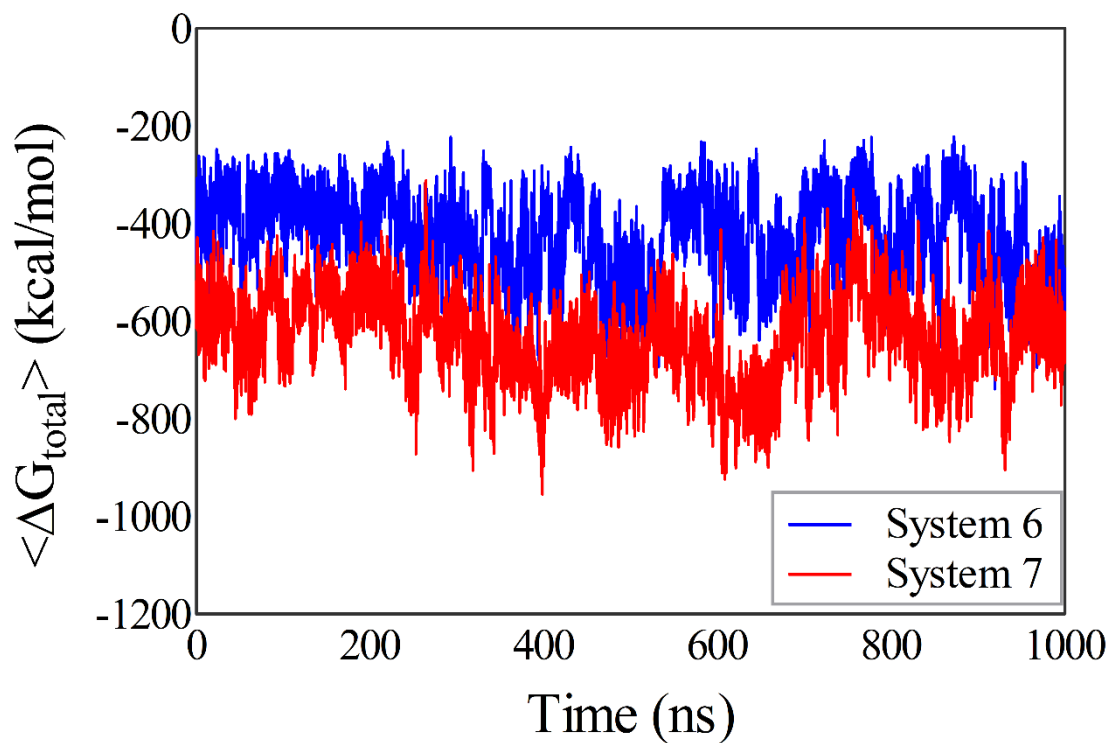
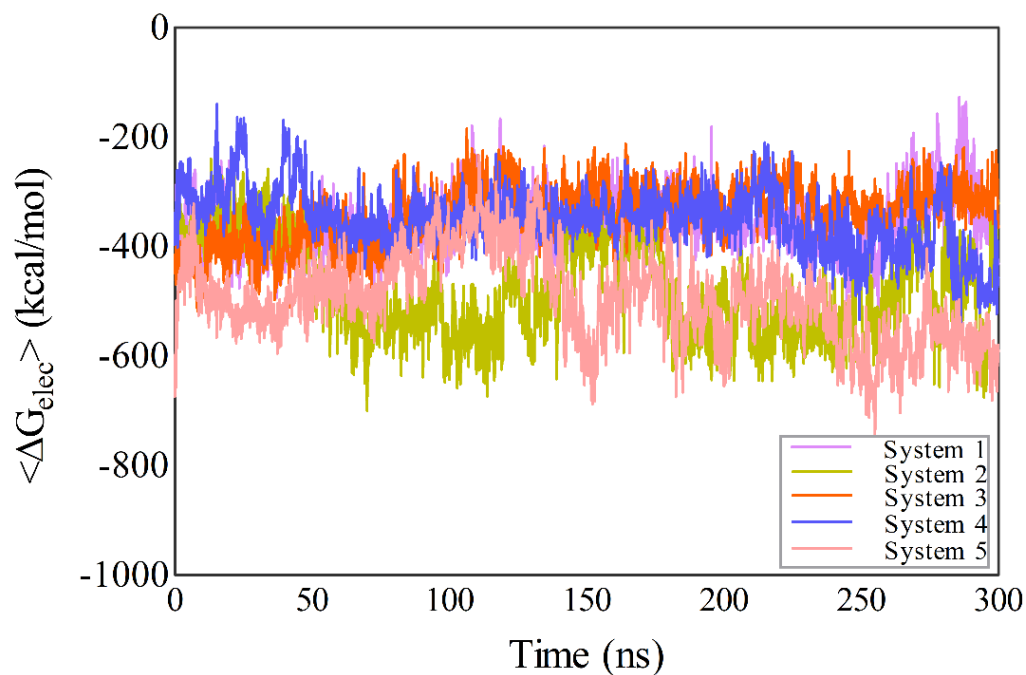


Figure 10. Total internal energies of Ral systems.

A.



B.

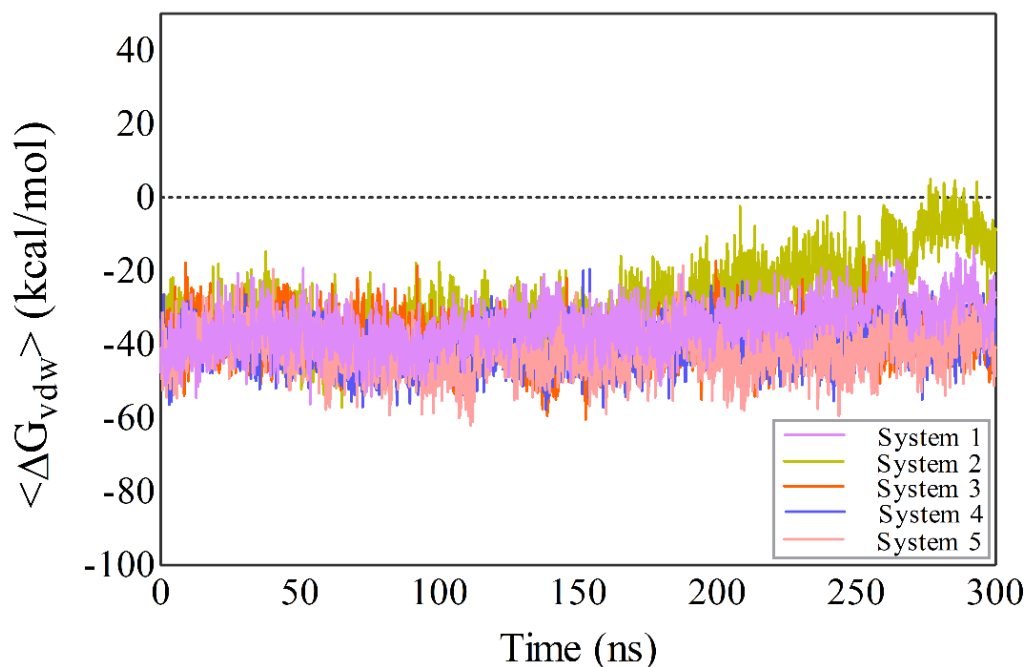


Figure 11. The electrostatic (A) and van der Waals (B) energy distributions as a function of time for five Ras systems; System 1, KRas^{wt}-RalGDS-RBD; System 2, KRas^{G12D}-RalGDS-RBD; System3, KRas^{G12V}-RalGDS-RBD; System 4, KRas^{G12V+E37G}-RalGDS-RBD; System 5, Rap1-RalGDS.

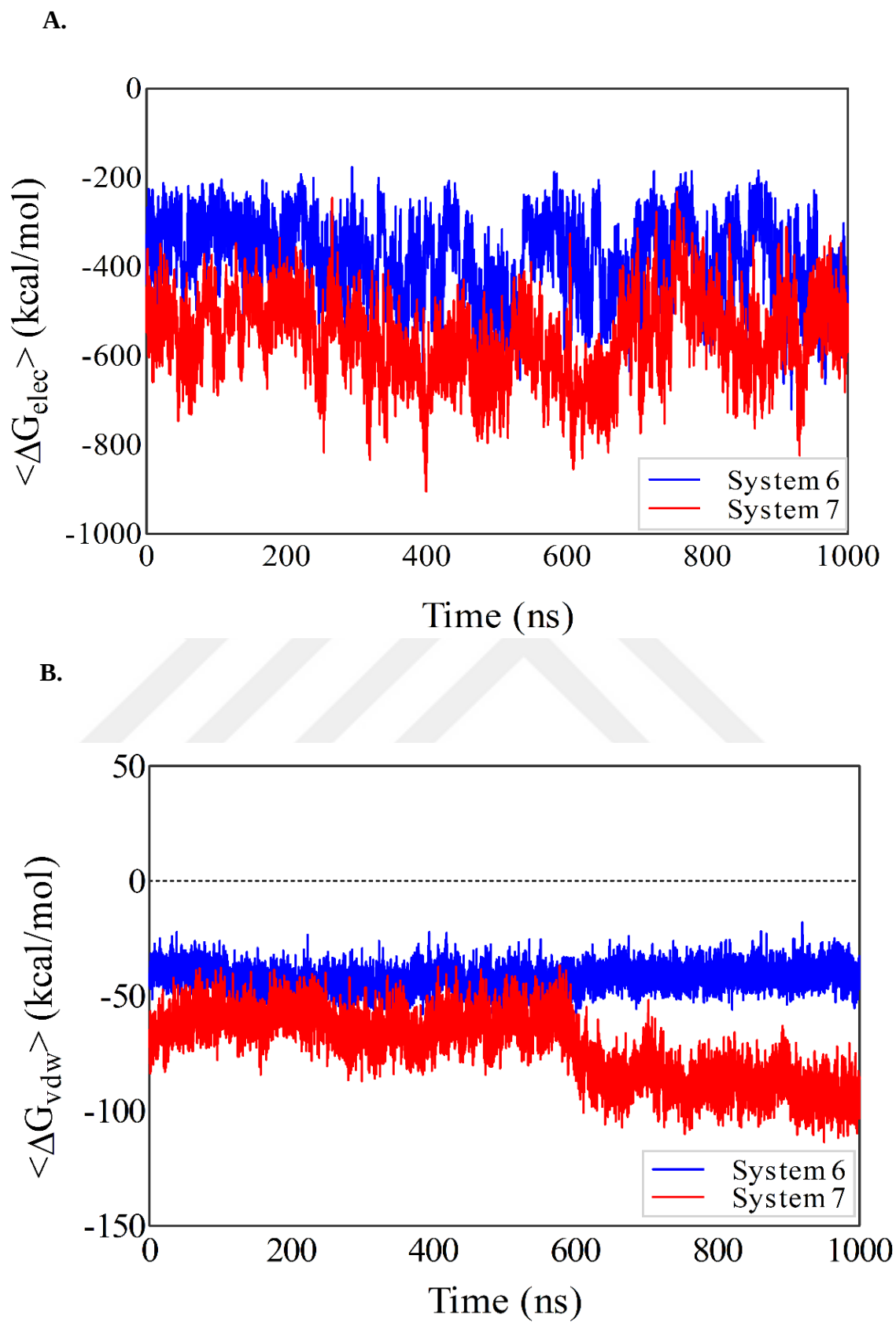


Figure 12. The electrostatic (A) and van der Waals (B) energy distributions as a function of a time for Ral systems; RalA-Sec5 and RalA-Exo84.

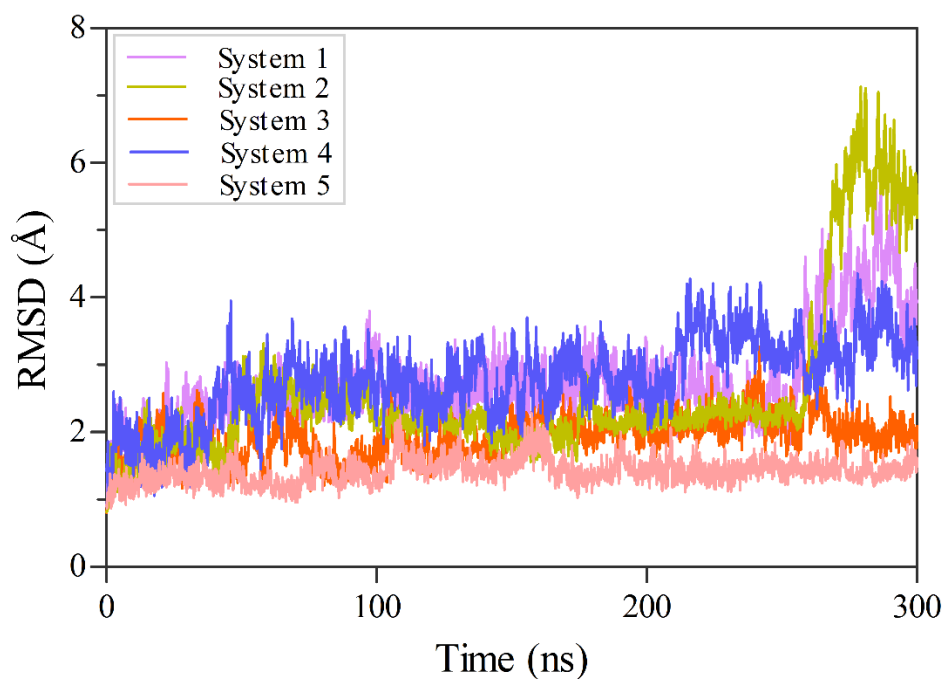


Figure 13. RMSD plots of five Ras-RalGDS-RBD systems throughout 300 ns trajectories. Each Ras system is represented in different color: System 1, KRas^{wt}-RalGDS-RBD, is in light purple; System 2, KRas^{G12D}-RalGDS-RBD, is in green; System 3, KRas^{G12V}-RalGDS-RBD, is in orange; System 4, KRas^{G12V+E37G}-RalGDS-RBD, is in blue; and System 5, Rap1-RalGDS-RBD, is in light pink.

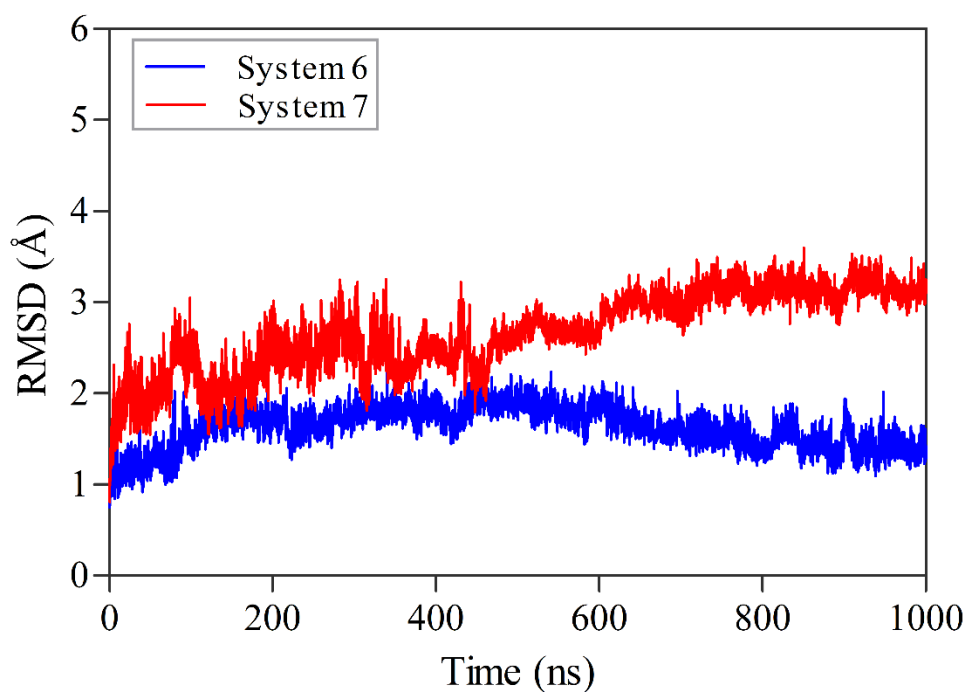


Figure 14. RMSD plots of two Ral systems throughout 1000 ns trajectories. System 6, RalA-Sec5, is represented in blue color; System 7, RalA-Exo84, is represented in red color.

Root mean squared fluctuation analyses discovered the average fluctuations of each amino acid throughout simulation trajectories. The sequence of Ras family members consists of P-loop (residues 10-17), Switch I (residues 30-38) and Switch II (residues 59-76) domains that are responsible for GTP/GDP and effector binding through conformational change. For each Ras systems, Switch II region fluctuates more than Switch I region (Figure 15 A). The highest picks in Switch II regions belong to the control groups; System 2 and System 3 that have Glycine to Aspartic acid and Glycine to Valine substitutions at residue 12 to keep Ras in constitutively active state. These mutations might have caused that much fluctuation in GTP/GDP binding region due to the conformational change.

Ras binding domain of RalGDS is responsible for Ras coupling and effector binding. The discovery of significant residues involved in Ras-binding is important for enlightening the RalGDS pathway. The RMSF plots show that the fewer fluctuations on RBD belong to wild-type system, whilst the higher fluctuations belong to System 3 and System 2; followed by System 4 and System 5.

