'IN SILICO ANALYSIS OF MOLECULAR EVOLUTION OF TRPV5 AND TRPV6'

Dissertation submitted in partial fulfillment for the degree of

Master of Science in Applied Microbiology

Submitted By

'SWETA AGARWAL'

School of Biotechnology (Campus 11)

KIIT

Deemed to be University

Bhubaneswar, Odisha, India

Under the Supervision of

Dr. Chandan Goswami

Associate Professor

School of Biological Science

NISER

Jatni , Khurda

Dr. Chandan Goswami
Associate Professor
School of Biological Sciences, NISER
Ph : (0674) 2494132
e-mail : chandan@niser.ac.in

CERTIFICATE

This is to certify the dissertation entitled **"*In Silico* analysis of Molecular Evolution of TRPV5 and TRPV6"** submitted by 'Sweta Agarwal' in partial fulfilment of the requirement for the degree of Master of Science in Applied Microbiology, KIIT School of Biotechnology, KIIT University, Bhubaneswar bearing Roll No. '16643351421' & Registration No. '16643351421' is a *bona fide* research work carried out by her under my guidance and supervision from '8th January 2018' to '13th May 2018'.

14/5/18

Chandan Goswami (Ph.D)
Supervisor
School of Biological Sciences
National Institute of Science Education and Research Bhubaneswar
P.O. Jatni, Khurda 752050, Odisha, India

Dr. Chandan Goswami
Associate Professor
School of Biological Sciences, NISER
Ph : (0674) 2494132
e-mail : chandan@niser.ac.in

CERTIFICATE

This is to certify that the dissertation entitled **"*In Silico* analysis of Molecular Evolution of TRPV5 and** "TRPV6" submitted by "Sweta Agarwal, 1662020, 16643351421" to the School of Biotechnology, KIIT University, Bhubaneswar-751024, for the degree of Master of Science in Applied Microbiology is her original work, based on the results of the experiments and investigations carried out independently by her during the period from "8th January 2018' to '13th May 2018' of study under my guidance.

Further, it is also to certify that the above said work has not been previously submitted for the award of any degree, diploma, or fellowship in any Indian or foreign University.

Date:

Place:

(*Supervisor name*) 14/5/18

Chandan Goswami (Ph.D)
Associate Professor
School of Biological Sciences
National Institute of Science Education and Research Bhubaneswar
P.O. Jatni, Khurda 752050, Odisha, India

DECLARATION

I hereby declare that the dissertation entitled **"*In Silico analysis of Molecular Evolution of TRPV5 and TRPV6*"** submitted by me, for the degree of Master of Science to KIIT Deemed to be University is a record of *bonafide* work carried by me under the supervision and guidance of ***'Dr, Chandan Goswami, Associate Professor, School of Biological Science, NISER'.***

Date: 14/05/2018

Place: Bhubaneswar

Signature

1. ABSTRACT

The Transient Receptor Potential Channels (TRP) are non selective cation channels, composed of six transmembrane helices along with intra and extracellular loops. Also, both the N- terminal and C- terminal regions are projected towards the cytosolic side. The subfamily of TRPV ion channels consists of six members, i.e. TRPV1 to TRPV6, which are activated and modulated by many endogenous molecules. These channels have been reported to be present in Yeast to the higher animals, but not in plants, and are expressed in diverse tissues and cells, performing several important functions.

In this study, we have compared the TRPV5 and TRPV6 sequences from different species available in the public data bank and analyzed the degree of conservation in details. Using statistical tools and other *in silico* methods, we analyzed the respective conservation and changes in different structural and functional motifs present in TRPV5 and TRPV6 throughout evolution, keeping human sequence as the sole reference. For example, conservation pattern of different transmembrane and cytoplasmic domains. Conservation pattern of amino acids present in Lipid Water Interface was also performed. This study indicates that different motifs and domains of TRPV5 and TRPV6 have undergone different selection pressure. In addition, we tried to characterize the plausible cholesterol sensing motifs like CRAC, CARC, CCM and SPHINGOLIPID BINDING motif in these channels and also the binding of different steroid hormones with the help of docking.

ACKNOWLEDGEMENT

I would like to express my pleasure in bringing my project entitled **"*In Silico analysis of Molecular Evolution of TRPV5 and TRPV6*"** and would like to acknowledge and thank to important peoples who have supported me throughout my dissertation period.

First of all , I am very grateful to my supervisor **Dr. Chandan Goswami,** Associate Professor, NISER for his valuable guidance, kind of cooperation and involving me throughout the project work.

I am thankful to **Dr. Luna Goswami,** Associate Professor, School of Biotechnology, KIIT Deemed to be University, Bhubaneswar for her continuous guidance and support.

I am also thankful to **Dr. Mrutyunjay Suar**, Director and Head, School of Biotechnology, KIIT Deemed to be University, Bhubaneswar for allowing me to carry out my dissertation in NISER.

I am grateful to my labmates Nikhil Tiwari, Rakesh K. Majhi, Manoj Yadav, Tushar Acharya, Rashmita Das, Somdatta Saha for helping me throughout my project work.

My sincere thanks to all my batch mates, seniors and all my teachers for inspiring me always. I acknowledge my parents, all my family members, relatives and all those who gave the possibility to complete my thesis.

Lastly, I apologize to all those who helped me directly or indirectly in completion of my work but whose names have not been mentioned , and I express my sincere regards to them.

Date: 14/05/2018 *Signature*

Place:Bhubaneswar

TABLE OF CONTENTS

ABSTRACT……..i
ACKNOWLEDGEMENT……..ii
TABLE OF CONTENTS…….…...iii
LIST OF ABBREVIATIONS……..…iv
LIST OF TABLES AND FIGURES……..vi
CHAPTER 1: INTRODUCTION…….……..…1
1.1 Background and Context……..…2
1.2 Scope and Objective…….4
1.3 Achievements……....4
1.4 Overview of Thesis……...5
CHAPTER 2: REVIEW OF LITERATURE……6
CHAPTER 3: MATERIALS AND METHODS……..17
CHAPTER 4: RESULTS……...22
CHAPTER 5: DISCUSSION…….44
CHAPTER 6: CONCLUSION……..48
6.1 SUMMARY……..49
6.2 FUTURE WORK…….49
REFERENCES
APPENDIX

LIST OF ABBREVIATIONS

CRAC	Cholesterol Recognition/Interaction Amino Acid Consensus Sequence
CARC	Inverted CRAC Domain
CCM	Cholesterol Consensus Motif
PIP_2	Phosphatidylinositol-4,5-bisphosphate
DAG	Diacylglycerol
IP_3	Inositol-1,4,5-triphosphate
DCT	Distal Convoluted Tubule
CNT	Connecting Tubule
MEGA	Molecular Evolutionary Genetic Analysis
TRP	Transient Receptor Potential
MUSCLE	Multiple Sequence Comparison by Log-Expectation
OPM	Orientation of Proteins in Membranes
B.E	Binding Energy

Amino Acids One Letter Codes and Abbreviations

Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic Acid	Asp	D
Cystiene	Cys	C
Glutamic Acid	Glu	E
Glutamine	Gln	Q
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	Z

In this thesis, both single letter codes and abbreviations are used. For example, D542 or Asp 542 indicates the Aspartate residue in position 542 of the corresponding sequence.

LIST OF TABLES AND FIGURES

Figure 1.1: Membrane alteration induced activation of TRP channels……..3
Figure 2.1.: Primary structures of the seven TRP channel subfamilies……..9
Figure 2.2.1: Architecture of TRP channels……...10
Figure 2.2.2: Phylogenetic tree of the TRP family…….10
Figure 2.4: Structure of TRPV5 and TRPV6…….12
Figure 4.1: Phylogenetic Tree of TRPV5 and TRPV6……...27
Figure 4.2: BoxPlot of Different Domains of TRPV5 and TRPV6……...28
Figure 4.3.1: BoxPlot of LWI Residues…….29
Figure 4.3.2: Sequence logo of TRPV5 and TRPV6…….30
Figure 4.4.1: BoxPlot of Cholesterol Binding motifs……31
Figure 4.4.2: Schematic Representation of probable Cholesterol Binding motifs in TRPV5 and TRPV6……..…32
Figure 4.5: Structures of TRPV5 and TRPV6……...34
Figure 4.6: TRPV5 and TRPV6 monomer in the Lipid Bilayer……35
Figure 4.7: Binding Energies of respective Global docking……..37
Figure 4.8 Various docking positions in TRPV5 and TRPV6 for both close and open conformations…….43

Table 2.6.1: Comparison of effects on deletion of different parts of *trpv6* exons..14
Table 2.7.1: Effect of different hormones on TRPV5…….15

CHAPTER 1

INTRODUCTION

1.1 Background and Context:

The Transient Receptor Potential (TRP) channel is a multigene superfamily which includes integral membrane proteins. These proteins function as ion channels **[1]**. Members of this family are conserved in yeast, invertebrates and vertebrates. TRP channels performs variety of cell functions like sensing, homeostatic processes, ion reabsorption etc. These are regulated by various external stimuli such as membrane potential, mechanical forces and endogenous stimuli including intracellular signaling components downstream of phospholipase C, lipid signaling molecules and lipid membrane components. Characteristically, most of the TRP channels can be activated by diverse group of chemical or physical stimuli, such as ligand binding, temperature, osmolarity fluctuations, mechanical forces or voltage.

As TRP channels are embedded in the lipid bilayer, the membrane environment might influence them. It is, however, known that some lipids can specifically activate and inhibit some of the TRP channels. For example, Cholesterol inhibits human TRPM3 **[2]**. Specific lipid binding proteins can also act as specific subunits of TRP channels and to a variable extent can thereby regulate the channel activity. For example, Pirt, which is a phosphoinositide-binding protein, has a role in regulating TRPV1 **[3]**. Also, enzymes regulating lipid metabolism are known to regulate TRP channels. For example, Phospholipase, PKC and sphingosine kinase are known to modulate the Ca^{2+} entry through TRP channels **[2]**. Altering the membrane composition, either by depletion or addition/saturation of the membrane cholesterol affects the properties of TRP channels and physiological functions mediated by them **[4]**. Infact, lipid packing of the membrane as well as the channel plasma membrane interface can be important for activation of these channels **[5, 6]**. The conversion of PIP2 to DAG by PLC stands as one of the major example for this. This conversion induces alteration in the membrane structure and forms a bended curvature in the small lipid membrane microdomain regions. This is due to the fact that DAG is smaller in structure when compared to the PIP_2, due to the loss of some head portion **[7]**. Furthermore, the alteration of the membrane composition may lead to the removal of Open channel block, a process by which ions bound to the inner side of a channel gets released **[5, 6]**.

TRP channels are also localized in specific regions of the plasma membrane known as lipid rafts **[8]**. For example, TRPM7 co-localizes with flotillin-2, a marker of lipid

rafts **[9]**. The store operated Ca^{2+} entry induced by TRPC1 is regulated by presence or absence in lipid raft **[10, 11]**.

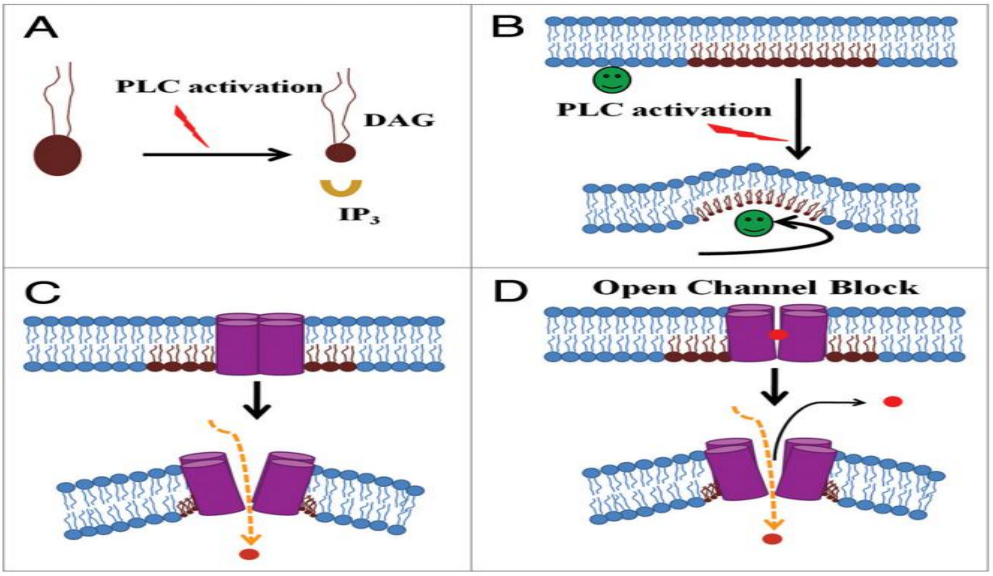


Figure 1.1 [12, 13]. Membrane alteration induced activation of TRP channels.
A. PLC activation converts membrane lipids to IP_3 and DAG.
B. This conversion causes shortening of certain membrane lipids, resulting in alteration of lipid packing at the inner surface of membrane.
C. Changes in membrane curvature and lipid-TRP channel interactions result in conformational changes leading to the opening of TRP channels.
D. Further opening of TRP channels due to open channel block.

Lipid rafts are highly dynamic, detergent resistant membrane fractions which contain high concentrations of cholesterol and glycosphingolipids. Infact, cholesterol is the main component of lipid rafts and is critical for its formation. However, not just cholesterol, some other types of lipid molecules are also important for different types of lipid rafts. For example, ceramide is one of the important components of lipid rafts. Inspite of their small size, these structures concentrate large number of signaling proteins in them. So, they are considered very important in regulating signaling pathways, cholesterol trafficking etc, through various different mechanisms. As lipid rafts have a distinct lipid composition and structure, all the ion channels, including TRP channels, particularly those involved in thermosensation/mechanosensation, behave differently when they are located outside the lipid rafts or when the composition of rafts are changed. But, as it is difficult to study these extremely small

and microdomains domains, the exact mechanism of ion channel regulation by lipid microdomains regulation is not known.

TRP channels have been linked to many pathological conditions including some disease syndromes **[75]**. Some hormones indeed regulates several TRP channels, both directly and indirectly by altering their expression level, with TRPV1 being the most common target **[14]**. For example, prolactin **[15]**, endothelin **[16]** and neurokinin **[17]** exert regulatory effect on TRPV1. In many cases, both development of hormone producing tissues and secretion of hormones are also regulated by TRP channels. Thus, a multidirectional regulation of TRP channels and hormonal actions are important as such cross talks regulate diverse functions like Ca^{2+} reabsorption, aging and many other biological processes.

TRP channels are specialized for the detection of taste, smell, pain, temperature, hormone, and pheromone, and are involved in many behavioral as well as other complex functions including olfaction **[2]**. By regulating Ca^{2+} and other second messengers, the TRP channels may contribute to the survival and differentiation of cells and tissues, specially in the early embryonic stages. The TRP channel mediated functions are critical for many developmental, neuronal and other functions.

1.2 Scope and Objectives:

The pattern of expression and selectivity for calcium permeation of TRPV5 and TRPV6 ion channels indicates their important role in the intracellular calcium homeostasis and proliferation activity of cells. Considering a direct role of TRPV5 and TRPV6 in calcium homeostasis and dietary intake, it can also be assumed that these channels may be involved in the pathogenesis of all types of osteoporosis. Over the last few years, the molecular structure and regulation of TRPV5 and TRPV6 has been studied in detail and studies of knockout animal models discussed below, strongly suggests that these ion channels are tightly regulated by hormonal stimuli. So, in this study, the evolutionary analysis and domain study was performed in detail. And the binding mode of different steroid hormones was investigated by *in silico* methods.

1.3 Achievements:

The nature of conservation of different domains of TRPV5 and TRPV6 indicates that *trpv6* gene was under selective pressure during the evolution of mammals. Also, the

possible binding regions of different steroid hormones in both open and closed conformation of protein were determined. This may be helpful to find, if these hormones play in role in TRPV5 and TRPV6 ion channel gating or not, which is directly related to calcium homeostasis and bone mineralization in the body.

1.4 Overview of Thesis:

The work presented in this thesis describes the detailed investigation of domain conservation of TRPV5 and TRPV6 ion channels, which provides insight into evolution of these channels from lower to higher vertebrates. This thesis also reports possible binding site of different steroid hormones with the protein in both open and closed conformation.

Chapter 1 includes a short introduction on TRP ion channels and their gating mechanism.

Chapter 2 gives a view of detailed study of TRPV channels, specially TRPV5 and TRPV6, along with their structure, function and regulation.

Chapter 3 reports on all the methods used in this work, for evolutionary analysis of TRPV5 and TRPV6, along with their nature of interaction with steroid hormones.