Ral proteins share highly similar catalytic G domain with Ras proteins (Figure 3). They have additional eleven residues at their N termini; so, their effector and GTP binding domains are shifted as in the following; P-loop (21-28 residues), Switch I region (41-52 residues), and Switch II (69-81 residues). Sec5 and Exo84 are well-validated Ral effectors that form exocyst complex.

The binding regions of RalA to Sec5 and Exo84 are the same, but the involved residues in Sec 5 and Exo84 are distinct. RMSF plots of Ral monomers for System 6 and System 7 demonstrate the synchronization between two systems for each residue. The Ral residues in the two systems are fluctuated at the same rate as expected (Figure 16 A). The most fluctuated residues belong to P-loop, Switch I and Switch II regions, respectively. It is expected to observe the fluctuations on GTP binding and effector interaction domains due to the conformational change.

Sec5 and Exo84 are two different Ral effectors; Sec5 has 92 amino acids, Exo84 has 109 amino acids. They have different binding affinities and interaction characteristics to RalA protein [56]. Root mean squared fluctuation plots of two proteins address the most fluctuated residues for Sec5 (blue) (Figure 16 B) and Exo84 (red) (Figure 16 C) proteins. Exo84 fluctuates from ~ 1 Å to ~ 9 Å; Sec5 fluctuates from ~ 1 Å to ~ 3 Å. The fluctuation range of Exo84 is more than Sec5 protein.

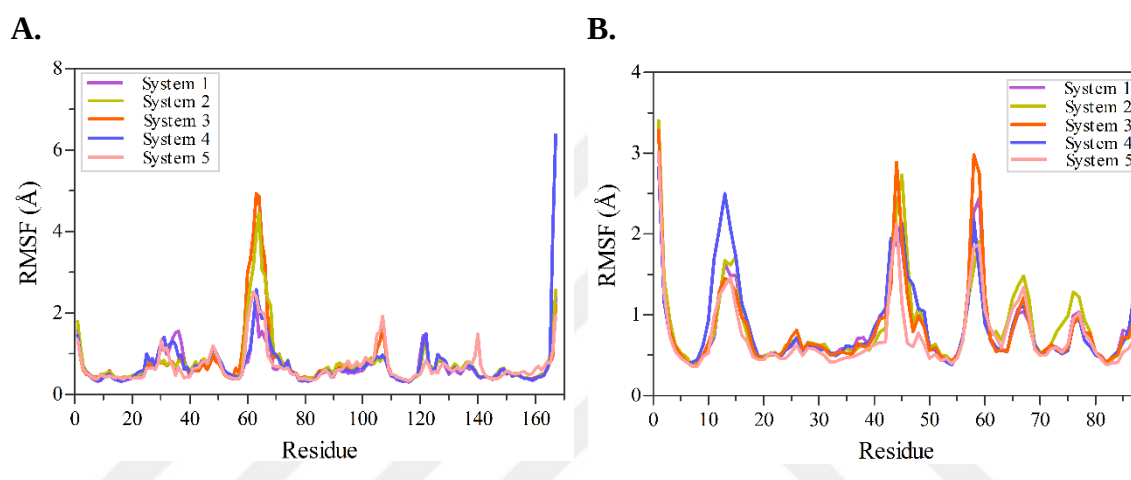


Figure 15. RMSF plots for five Ras systems. Each system is represented by different color: Light purple, KRas^{wt}-RalGDS-RBD complex; green, KRas^{G12D}-RalGDS-RBD complex; orange, KRas^{G12V}-RalGDS-RBD complex; light blue, KRas^{G12V+E37G}-RalGDS-RBD complex; and light pink, Rap1-RalGDS complex. A. The fluctuations of each Ras residue; B. the fluctuations of each RBD residue.

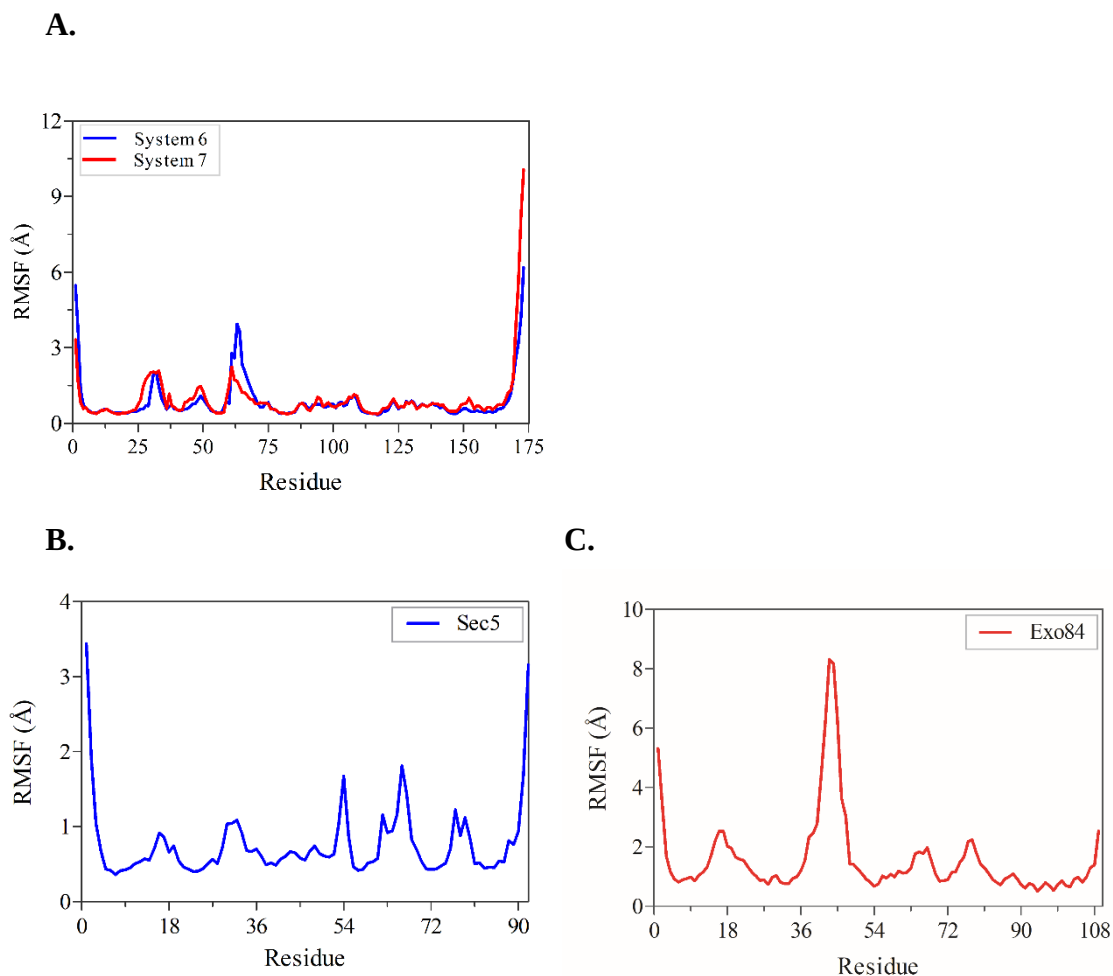


Figure 16. RMSF plots for Ral systems. **A.** RMSF of RalA monomers in System 6, (blue) and System 7 (red). **B.** RMSF of Sec5 (92 aa) residues in System 6. **C.** RMSF of Exo84 (109 aa) residues in System 7.

RMSF analysis on its own is not sufficient to determine significant residue interactions. To elaborate involved residues in Ras–RalGDS-RBD coupling, salt-bridge and H-bond analysis were performed.

There are conserved bond formations within five Ras systems. The strong bond interactions between ASP33-LYS52 (light pink box in Figure 17 and grey box in Figure 18), ARG41-GLY28 (pink box), and SER39-MET30 (light green) are observed with high occupancy percentages in all five Ras systems (Figure 17 and Figure 18). The residue interactions between Ras-RalGDS; GLU37-ARG20 (blue box), GLU37-SER33 (dark yellow box), and GLU37- TYR31 (light purple box) were observed in all systems except

System 4, which includes Glutamic acid to Glycine substitution mutation at residue 37 that is the reason of lacking the interactions that other systems have.

The salt-bridge and h-bond formations between ASP38-LYS32 (dark pink box in Figure 17, red box in Figure 18) was observed in all Ras protein systems except Rap1 complex with RalGDS-RBD.

System 5 has three additional hydrogen bond formations between ASP38-LYS52 (black box), ARG41-ASP26 (dark green box), and LYS31-ASN54 (neon blue box) residues (Figure 17): two salt-bridge formations between ASP38-LYS52 (dark blue box) and LYS31-ASP56 (pink box) amino acids (Figure 18).

System 4 and System 5 are expected to have better binding affinities to RalGDS-RBD [106]. The strong hydrogen bond formations between ASP38-SER33 (gray box) and SER39-TYR31 (reddish orange box) residues were observed specifically in System 4 and System 5 (Figure 17). These are striking residues that might have significance in the formation of stable complex.

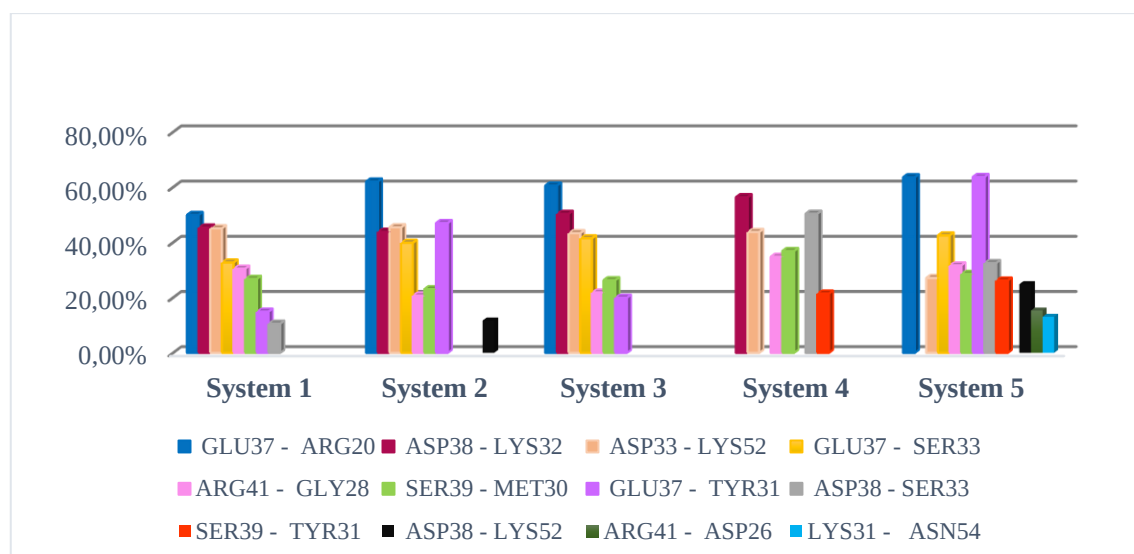


Figure 17. Hydrogen bond analysis of five simulated Ras systems.

These are significant H-bond formations for each system between the residue pairs throughout 300 ns MD simulations. The residue pairs that have higher than 10% occupancy were selected as significant. Each residue pair is represented in different color. The first residue in a pair denotes amino acid from Ras monomer; the second residue in a pair denotes amino acid from RalGDS-RBD monomer. System 1, KRas^{wt}-RalGDS-RBD; System 2, KRas^{G12D}-RalGDS-RBD; System 3, KRas^{G12V}-RalGDS-RBD; System 4, KRas^{G12V+E37G}-RalGDS-RBD; System 5, Rap1-RalGDS.

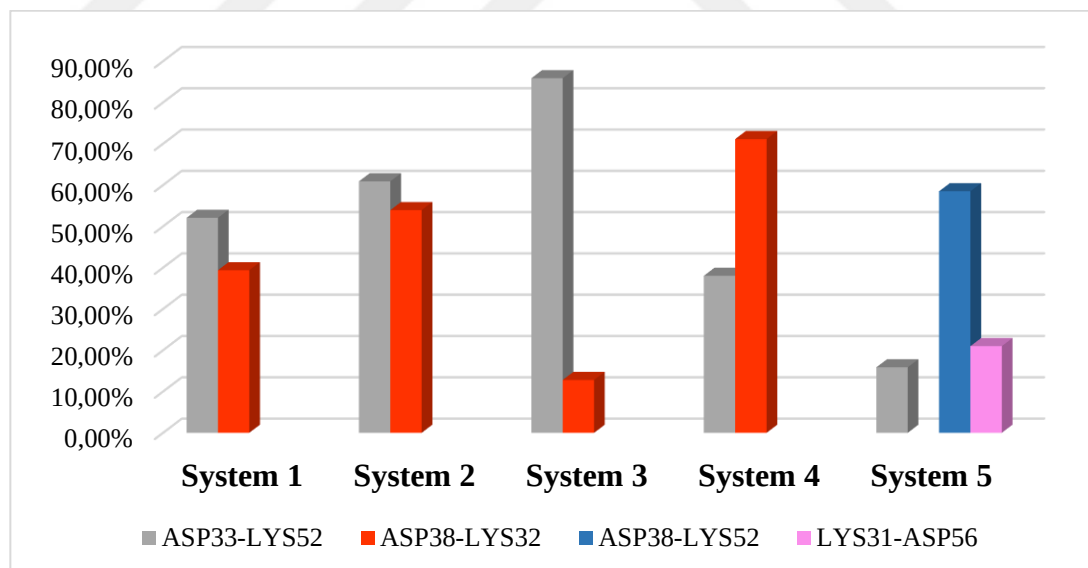


Figure 18. Salt-bridge analysis of five simulated Ras systems.

These are significant salt-bridge formations for each system between the residue pairs throughout 300 ns MD simulations. The residue pairs that have higher than 10% occupancy were selected. Each residue pair is represented in different color. The first residue in a pair denotes amino acid from Ras monomer; the second residue in a pair denotes amino acid from RalGDS-RBD monomer. System 1, KRas^{wt}-RalGDS-RBD; System 2, KRas^{G12D}-RalGDS-RBD; System 3, KRas^{G12V}-RalGDS-RBD; System 4, KRas^{G12V+E37G}-RalGDS-RBD; System 5, Rap1-RalGDS.

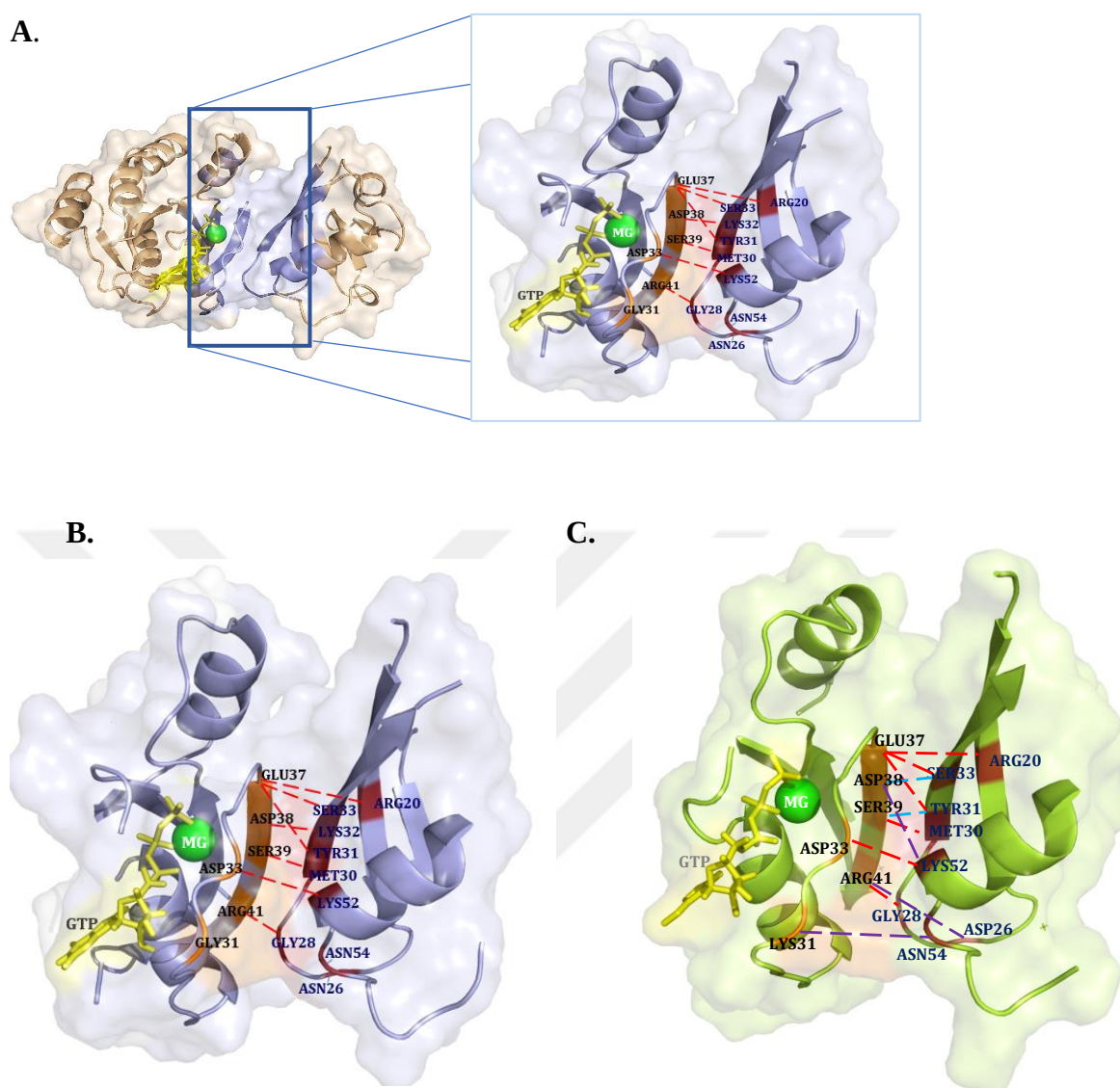


Figure 19. Representations of hydrogen bond formations for Ras systems. Left monomers are from Ras proteins, right monomers are RalGDS-RBD. Red lashes indicate the bond formations. Blue dashes indicate the bond formations occurred only in the most stable systems, System 4 and System 5; purple dashes indicate the bond formations occurred only in the System 5.