Chapter 4 reports on data and results of evolutionary study of both the proteins, conservation of domains, their structure and docking with steroid hormones. It also validates somehow, previous reports on gene duplication of *trpv6* and evolution of *trpv5*.

Chapter 5 discusses stepwise, on each result obtained, providing evidence on evolution of both the proteins from lower to higher vertebrates. It also throws light on possible binding of steroid hormones, which may affect channel gating and regulation.

Chapter 6 summarises the findings of this work and also proposes a general idea on possibility of interaction of steroid hormones with the protein.

CHAPTER 2

REVIEW OF LITERATURE

TRP Channels were initially discovered in the late 1970s and early 1980s in the *trp* mutant of the fruit fly *Drosophila.* They showed the property of transient elevation of potential in response to light stimuli and so were named “transient receptor potential” channels. Since then, a number of TRP Channels have been identified in a variety of organisms, from invertebrates to humans, and were then classified into many classes not only depending on potential elevation, but on the basis of similarity in gene sequence and protein structure. In lower order, only yeast has been reported to have the TRP channels, where they have a role in detection and response of hyper-tonicity. So far, no report is available that suggest presence of TRP channels in plant kingdom or in prokaryotes.

Among these, TRPV5 and TRPV6, members of TRPV subfamily, were found to be highly selective for Ca^{2+} ions, a property strikingly different from other members of the same family. Expression of TRP channels can be seen both in neuronal and non-neuronal cells like heart, endothelium, liver, lungs, placenta, trachea and salivary gland. These channels were found to be vital for epithelial calcium uptake and bone formation **[53]**. These ion channels are thoroughly regulated in order to fine-tune the amount of Ca^{2+} reabsorption. Many steroid hormones have also been shown to be involved in this process. Here, we investigate this binding detail and find significant difference and similarities between TRPV5 and TRPV6.

2.1 General domain and motif structures of TRP Channels:

Like voltage-gated Na^+ channels, all TRP channels shares similar topology- the monomer has six transmembrane spanning domains (S1-S6) with cation permeable pore region between the 5^{th} (S5) and 6^{th} (S6) transmembrane regions **[19][Figure 5.1.1, 5.1.2]**. Both N- and C- termini are located within the intracellular regions. The N-terminal cytoplasmic domain of the TRPV and TRPC channels contain single to multiple Ankyrin Repeat Domain (ARD), while the C- terminal cytoplasmic domain contains a TRP-box (in many TRP channels), This domain of the protein is well conserved in members of TRPC subfamily, but is less conserved in members of TRPM and TRPV subfamilies. ARD are 33 residue sequence motifs, consisting of pairs of antiparallel alpha-helices connected by beta-hairpin motifs, often involved in protein protein interactions and present in many eukaryotic and prokaryotic proteins with functions including signaling, cytoskeleton integrity, transcription and cellular localization. TRP-box, a typical structural signature characteristics of many TRP channels, contains a sequence of 25 amino acids located immediately after the 6^{th} transmembrane (S6) helix. Cytoplasmic domains are involved in regulation and modulation of channel function and trafficking. The length of N- termini, C- termini and structural domains (ARD) varies between members.

TRP channels generally form homotetramer, which acts as functional ion channels. But, depending on the degree of sequence homology and the expression pattern, some of them can form heteromers as well, which are functional in terms of ionic conductivity **[18]**. For example, close homologues like TRPM1 and TRPM3 can form functional heteromeric channel as confirmed by immuno-precipitation and ionic conductance measurement. Heteromultimer formation is possible even when two channels are not closely related. For example, TRPC1 can form functional heteromeric complex with TRPV4. However, it is difficult to conclude any reason for heteromerization, whether it occurs due to overexpression of the two related or unrelated TRP channels.

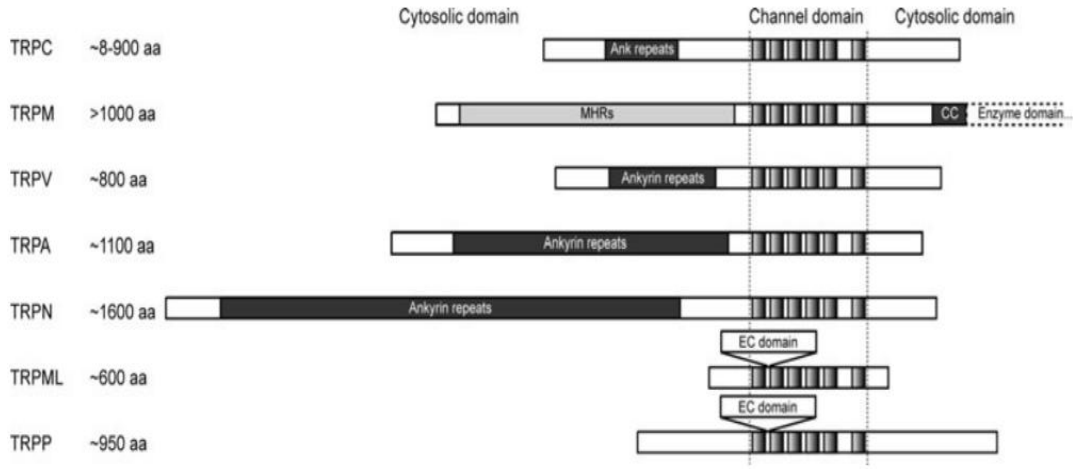


Figure 2.1 [47]. Primary structures of the seven TRP channel subfamilies. Lengths are approximately to scale. CC is coiled-coil region, EC domain is an extracellular domain and the dotted lines indicate C-terminal extensions containing enzymatic domains in some TRPM channels.

2.2 The TRP super family:

TRP channels is subdivided into seven subfamilies:

TRP Canonical channels (TRPC1-TRPC7), Vanilloid Receptor-related TRP channels (TRPV1-TRPV6) and Melastatin-related TRP channels (TRPM1-TRPM8) constitutes the TRP channel core family. While, Polycystin-related channels (TRPP), Mucolipin-related proteins (TRPML), Ankyrin-related proteins (TRPA1 or ANKTM1) and No Mechanoreceptor proteins (TRPN1) are more distantly related TRP homologs that, together with TRPC, TRPV and TRPM channels constitute the extended family of TRP channels **[1][Figure 5.2.1]**. TRPN is found only in invertebrates and fish. 28 TRP genes are present in humans and mouse. Some chromosomes carry one to three or four genes. Only TRPV5 and TRPV6, TRPV1 and TRPV3 genes are located side by side as well as in same transcriptional orientation suggesting gene duplication event.

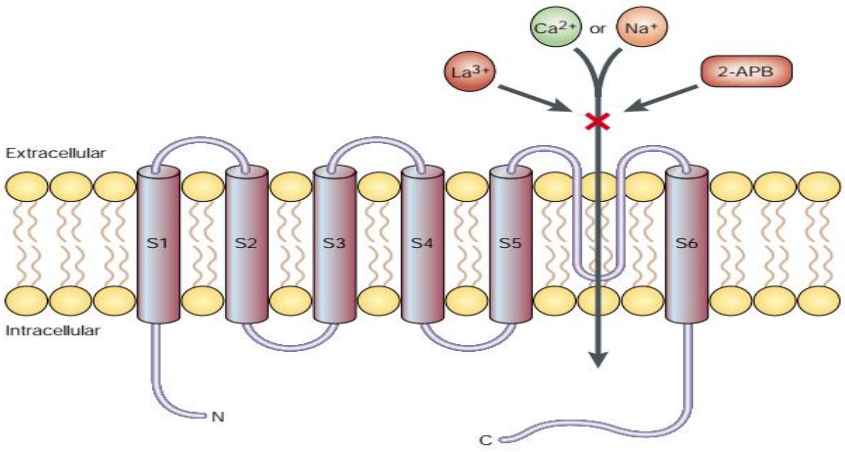


Figure 2.2.1 [1]. Architecture of TRP channels. S1 to S6 are transmembrane domains. Lanthanum ions (La^{3+}) and 2-aminodiethyldiphenyl borate (2-APB) are found to block these channels, although not specifically.

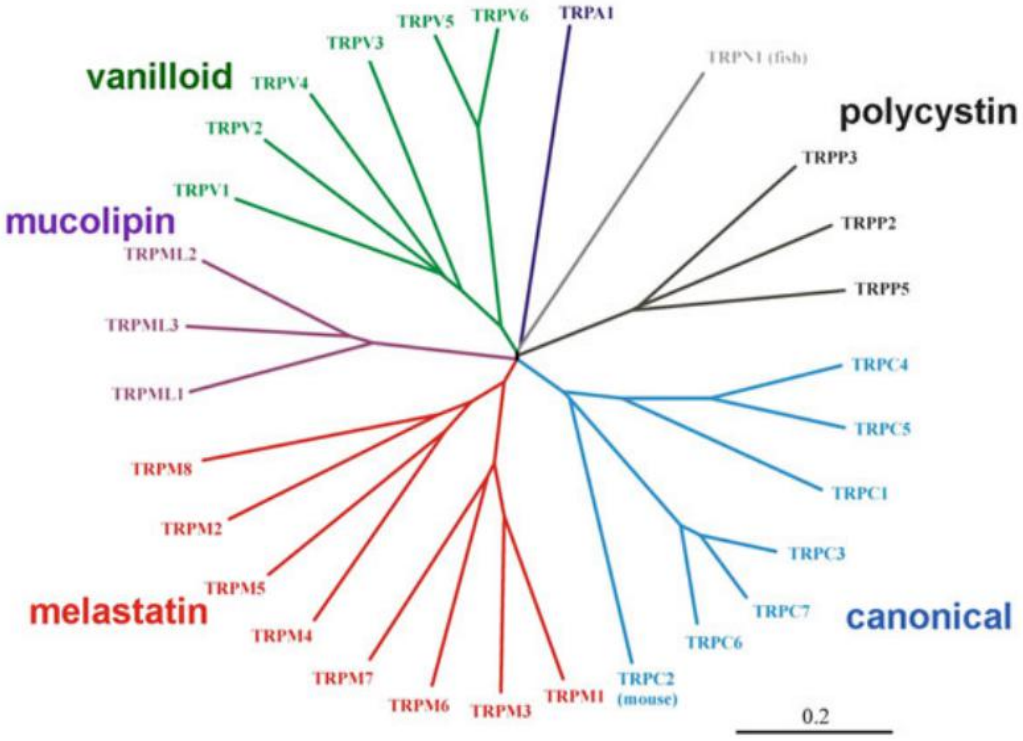


Figure 2.2.2 [11] Phylogenetic tree of the TRP family. Whole TRP family is divided into 7 subfamilies. TRPC (Canonical), TRPM (Melastatin), TRPA1 (ANKTM1), TRPP (Polycystin), TRPML (Mucolipin), TRPN (NOMPC-like) only found in lower organisms like fish and TRPV (Vanilloid).

2.3 The TRPV sub family:

The TRPV family has six members, i.e TRPV1 to TRPV6. Among the six TRPV members, only TRPV1 to TRPV4 are thermosensitive and non-selective, while the rest two, i.e TRPV5 and TRPV6 are non-thermosensitive, rather they are highly calcium selective, so called ECaC1/CaT2 (Epithelial Calcium Channel/Calcium Transporter) and ECaC2/CaT1 respectively. The expression of members differs according to the cell type. TRPV1 to TRPV4 are usually expressed in brain, peripheral sensory neurons, lung, kidney and liver. TRPV5 is found to be present in distal tubule of kidney, human placenta, osteoclasts, retinal pigment, lymphocyte, Jurkat leukemia T- cells and leukemia K562 cells. TRPV6 is expressed in rat small intestine, in human and mouse placenta and to a lower extent in human prostate cancer and mouse epididymis.

2.4. Structure of TRPV5/V6:

Human TRPV5 is a 729 amino acid long protein. In intracellular portion, six Ankyrin repeats are present in the N- terminal region and PDZ binding motif in C- terminal region. Except the 1st Ankyrin Repeat, all N terminal Ankyrin repeats are important for channel assembly. It is glycosylated at 358 residue amino acid, in the first extracellular loop, which codes for Asparagine. Binding sites for Protein Kinase A (PKA), Protein Kinase C (PKC) and Calmodulin is present in both amino and carboxy terminal regions. Just like Potassium channels, for TRP channels also, it is found that glycosylation has a role in determining the stability and assembly of TRPV5 and TRPV6 **[22]**. Tetramerization of TRPV5 allows for D542 residues, in the pore forming loop, to form Calcium selective ring, also involved in Mg^{2+} blockade.

Endogenous human full length TRPV6 consists of 765 amino acids, compared to the 725 amino acid annotated TRPV6 protein, which is truncated. The additional 40 amino acid sequence shows no similarity to any known protein sequence; nine out of 40 amino acid residues are proline residues, and they might well constitute motifs involved in protein protein interaction. Like TRPV5, TRPV6 also contains 6 Ankyrin repeats in the N- terminal region. Ankyrin repeat 3 and 5 are found to be critical components involved in assembly of functional channel complexes. It is glycosylated at Asn357, which is located within extracellular S1-S2 linker. Calmodulin binding site is present at the C- terminus. A single aspartate residue at

position 581 (541 in truncated protein), is a critical constituent of TRPV6 selectivity filter, whose replacement completely abolishes Ca^{2+} permeability **[32].**

The epithelial calcium channels, TRPV5 and TRPV6 can combine with each other to form heteromultimeric channels, which displays properties, intermediate between these two channels, but exhibit voltage-dependent gating like the individual homotetramers channels **[21]**.

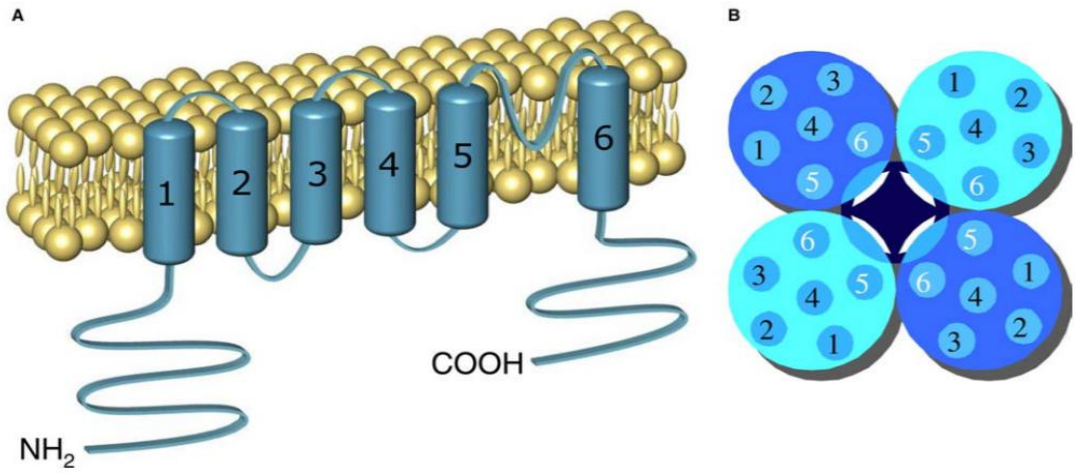


Figure 2.4 [60] Structure of TRPV5 and TRPV6 (A). Six TM domains, with one putative pore forming region between TM5 and TM6. **(B).** The six TM units are presumed to surround a central pore in homo or heterotetrameric configuration.

2.5. Cations Channeling:

The divalent selectivity cation profile is $Ca^{2+} > Mn^{2+} > Ba^{2+} > \sim Sr^{2+}$ **[20]**. In the absence of divalent cations, monovalent cations pass through in a profile $Na^{+} > Li^{+} > K^{+} > Cs^{+}$. But the permeability ratio between Ca^{+} and Na^{+} is over 100. In TRPV5, as amplitude of Na^{+} current is as same as that of Ca^{2+}, so is used for activity measurement. While in TRPV6, Ca^{2+} uptake measurement is used for activity study. Also, permeability and activation properties of Ba^{2+} is different for these two channels.

Some ions also acts as current blockers in the following order-

$Pb^{2+} = Cu^{2+} = Gd^{3+} > Cd^{2+} > Zn^{2+} > La^{3+} > Co^{2+} > Fe^{2+} > Fe^{3+}$

Mg^{2+} also acts as current blocker, but in a voltage dependent manner. Urinary Ca^{2+} excretion is proportional to changes in Mg^{2+} excretion, which suggests blockage by this ion. Also, in TRPV5, a SNP variation A563T, close to D542 increases sensitivity to extracellular Mg^{2+}, which results in suppressed Na^{+} permeation.