A. The interface binding region of System 1 was represented for Ras systems. The most frequently identified bond formations in Ras systems were represented in K-Ras^{wt}-RalGDS-RBD structure (right panel). **B.** These are the representations of identified bond formations for Ras systems. The bond formations including Glu37 were not found in System 4. **C.** These are the illustration of the most frequently bond formations for Rap1-RalGDS-RBD system. K-Ras4B and Rap1 proteins are highly similar, they differ at residue 31; Gly in KRas, Lys in Rap1. In System 5, there is a bond formation at residue 31 with Asn54 in RBD. The bond formations Asp38-Ser33, Ser39-Tyr31 (blue dashes) occur in the most stable systems (System 4-5). The

purple dashes, Arg41-Asp26, Lys31-Asn54, and Asp38-Lys52 occur in the System 5, which yield the best binding energy for Ras-RBD complex formation.

The strength of the bond interactions in Ral-effector systems was elaborated with salt-bridge and hydrogen bond analysis. As observed in RMSF analysis of System 6 and System 7, the frequently involved residues on RalA proteins are almost the same for two systems. The bold RalA residues in Table 2 and Table 3 address the common amino acids involved in the complex formation with Sec5 and Exo84.

RalA protein establish strong bond interactions with Sec5 and Exo84 through Asp42, Ala48, Asp49, Ser50, Arg52, Tyr82 amino acids (Figure 20). RalA structure is composed of P-loop (21-28 residues), Switch I region (38-50 residues), and Switch II (69-85 residues). Ala42, Ala48, Asp49, and Ser50 residues correspond to the Switch I domain; and Tyr82, Switch II.

In RalA-Sec5 complex, the most stable bond formations are observed in the prominent residues located in the Switch I region; Glu38, Asp49 and Tyr36 (Table 2). The Arg52 residue contributes the stable RalA-Sec5 complex formation through two strong hydrogen bond formations with Thr87 and Thr11 on Sec5.

Sec5 and Exo84 are different Ral effectors having different affinities to RalA. The bond formations will consequently occur at different residues on Sec5 and Exo84. The essential interacting residues on RalA located in the effector binding region, the corresponding residues on Sec5 and Exo84 are important to elucidate downstream mechanism of Ral-effector pathway.

In RalA-Exo84, the most stable bond formations are observed between the common residues of RalA, Ser50, Glu44, Ala48, Tyr82, Asp49, Arg52 and their corresponding residues on Exo84. The hydrogen bond interactions indicated in Table 3 show that there are residues involved in the formation of multiple stable bonds, such as Glu44 binds to Arg262 and Lys233; Ser50 binds to Asn231 and Lys233; Asp49 binds to Lys233 and Asn231 with high occupancy rate throughout 1000 ns trajectories. The frequently involved residues of RalA present in the effector binding and GTP binding domains.

Some of the corresponding residues on Exo84 are contacted with multiple RalA residues such as Asn231^{Exo84} binds to Ala48^{RalA}, Asp49^{RalA} and Ser50^{RalA}; Lys233^{Exo84} binds to Glu44^{RalA}, Asp49^{RalA}, and Ser50^{RalA}. These replicated residues on RalA and Exo84 are the outstanding amino acids for stable complex formation.

The salt bridge analysis shows that salt bridge formations occur at the residues located in the GTP and effector binding regions (Table 2 B and Table 3 B). The residues of RalA Asp42 and Glu44 in RalA-Sec5 system; Lys47, Glu73, Glu44, Lys16 and Asp49 residues in RalA-Exo84 are involved in the stable complex formations with high occupancy rate throughout 1000 ns simulation trajectories.

Table 2. Hydrogen bond (A) and Salt bridge (B) analysis of RalA-Sec5 complex. The bond interactions that have higher than 10% occupancy rate were selected.

A.

RalA	Sec5	Occupancy Percentage
GLU38	ARG27	84,34%
ASP49	SER14	72,06%
TYR36	THR11	58,91%
SER50	ILE13	39,93%
ARG52	THR87	39,13%
ALA48	ASN16	38,51%
TYR82	GLU17	37,97%
ARG52	THR11	36,54%
TYR75	GLU17	22,65%
GLU44	LYS23	14,68%
ASP42	LYS59	13,41%

B.

RalA	Sec5	Occupancy Percentage
ASP42	LYS59	28,51 %
GLU44	LYS23	22,22 %

Table 3.Hydrogen bond (A) and Salt bridge (B) analysis of RalA-Exo84 complex. The bond interactions that have higher than 10% occupancy rate were selected.

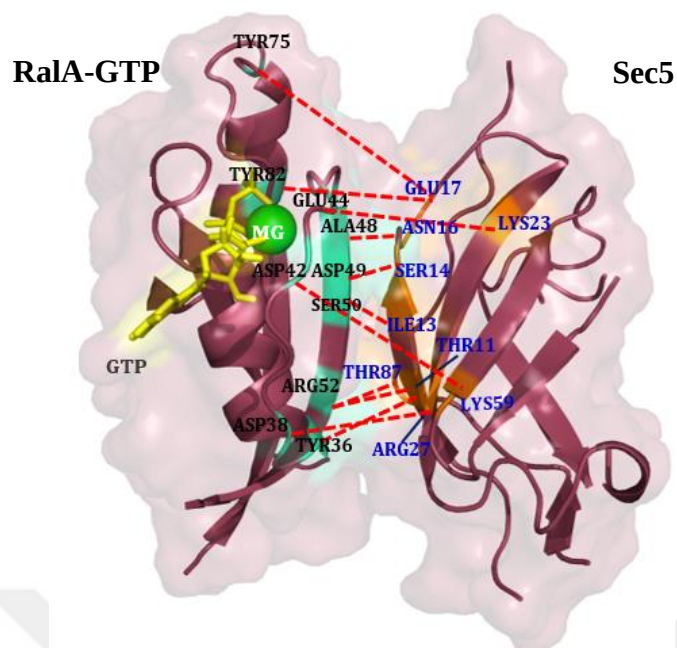
A.

RalA	Exo84	Occupancy Percentage
SER50	ASN231	50,61%
SER50	LYS233	46,98%
GLU44	ARG262	42,15%
ASP74	SER276	40,70%
GLU73	LYS272	36,83%
ALA48	ASN231	35,96%
TYR82	VAL229	34,65%
ASP74	LYS272	25,96%
ASP49	LYS233	25,82%
GLU44	LYS233	19,59%
ASP49	ASN231	13,90%
ARG52	ALA185	13,78%
ASN81	PHE247	13,65%
ASN81	ARG226	12,74%
ASN81	LEU245	10,84%

B.

RalA	Exo84	Occupancy Percentage
LYS47	GLU269	76%
GLU73	LYS272	58%
GLU44	LYS233	14%
LYS16	ASP186	12,71%
ASP49	LYS233	9,87%

A.



B.

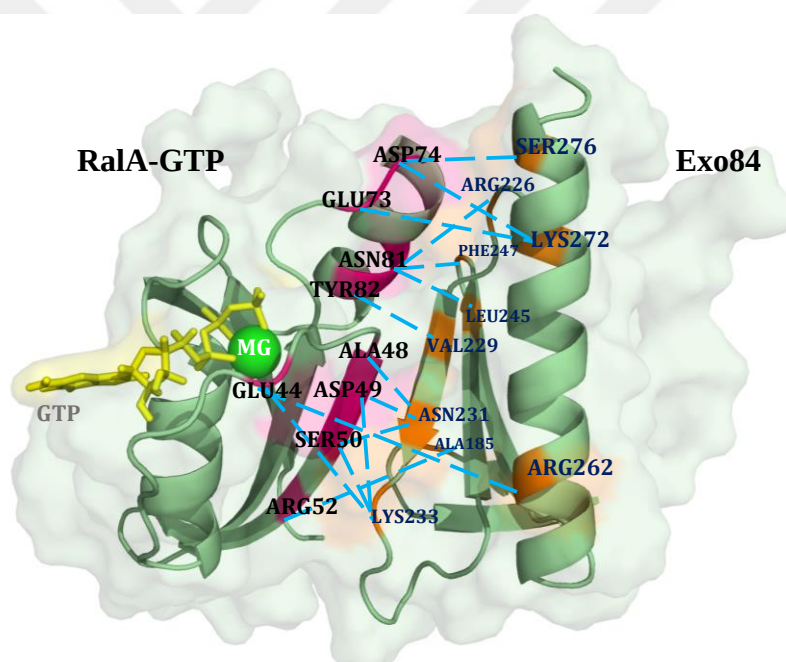


Figure 20. 3D representations of hydrogen bond formations in the interface region of Ral systems. **A.** The bond formations in System 6, RalA-Sec5. Red dashes indicate the interactions between RalA and Sec5. **B.** The bond formations in System 7, RalA-Exo84. Blue dashes indicate the interactions between RalA and Exo84. The frequently common involved residues in RalA for both systems are Glu44, Ala48, Ala49, Ser50, Arg52, and Tyr82, that correspond Switch I and II regions in RalA. The residues Glu73, Asp74, and Asn81 are additional involved residues on RalA in System 7, which might contribute the increase in the binding affinity.

4.1 Binding free energy analysis

The strength of the interactions of each system were investigated through calculation of binding free energies and the energy contributions using the molecular mechanics energies combined with the Poisson–Boltzmann surface area continuum solvation (MM-PBSA) method (Figure 21 and Figure 22). The calculated binding free energy values of five Ras systems are indicated in Table 4. High negative binding free energy values denote low stability of the complexes. The order of binding affinities of five Ras systems from the highest to the lowest are in the following: System 5, System 4, System 2, System 3, and System 1.

The Rap1 protein is expected to have more affinity to RalGDS than other Ras proteins have [106]. The binding free energy results show that Rap1 (27.81 ± 11.05 kcal/mol) yields the most stable complex with RalGDS-RBD, followed by System 4 (-14.04 ± 6.225 kcal/mol), whilst the wild-type KRas (-4.30 ± 9.734 kcal/mol) forms the less stable complex with RalGDS-RBD.

System 4, KRas^{G12V+E37G}–RalGDS-RBD, contains RalGDS targeting mutations to effectuate strong interaction with RalGDS-RBD. It has the lowest energy value, highest binding affinity among KRas-driven systems (System 1-4).

The binding free energy values of System 6 and System7 reveals that Exo84 effector protein ($-58,87 \pm 16,66$ -kcal/mol) has the highest affinity to RalA compared with Sec5 ($-30,75 \pm 10,07$ kcal/mol) (Table 5).

Table 4. Gibbs binding free energy values of five Ras systems.

	$\Delta G_b = G_b^{complex} - G_b^{Ras} - G_b^{RBD}$
System 1	-4.30 ± 9.734 kcal/mol
System 2	-10.60 ± 13.77 kcal/mol
System 3	-8.02 ± 6.005 kcal/mol
System 4	-14.04 ± 6.225 kcal/mol
System 5	-27.81 ± 11.05 kcal/mol

Table 5. Gibbs binding free energy values of two Ral systems.

	$\Delta G_b = G_b^{complex} - G_b^{Ral} - G_b^{Exo84/Sec5}$
System 6	$-30,75 \pm 10,07$ kcal/mol
System 7	$-58,87 \pm 16,66$ kcal/mol

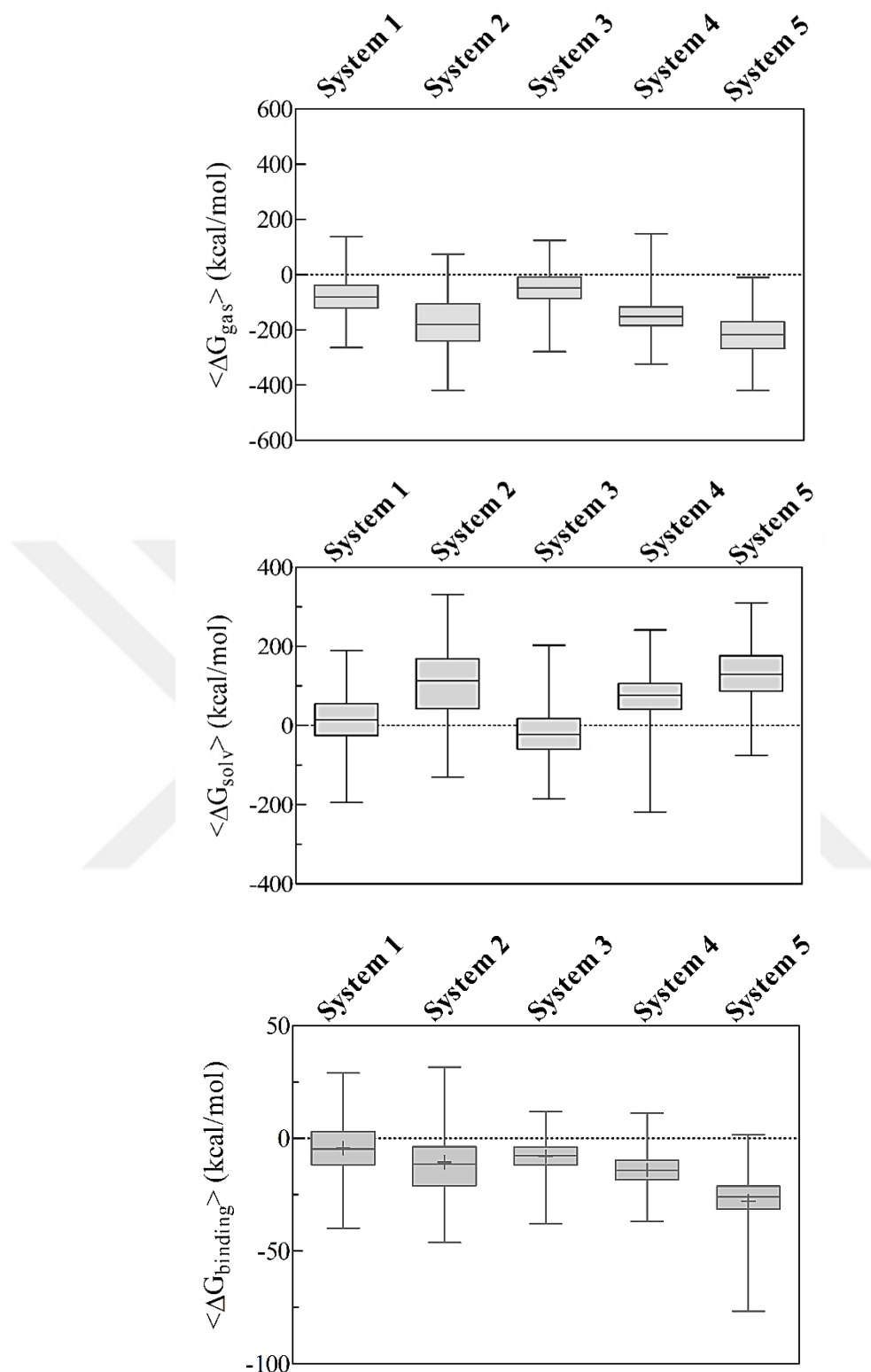


Figure 21. The energy contributions of five Ras-RalGDS systems.

The energy of the system consists of three major components: $\langle \Delta G_{\text{gas}} \rangle$, gas-phase contribution; $\langle \Delta G_{\text{solv}} \rangle$, solvation energy contribution; and $\langle \Delta G_{\text{bind}} \rangle$, binding free energy contribution. System 1, KRas^{wt}-RalGDS-RBD; System 2, KRas^{G12D}-RalGDS-RBD; System 3, KRas^{G12V}-RalGDS-RBD; System 4, KRas^{G12V+E37G}-RalGDS-RBD; and System 5, Rap1-RalGDS. The (+) sign inside the boxes in bottom graph indicates the mean values.