2.6. Functions of TRPV5/V6:

Intestinal Ca^{2+} absorption depends on two major pathway- paracellular pathway and a transcellular pathway **[40]**. The transcellular pathway involves a Ca^{2+} uptake channel at the luminal or apical membrane, soluble cytosolic Ca^{2+} binding proteins and a Ca^{2+} extrusion mechanism at the basal membrane. This process involves both TRPV5 and TRPV6. But, TRPV5 is a rate-limiting component in the transcellular pathway of Ca^{2+} reabsorption in the apical membrane of tubular cells in the DCT and CNT of the kidney, where it is constitutively expressed **[22, 23]**. There is also indication of malfunction of osteoclast to some degree in the absence of TRPV5 (i.e. in TRPV5 knockout mice). But, bone matrix mineralization is found to be decreased in TRPV5 knockout mice in the presence of Vitamin D analog, suggesting an indirect role of TRPV5 in bone resorption **[24]**. The role is still not well understood but, it is involved in receptor activator of NF-kappaβ ligand-induced rise in Ca^{2+} in human osteoclast, which is likely to be a part of negative feedback loop to terminate bone resorption **[25]**. Although, both TRPV5 and TRPV6 are present in osteoclasts, TRPV6 did not compensate the reduced bone resorption due to lack o TRPV5, which may be due to their less no. in osteoclasts **[30]**. TRPV5 plays only a minor role, as compared to TRPV6, in intestine and placental Ca^{2+} transport due to its low level of expression in these organs **[26]**. Humans having mutation or defect in *trpv5* gene expression may have imbalanced Ca^{2+} homeostasis, but could be clinically asymptomatic, except for hypercalciuria **[27]**. Moreover, any change in *trpv5* expression may lead to alteration in urinary Ca^{2+} excretion, while on the other hand, alteration in Ca^{2+} resorption elsewhere in the nephron leads to increased/decreased Ca^{2+} delivery to the DCT and CNT and in turn causes elevation or retardation in *trpv5* gene expression. TRPV5/TRPV6 modulators can help in heating abnormality in Ca^{2+} homeostasis in case of a disease. For example, diseased condition of Vitamin D signaling defect as in Vitamin D dependent rickets, where intestinal and renal TRPV5/TRPV6 expression are likely disrupted, can be treated by enhancing TRPV5/TRPV6 mediated Ca^{2+} absorption to achieve a positive Ca^{2+} level under this condition **[28]**. Also, enhancers can prevent osteoporosis in women after menopause, due to reduced estrogen level **[29]**, and preventing kidney stone formation in patients with renal Ca^{2+} leak. While, on the other hand, TRPV6 inhibitor would prevent kidney stone disease and absorptive hypercalciuria.

Transcellular Ca^{2+} uptake involves TRPV6 channels in the luminal intestinal side as Ca^{2+} uptake mechanism, but not as the essential component of intestinal Ca^{2+} uptake. Rather, it is involved in mechanisms preventing the loss of Ca^{2+} already present in the organism by reabsorbing Ca^{2+}. Though, clear role of TRPV6 is not yet understood, but it is found to be in placental Ca^{2+} transport, where it is highly expressed in the foetal side of placenta and only weakly expressed on the maternal side during the last trimester of gestation. But, *trpv6* expression was highly upregulated from embryonic day 15 to day 18, which indicates its involvement in the increase of maternal-foetal Ca^{2+} transport which supports fetal bone mineralization **[31]**. Different knockout experiments suggests, that mice lacking *trpv6* shows dermatitis, decreased intestinal Ca^{2+} absorption, lower bone density, lower body weight, growth retardation and reduced fertility specially in males **[Table 5.6.1] [32, 33]**. Not only this, but *trpv6* gene expression is also upregulated in many malignancies like breast cancer, ovarian cancer, thyroid cancer, leukemia, endometrial cancer and specially prostate cancer/tumor. Here, it not only represents a marker of cancer progression, but can also serve as a target for therapeutic strategies **[19]**.

Analyzed parameter	*Trpv6*$^{-/-}$ **[32]**	*Trpv6*$^{-/-}$ **[33]**
Femoral bone mineral density	Not analyzed	Decreased
Body weight	No difference	Decreased in homozygous males and females
Growth retardation	Not detectable	Decreased in homozygous males and females
Fertility defect	Decreased in Homozygous males	Decreased in Homozygous males and females
Sperm motility	Impaired	Not analyzed
Sperm viability	Impaired	Not analyzed
Alopecia	Not detectable	Yes
Dermatitis	Not detectable	Yes

Table 2.6.1: Comparison of effects on deletion of different parts of *trpv6* exons

2.7. <u>Regulation by hormones</u>:

A no. of hormones have been found to regulate TRPV5. 1,25-Dihydroxy Vitamin D_3 increases transcellular Ca^{2+} absorption, which then upregulates *trpv5* transcription as a result of increased Ca^{2+} level in DCT **[34]**. PTH regulates both expression/activity of TRPV5 by PKA or PKC signalling cascade. Both the signalling cascades acts by phosphorylating TRPV5 at different sites, i.e. PKA signalling cascade increases TRPV5 open probability by phosphorylation of Threonine(709) **[35]**, while PKC phosphorylation at S299/S654 **[36]**. Mutation of PKC phosphorylation sites abolishes the regulation. Estrogen shows a positive effect on TRPV5 **[37]**. Most potent estrogen, i.e. 17β-estradiol, increases TRPV5 mRNA and protein expression in kidney, including elevation in TRPV5 current and activity. In contrast, male hormone testosterone shows negative effect on TRPV5 **[38]**. Testosterone treatment can be used to normalize decreased urinary Ca^{2+} excretion as a result of androgen deficiency. Vasopressin also induces an increase in Ca^{2+} uptake, indicating that TRPV5 is its target **[39]**.

Hormones	Effects
$1,25(OH)_2D_3$	Increases Trpv5 transcription **[34]**
PTH	Increases TRPV5 expression and activity via PKA and/or PKC mediated signalling cascade **[35, 36]**
Estrogen	Increases both TRPV5 activity and expression **[37]**
Testosterone	Decreases TRPV5 expression **[38]**
Vasopressin	Increase transepithelial Ca2+ transport by the activation of cAMP/PKA pathway **[39],**

Table 2.7.1: Effect of different hormones on TRPV5

TRPV6 is also regulated by Vitamin D_3, as evident from the binding sites for 1,25-Dihydroxy Vitamin D_3 identified in *trpv6* promoter region **[41]**. *trpv6* expression level is found to be hampered in Vitamin D-receptor deficient mice, and also suffered from hypocalcaemia. Vitamin D supplementation in the mice results in increased expression of TRPV6 and normalization of serum Ca^{2+} **[42]**. Prolactin had been shown to enhance intestinal Ca^{2+} absorption in case of Vitamin D_3 deficiency, to directly stimulate the transcellular Ca^{2+} transport mechanism, regulate Vitamin D metabolism

and induce *trpv6* expression **[43]**. *trpv6* expression is found to be increased in the presence of estradiol, progesterone and to be reduced in presence of dihydrotestosterone **[45]** and tamoxifen, which is an oestrogen receptor modulator **[44]**.

While, Parathyroid hormone related protein regulates active Ca^{2+} transport through the placenta, but did not affects *trpv6* expression **[46]**.

CHAPTER 3

MATERIALS AND METHODS

3.1 Sequence retrieval and alignment:

The TRPV5 and TRPV6 sequences were retrieved from National Centre for Biotechnology Information (NCBI) database **[49]**. Details of each ion channel protein are given in the material and method section as tabular form **[Table 7.1.1, 7.1.2]**. The sequence alignment was done by using MUSCLE (Multiple Sequence Comparison by Log Expectation) alignment software with its default values **[50]**. As a highly conserved protein. Sequences for histone H4 from different species were downloaded from the Ensembl site (http://www.ensembl.org/index.html) **[51,52]**.

3.2 Molecular Phylogenetic analysis by Maximum Likelihood Method:

The evolutionary history was inferred by using the Maximum Likelihood Method based on the JTT matrix-based model **[53]**. The tree for TRPV5 sequences with the highest log likelihood (-2109.39) and the tree for TRPV6 sequences (-21.85) is shown. Initial tree(s) for the heuristic search was obtained automatically by applying Neighbour-Join and BioNJ algorithms to a matrix of pairwise distances estimated using a JTT model and then selecting the topology with with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 122 amino acid sequences, for TRPV5 and 128 amino acid sequences for TRPV6. All positions containing gaps and missing data were eliminated. There were total of 92 positions (for TRPV5) and 3 positions(for TRPV6) in the final dataset. Evolutionary analysis were conducted in MEGA7 **[54]**.

3.3 Fragmentation of TRPV5 and TRPV6 in different domains and motifs:

In order to analyse the degree of conservation of the different domains present in TRPV5 and TRPV6, their various structural regions, domains and motifs, like Cytoplasmic domains, Transmembrane domains, Extracellular domains and Pore forming regions were analyzed separately. Aligned protein sequences of different species were taken, and their domains were considered individually for analysis. Domain information of Human TRPV5 and TRPV6 protein was taken from Uniprot and Protein Database (PDB) **[Table 8.3.1]**. MUSCLE software was used to align these domain sequences and aligned data were subsequently imported into ‘R’ statistical tool for statistical analysis.

3.4 Determination of Cholesterol Binding Motif:

Cholesterol binding motifs has been reported for TRPV1 **[56]**. We scanned this motif to evaluate its presence in TRPV5 and TRPV6. To find different Cholesterol Binding motifs, PATTINPROT online server was used with 95% sequence similarity.

CRAC motif - **(L/V)-$X_{(1-5)}$-(Y)-$X_{(1-5)}$-(K/R)**

CARC motif - **(K/R)-$X_{(1-5)}$-(Y/F)-$X_{(1-5)}$-(L/V)**

CCM motif - **(R/K)-$X_{(7-10)}$** or **(W/Y)-$X_{(4)}$-(I/V/L)**, along with **(F/Y)** in the nearby helix

SPHINGOLIPID BINDING motif **[61]** -

(V/I/T/L)-X-X-(V/I/T/L)-(V/I/T/L)-X-X-(V/I/T/L)-(F/W/Y) OR

(V/I/T/L)-$X_{(1-2)}$-(V/I/T/L)-(V/I/T/L)-$X_{(1-2)}$-(V/I/T/L)-(F/W/Y)

3.5 Determination of LWI Residues:

What was investigated was whether a bias is present in the lipid water interface amino acid conservation in both the proteins. In that context, a lipid water interface was taken as a region of 5 amino acids. The core was confirmed as the region which has been annotated as a TM region.

3.6 Distance Matrix Generation and Statistical Tests:

Using the saved alignment files in MEGA7 distance matrices were generated for different aligned datasets, i.e. Cholesterol binding motifs, LWI residues and Different Domains of the protein. Using this method, pair-wise distances of any two different amino acid sequences within a group can be measured. To estimate the variance, bootstrap method was used. In substitution method, amino acid p-distance was used. In case of data gaps/data missing pair wise deletion method was used. For each dataset there will be one matrix which informs about the pair-wise distances of all sequences in a group. In the matrix window, distances between each sequence with another is calculated along with overall mean distance of all sequences. Then the pair-wise distance values (generated in the distance matrix) were imported in 'R' software for statistical analysis and graphical representation. Using 'R' software, boxplots were generated to represent the evolutionary relationship of different protein sequences. As we have measured the pair-wise evolutionary distances of protein sequences, the graphical representation reflects the values in the Y-axis, which is

inversely proportional to the conservation of that group of sequence. Therefore, the conserved sequences show lower values and divergent sequences show higher values in the Y-axis. Along with this calculation, the median values of each dataset were calculated and also represented along with conservation.

3.7 Sequence Logo Generation:

A sequence logo is a graphical representation of an amino acid or nucleic acid multiple sequence alignment **[58]**. Each logo consists of stacks of symbols, where one stack represents each position in the sequence. The overall height of the stack denotes the conservation of sequence at that position, while the height of the symbols within the stack determines the relative frequency of each amino or nucleic acid at that position. Weblogo is an online server, designed to generate sequence logos easily and. Here, it is used to generate sequence logo of LWI residues of both the proteins.

3.8 Protein data analysis by visualization:

Protter is an online server which supports analysis of protein data and generation of hypothesis, which is achieved by visualizing both sequence features and experimental proteomic data in the context of protein topology **[55]**. Protter supports various formats of proteomic files and then automatically integrates a variety of reference protein annotation sources, which can be readily extended via modular plug-ins. It also contains a built-in export function. This function plays a role in producing protein illustrations, even for large datasets. Visualizations of datasets shows the specific utility of Protter for the integrated visual analysis of membrane proteins and selection of peptide for targeted proteomics. Cholesterol Binding motifs in both the proteins were visualized using Protter, and then their positions were compared.

3.9 Homology Modeling:

This step was performed only for TRPV5 open structure, since other required structures for docking, i.e TRPV5 closed structure, TRPV6 open and closed structure, were already available in PDB. TRPV5 Human protein sequences was copied from NCBI in FASTA format, and fed to YASARA, a molecular graphics, modeling and simulation software. 6BOB (PDB ID of TRPV6 open structure), was imported to

YASARA as a template, and algorithms were set to start the modeling. On completion of this task, the new modeled structure was finally refined for a proper resolution.

3.10 Protein visualization in Lipid bilayer:

This is done using the online PPM Server, which is a part of OPM (Orientations of Proteins in Membranes) database **[62]**. The PPM Server provides both rotational and transitional positions of peripheral or transmembrane domains within the lipid bilayer using their 3D structure, i.e PDB file as input. It can be applied to both newly determined protein structures and theoretical models.

3.11 *In silico* Docking of Cholesterol and its derivatives:

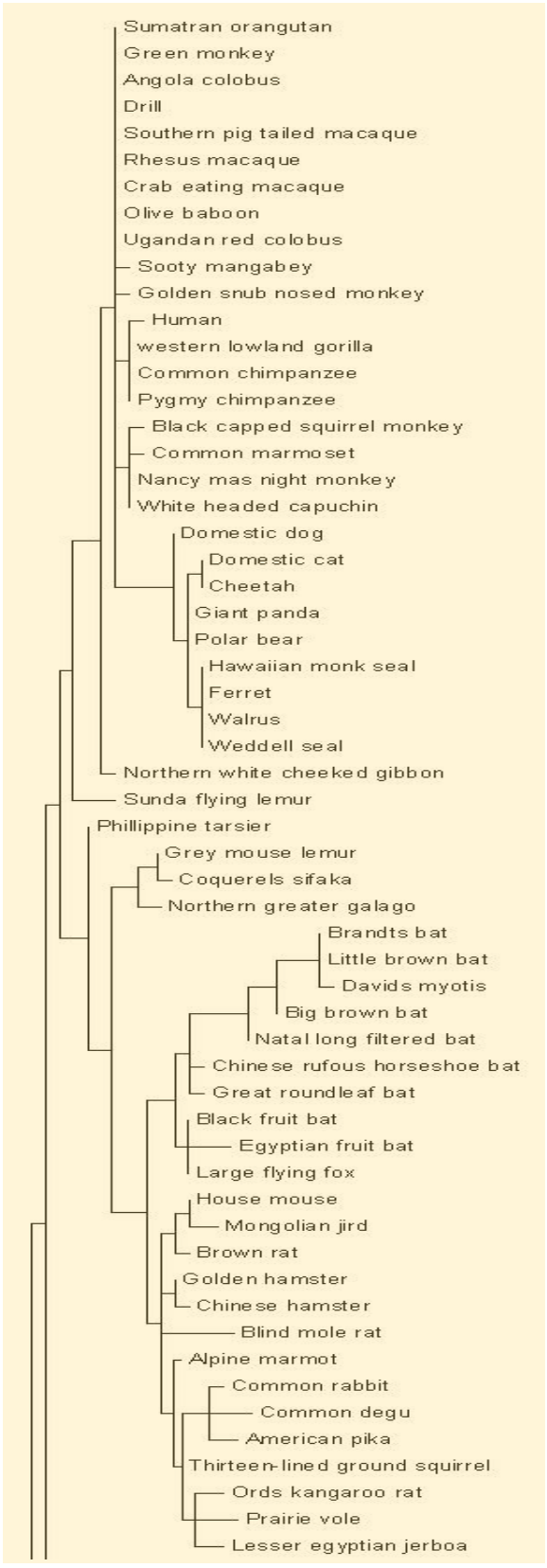
Receptor-Ligand docking was performed with VINA, keeping the default parameters intact. The entire framework was done with YASARA Molecular Modeling Program, out of which the best structures were chosen manually. Docking was performed keeping the internal degrees of freedom of ligand into account, maintaining the flexibility. Ligands chosen for the study includes Cholesterol, PIP_2, Cortisol, Estradiol, Progesterone, Testosterone and Aldosterone. All ligand structures were obtained from PUBCHEM, in SDF format. Both Global and Local docking were performed. For Cholesterol, fully conserved CRAC and CRAC-like motifs were selected as local binding sites, while for other molecules, TM5-TM5 loop region was selected. The interaction of all the ligands with both TRPV5 and TRPV6 by structural docking displayed many forms. Binding energies of all possible orientations were plotted on a graph, using GraphPad Prism, and the number of possible binding sites and average binding energy was compared between closed and open conformation of the protein for each hormone. Finally, only some were selected depending upon their binding energies and other thermodynamic conditions.

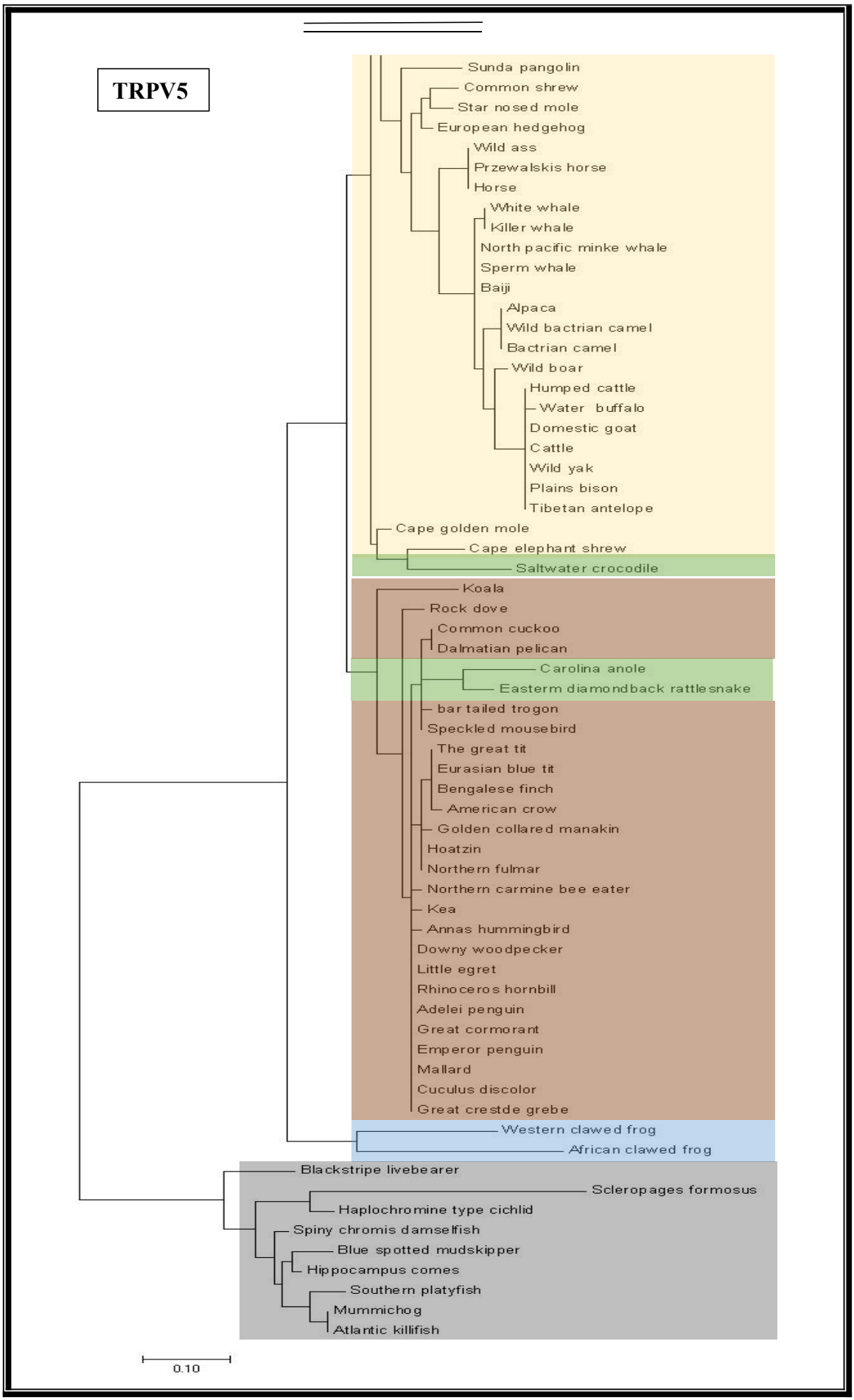
CHAPTER 4

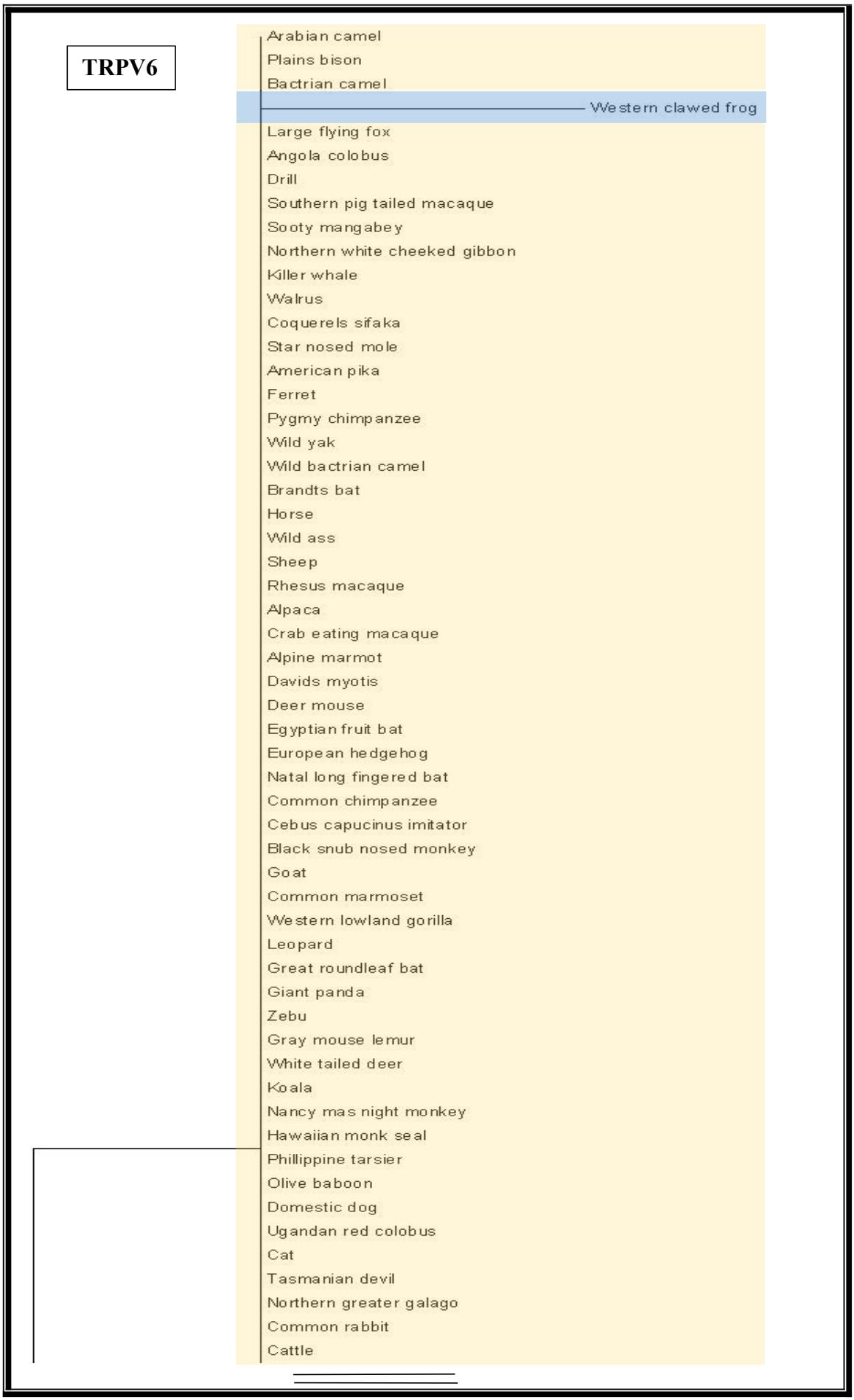
RESULTS

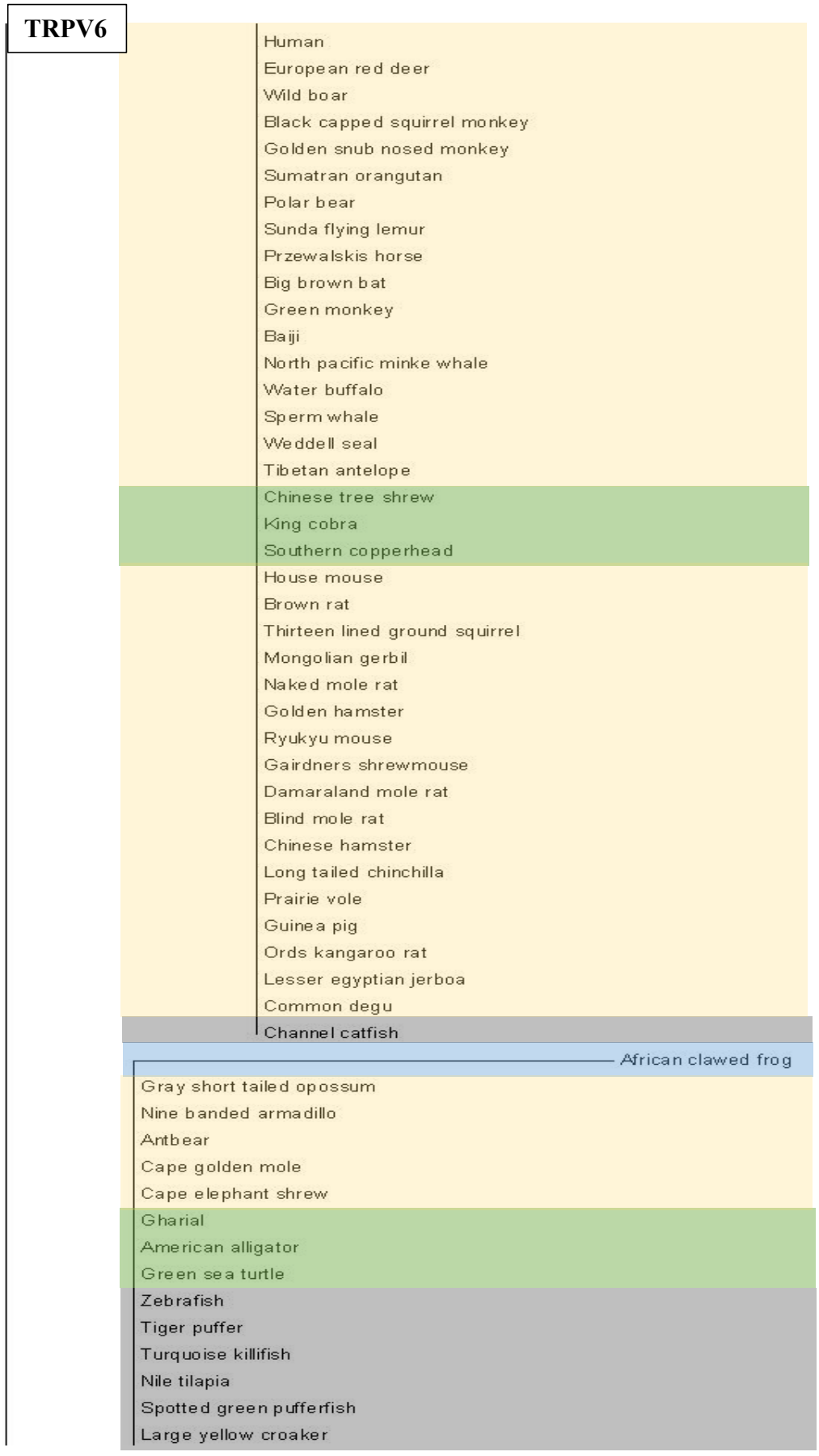
4.1 Molecular Phylogenetic analysis by Maximum Likelihood Method:

TRPV5




TRPV5
Sunda pangolin
Common shrew
Star nosed mole
European hedgehog
Wild ass
Przewalskis horse
Horse
White whale
Killer whale
North pacific minke whale
Sperm whale
Baiji
Alpaca
Wild bactrian camel
Bactrian camel
Wild boar
Humped cattle
Water buffalo
Domestic goat
Cattle
Wild yak
Plains bison
Tibetan antelope
Cape golden mole
Cape elephant shrew
Saltwater crocodile
Koala
Rock dove
Common cuckoo
Dalmatian pelican
Carolina anole
Easterm diamondback rattlesnake
bar tailed trogon
Speckled mousebird
The great tit
Eurasian blue tit
Bengalese finch
American crow
Golden collared manakin
Hoatzin
Northern fulmar
Northern carmine bee eater
Kea
Annas hummingbird
Downy woodpecker
Little egret
Rhinoceros hornbill
Adelei penguin
Great cormorant
Emperor penguin
Mallard
Cuculus discolor
Great crestde grebe
Western clawed frog
African clawed frog
Blackstripe livebearer
Scleropages formosus
Haplochromine type cichlid
Spiny chromis damselfish
Blue spotted mudskipper
Hippocampus comes
Southern platyfish
Mummichog
Atlantic killifish
0.10


TRPV6
Arabian camel
Plains bison
Bactrian camel
Western clawed frog
Large flying fox
Angola colobus
Drill
Southern pig tailed macaque
Sooty mangabey
Northern white cheeked gibbon
Killer whale
Walrus
Coquerels sifaka
Star nosed mole
American pika
Ferret
Pygmy chimpanzee
Wild yak
Wild bactrian camel
Brandts bat
Horse
Wild ass
Sheep
Rhesus macaque
Alpaca
Crab eating macaque
Alpine marmot
Davids myotis
Deer mouse
Egyptian fruit bat
European hedgehog
Natal long fingered bat
Common chimpanzee
Cebus capucinus imitator
Black snub nosed monkey
Goat
Common marmoset
Western lowland gorilla
Leopard
Great roundleaf bat
Giant panda
Zebu
Gray mouse lemur
White tailed deer
Koala
Nancy mas night monkey
Hawaiian monk seal
Phillippine tarsier
Olive baboon
Domestic dog
Ugandan red colobus
Cat
Tasmanian devil
Northern greater galago
Common rabbit
Cattle


TRPV6
Human
European red deer
Wild boar
Black capped squirrel monkey
Golden snub nosed monkey
Sumatran orangutan
Polar bear
Sunda flying lemur
Przewalskis horse
Big brown bat
Green monkey
Baiji
North pacific minke whale
Water buffalo
Sperm whale
Weddell seal
Tibetan antelope
Chinese tree shrew
King cobra
Southern copperhead
House mouse
Brown rat
Thirteen lined ground squirrel
Mongolian gerbil
Naked mole rat
Golden hamster
Ryukyu mouse
Gairdners shrewmouse
Damaraland mole rat
Blind mole rat
Chinese hamster
Long tailed chinchilla
Prairie vole
Guinea pig
Ords kangaroo rat
Lesser egyptian jerboa
Common degu
Channel catfish
African clawed frog
Gray short tailed opossum
Nine banded armadillo
Antbear
Cape golden mole
Cape elephant shrew
Gharial
American alligator
Green sea turtle
Zebrafish
Tiger puffer
Turquoise killifish
Nile tilapia
Spotted green pufferfish
Large yellow croaker

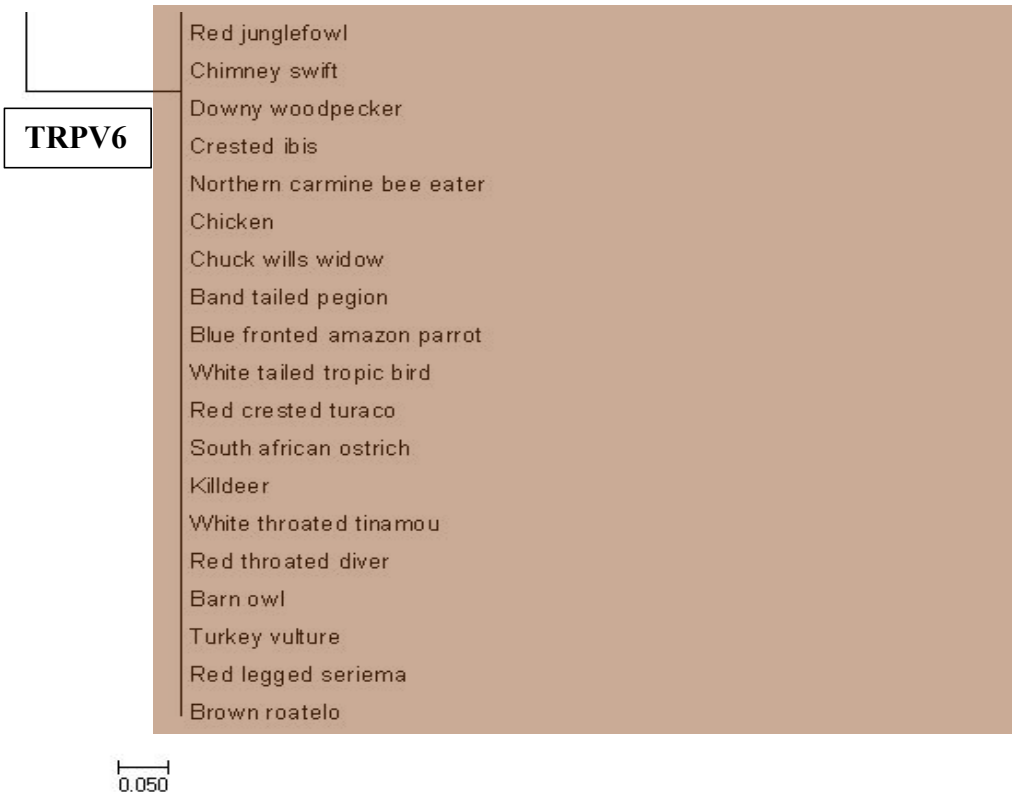


Figure 4.1. Phylogenetic Tree of TRPV5 and TRPV6. Horizontal lines are branches and represents evolutionary lineages changing over time. The longer the branch in the horizontal dimension, the larger the amount of change. The bar at the bottom with the no. 0.01 (in TRPV5) and 0.050 (in TRPV6), is the scale, which shows length of branch that represents an amount of genetic change of 0.01 and 0.05 respectively. The unit of branch length are usually nucleotide substitution per site. The vertical line simply tells, which horizontal line connects to which and how long they are irrelevant. Mammals (yellow), Reptiles (green), Aves (brown), Amphibians (blue) and Fishes (grey).