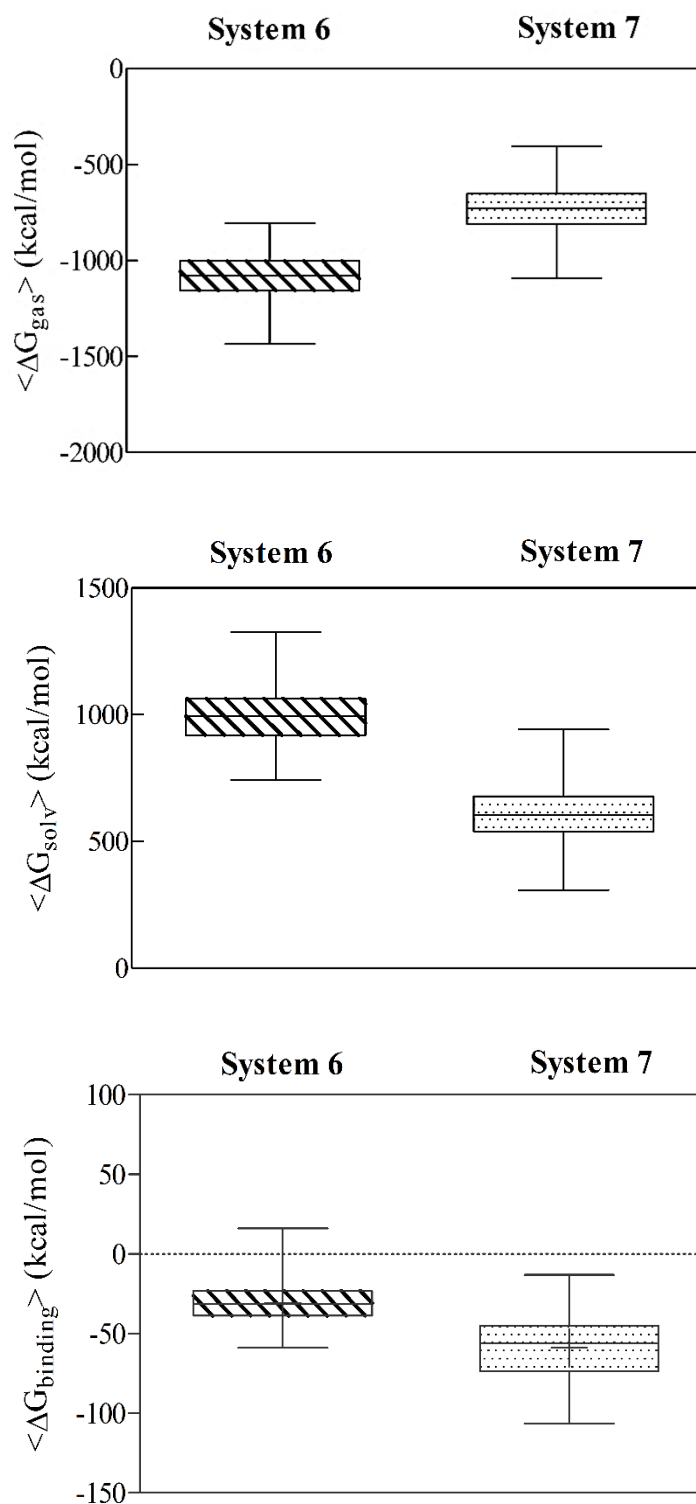


Figure 22. The binding energy contributions of two Ral systems.

The energy contributions of the system have three components: $\langle \Delta G_{\text{gas}} \rangle$, gas-phase contribution; $\langle \Delta G_{\text{solv}} \rangle$, solvation energy contribution; and $\langle \Delta G_{\text{bind}} \rangle$, binding free energy contribution. System 6 denotes RalA-Sec5 complex; System 7 indicates RalA-Exo84 complex. The (+) sign inside the boxes indicate the mean values.

4.2 Discussion

RAS family members including K-Ras, H-Ras and N-Ras oncogenic proteins, have been the focus of intense cancer researches due to the presence of their mutagenic isoforms in ~30% of human cancer types. These are evolutionarily conserved proteins; in turn they have all crucial regulatory roles in life-sustaining cellular processes as gene transcription, cell survival, and cellular proliferation through physical interaction with their numerous effector proteins [4].

The RAS mutations were first discovered in human cancerous cells in 1982. Since then, the substantial intense research scrutiny has been in progress to develop Ras suppressing drugs. RAS has been attempted to be targeted directly by small molecule that inhibits their activity. The efforts of developing small compound that targets directly RAS has failed to enter clinical trials. Despite tremendous research and pharmaceutical effort, Ras proteins are yet to be drugged [3].

The distribution of the oncogenic Ras proteins in Ras-driven cancers are distinct, even they share conserved catalytic domain. KRAS is the frequently mutated isoform (%86), followed by NRAS (11%), and HRAS (3%). The reason of why KRAS is particularly the most expressed isoform among other oncogenic Ras members is still mysterious [107]. The oncogene KRAS remain harboring threats for cancer treatments.

The failure in the development of direct Ras suppressors has shifted the focus on developing small molecules that inhibit effector pathways, in particular the Raf-MEK-ERK, PI3K-Akt-mTOR, and RalGEF-Ral signaling pathways. The Raf kinases and PI3K are well-characterized Ras effectors compared with RalGEF family member RalGDS. The Raf and PI3K cascades have been known with their crucial role in Ras-mediated tumorigenesis, but also there have been substantially accumulating studies that address the significant contributions of other Ras effector downstream as RalGEF-Ral signaling in tumor growth, in particularly K-Ras driven cancers [9].

The RalGDS is the first established RalGEF, which is an exchange factor specifically for Ral proteins. The Ras family members, K-Ras, H-Ras, N-Ras, R-Ras and Rap1, can activate RalGDS upon GTP binding. RalGDS have been taken attention with its crosstalk role as an effector of Ras and an activator of Ral proteins (Figure 7). It is a unique case, in which one Ras family member activates another family member [43].

The reason of how Ras family members favor so distinctive to RalGDS is yet to be understood. To elaborate this mystery, five Ras systems coupling with RalGDS-RBD and two Ral systems Ral-effector coupling were generated. The five Ras systems are composed of KRas4B^{wt}, KRas4B^{G12D}, KRas4B^{G12V}, KRas4B^{G12V+E37G}, and Rap1 proteins coupling with Ras binding domain (RBD) of RalGDS.

The oncogenic substitutions G12V, G12C, and G12D on K-Ras4B are found in ~90% human cancers [91], as they keep Ras proteins in their constitutively active forms [108]. It is the reason that our systems contain G12D and G12V mutations for control systems. The control systems, System 2-3, have better binding affinity (-10.60 ± 13.77 kcal/mol and -8.02 ± 6.005 kcal/mol) than wild type system (-4.30 ± 9.734 kcal/mol).

Rap1 proteins have been reported as the strongest interaction seen for the complex formation of RalGDS-RBD in Ras family members. System 5 is generated to compare Rap1-RalGDS-RBD with other Ras-RalGDS-RBD complexes to identify significant residues.

The experimental studies showed that G12V+E37G mutations on Ras protein target particularly RalGDS, not the other effectors [109]. Mutation E37G was showed as an abrogator in Ras-Raf interaction [88], and an activator in Ras-RalGDS coupling [110]. System 4, KRas4B^{G12V+E37G} –RalGDS-RBD, is expected to yield better affinity values than any other KRas4B systems. The binding free energy values show that System 4 (14.04 ± 6.225 kcal/mol) constitutes the most stable complex formation with RalGDS-RBD, followed by System 2 (-10.60 ± 13.77 kcal/mol), System3 (-8.02 ± 6.005 kcal/mol), and System 1 (-4.30 ± 9.734 kcal/mol) (Table 4).

The salt-bridge and hydrogen bond analysis revealed the frequently involved residues that form strong complex interactions within four KRas4B systems as in the following; Asp38, Asp33, Arg41, Ser39. The Glu37 residue is observed in all systems except System4 in which Glu was mutated to Gly. The residue 33, 37, 38, and 39 on Ras were previously showed in Ras-effector interactions [40]. These residues are in the Switch I, effector binding domain of Ras proteins, so our results confirm that Switch I region is responsible for effector interactions. Studies have shown that Glu37 and Asp38 are conserved in all Ras family members for effector coupling [111]. In this study, we demonstrated additional conserved residues on Ras members to establish stable complex formations between Ras-effector interactions.

Ras and Rap proteins have common effector binding region (residues 32-40). There are fundamental residue interactions observed in Ras-RalGDS-RBD complex formations within five systems in that effector region. The interactions between Asp33-Lys52, Arg41-Gly28, and Ser39-Met30 were observed in all Ras systems. Additionally, Glu37-Arg20, Glu37-Ser33, Glu37-Tyr31 residue interactions were observed in four systems, except System 4, that is expected because of the substitution at residue 37.

Ras and Rap proteins have difference at residue 31 with oppositely charged amino acids; Lys in Rap1, Glu in Ras. This difference was showed to procure Ras interaction with Raf [112]. The residue 31 generates additional bond formation particularly in System 5 (Figure 20 C). Our results proposed that this residue might have strong contribution in Rap-RalGDS binding affinity.

The residues, Ser33^{RalGDS-RBD} and Tyr31^{RalGDS-RBD} tend to form bond formations with Glu37 in all systems. In the absence of Glu37 as in System 4, the Ser33^{RalGDS-RBD} binds to Asp38^{Ras}, and Tyr31^{RalGDS-RBD} binds to Ser39^{Ras}. Moreover, the residue interactions of Asp38-Ser33 (gray) and Ser39-Tyr31 (red) are observed only in the most favorable systems, System 4 and System 5 (Figure 17). Even System 5 has glutamic acid at residue 37, the Ser33^{RalGDS-RBD} not only binds to Glu37^{Ras}, also binds to Asp38^{Ras}. The Tyr31^{RalGDS-RBD} residue favors binding to Glu37^{Ras} in all Ras systems.

In the absence of Glu37 as in System 4, Tyr31^{RalGDS-RBD} forms bond formation with Ser39^{Ras}. Surprisingly, the interaction between Ser39-Tyr31 (red) occurs only in the most favorable systems; System 4 and System 5 (Figure 17).

There are some residues on both Ras and RalGDS-RBD involved in the constitution of more than one residue pairs in the systems with the lowest binding free energy values; System 2, System 4, and System 5. The residues Asp38, Asp33, Ser39, and Glu37 on Ras proteins; Lys52, Ser33, and Tyr31 on RBD have tendency to form more than one strong bond interactions in the most favorable systems. These are the prominent residues in the complex formation of Ras and RalGDS-RBD.

System 5, Rap1–RalGDS-RBD, yields better binding free energy value than other KRas systems. The energy value might be the description of Ras antagonistic activity of Ras [113]. Our results are compelling with the hypothesis that Rap1 proteins inhibit the Ras activity through competition for the same effector proteins [106].

The salt-bridge and hydrogen bond analysis revealed the frequently involved residues of RalA within System 6, RalA-Sec5, and System 7, RalA-Exo84. The common involved residues on RalA in both systems allow us to identify critical amino acid information on the effector binding region of Ral. The fundamental amino acids on RalA in the stable complex formation between its effectors, Sec5 and Exo84, are in the following; Glu44, Ala48, Asp49, Ser50, Arg52 and Tyr82. They constitute strong bond interactions in the stable Ral-effector complexes with high occupancy rates. These residues might be considered as the fundamental amino acids on RalA that permit strong bond formations in stable effector coupling.

Some residues on Ral proteins have tendency to bind more than one residue on either Sec5 or Exo84. The residue, Arg52^{RalA} binds to Thr87^{Sec5} and Thr11^{Sec5} with high occupancy percentages. Some residues on Sec5 protein also couple with more than one amino acid, such as Thr11^{Sec5}, coupling with Tyr36^{RalA} and Arg52^{RalA}; Glu17^{Sec5}, coupling with Tyr82^{RalA} and Tyr75^{RalA}.

In the RalA-Exo84 complex, the residues Glu44, Asp49, Ser50, Asp74 on RalA; constitute more than one strong bond formation. The Glu44^{RalA} contacts with Arg262^{Exo84} and Lys233^{Exo84}; Asp49^{RalA} contacts with Lys233^{Exo84} and Asn231^{Exo84}; Ser50^{RalA} contacts with Asn231^{Exo84} and Lys233^{Exo84}; Asp74^{RalA} contacts with Ser276^{Exo84} and Lys272^{Exo84}. As seen, some residues on Exo84 produce more than one bond formation as Asn231^{Exo84}, interacts with Ala48^{RalA} and Ser50^{RalA}; and Lys233^{Exo84}, interacts with Glu44^{RalA} and Asp49^{RalA}.

The residues that form multiple residue interactions between Ral and effectors might be considered as the fundamental residues for the development of stable complex formation. Moreover, some residues among common involved amino acids on RalA in both systems, Glu44, Asp49, Ser50, and Arg52 were found in the multiple strong bond formations. So, these residues from common amino acids might be considered as the most frequently involved significant residues on RalA coupling with its effectors.

Sec5 and Exo84 protein locate in different subcomplexes. Ral protein was proposed acting as a bridge between these subcomplexes. The involvement of common residues in the strong bond formation confirms the competitive interaction of Sec5 and Exo84 to RalA. The binding energy values denote that Exo84 ($-58,87 \pm 16,66$ -kcal/mol) has more affinity to RalA than Sec5 ($-30,75 \pm 10,07$ kcal/mol). These energy values might explain the hypothesis that activated Ral recruits Exo84 containing complex to the place that was tagged by Sec5 containing subunit [56], [57].

Our results are compelling with the previous studies, that identify the partially overlapping residues in the effector binding domain of RalA as Ala48, Ser50, and Arg52 [56]. We discovered additional overlapping residues on RalA as Glu44 and Asp49 in System 6 and System 7.

The previous studies on Ral-Sec5 interaction revealed some interacting residues on Sec5 collide with our results; Thr11, Ile13, Asn16, Arg27 [55]. Additionally, we discovered more residues that frequently involve in strong complex formation as Ser14, Glu17, Lys23, Lys57, Thr87 with high occupancy rates.

Chapter 5: **CONCLUSION**

The main purpose of this study is to elaborate Ras-RalGDS pathway, and in turn investigate the downstream mechanism of Ral-effector couplings upon RalGDS activation. This study is aimed to characterize the residue specificities of K-Ras, Rap1 with RalGDS-RBD, and RalA protein with Sec5 and Exo84 effector proteins.

Rap1, Ral and Ras proteins are similar small GTPases that are activated in response to the various extracellular stimuli. Activated Ras family members mediate life-sustaining cellular processes through interactions with their effector proteins. Ras proteins have three well-known effectors: Raf kinase, PI3K, and RalGDS. RalGDS is an effector of K-Ras and Rap1 proteins, whilst a guanine nucleotide exchange factor for Ral protein [26].

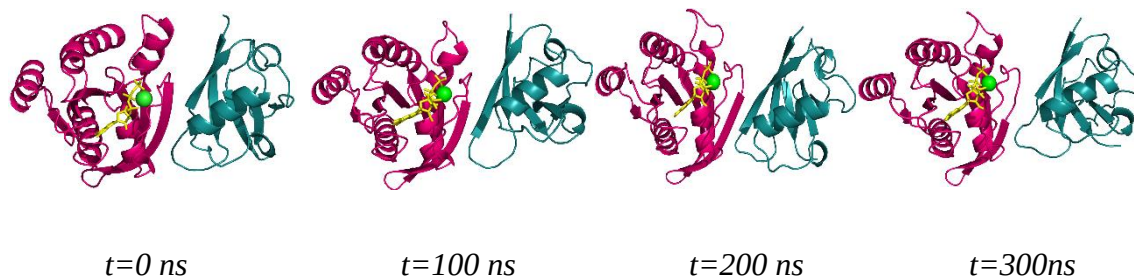
For several decades, the investigation of the role of oncogenic Ras proteins in cancer cell lines have been the focus of researches. The aberrant consequences of Ras interactions with Raf and PI3K pathways have elaborated. The contributions of other Ras effectors as RalGDS in tumorigenesis remain elusive [3]. This dissertation elaborates RalGDS interactions with K-Ras and Rap1, and in turn RalGDS activated Ral signaling pathway. K-Ras has been taken attention, since it is the most frequently mutated isoform in Ras-driven cancer [7]; Rap1 has been taken attention because of antagonistic effect on Ras activity [8]; Ral has been taken attention due to its GEF factor is an effector of Ras [9]. Our study revealed the residue specificities in the effector binding region of K-Ras, Rap1, RalGDS-RBD; RalA, Sec5, and Exo84 proteins through well-designated seven systems using molecular dynamics method.

The designed systems provide us better understanding of RalGDS-RBD interaction as an effector of Ras and an activator of Ral protein with its GEF function. In this study, we confirmed that Rap1 and K-Ras competes to bind RalGDS-RBD. Even they have high sequence homology, the differences in their sequence as residue 31, yield different binding proximities to RalGDS-RBD through formation of strong bond interaction. We discovered the significant residues on proteins K-Ras, Rap1, RalGDS-RBD; RalA, Sec5, and Exo84 in the complex formation. Our results validated that Sec5 and Exo84 locate in different complexes, and they compete to bind RalA. The identified overlapping residues on RalA denotes their competition. Using computational methods, we further investigated that the affinity of Exo84 to RalA is better than Sec5. The binding energy values validate the hypothesis [57] that activated RalA recruit Exo84 containing complex to the target that is marked by Sec5 containing complex.