4.2 Conservation of Different domains:

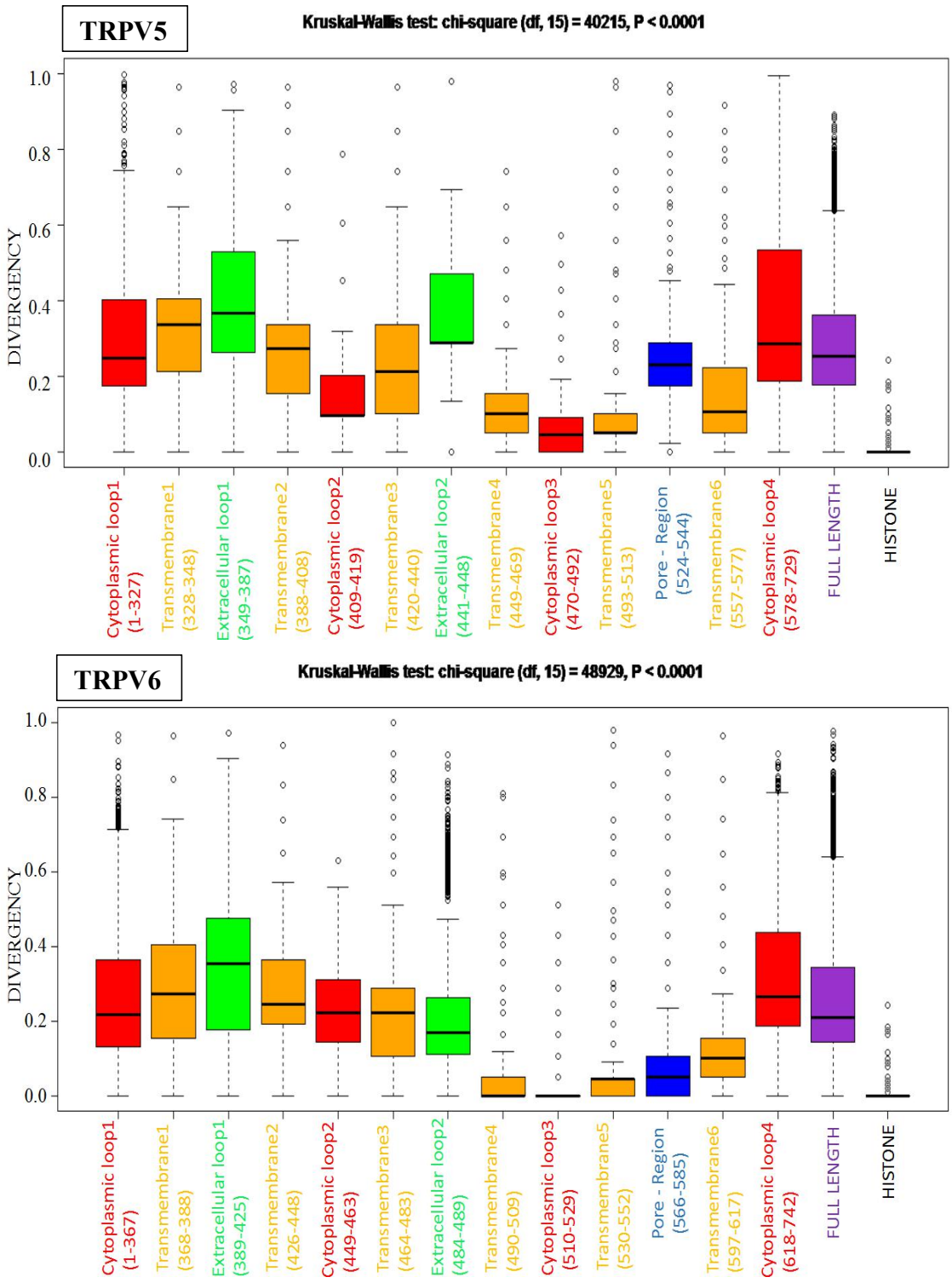


Figure 4.2 BoxPlot of Different Domains of TRPV5 and TRPV6. BoxPlot showing conservation of Different domains of TRPV5 and TRPV6. Different domains are indicated by different colors. The lower value indicates more conservation, while the higher value indicates less conservation.

4.3.1 Determination of LWI Residues:

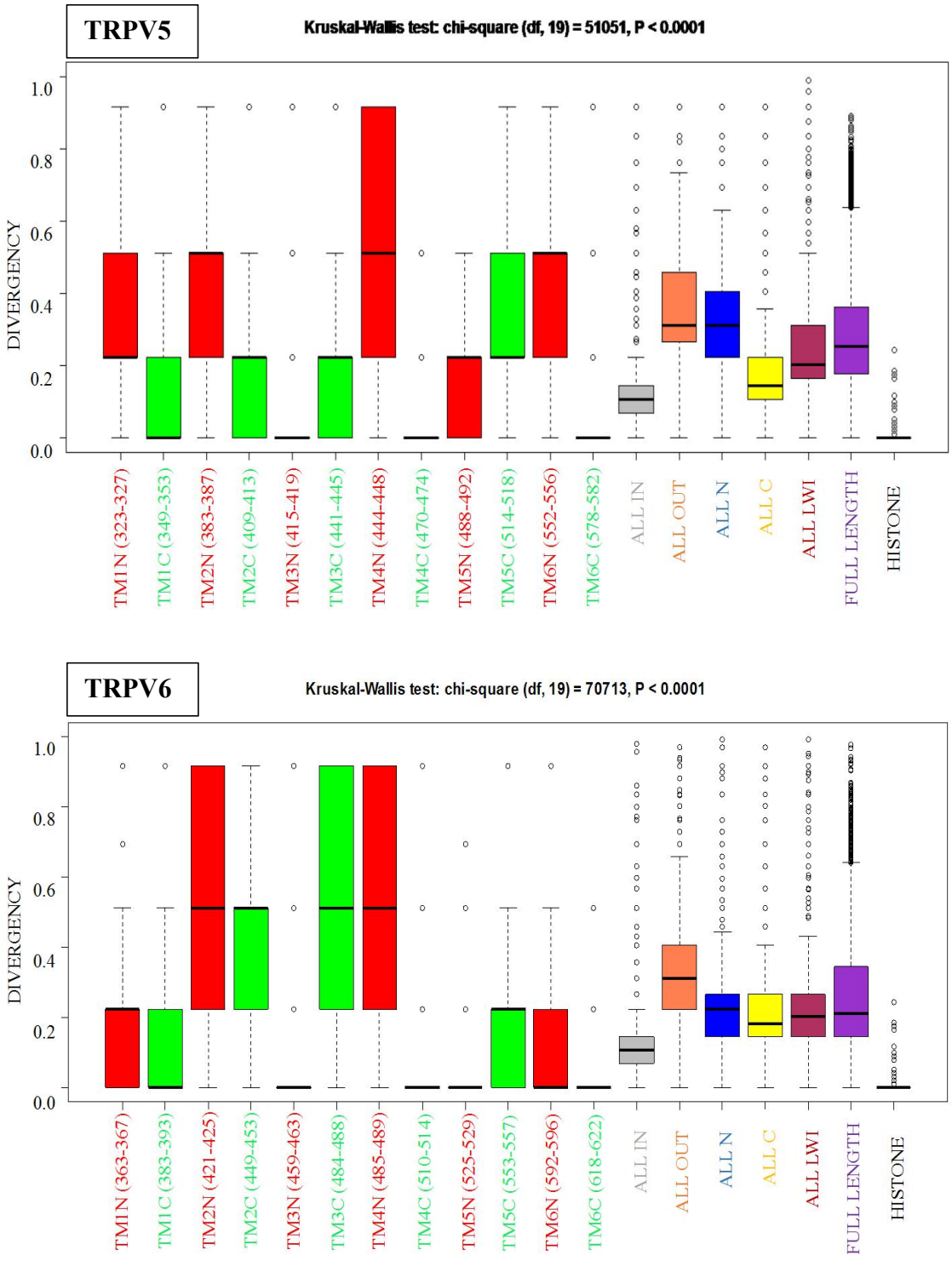


Figure 4.3.1 BoxPlot of LWI Residues. BoxPlot showing conservation of LWI Residues of TRPV5 and TRPV6. Different motifs are indicated by different colors. The lower value indicates more conservation, while the higher value indicates less conservation.

4.3.2 Sequence Logo Generation:

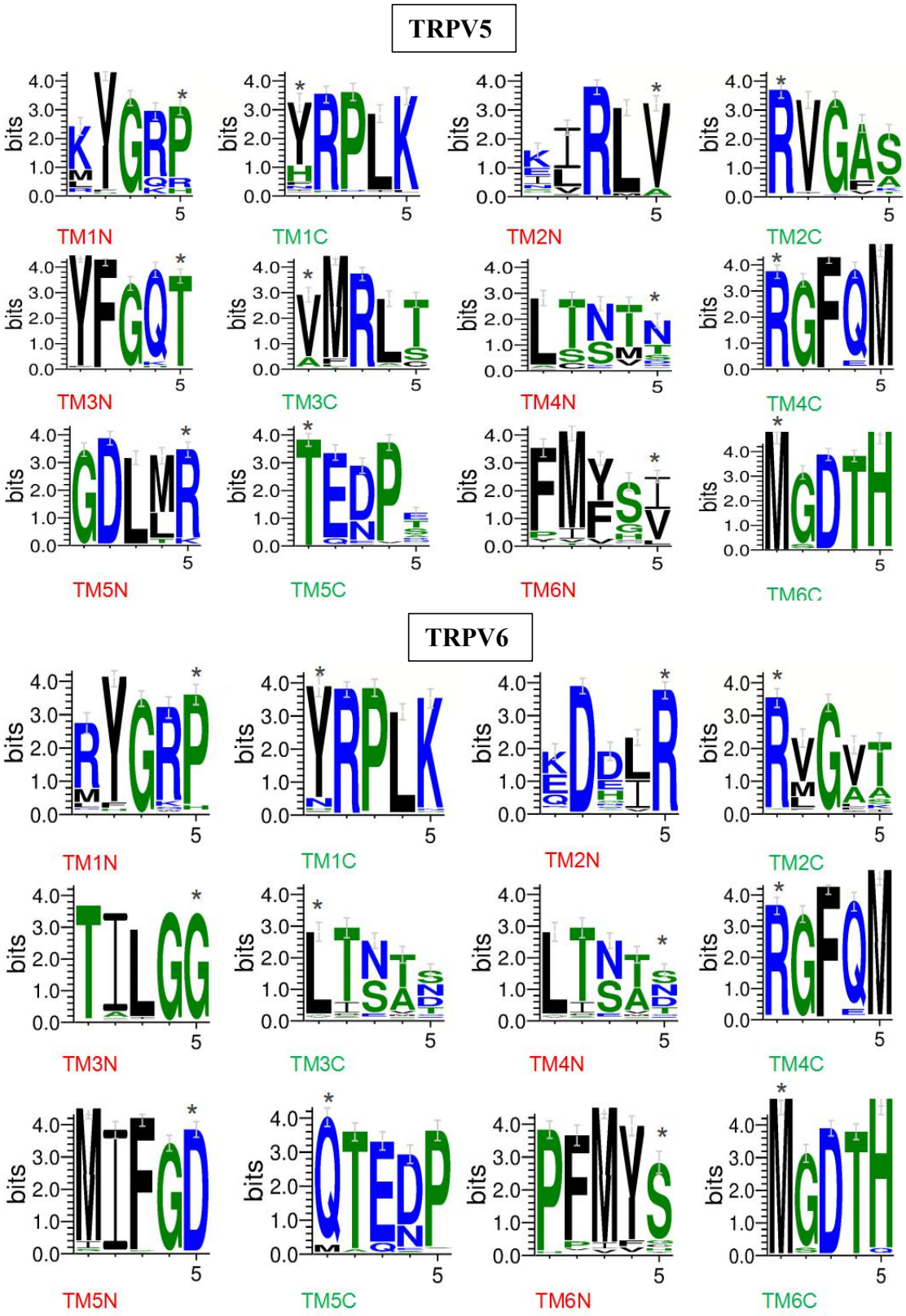


Figure 4.3.2 Sequence logo of TRPV5 and TRPV6. The black colored arterisk marks the amino acid, whose conservation is studied. The Y-axis denotes the degree of conservation.

4.4.1 Determination of Cholesterol Binding motifs:

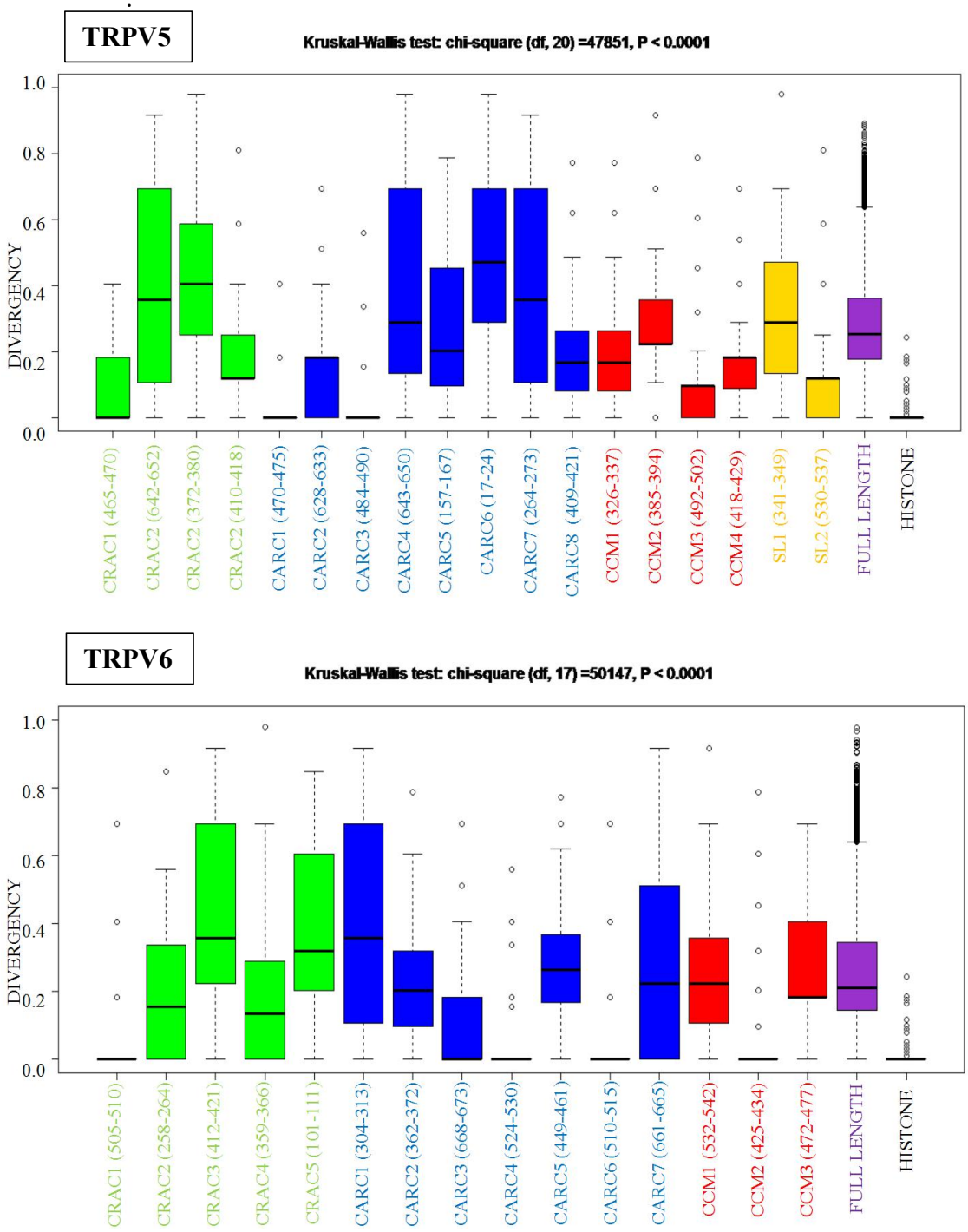


Figure 4.4.1 BoxPlot of Cholesterol Binding motifs. BoxPlot showing conservation of Cholesterol Binding motifs of TRPV5 and TRPV6. Different motifs are indicated by different colors. The sequence of motifs in the BoxPlot are in decreasing order of similarity with known motif sequence, as generated by Pattinprot server. 'SL' denotes Sphingolipid binding motif. The lower value indicates more conservation, while the higher value indicates less conservation.