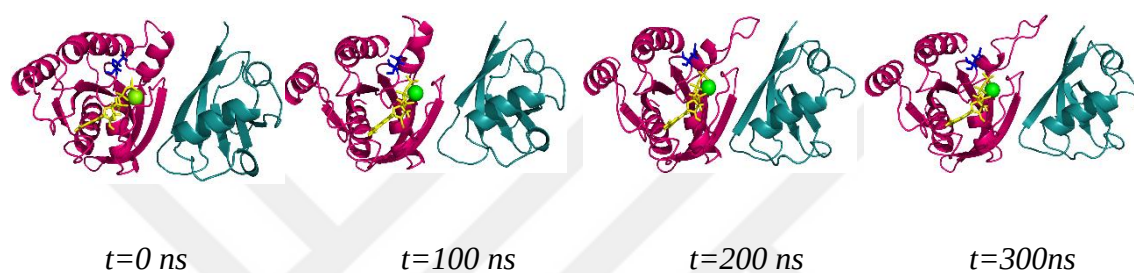
These observations enlighten the underlying mechanism behind oncogenic Ras-RalGDS pathway, and RalGDS activated Ral signaling in Ras mediated cancer. In the long term, the consequences of this dissertation as identified residues, might provide new insights into developing potential anti-cancer small molecules for cancer treatment.

APPENDIX A

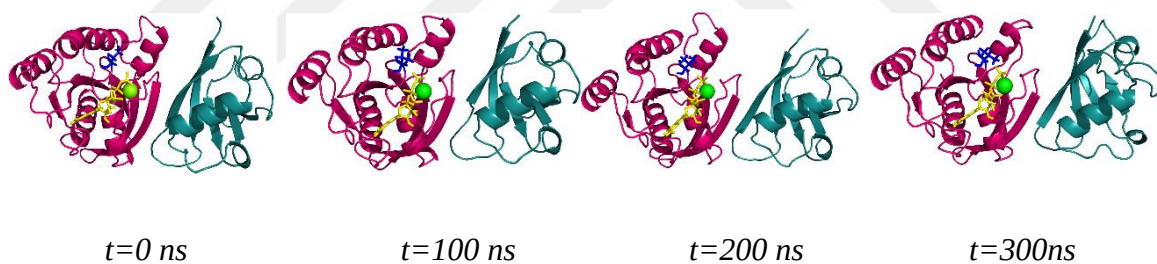
A.



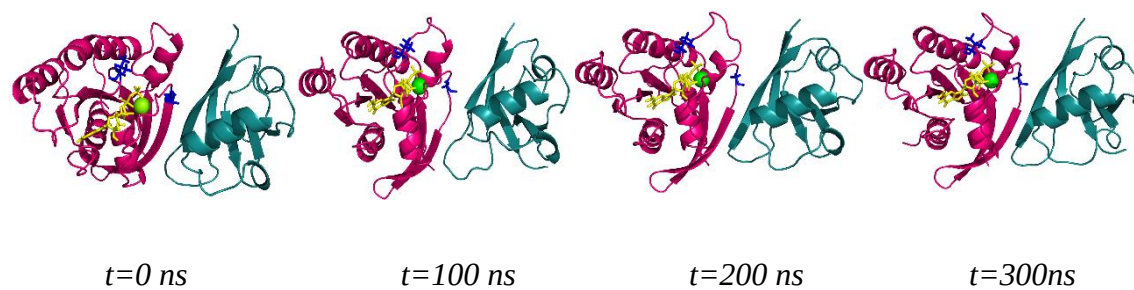
B.



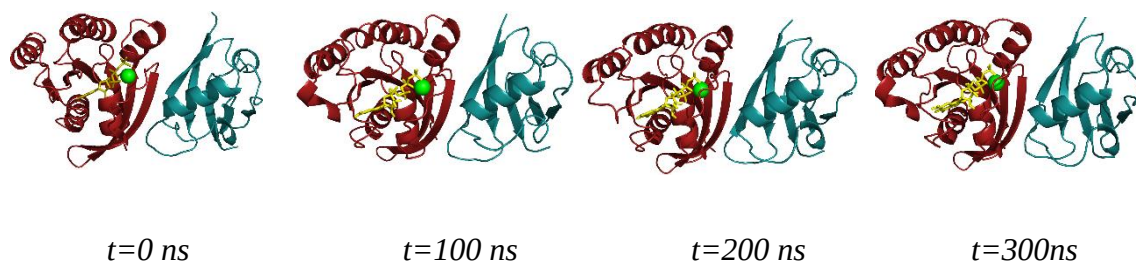
C.



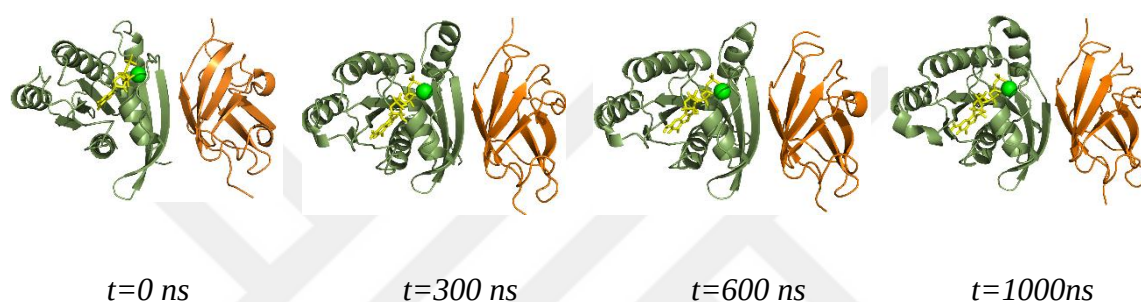
D.



E.



F.



G.

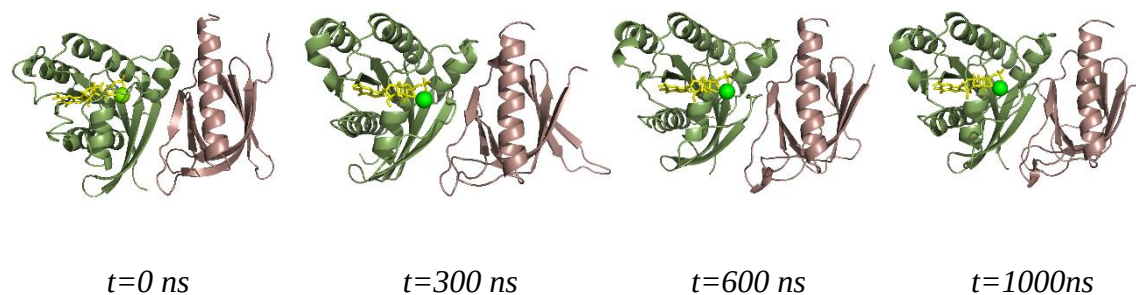
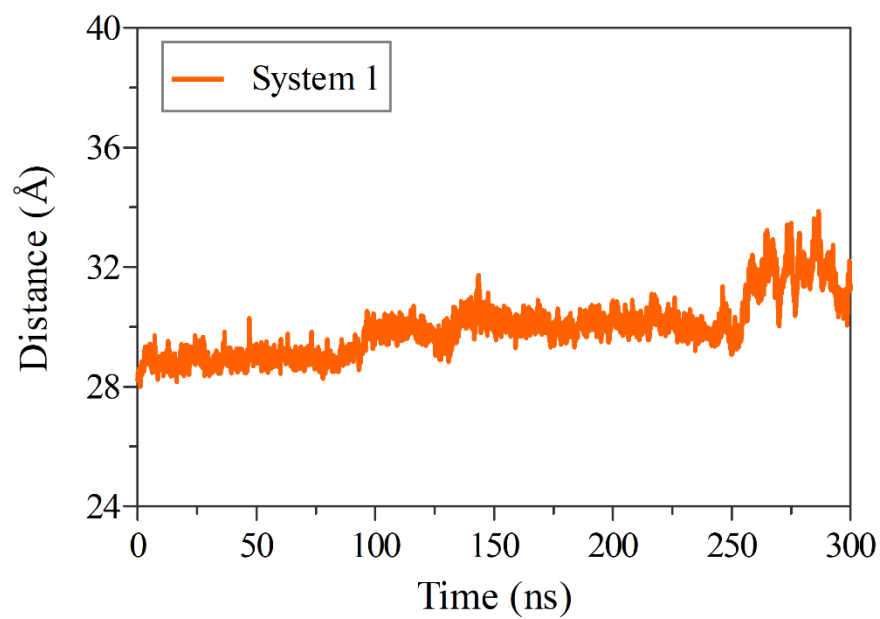
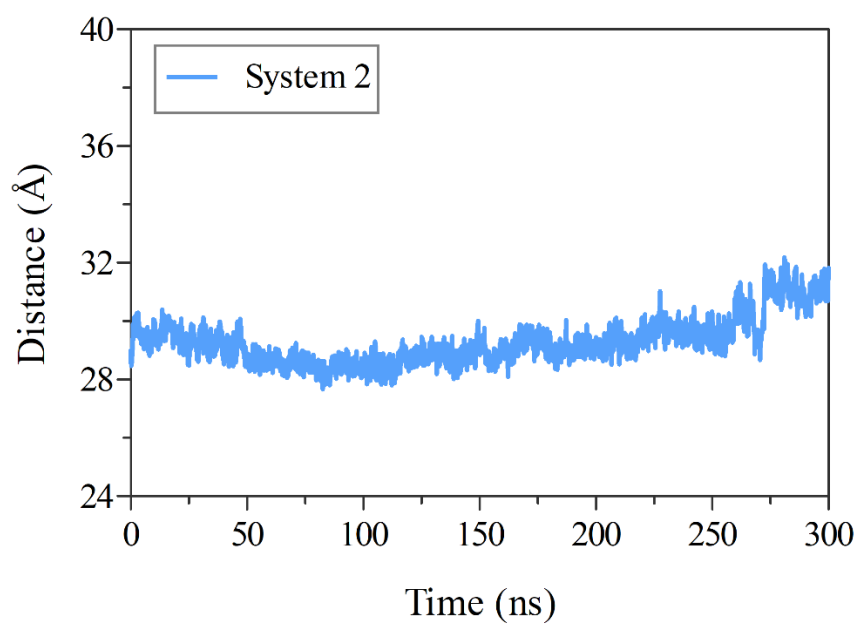
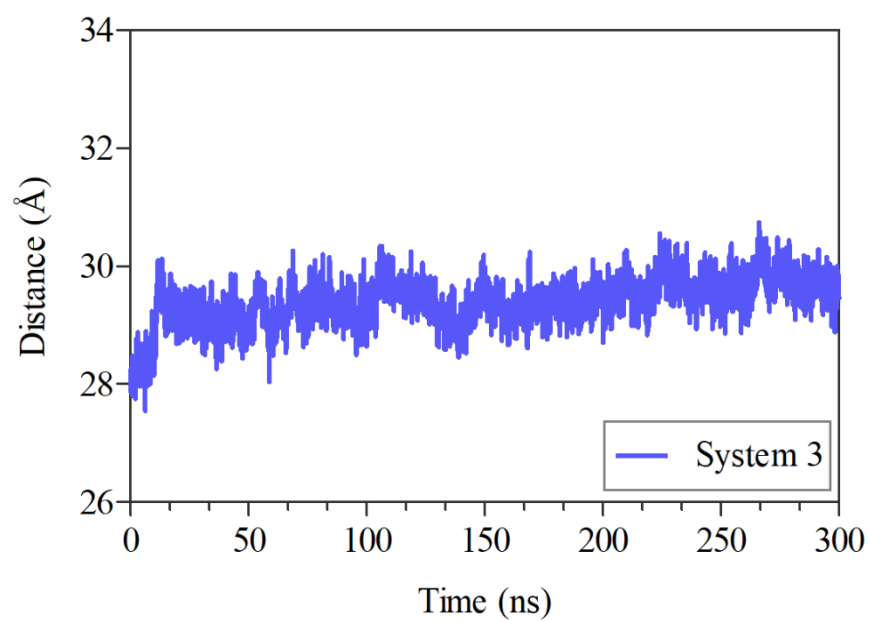


Figure A. 1. Snapshots of each system throughout MD trajectories.

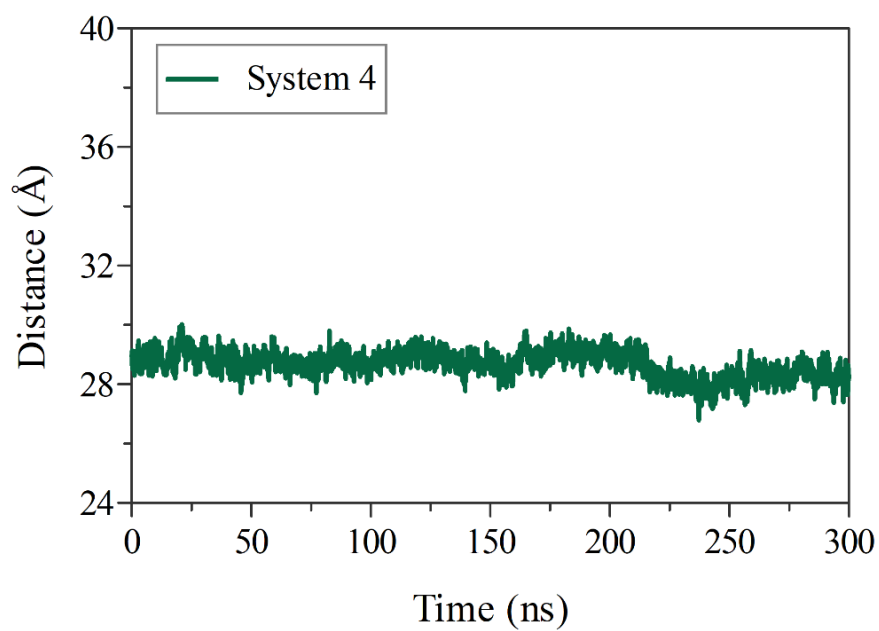
A Snapshots of System1, K-Ras4B^{WT}–RalGDS-RBD, B. Snapshots of System 2, K-Ras4B^{G12D}–RalGDS-RBD, C. Snapshots of System 3, K-Ras4B^{G12V}–RalGDS-RBD, D. Snapshots of K-Ras4B^{G12V+E37G}–RalGDS-RBD, E. Snapshots of System 5, Rap1^{WT}–RalGDS-RBD; F. Snapshots of System 6, RalA-Sec5, G. Snapshots of System 7, RalA-Exo84.

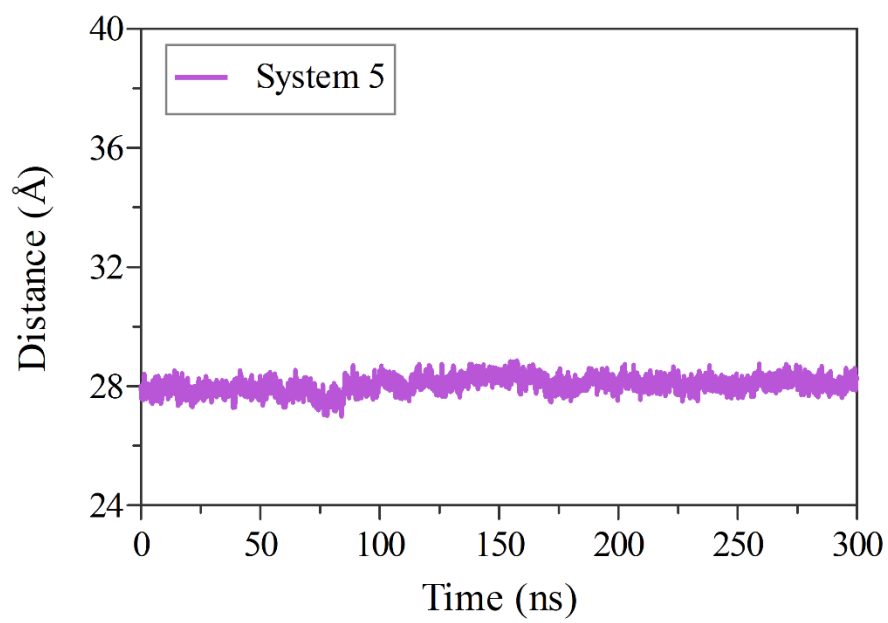
A.**B.**

C.

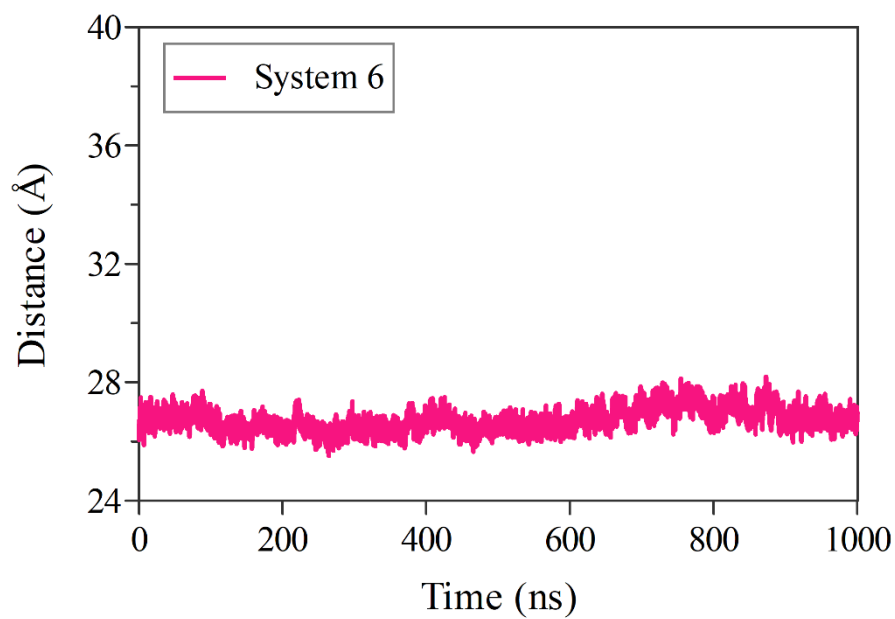


D.



E.

F.



G.

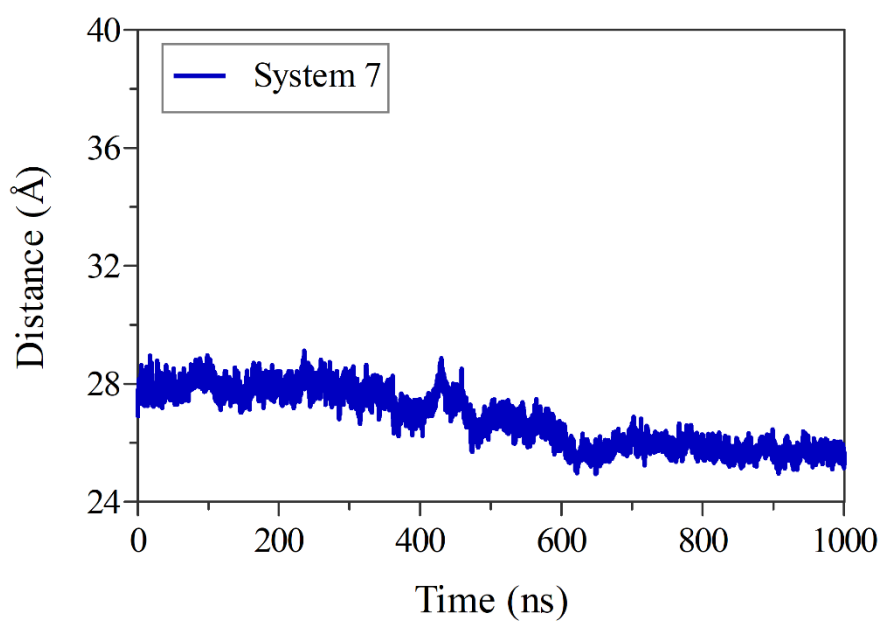


Figure A. 2. Distance measurements of seven systems.

A-D) The center of mass distances between K-Ras and RalGDS-RBD (System 1-4),

E) The center of mass distance between Rap1 and RalGDS-RBD (System 5),

F-G) The center of mass distances between RalA-Sec5 (System 6) and RalA-Exo84 (System 7), respectively.

APPENDIX B

A sample script of psf/pdb generation using CHARMM

```

* File: krwtgtp_ralgds_mk_psf_salt.inp
* Generate combined coordinates for NAMD simulation
*
!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!
! set directory
set type krwtgtp_ralgds
set dir data/omurme/@type
!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!
bomlev -2

! read topology
open read card unit 10 name /data/omurme/charmm_input/top_all36_ras_c39b2_hbj.inp
read rtf card unit 10
close unit 10

! read parameters
open read card unit 10 name /data/omurme/charmm_input/par_all36_ras_c39b2_hbj.inp
read param card unit 10
close unit 10

!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!

! Get KRas

read sequence card
* The sequence is KRas-GTP 1 to 367
*
167
MET THR GLU TYR LYS LEU VAL VAL VAL GLY ALA GLY GLY
VAL GLY LYS SER ALA LEU THR ILE GLN LEU ILE GLN ASN
HSD PHE VAL ASP GLU TYR ASP PRO THR ILE GLU ASP SER
TYR ARG LYS GLN VAL VAL ILE ASP GLY GLU THR CYS LEU
LEU ASP ILE LEU ASP THR ALA GLY GLN GLU GLU TYR SER
ALA MET ARG ASP GLN TYR MET ARG THR GLY GLU GLY PHE
LEU CYS VAL PHE ALA ILE ASN ASN THR LYS SER PHE GLU
ASP ILE HSD HSD TYR ARG GLU GLN ILE LYS ARG VAL LYS
ASP SER GLU ASP VAL PRO MET VAL LEU VAL GLY ASN LYS
CYS ASP LEU PRO SER ARG THR VAL ASP THR LYS GLN ALA
GLN ASP LEU ALA ARG SER TYR GLY ILE PRO PHE ILE GLU
THR SER ALA LYS THR ARG GLN GLY VAL ASP ASP ALA PHE
TYR THR LEU VAL ARG GLU ILE ARG LYS HSD LYS

generate KRAS first NTER last CTER setup

```

```

! Get GTP
read sequence GTP 1
generate GTP setup first none last none

! Get Mg
read sequence MG 1
generate MAGN noangle nodihedral

! Get RalGDS

read sequence card
* The sequence is RALG 1 to 87
*
87
GLY ASP CYS CYS ILE ILE ARG VAL SER LEU ASP VAL ASP
ASN GLY ASN MET TYR LYS SER ILE LEU VAL THR SER GLN
ASP LYS ALA PRO THR VAL ILE ARG LYS ALA MET ASP LYS
HSD ASN LEU ASP GLU ASP GLU PRO GLU ASP TYR GLU LEU
LEU GLN ILE ILE SER GLU ASP HSD LYS LEU LYS ILE PRO
GLU ASN ALA ASN VAL PHE TYR ALA MET ASN SER ALA ALA
ASN TYR ASP PHE ILE LEU LYS LYS ARG

generate RALG first GLYP last CTER setup

open read card unit 1 name /@dir/coord/@type_add_salt_system.crd
read coord card unit 1
close unit 1

set N ?nres

open read card unit 1 name /@dir/coord/temp_blk1.crd
read sequence coord unit 1
generate BLK1 nodihedrals noangles first none last none
close unit 1
open read card unit 1 name /@dir/coord/temp_blk1.crd
read coord card unit 1 offset @N
close unit 1

set N ?nres

open read card unit 1 name /@dir/coord/temp_blk2.crd
read sequence coord unit 1
generate BLK2 nodihedrals noangles first none last none
close unit 1
open read card unit 1 name /@dir/coord/temp_blk2.crd
read coord card unit 1 offset @N
close unit 1

set N ?nres

open read card unit 1 name /@dir/coord/temp_blk3.crd
read sequence coord unit 1
generate BLK3 nodihedrals noangles first none last none
close unit 1
open read card unit 1 name /@dir/coord/temp_blk3.crd

```

```
read coor card unit 1 offset @N
close unit 1

set N ?nres

open read card unit 1 name /@dir/coor/temp_blk4.crd
read sequence coor unit 1
generate BLK4 nodihedrals noangles first none last none
close unit 1
open read card unit 1 name /@dir/coor/temp_blk4.crd
read coor card unit 1 offset @N
close unit 1

set N ?nres

open read card unit 1 name /@dir/coor/temp_sod.crd_mod
read sequence coor unit 1
generate SODI noangle nodihedral
close unit 1
open read card unit 1 name /@dir/coor/temp_sod.crd_mod
read coor card unit 1 offset @N
close unit 1

set N ?nres

open read card unit 1 name /@dir/coor/temp_cla.crd_mod
read sequence coor unit 1
generate CHLO noangle nodihedral
close unit 1
open read card unit 1 name /@dir/coor/temp_cla.crd_mod
read coor card unit 1 offset @N
close unit 1

!!!!!!!!!!!!!!!!!!!!

! Stat for protein
coor stat sele segid KRAS end
coor stat sele segid KRAS .and. type CA end
coor stat sele segid GTP end
coor stat sele segid MAGN end
coor stat sele segid RALG end
coor stat sele segid RALG .and. type CA end

! Stat for salt
coor stat sele resn SOD end
coor stat sele resn CLA end

! Stat for water
coor stat sele segid BLK1 .and. type OH2 end
coor stat sele segid BLK2 .and. type OH2 end
coor stat sele segid BLK3 .and. type OH2 end
coor stat sele segid BLK4 .and. type OH2 end
```

!!!!!!!!!!!!!!!

!IOFormat NOEXtended

!!!!!!!!!!!!!!!

! Write psf file in charmm format

open write card unit 1 name /@dir/psf/@type_namd_salt_charmm.psf

write psf card unit 1

close unit 1

! Write psf file in charmm format

open write card unit 1 name /@dir/psf/@type_namd_salt_xplor.psf

write psf card xplor unit 1

close unit 1

!!!!!!!!!!!!!!!

! Get the final coordinates in pdb

open write form unit 1 name /@dir/coord/@type_namd_salt_charmm.pdb

write coord pdb unit 1

close unit 1

stop

A sample script of minimization configuration file (for CHARMM generated pdb/psf files)

```
#####
##JOB DESCRIPTION
#####
FILE: krwtgtp_ralgds_namd_preequ_mini.cnfg
# Minimization of solvent with protein fix
#####
# Set variable values
set temp      310
set dir       /data/omurme/krwtgtp_ralgds
set type      krwtgtp_ralgds

#####
#Parameters
paraTypeCharmm    on
parameters        par_all36_ras_c39b2_hbj.inp

#####
# Input files
structure          $../psf/..._namd_salt_xplor.psf
coordinates        $../coord/..._namd_salt_charmm.pdb

#####
# Restart files
NOTE: Do not set the initial restart velocity if you have also specified a temperature!
if {0} {
  velocities        ${dir}/vel/                #Initial velocities in pdb file
  binVelocities     ${dir}/vel/
  binCoordinates    ${dir}/coord/
  extendedSystem    ${dir}/xsc/
}

#####
# Simulation temperature
# NOTE: Do not set the initial velocity temperature if you have also specified a .vel restart
file!
temperature        $temp

#####
# Output files
outputName         $../coord/..._namd_preequ_mini_pdb
binaryoutput       no
restartname        $../coord/..._namd_preequ_mini
binaryrestart       yes
restartfreq        10000
restartsave        yes
dcdfile            $../coord/..._namd_preequ_mini.dcd
dcdfreq            100
dcdUnitCell        yes
```

```
#####
# Output configurations
outputEnergies    100                # Freq for printing Energies
outputTiming      100                # Freq for printing Timing info
outputPressure    100                # Freq for printing Pressure

#####
# Periodic Boundary conditions
# NOTE: Do not set the periodic cell basis if you have specified an .xsc restart file!
if {1} {
cellBasisVector1  90.0 0. 0.
cellBasisVector2  0. 90.0 0.
cellBasisVector3  0. 0. 90.0
cellOrigin        0. 0. 0.
}
#xstFile           ${dir}/xsc/${type}_namd_preequ_mini.xst
#xstfreq           100
wrapWater          on
wrapAll            on

#####
# Force-field parameters
cutoff             12.
switching          on
switchdist         10.
pairlistdist       14.
exclude            scaled1-4
1-4scaling         1.0
COMmotion          no
splitPatch         hydrogen
hgroupCutoff       2.5

#####
# Time step parameters
timestep           2.                # 2 fs/step
firsttimestep      0                # Starting time step value
stepspcycle        20               # Freq updating nonbond list

#####
# Multiple time step parameters for long range elec
fullElectFrequency 1                # Calc long range elec stat every time step
nonbondedFreq      1                # Freq for calc short range nonbond interac
MTSAlgorithm        impulse          # Integr method for long range elec
longsplitting       c1              # Method for split elec forces between long and
short

#####
# PME parameters
if {0} {
PME                no
PMETolerance        1.0e-6
PMEInterpOrder      4
PMEGridSizeX        92
}
```

```

PMEGridSizeY      92
PMEGridSizeZ      92
}
#####
# Constant temperature control
if {0} {
  langevin          on
  langevinTemp      $temp
  langevinDamping    2.5
  langevinHydrogen   off
}

#####
# Temperature control for heating or cooling
if {0} {
  reassignFreq      50000
  reassignTemp       10.0
  reassignIncr       30.0
  reassignHold       $temp
}

#####
# Constant pressure control
useGroupPressure    yes
useFlexibleCell      no

useConstantArea      no

if {1} {
  LangevinPiston      on
  LangevinPistonTarget 1.01325
  LangevinPistonPeriod 200
  LangevinPistonDecay 100
  LangevinPistonTemp  $temp
  SurfaceTensionTarget 0.0
}

#####
# Fix hydrogens
if {1} {
  rigidBonds          all
  rigidTolerance       1.0e-8
  rigidIterations       100
  rigidDieOnError       on
  useSettle            off
}

#####
# Harmonic constraints: constraint protein backbone with k=5
if {1} {
  constraints          on
  consexp              2
  consref              ${dir}/cons/${type}_preequ_constr_atoms_ref_5.pdb
  conskfile            ${dir}/cons/${type}_preequ_constr_atoms_kfile_5.pdb
}

```

```

conskcol      B
}

#####
# Fixed atoms constraint: fix protein
if {1} {
  fixedAtoms      on
  fixedAtomsForces on
  fixedAtomsFile   ${dir}/cons/${type}_preequ_fix_protein.pdb
  fixedAtomsCol    O
}

#####
# Minimization parameters
if {1} {
  minimization      on
  minTinyStep       1.0e-8
  minBabyStep       1.0e-2
  minLineGoal       1.0e-4
}

#####
# Extra parameters
#####
# Put here any custom parameters that are specific to this job

# Execution script
#####
#-----#
minimize      10000                      # Minimization step
#-----#
#-----#
#run          10000                      # Dynamics step
#-----#
#####
# End of script
#####

```

A sample script of production run configuration file

```
#####
# FILE: krwtgtp_ralgds_namd_preequ_dyna.cnfg
# 100ps dynamics and equilibration with protein fix
#####

#####
# Set variable values
#####
set temp      310
set dir       /data/omurme/krwtgtp_ralgds
set type      krwtgtp_ralgds

#####
# Parameters
paraTypeCharmm on
parameters    /data/omurme/charmm_input/par_all36_ras_c39b2_hbj.inp

#####
# Input files

structure     ${dir}/psf/${type}_namd_salt_xplor.psf  ;# Name of structure file
coordinates   ${dir}/coor/${type}_namd_salt_charmm.pdb ;# Name of input pdb file

#####
# Restart files

# NOTE: Do not set the initial restart velocity if you have also specified temperature!
if {1} {
#velocities      ${dir}/vel/          ;# Initial velocities in pdb file
#binVelocities   ${dir}/vel/          ;# Initial velocities in binary format
# Remove the "temperature" entry if you use this!
binCoordinates  ${dir}/coor/${type}_namd_preequ_mini.10000.coor ;# Initial coor in
binary format
extendedSystem  ${dir}/xsc/${type}_namd_preequ_mini_pdb.xsc    ;# Input periodic cell
para read from
}

#####
# Simulation temperature
#####
# NOTE: Do not set the initial velocity temperature if you have also specified a .vel restart
file!
temperature     $temp

#####
# Output files

outputName      ${dir}/coor/${type}_namd_preequ_dyna_pdb  ;# Name of final
output coor file
binaryoutput    no          ;# Save final coor in binary format
restartname     ${dir}/coor/${type}_namd_preequ_dyna      ;# Name of output(restart)
coor & vel file
binaryrestart   yes          ;# Save restart file in binary format
```

```

restartfreq      50000                ;# Freq saving coor & vel (1000 steps = every 1ps)
restartsave      yes                  ;# Append time step at end of file name
dcdfile          ${dir}/trj/${type}_namd_preequ_dyna.dcd ;# Name of trajectory file
dcdfreq          1000                ;# Freq saving trajectory
dcdUnitCell      y                    ;# Write unit cell data to dcd file
#velcdcdfile     ${dir}/vel/${type}_namd_preequ_dyna_vel.dcd ;# Name of vel
trajectory file
#velcdcfreq      1000                # Freq saving vel trajectory

#####
# Output configurations

outputEnergies   1000                ;# Freq for printing Energies
outputTiming     1000                ;# Freq for printing Timing info
outputPressure    1000                ;# Freq for printing Pressure

#####
# Periodic Boundary conditions

# NOTE: Do not set the periodic cell basis if you have specified an .xsc restart file!
if {0} {
  cellBasisVector1 90.0 0. 0.
  cellBasisVector2 0. 90.0 0.
  cellBasisVector3 0. 0. 90.0
  cellOrigin        0. 0. 0.
}
#xstFile          ${dir}/xsc/${type}_namd_preequ_dyna.xst ;# Name of cell param
trajectory file
#xstfreq          1000                ;# Freq for writing cell param trajectory
wrapWater         on                  ;# Wrap water around periodic boundary
wrapAll           on                  ;# Wrap all atoms around periodic boundary

#####
# Force-field parameters

cutoff            12.                 ;# Local interaction dist to elec & VdW
calculation
switching         on                  ;# Smoothing func for cutoff, otherwise
truncated
switchdist        10.                 ;# Should be less than or equal to cutoff value
pairlistdist      14.                 ;# Cutoff for generating pair list, larger than
cutoff
exclude           scaled1-4           ;# Exclude pairs in nonbonding interactions
1-4scaling        1.0                 ;# Scaling factor for 1-4 interaction
COMmotion         no                  ;# No center of mass motion for entire system
splitPatch        hydro               ;# Set hydrogen to allow faster distance check
hgroupCutoff      ;# Use for group-based distance testing

#####
# Time step parameters

timestep          2.                  ;# 2 fs/step
firsttimestep      0                  ;# Starting time step value
stepspercycle      20                 ;# Freq updating nonbond list