4.4.2 Visualization of Cholesterol Binding motifs:

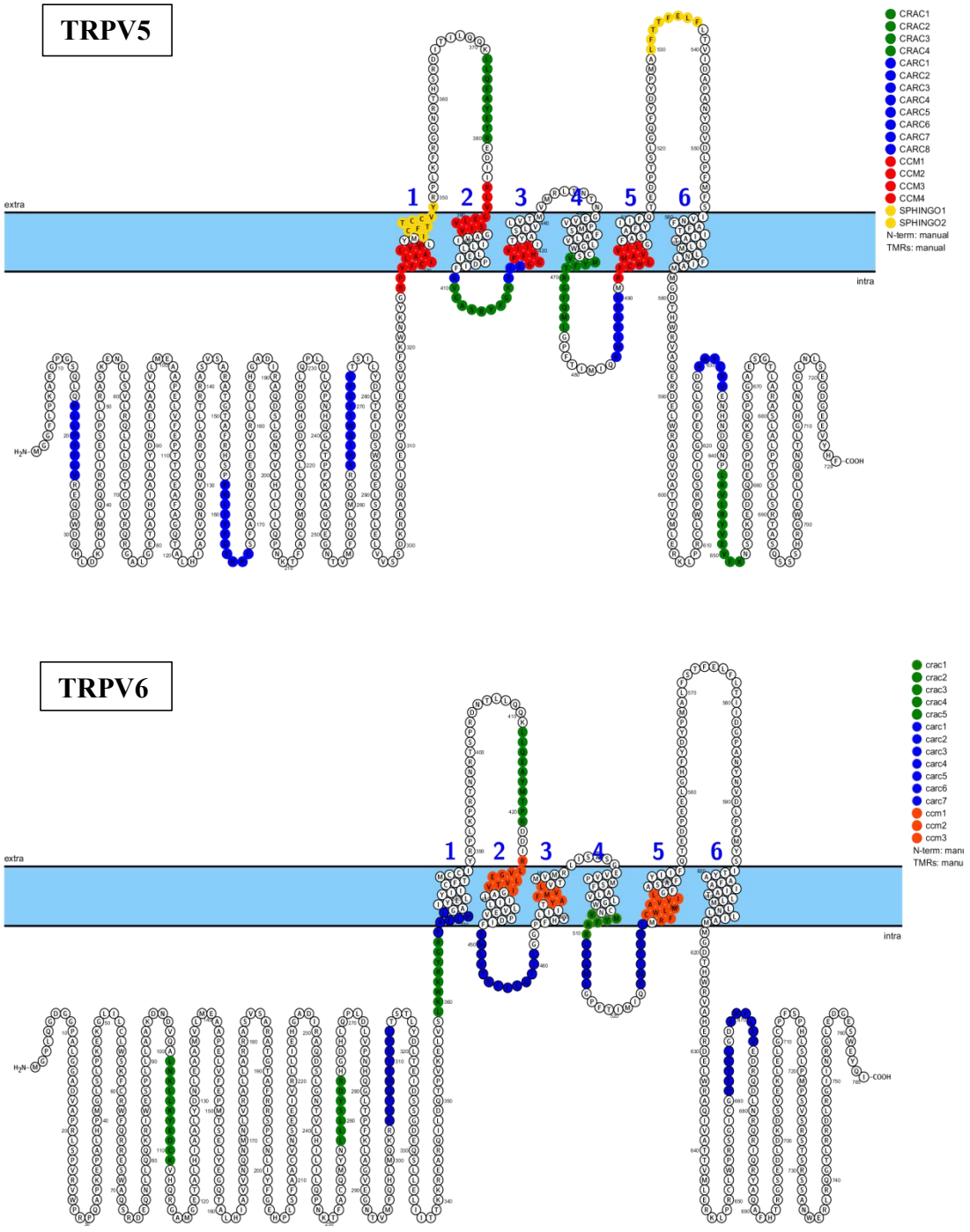
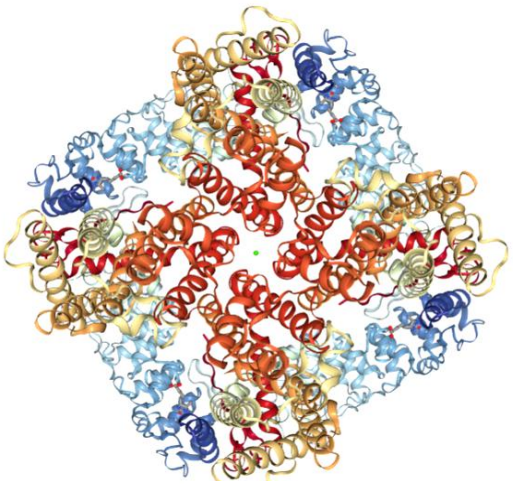


Figure 4.4.2 Schematic Representation of probable Cholesterol Binding motifs in TRPV5 and TRPV6. Different motifs are indicated by different colors.

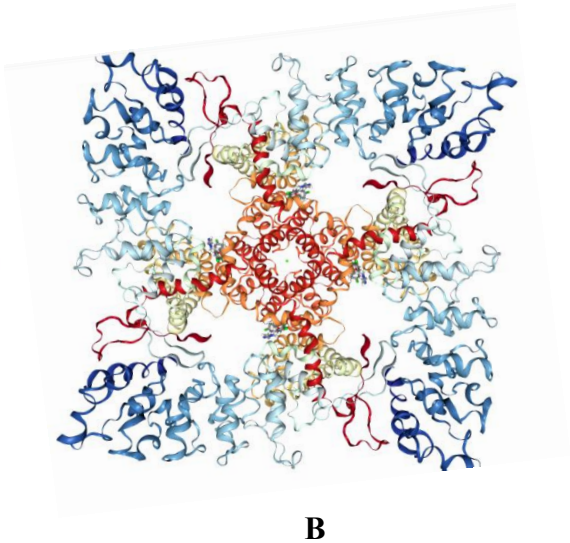
CRAC - green, CARC - blue, CCM - red, Sphingolipid binding motif - yellow

The numbering of motifs is according to the their occurence in the protein sequence.

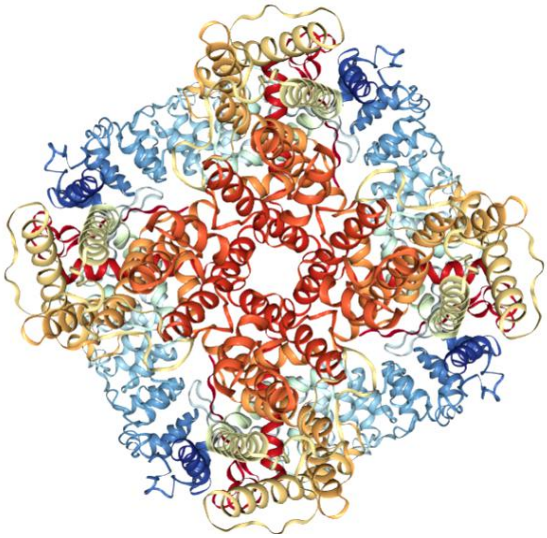
4.5 Homology Modeling:



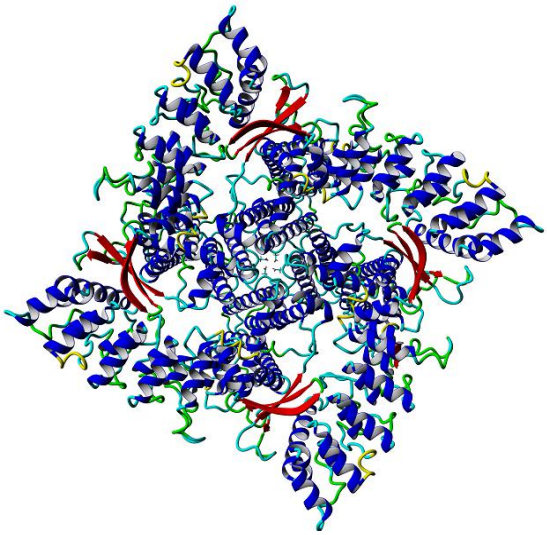
A



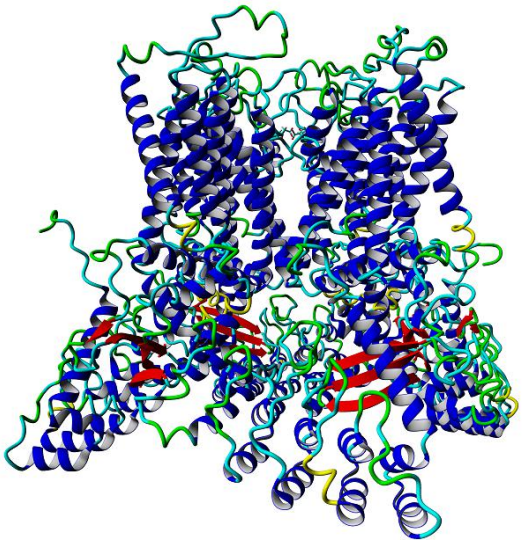
B



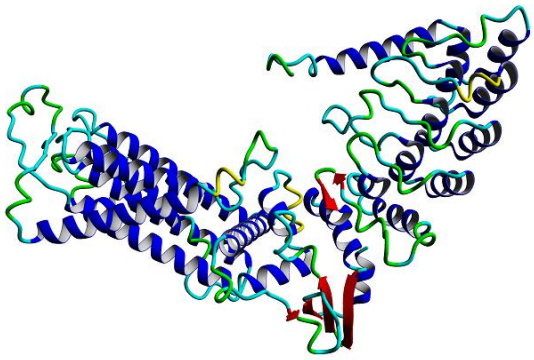
C



D



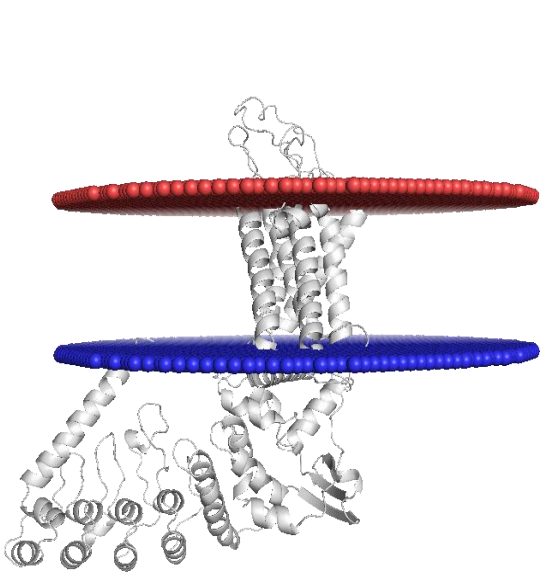
E



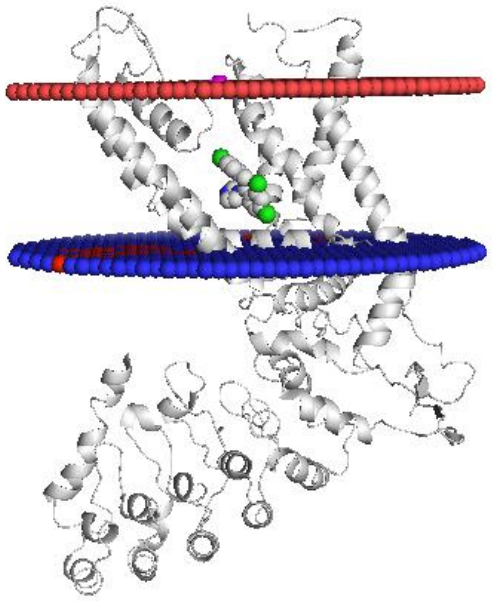
F

Figure 4.5 Structures of TRPV5 and TRPV6: (A) Closed structure of TRPV6 homotetramer (PDB ID '5IWK'). **(B)** Closed structure of TRPV5 homotetramer (PDB ID '6B5V'). **(C)** Open structure of TRPV6 tetramer (PDB ID '6BOB'). **(D)** Homology modeled structure of TRPV5 homotetramer in Open conformation-Top view. **(E)** Homology modeled structure of TRPV5 homotetramer in Open conformation-Side view. **(F)** Homology modeled structure of TRPV5 monomer in Open conformation.

4.6 Protein Visualization in Lipid Bilayer:



A



B

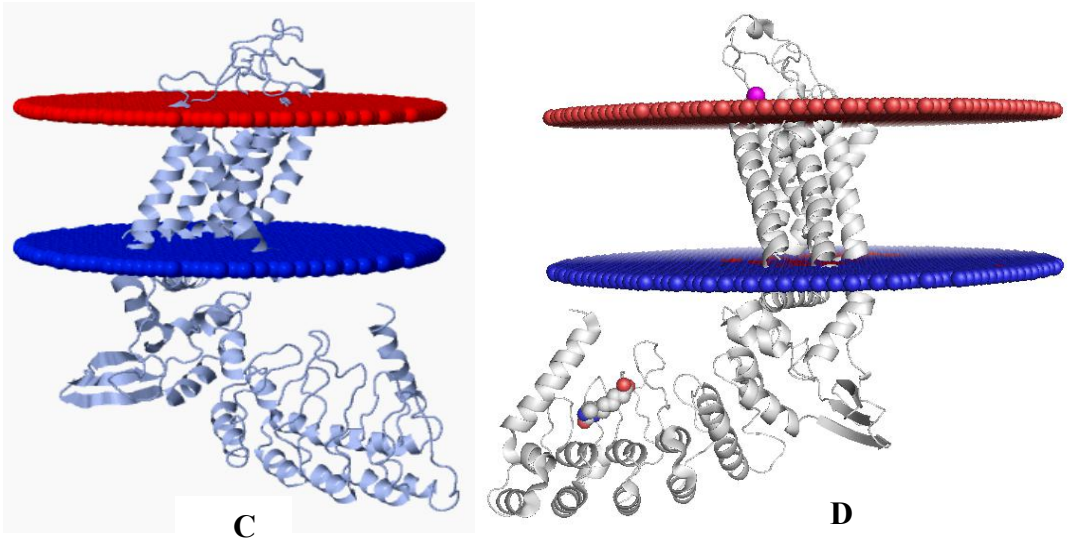


Figure 4.6 TRPV5 and TRPV6 monomer in the Lipid Bilayer: (A) TRPV5 in open conformation. **(B)** TRPV5 in closed conformation, bound to Calcium (magenta coloured) and its inhibitor Econazole (green and white coloured molecule). **(C)** TRPV6 in open conformation. **(D)** TRPV6 in closed conformation, bound to Calcium (magenta coloured) and its inhibitor D-Desthibiotin (red and white coloured molecule).