```

```
#####
# Multiple time step parameters for long range elec

fullElectFrequency 1                ;# Calc long range elec stat every time step
nonbondedFreq      1                ;# Freq for calc short range nonbond interac
MTSAlgorithm        impulse          ;# Integr method for long range elec
longsplitting      c1                ;# Method for split elec forces between long
and short

#####
# PME parameters

if {0} {
PME                no                ;# Use particle mesh Ewald for electrostatics
PMETolerance       1.0e-6            ;# Affect value of Ewald coefficient
PMEInterpOrder     4                 ;# For cubic 4
PMEGridSizeX       92                ;# Number of grid ponts in x
PMEGridSizeY       92                ;# Number of grid ponts in y
PMEGridSizeZ       92                ;# Number of grid ponts in z
}

#####
# Constant temperature control

if {1} {
langevin           on                ;# Use langeving dynam for temp control
langevinTemp       $temp
langevinDamping     2.5              ;# Friction coeff (gamma) (1/ps), use a
small value
langevinHydrogen    off              ;# Don't couple langevin bath to hydrogens
}

#####
# Temperature control for heating or cooling          #
#####
if {0} {
reassignFreq       50000             ;# Frequency to reassign temperature
reassignTemp        10.0              ;# Initial temperature
reassignIncr        30.0              ;# Temperature increment or decrement
reassignHold        $temp             ;# Target temperature
}

#####
# Constant pressure control

useGroupPressure    yes                ;# Should use w/SHAKE, needed for 2fs
steps
useFlexibleCell     no                ;# Allows dimensions of cell to fluctuate
;# no for water box, yes for membrane
useConstantArea     no                ;# x-y fixed and z dim can vary
;# no for water box, yes for membrane

if {1} {
LangevinPiston      on                ;# Use Nose-Hoover Langeving Piston
Algorithm
LangevinPistonTarget 1.01325          ;# Target pressure to run simu at (bars)
}
```

```

LangevinPistonPeriod 200           ;# Oscillation period
LangevinPistonDecay 100            ;# Damping time scale
LangevinPistonTemp $temp           ;# Should be set equal to the target temperature
SurfaceTensionTarget 0.0           ;# Apply surface tension in xy plane (dyn/cm)
}

#####
# Fix hydrogens

if {1} {
  rigidBonds      all                ;# Fix h-bonds to none, water, or all h-atoms
  rigidTolerance  1.0e-8             ;# Allowable bond-length error for ShakeH
  rigidIterations 100                ;# Maximum ShakeH iterations
  rigidDieOnError  on                ;# Exit and report an error
  useSettle       off               ;# Keep waters rigid
}

#####
# Harmonic constraints: constraint protein backbone with k=5

if {1} {
  constraints      on                ;# Turn on the use of constraints
  consexp          2                ;# Exponent to be use in harmonic constraint
  energy
  consref          ${dir}/cons/${type}_preequ_constr_atoms_ref_5.pdb ;# A pdb file to use for
  reference positions
  conskfile        ${dir}/cons/${type}_preequ_constr_atoms_kfile_5.pdb ;# A pdb file to use
  for k values
  conskcol         B                ;# Column of pdb file to use for harmonic
  constraint
}

#####
# Fixed atoms constraint: fix protein

if {1} {
  fixedAtoms      on                ;# Turn on the use of constraints
  fixedAtomsForces on                ;# Calculate forces between fixed atoms
  fixedAtomsFile   ${dir}/cons/${type}_preequ_fix_protein.pdb ;# A pdb file to use
  for reference positions
  fixedAtomsCol    O                ;# Set O to read occupancy column in pdb file
}

#####
# Minimization parameters

if {0} {
  minimization     on                ;# Perform conjugate gradient energy minimization
  minTinyStep      1.0e-8            ;# First initial step for line minimizer
  minBabyStep      1.0e-2            ;# Max initial step for line minimizer
  minLineGoal      1.0e-4            ;# Gradient reduction factor for line minimizer
}

```

```
#####  
# Extra parameters  
#####  
# Put here any custom parameters that are specific to this job  
  
#####  
# Execution script  
#####  
#-----#  
#minimize      5000                # Minimization step  
#-----#  
#-----#  
run            50000                ;# Dynamics step  
#-----#  
#####  
# End of script  
#####
```

A sample script of MM-PBSA analysis

```

* File: mmgbbsa_ras1.inp
* Use protein only coordinates
*

!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!
set type krwtgtp_ralgds
set dir data/omurme/analysis/@type_analysis
set sdir data/omurme/@type

!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!
set tmole dimer ! Set file name for complex, protein, or ligand
!set tmole mono1
!set tmole mono2
!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!

bomlev -2

! read topology
open read card unit 10 name /data/omurme/charmm_input/top_all36_ras_c39b2_hbj.inp
read rtf card unit 10
close unit 10

! read parameters
open read card unit 10 name /data/omurme/charmm_input/par_all36_ras_c39b2_hbj.inp
read param card unit 10
close unit 10

! read new psf file
open read card unit 10 name /@dir/psf/protein_only_@tmole.psf
read psf card unit 10
close unit 10

!!!!!!!!!!!!!!!!!!!!!!

! make dat files

open write card unit 11 name /@dir/dat/@type_mmgbbsa_@tmole.dat
write title unit 11
*mm-gbsa analysis
*time dG Hene Hint Hext SolvationE

!!!!!!!!!!!!!!!!!!!!!!

! Read trj

set time 0.1
set ifile 100
set inunt 80

label loop_file

! each 100 ns trajectory - merged 1000 frames

```

```

open read uniform unit @inunt name /@dir/trj/@type_mmgbsa_trj_@tmole_@ifile_ns.dcd

set j 1
label loop_trj

    if j eq 1 read coor file unit @inunt ifile 1
    if j gt 1 read coor file unit @inunt ifile -1

    coor orient norot ! center coordinates

    ! Internal energy in vacuum

    energy cutnb 999.0 ctofnb 997.0 ctonnb 995.0 ctexnb 999.0

    set HTOT ?ener
    calc HEXT = ?elec + ?vdw
    calc HINT = ?ener - ?elec - ?vdw

    ! Nonpolar contrubution of sol free ene using surface area,
    ! HGNP = (gamma1*Area + beta), where gamma1 = 0.00542, beta = 0.92
    coor surf rprobe 1.4
    calc HGNP = 0.00542 * ?area + 0.92

    ! GB calculations
    ! Set nonbonded interactions
    NBOND atom switch cdie vdw vswitch -
    ctonnb 100.000 ctofnb 110.000 cutnb 120.000

    ! Read optimized PB radii
    stream /data/omurme/charmm_input/radius_gbsw.str

    ! Fill proper set of radii
    scalar wmain statistics select .not. type H* end
    define check select (.not. type H* ) .and. ( prop wmain .eq. 0.0 ) show end
    if ?nsel ne 0 stop !some heavy atom have a zero radius

    ! Request GBSW implicit membrane
    GBSW sw 0.3 sgamma 0.003 nang 50 dgp 1.5 GBenergy TEMP 310 CONC 0.1

    ! Invoke energy
    ENERGY switch vswitch cdie ctonnb 100.000 ctofnb 110.000 cutnb 120.000

    calc PBEQ = ?ener - ?vdw - ?elec - ?bond - ?angl - ?urey - ?dihe - ?impr - ?cmap -
?asp
    calc SOLV = @PBEQ + @HGNP

    !
    calc SOLV = ?ener - ?vdw - ?elec - ?bond - ?angl - ?urey - ?dihe - ?impr - ?cmap

    GBSW reset

    ! Free energy at each frame

    calc DGI = @HTOT + @SOLV

    write title unit 11

```

```
* @time @DGI @HTOT @HINT @HEXT @SOLV @PBEQ @HGNP  
*
```

```
incr time by 0.1  
incr j by 1  
if j le 1000 goto loop_trj
```

```
incr inunt by 1  
incr ifile by 100  
if ifile le 300 goto loop_file
```

```
!!!!!!!!!!!!!!!!!!  
stop
```



A sample script of entropy calculations

```

* File: entropy_dimer.inp
* Quasiharmonic dynamics over CA trajectories
* Calculate entropy change, delta S
*

!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!
set type krwtgtp_ralgds
set dir data/omurme/analysis/@type_analysis
set sdir data/omurme/@type
!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!
set tmole dimer ! Set file name for complex, protein, or ligand
!set tmole mono1
!set tmole mono2
!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!

bomlev -2

! read topology
open read card unit 10 name /data/omurme/charmm_input/top_all36_ras_c39b2_hbj.inp
read rtf card unit 10
close unit 10

! read parameters
open read card unit 10 name /data/omurme/charmm_input/par_all36_ras_c39b2_hbj.inp
read param card unit 10
close unit 10

! read psf file, merged coor
open read card unit 10 name /@dir/psf/@type_trj_ca_@tmole.psf
read psf card unit 10
close unit 10

!!!!!!!!!!!!!!!!!!!!!!

! Open average coordinate positions as a reference

open read card unit 10 name /@dir/coor/@type_ave_crd_ca_@tmole.crd
read coor card unit 10
close unit 10

coor copy comp

!!!!!!!!!!!!!!!!!!!!!!

! make dat files

open write card unit 9 name /@dir/dat/@type_entropy_ca_@tmole.dat
write title unit 9
*Entropy
*tra rot vib stot
*

!!!!!!!!!!!!!!!!!!!!!!

```

```

! Read coordinates from merged single trajectory

open read uniform unit 50 name /@dir/trj/@type_combine_trj_ca_@tmole.dcd

!!!!!!!!!!!!!!!!!!!!

! Average coordinate positions are used as a reference
coor dyna firstu 50 nunit 1 -
  orie mass sele all end sele all end

! Remove translation and rotation motions
!coor orient rms mass

! Calculate translational and rotational entropie in [cal/(mol*T)], be careful the unit!
Coor inertia entropy temp 310
set dtra ?STRA
set drot ?SROT

! Calculate number of vectors to use for vibrational analysis
coor stat sele all end
set nmod ?natom
calc nmod = @nmod * 3

! Quasiharmonic mode calculation to evaluate vibrational entropy in [kcal/(mol*T)], be
careful the unit!
vibran nmod @nmod
  quasi first 50 nunit 1 temp 310
  print norm
  thermo temp 310 step 10
  set dvib ?stot
end

! Convert the unit in [kcal/(mol*T)]
calc dtra = @dtra/1000.0
calc drot = @drot/1000.0
calc dsum = @dtra + @drot + @dvib

write title unit 9
* @dtra @drot @dvib @dsum
*

! Calculate -TdeltaS
calc tdtra = -310.0 * @dtra
calc tdrot = -310.0 * @drot
calc tdvib = -310.0 * @dvib
calc tdsun = -310.0 * @dsum

write title unit 9
* @tdtra @tdrot @tdvib @tdsum
*

!!!!!!!!!!!!!!!!!!!!
Stop

```

BIBLIOGRAPHY

- [1] A. L. Tetlow and F. Tamanoi, “The ras superfamily g-proteins,” in *Enzymes*, 2013.
- [2] C. V. Dang, E. P. Reddy, K. M. Shokat, and L. Soucek, “Drugging the ‘undruggable’ cancer targets,” *Nature Reviews Cancer*. 2017.
- [3] J. M. Shields, K. Pruitt, A. McFall, A. Shaub, and C. J. Der, “Understanding Ras: ‘It ain’t over ‘til it’s over,’” *Trends in Cell Biology*. 2000.
- [4] I. A. Prior, P. D. Lewis, and C. Mattos, “A comprehensive survey of ras mutations in cancer,” *Cancer Research*. 2012.
- [5] A. González-García, C. A. Pritchard, H. F. Paterson, G. Mavria, G. Stamp, and C. J. Marshall, “RalGDS is required for tumor formation in a model of skin carcinogenesis,” *Cancer Cell*, vol. 7, no. 3, pp. 219–226, 2005.
- [6] D. J. Reiner and E. A. Lundquist, “Small GTPases,” *Encycl. Biol. Chem. Second Ed.*, 2016.
- [7] S. A. Forbes *et al.*, “COSMIC: Mining complete cancer genomes in the catalogue of somatic mutations in cancer,” *Nucleic Acids Res.*, 2011.
- [8] J. Downward, “New exchange, new target,” *Nature*. 1998.
- [9] N. F. Neel, T. D. Martin, J. K. Stratford, T. P. Zand, D. J. Reiner, and C. J. Der, “The RalGEF-ral effector signaling network: The road less traveled for anti-ras drug discovery,” *Genes and Cancer*. 2011.
- [10] G. Vandal, B. Geiling, and D. Dankort, “Ras effector mutant expression suggest a negative regulator inhibits lung tumor formation,” *PLoS One*, 2014.
- [11] K. Wennerberg, “The Ras superfamily at a glance,” *J. Cell Sci.*, vol. 118, no. 5, pp. 843–846, 2005.
- [12] T. J. P. van dam, J. L. Bos, and B. Snel, “Evolution of the Ras-like small GTPases and their regulators,” *Small GTPases*, 2011.
- [13] J. Colicelli, “Human RAS Superfamily Proteins and Related GTPases,” *Sci. Signal.*, 2004.
- [14] A. E. Karnoub and R. A. Weinberg, “Ras oncogenes: Split personalities,” *Nature Reviews Molecular Cell Biology*. 2008.
- [15] B. E. Hall, D. Bar-Sagi, and N. Nassar, “The structural basis for the transition

- from Ras-GTP to Ras-GDP,” *Proc. Natl. Acad. Sci.*, 2002.
- [16] J. L. Bos, H. Rehmann, and A. Wittinghofer, “GEFs and GAPs: Critical Elements in the Control of Small G Proteins,” *Cell*. 2007.
- [17] G. Aaron Hobbs *et al.*, “NIH Public Access,” *Proc. Natl. Acad. Sci. U. S. A.*, 2012.
- [18] P. A. Boriack-Sjodin, S. M. Margarit, D. Bar-Sagi, and J. Kuriyan, “The structural basis of the activation of Ras by Sos,” *Nature*, 1998.
- [19] G. W. Reuther and C. J. Der, “The Ras branch of small GTPases: Ras family members don’t fall far from the tree,” *Current Opinion in Cell Biology*. 2000.
- [20] D. Vigil, J. Cherfils, K. L. Rossman, and C. J. Der, “Ras superfamily GEFs and GAPs: Validated and tractable targets for cancer therapy?,” *Nature Reviews Cancer*. 2010.
- [21] B. E. Hall, D. Bar-Sagi, and N. Nassar, “The structural basis for the transition from Ras-GTP to Ras-GDP,” *Proc. Natl. Acad. Sci.*, 2002.
- [22] K. Zenonos, “RAS signaling pathways, mutations and their role in colorectal cancer,” *World J. Gastrointest. Oncol.*, 2013.
- [23] K. Rajalingam, R. Schreck, U. R. Rapp, and Š. Albert, “Ras oncogenes and their downstream targets,” *Biochimica et Biophysica Acta - Molecular Cell Research*. 2007.
- [24] G. A. Hobbs, C. J. Der, and K. L. Rossman, “RAS isoforms and mutations in cancer at a glance,” *J. Cell Sci.*, 2016.
- [25] A. Rangarajan, S. J. Hong, A. Gifford, and R. A. Weinberg, “Species- and cell type-specific requirements for cellular transformation,” *Cancer Cell*, 2004.
- [26] J. L. Bos, “All in the family? New insights and questions regarding interconnectivity of Ras, Rap1 and Ral,” *EMBO J.*, vol. 17, no. 23, pp. 6776–6782, 1998.
- [27] R. M. Wolthuis and J. L. Bos, “Ras caught in another affair: The exchange factors for Ral,” *Curr. Opin. Genet. Dev.*, 1999.
- [28] L. R. Gentry, T. D. Martin, D. J. Reiner, and C. J. Der, “Ral small GTPase signaling and oncogenesis: More than just 15 minutes of fame,” *Biochim. Biophys. Acta - Mol. Cell Res.*, vol. 1843, no. 12, pp. 2976–2988, 2014.
- [29] F. J. T. Zwartkruis, R. M. F. Wolthuis, N. M. J. M. Nabben, B. Franke, and J. L. Bos, “Extracellular signal-regulated activation of Rap1 fails to interfere in Ras effector signalling,” *EMBO J.*, 1998.

- [30] J. L. Meinkoth *et al.*, “Signal transduction through the cAMP-dependent protein kinase,” *Mol. Cell. Biochem.*, 1993.
- [31] E. P. Brandon, R. L. Idzerda, and G. S. McKnight, “PKA isoforms, neural pathways, and behaviour: Making the connection,” *Curr. Opin. Neurobiol.*, 1997.
- [32] D. L. Altschuler, S. N. Peterson, M. C. Ostrowski, and E. G. Lapetina, “Cyclic AMP-dependent activation of Rap1b,” *J. Biol. Chem.*, 1995.
- [33] J. De Rooij *et al.*, “Epac is a Rap1 guanine-nucleotide-exchange factor directly activated by cyclic AMP,” *Nature*, 1998.
- [34] H. Kawasaki *et al.*, “A Rap guanine nucleotide exchange factor enriched highly in the basal ganglia,” *Proc. Natl. Acad. Sci.*, 2002.
- [35] B. Franke, J. W. N. Akkerman, and J. L. Bos, “Rapid Ca²⁺-mediated activation of Rap1 in human platelets,” *EMBO J.*, 1997.
- [36] K. Matsubara, S. Kishida, Y. Matsuura, H. Kitayama, M. Noda, and A. Kikuchi, “Plasma membrane recruitment of RalGDS is critical for Ras-dependent Ral activation,” *Oncogene*, 1999.
- [37] S. Kishida, S. Koyama, K. Matsubara, M. Kishida, Y. Matsuura, and A. Kikuchi, “Colocalization of Ras and Ral on the membrane is required for Ras-dependent Ral activation through Ral GDP dissociation stimulator,” *Oncogene*, 1997.
- [38] H. Koide, T. Satoh, M. Nakafuku, and Y. Kaziro, “GTP-dependent association of Raf-1 with Ha-Ras: identification of Raf as a target downstream of Ras in mammalian cells,” *Proc. Natl. Acad. Sci.*, 2006.
- [39] M. J. Smith and M. Ikura, “Integrated RAS signaling defined by parallel NMR detection of effectors and regulators,” *Nat. Chem. Biol.*, 2014.
- [40] I. R. Vetter *et al.*, “Structural and biochemical analysis of Ras-effector signaling via RalGDS,” *FEBS Lett.*, vol. 451, no. 2, pp. 175–180, 1999.
- [41] H. Shao and D. A. Andres, “A novel RalGEF-like protein, RGL3, as a candidate effector for Rit and Ras,” *J. Biol. Chem.*, 2000.
- [42] R. Wolthuis *et al.*, “RalGDS-like factor (Rlf) is a novel Ras and Rap 1A-associating protein,” *Oncogene*, 1996.
- [43] E. Ferro and L. Trabalzini, “RalGDS family members couple Ras to Ral signalling and that’s not all,” *Cellular Signalling*. 2010.
- [44] A. Kikuchi, S. D. Demo, Z. H. Ye, Y. W. Chen, and L. T. Williams, “ralGDS family members interact with the effector loop of ras p21,” *Mol. Cell. Biol.*, 2015.

- [45] X. Tian, G. Rusanescu, W. Hou, B. Schaffhausen, and L. A. Feig, "PDK1 mediates growth factor-induced Ral-GEF activation by a kinase-independent mechanism," *EMBO J.*, 2002.
- [46] L. E. Rameh and L. C. Cantley, "The role of phosphoinositide 3-kinase lipid products in cell function," *Journal of Biological Chemistry*. 1999.
- [47] K. L. Wang, M. T. Khan, and B. D. Roufogalis, "Identification and characterization of a Calmodulin-binding Domain in Ral-A, a Ras-related GTP-binding protein purified from human erythrocyte membrane," *J. Biol. Chem.*, 1997.
- [48] R. R. Clough, R. S. Sidhu, and R. P. Bhullar, "Calmodulin Binds RalA and RalB and is Required for the Thrombin-Induced Activation of Ral in Human Platelets," *J. Biol. Chem.*, 2002.
- [49] F. Hofer, R. Berdeaux, and G. Steven Martin, "Ras-independent activation of Ral by a Ca²⁺-dependent pathway," *Curr. Biol.*, vol. 8, no. 14, pp. 839–844, 2004.
- [50] M. G. Rondaij *et al.*, "Guanine exchange factor RalGDS mediates exocytosis of Weibel-Palade bodies from endothelial cells," *Blood*, 2008.
- [51] R. M. F. Wolthuis *et al.*, "Activation of the Small GTPase Ral in Platelets," *Mol. Cell. Biol.*, 2015.
- [52] R. Shirakawa and H. Horiuchi, "Ral GTPases: Crucial mediators of exocytosis and tumorigenesis," *J. Biochem.*, 2015.
- [53] A. Brymora, V. A. Valova, M. R. Larsen, B. D. Roufogalis, and P. J. Robinson, "The brain exocyst complex interacts with RalA in a GTP-dependent manner. Identification of a novel mammalian Sec3 gene and a second Sec15 gene," *J. Biol. Chem.*, 2001.
- [54] K. Sugihara, S. Asano, K. Tanaka, A. Iwamatsu, K. Okawa, and Y. Ohta, "The exocyst complex binds the small GTPase RalA to mediate filopodia formation," *Nat. Cell Biol.*, 2002.
- [55] S. Fukai, H. T. Matern, J. R. Jagath, R. H. Scheller, and A. T. Brunger, "Structural basis of the interaction between RalA and Sec5, a subunit of the sec6/8 complex," *EMBO J.*, 2003.
- [56] R. Jin, J. R. Junutula, H. T. Matern, K. E. Ervin, R. H. Scheller, and A. T. Brunger, "Exo84 and Sec5 are competitive regulatory Sec6/8 effectors to the RalA GTPase," *EMBO J.*, vol. 24, no. 12, pp. 2064–2074, 2005.
- [57] S. Moskalenko *et al.*, "Ral GTPases Regulate Exocyst Assembly through Dual

- Subunit Interactions,” *J. Biol. Chem.*, 2003.
- [58] D. A. Barbie *et al.*, “Systematic RNA interference reveals that oncogenic KRAS-driven cancers require TBK1,” *Nature*, 2009.
- [59] L. Seguin *et al.*, “An integrin β 3-KRAS-RalB complex drives tumour stemness and resistance to EGFR inhibition,” *Nat. Cell Biol.*, 2014.
- [60] J. H. Camonis and M. A. White, “Ral GTPases: Corrupting the exocyst in cancer cells,” *Trends in Cell Biology*. 2005.
- [61] S. B. Cantor, T. Urano, and L. A. Feig, “Identification and characterization of Ral-binding protein 1, a potential downstream target of Ral GTPases,” *Mol. Cell. Biol.*, 1995.
- [62] V. Jullien-Flores *et al.*, “Bridging Ral GTPase to Rho pathways: RLIP76, a Ral effector with CDC42/Rac GTPase-activating protein activity,” *J. Biol. Chem.*, 1995.
- [63] D. F. Kashatus, K. H. Lim, D. C. Brady, N. L. K. Pershing, A. D. Cox, and C. M. Counter, “RALA and RALBP1 regulate mitochondrial fission at mitosis,” *Nat. Cell Biol.*, 2011.
- [64] B. G. Coon, J. Burgner, J. H. Camonis, and R. C. Aguilar, “The Epsin family of endocytic adaptors promotes fibrosarcoma migration and invasion,” *J. Biol. Chem.*, 2010.
- [65] Z. Wu, C. Owens, N. Chandra, K. Popovic, M. Conaway, and D. Theodorescu, “RalBP1 Is Necessary for Metastasis of Human Cancer Cell Lines,” *Neoplasia*, 2015.
- [66] W. C. Colley *et al.*, “Phospholipase D2, a distinct phospholipase D isoform with novel regulatory properties that provokes cytoskeletal reorganization,” *Curr. Biol.*, 1997.
- [67] D. A. Foster *et al.*, “Phospholipase D Mediates Nutrient Input to Mammalian Target of Rapamycin Complex 1 (mTORC1),” *J. Biol. Chem.*, 2011.
- [68] L. A. Feig *et al.*, “Phospholipase D and RalA Cooperate with the Epidermal Growth Factor Receptor To Transform 3Y1 Rat Fibroblasts,” *Mol. Cell. Biol.*, 2002.
- [69] K. L. Wang and B. D. Roufogalis, “Ca²⁺/calmodulin stimulates GTP binding to the Ras-related protein Ral- A,” *J. Biol. Chem.*, 1999.
- [70] R. S. Sidhu, R. R. Clough, and R. P. Bhullar, “Regulation of phospholipase C- δ 1 through direct interactions with the small GTPase Ral and calmodulin,” *J. Biol.*

- Chem.*, 2005.
- [71] E. M. van Dam and P. J. Robinson, "Ral: Mediator of membrane trafficking," *International Journal of Biochemistry and Cell Biology*. 2006.
- [72] P. Frankel *et al.*, "RalA interacts with ZONAB in a cell density-dependent manner and regulates its transcriptional activity," *EMBO J.*, 2005.
- [73] Y. Ohta, N. Suzuki, S. Nakamura, J. H. Hartwig, and T. P. Stossel, "The small GTPase RalA targets filamin to induce filopodia.," *Proc. Natl. Acad. Sci. U. S. A.*, 1999.
- [74] N. M. Hamad *et al.*, "Distinct requirements for Ras oncogenesis in human versus mouse cells," *Genes Dev.*, 2002.
- [75] W. C. Hahn, C. M. Counter, A. S. Lundberg, R. L. Beijersbergen, M. W. Brooks, and R. A. Weinberg, "Creation of human tumour cells with defined genetic elements," *Nature*, 1999.
- [76] G. Oxford *et al.*, "RalA and RalB: Antagonistic relatives in cancer cell migration," *Cancer Res.*, 2005.
- [77] K. H. Lim *et al.*, "Activation of RalA is critical for Ras-induced tumorigenesis of human cells," *Cancer Cell*, 2005.
- [78] Y. Chien and M. A. White, "RAL GTPases are linchpin modulators of human tumour-cell proliferation and survival," *EMBO Rep.*, 2003.
- [79] L. A. Feig, "Ral-GTPases: Approaching their 15 minutes of fame," *Trends in Cell Biology*. 2003.
- [80] Y. Ward, W. Wang, E. Woodhouse, I. Linnoila, L. Liotta, and K. Kelly, "Signal Pathways Which Promote Invasion and Metastasis: Critical and Distinct Contributions of Extracellular Signal-Regulated Kinase and Ral-Specific Guanine Exchange Factor Pathways," *Mol. Cell. Biol.*, 2002.
- [81] K.-H. Lim *et al.*, "Aurora-A phosphorylates, activates, and relocalizes the small GTPase RalA," *Mol Cell Biol*, 2010.
- [82] A. A. Sablina *et al.*, "The Tumor Suppressor PP2A A β Regulates the RalA GTPase," *Cell*, 2007.
- [83] J. Yin *et al.*, "Activation of the RalGEF/Ral Pathway Promotes Prostate Cancer Metastasis to Bone," *Mol. Cell. Biol.*, vol. 27, no. 21, pp. 7538–7550, 2007.
- [84] T. D. Martin, J. C. Samuel, E. D. Routh, C. J. Der, and J. J. Yeh, "Activation and involvement of Ral GTPases in colorectal cancer," *Cancer Res.*, 2011.
- [85] P. A. Zipfel, D. C. Brady, D. F. Kashatus, B. D. Ancrile, D. S. Tyler, and C. M.

- Counter, "Ral activation promotes melanomagenesis," *Oncogene*, 2010.
- [86] A. G. Sowalsky, A. Alt-Holland, Y. Shamis, J. A. Garlick, and L. A. Feig, "RalA suppresses early stages of Ras-induced squamous cell carcinoma progression," *Oncogene*, 2010.
- [87] D. Vigil, T. D. Martin, F. Williams, J. Jen Yeh, S. L. Campbell, and C. J. Der, "Aberrant overexpression of the Rgl2 Ral small GTPase-specific guanine nucleotide exchange factor promotes pancreatic cancer growth through Ral-dependent and Ral-independent mechanisms," *J. Biol. Chem.*, 2010.
- [88] M. A. White, T. Vale, J. H. Camonis, E. Schaefer, and M. H. Wigler, "A role for the Ral guanine nucleotide dissociation stimulator in mediating Ras-induced transformation," *J. Biol. Chem.*, 1996.
- [89] K. Tomczak, P. Czerwińska, and M. Wiznerowicz, "The Cancer Genome Atlas (TCGA): An immeasurable source of knowledge," *Współczesna Onkologia*. 2015.
- [90] J. Zhang *et al.*, "International cancer genome consortium data portal-a one-stop shop for cancer genomics data," *Database*, 2011.
- [91] A. G. Stephen, D. Esposito, R. G. Bagni, and F. McCormick, "Dragging ras back in the ring," *Cancer Cell*. 2014.
- [92] C. Herrmann, G. Horn, M. Spaargaren, and A. Wittinghofer, "Differential interaction of the Ras family GTP-binding proteins H-Ras, Rap1A, and R-Ras with the putative effector molecules Raf kinase and Ral-Guanine nucleotide exchange factor," *J. Biol. Chem.*, 1996.
- [93] D. F. Kashatus, "The Many Roles of Ral GTPases in Ras-Driven Cancer," in *Conquering RAS: From Biology to Cancer Therapy*, 2016.
- [94] X. Li, R. Zhang, K. M. Draheim, W. Liu, D. A. Calderwood, and T. J. Boggon, "Structural basis for small G protein effector interaction of ras-related protein 1 (Rap1) and adaptor protein krev interaction trapped 1 (KRIT1)," *J. Biol. Chem.*, 2012.
- [95] B. R. Brooks, R. E. Bruccoleri, B. D. Olafson, D. J. States, S. Swaminathan, and M. Karplus, "CHARMM: A program for macromolecular energy, minimization, and dynamics calculations," *J. Comput. Chem.*, 1983.
- [96] A. D. and Brooks, B. R. and Brooks, III, C. L. and Mackerell, Jr. *et al.*, "CHARMM: The Biomolecular Simulation Program," *J. Comput. Chem.*, 2009.
- [97] S. R. Durell, B. R. Brooks, and A. Ben-Naim, "Solvent-induced forces between

- two hydrophilic groups,” *J. Phys. Chem.*, 1994.
- [98] T. Darden, D. York, and L. Pedersen, “Particle mesh Ewald: An $N \cdot \log(N)$ method for Ewald sums in large systems,” *J. Chem. Phys.*, 1993.
- [99] James C. Phillips *et al.*, “Scalable molecular dynamics with NAMD,” *J. Comput. Chem.*, 2005.
- [100] W. Humphrey, A. Dalke, and K. Schulten, “VMD: Visual molecular dynamics,” *J. Mol. Graph.*, 1996.
- [101] V. Zoete, M. Meuwly, and M. Karplus, “Study of the insulin dimerization: Binding free energy calculations and per-residue free energy decomposition,” *Proteins Struct. Funct. Genet.*, 2005.
- [102] W. Wang and P. A. Kollman, “Free energy calculations on dimer stability of the HIV protease using molecular dynamics and a continuum solvent model,” *J. Mol. Biol.*, 2000.
- [103] R. M. Levy, L. Y. Zhang, E. Gallicchio, and A. K. Felts, “On the nonpolar hydration free energy of proteins: Surface area and continuum solvent models for the solute-solvent interaction energy,” *J. Am. Chem. Soc.*, 2003.
- [104] M. R. Lee, Y. Duan, and P. A. Kollman, “Use of MM-PB/SA in estimating the free energies of proteins: Application to native, intermediates, and unfolded villin headpiece,” *Proteins Struct. Funct. Genet.*, 2000.
- [105] F. E. Cohen and M. J. E. Sternberg, “On the prediction of protein structure: The significance of the root-mean-square deviation,” *J. Mol. Biol.*, 1980.
- [106] T. Linnemann, C. Kiel, P. Herter, and C. Herrmann, “The activation of RalGDS can be achieved independently of its Ras binding domain: Implications of an activation mechanism in Ras effector specificity and signal distribution,” *J. Biol. Chem.*, 2002.
- [107] A. D. Cox, S. W. Fesik, A. C. Kimmelman, J. Luo, and C. J. Der, “Drugging the undruggable RAS: Mission Possible?,” *Nature Reviews Drug Discovery*. 2014.
- [108] N. T. Ihle *et al.*, “Effect of KRAS oncogene substitutions on protein behavior: Implications for signaling and clinical outcome,” *J. Natl. Cancer Inst.*, 2012.
- [109] P. A. Madureira *et al.*, “Cell surface protease activation during RAS transformation: Critical role of the plasminogen receptor, S100A10,” *Oncotarget*, 2016.
- [110] D. Esser, B. Bauer, R. M. F. Wolthuis, A. Wittinghofer, R. H. Cool, and P. Bayer, “Structure determination of the ras-binding domain of the Ral-specific

- guanine nucleotide exchange factor Rlf,” *Biochemistry*, 1998.
- [111] H. Nakhaeizadeh, E. Amin, S. Nakhaei-Rad, R. Dvorsky, and M. R. Ahmadian, “The RAS-effector interface: Isoform-specific differences in the effector binding regions,” *PLoS One*, 2016.
- [112] N. Nassar, G. Horn, C. Herrmann, C. Block, R. Janknecht, and A. Wittinghofer, “Ras/Rap effector specificity determined by charge reversal,” *Nat. Struct. Biol.*, 1996.
- [113] H. Kitayama, Y. Sugimoto, T. Matsuzaki, Y. Ikawa, and M. Noda, “A ras-related gene with transformation suppressor activity,” *Cell*, 1989.