4.7 *In silico* Docking(Global) study:

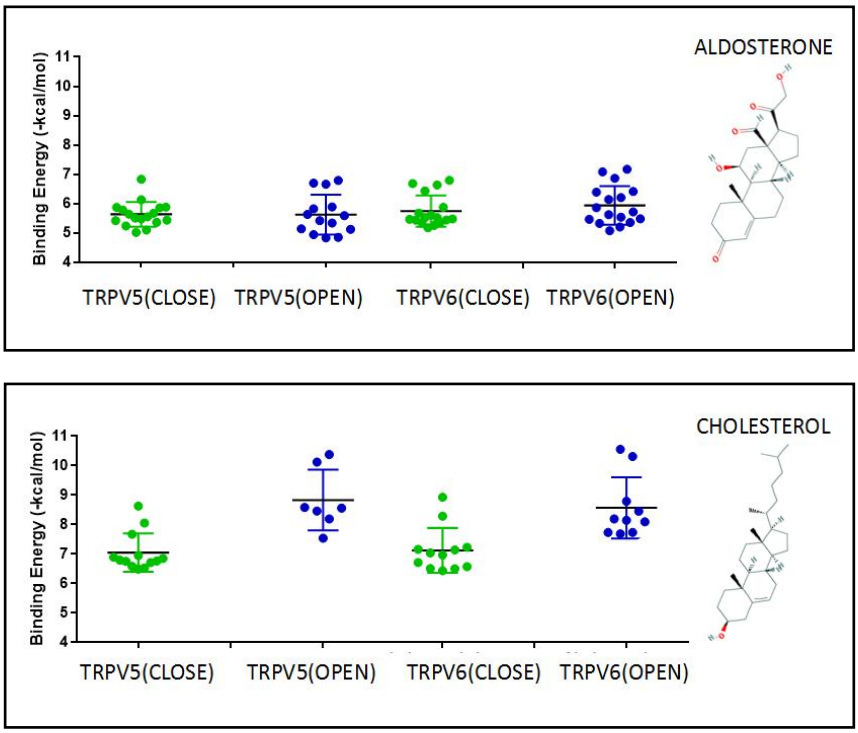
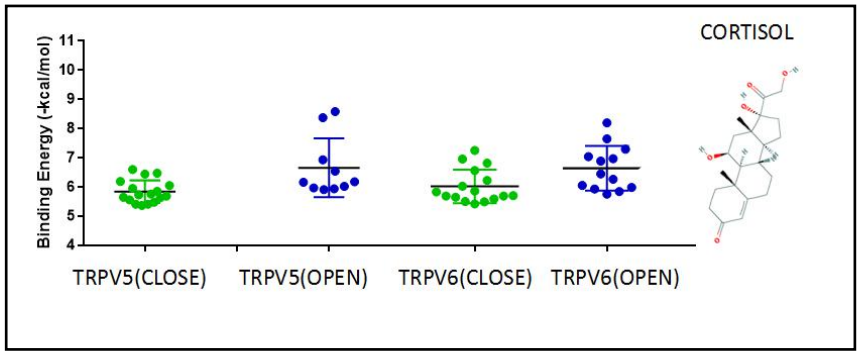
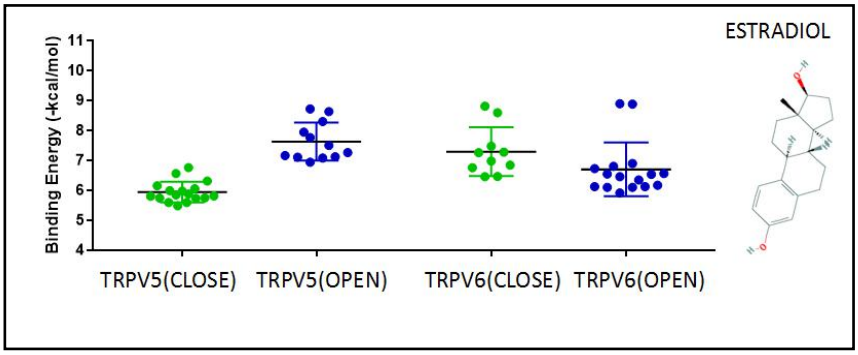
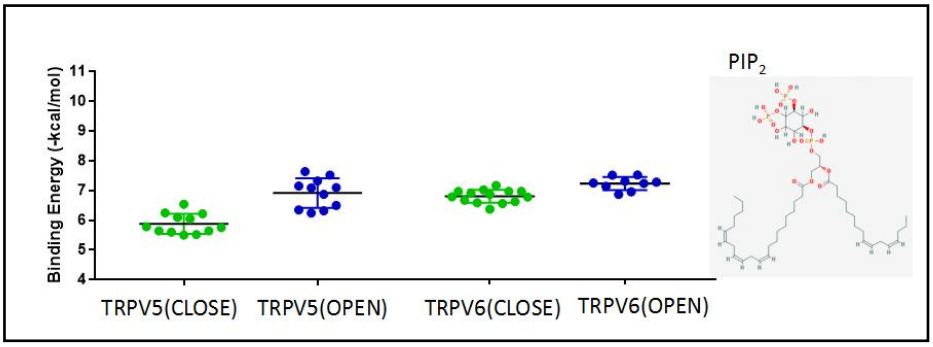
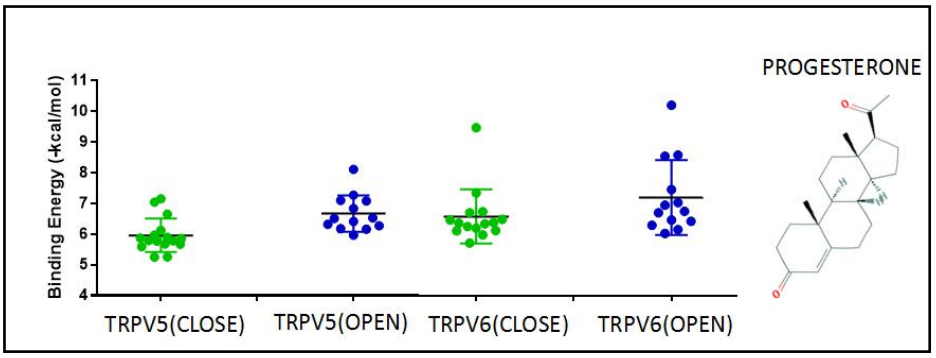


Figure 4.7 Binding Energies of respective Global docking:









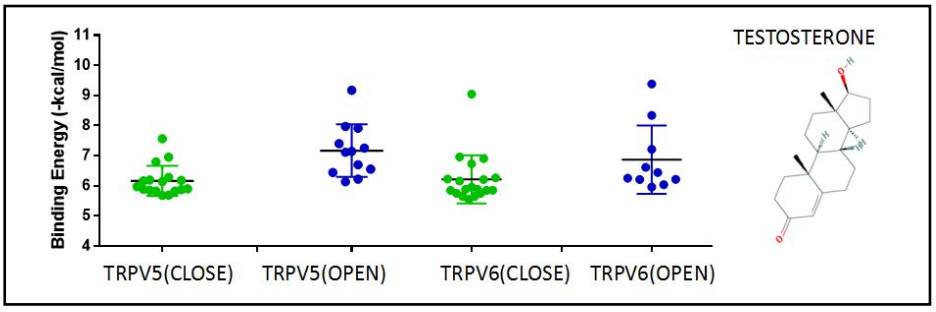


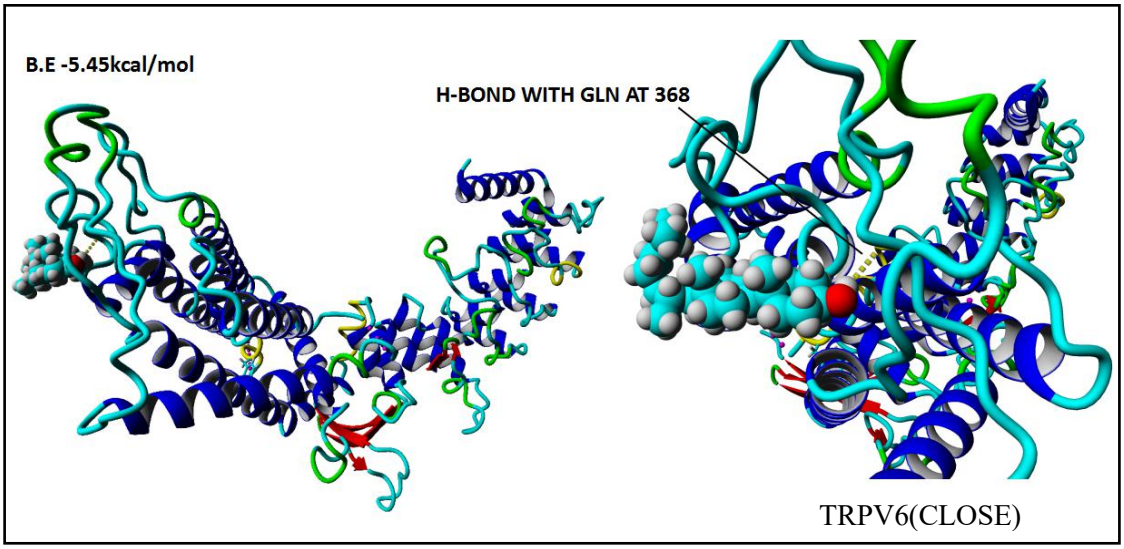
Figure 4.7 Binding Energies of respective Global docking:

Figure 4.7 Binding Energies of respective Global docking: Graphs showing comparison of average binding energy (black line) of respective hormones in both close and open conformation of TRPV5 and TRPV6. Green dots denotes binding energies of respective hormone with closed conformation of the protein, while blue dots denotes the same with open conformation.

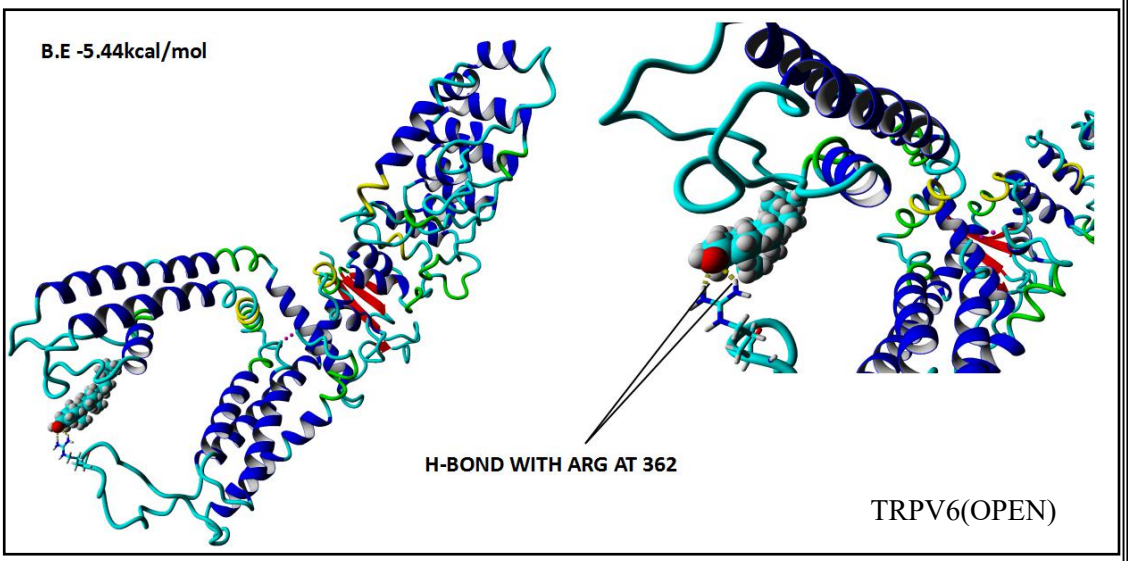
4.8 *In silico* Docking(Local) study:

CHOLESTEROL

No Hydrogen bonding found in case of both TRPV5 closed and open conformation.

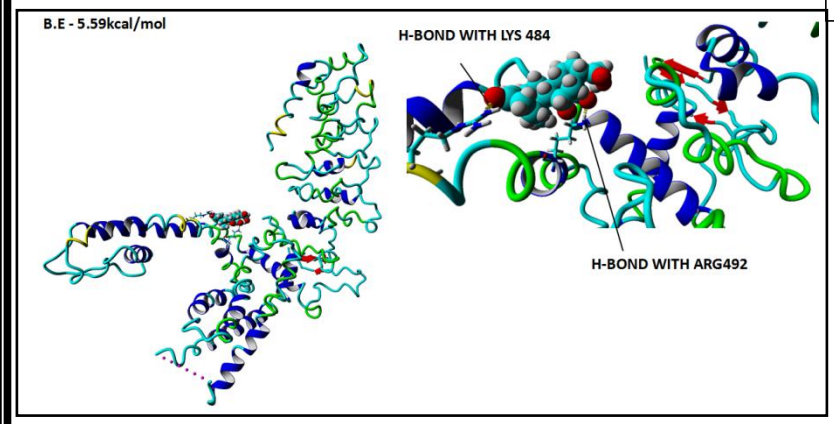


TRPV6(CLOSE)

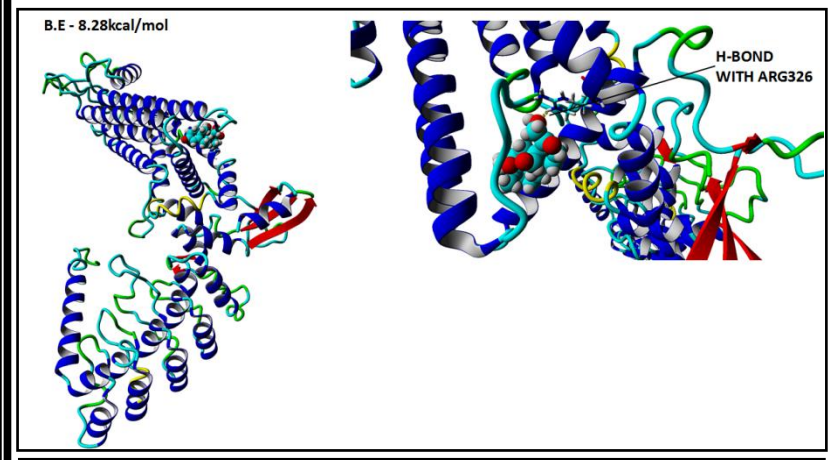


TRPV6(OPEN)

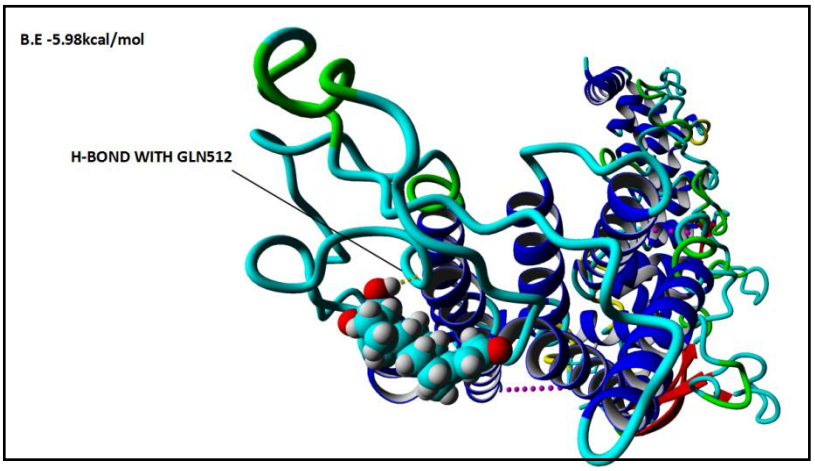
ALDOSTERONE



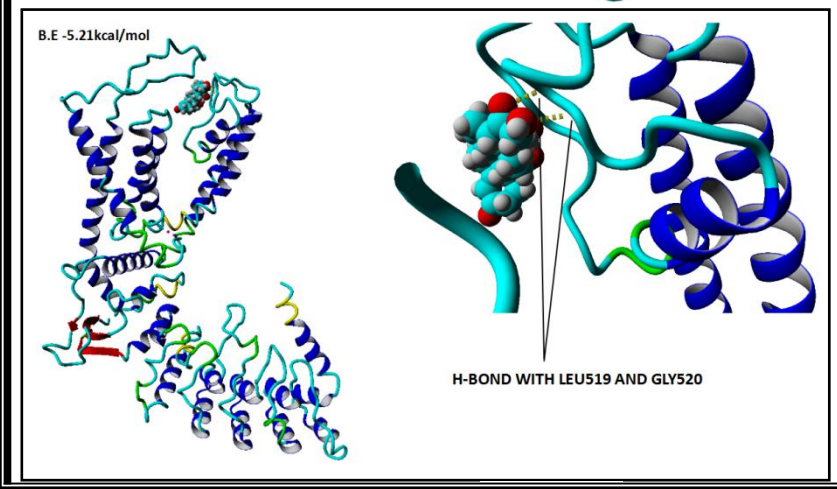
TRPV5(CLOSE)



TRPV5(OPEN)

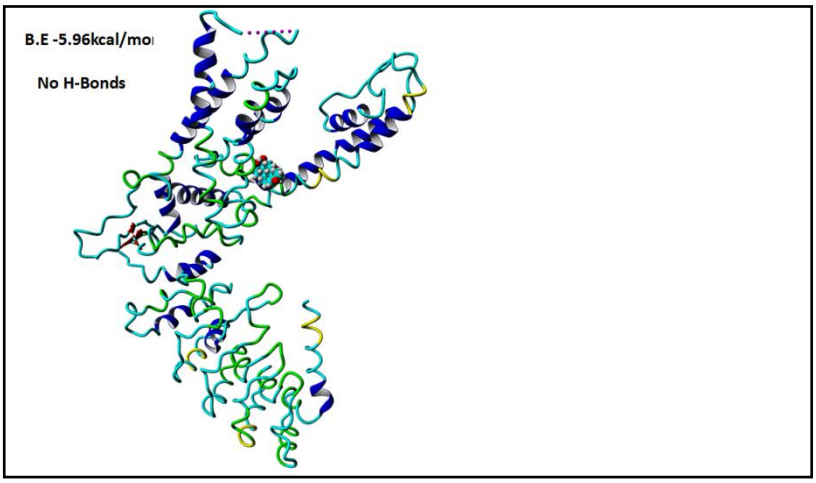


TRPV6(CLOSE)

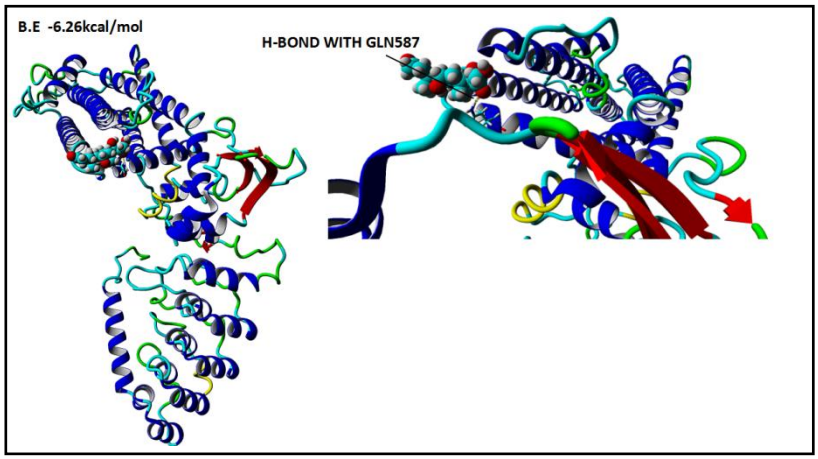


TRPV6(OPEN)

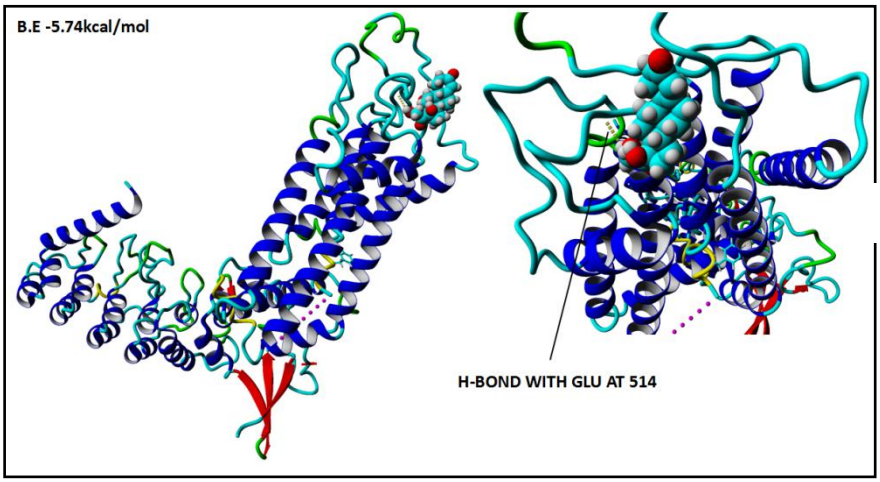
CORTISOL



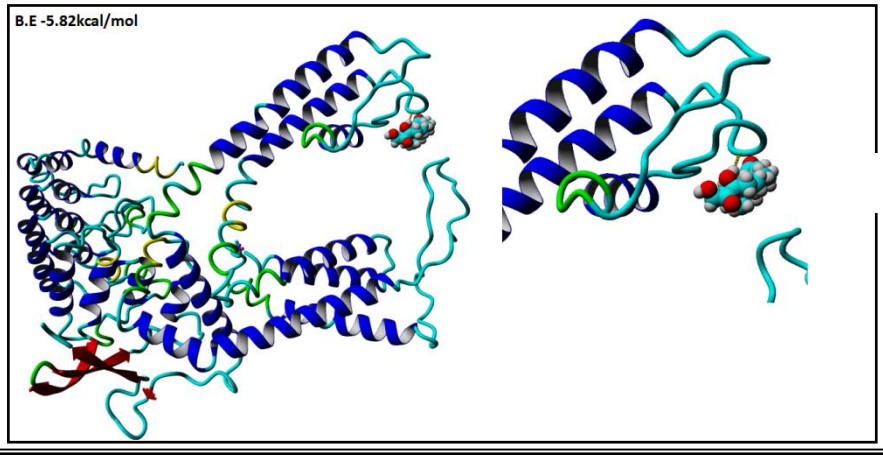
TRPV5(CLOSE)



TRPV5(OPEN)

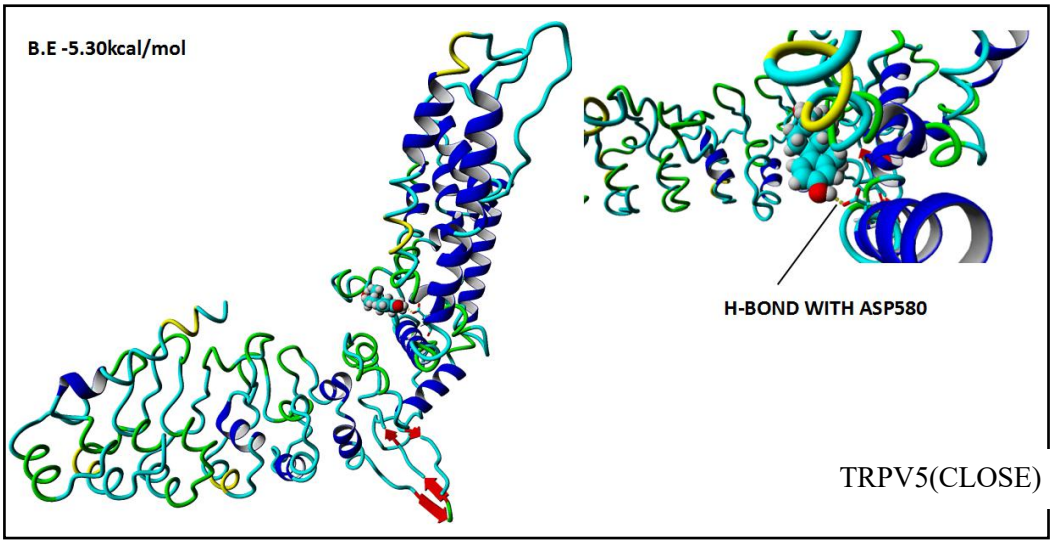


TRPV6(CLOSE)

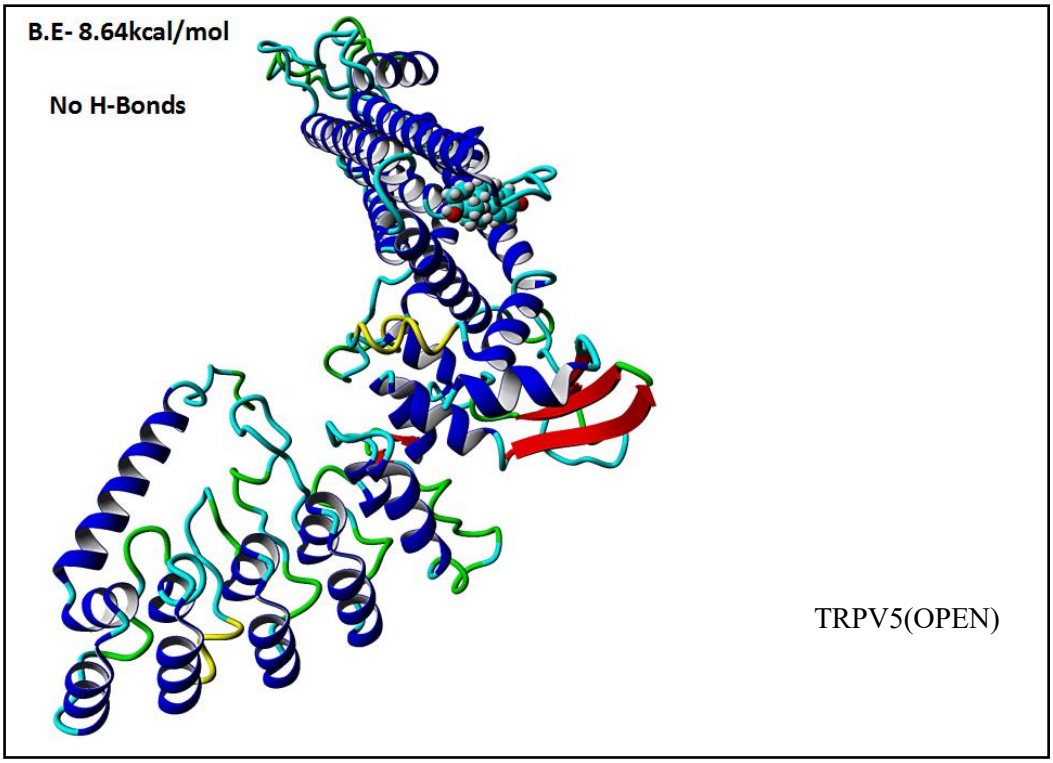


TRPV6(OPEN)

ESTRADIOL

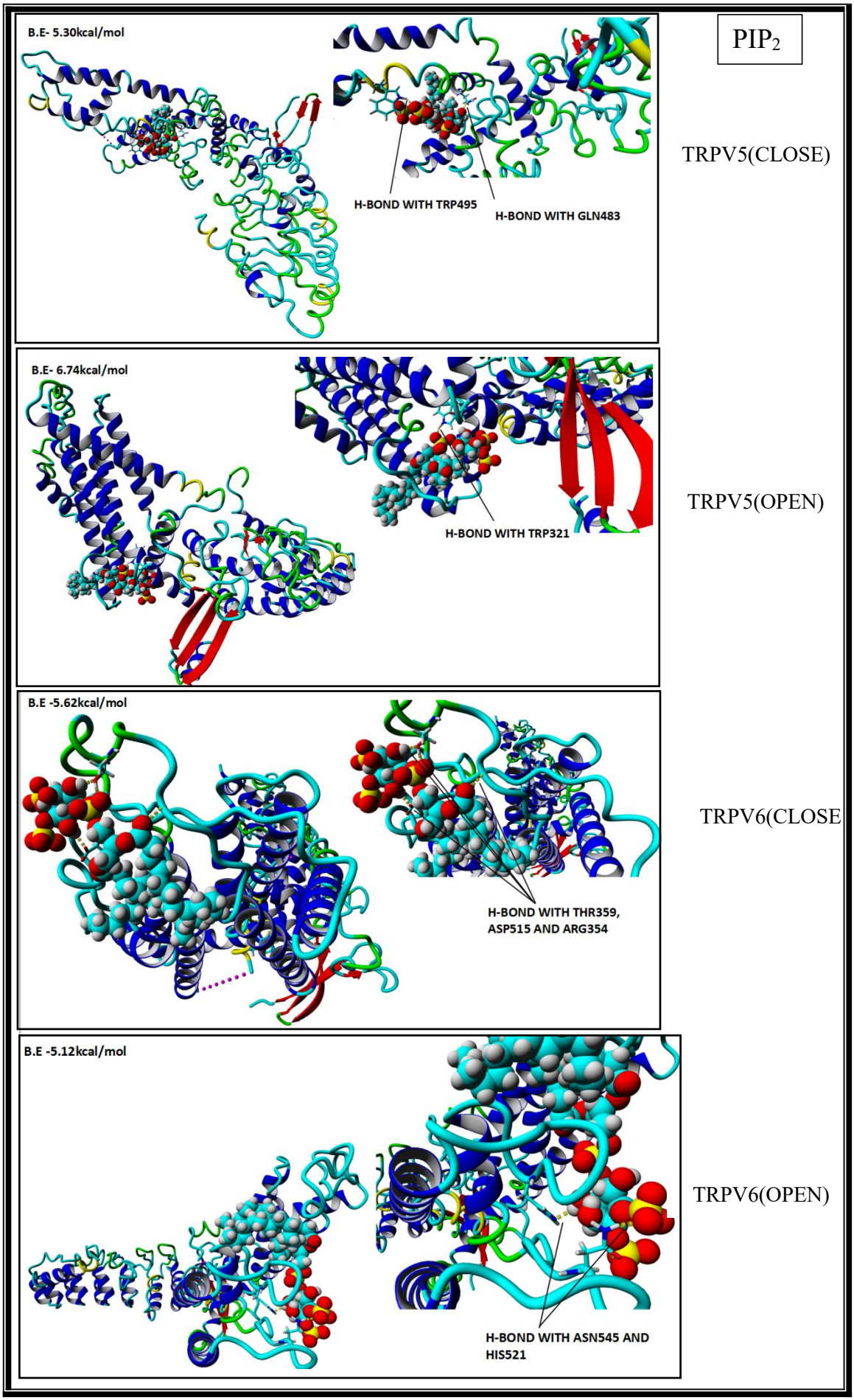


TRPV5(CLOSE)

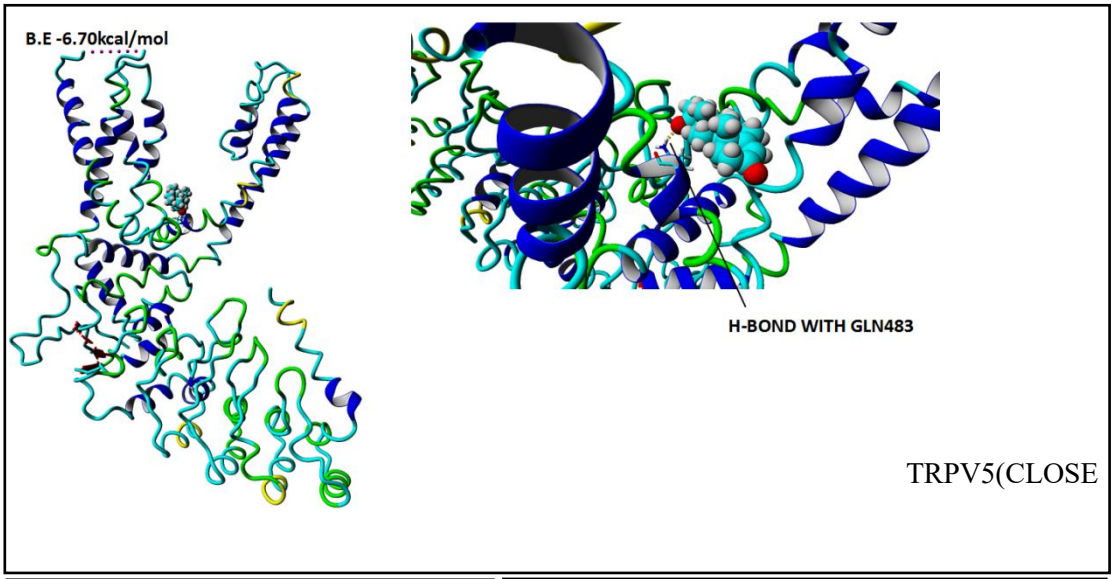


TRPV5(OPEN)

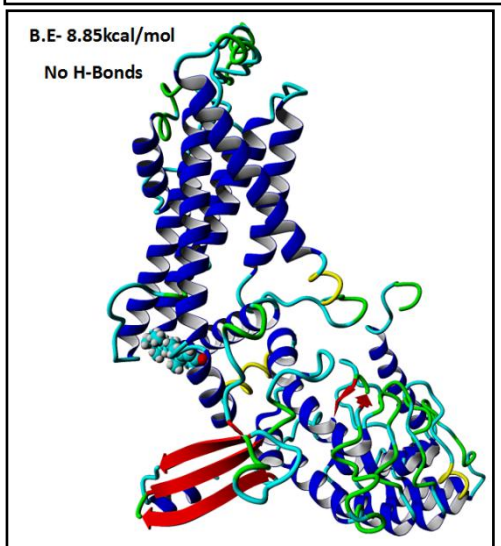
No Hydrogen Bonding is found in case of both TRPV6 close and open conformation.


PIP2
B.E- 5.30kcal/mol
H-BOND WITH TRP495
H-BOND WITH GLN483
TRPV5(CLOSE)
B.E- 6.74kcal/mol
H-BOND WITH TRP321
TRPV5(OPEN)
B.E -5.62kcal/mol
H-BOND WITH THR359,
ASP515 AND ARG354
TRPV6(CLOSE
B.E -5.12kcal/mol
H-BOND WITH ASN545 AND
HIS521
TRPV6(OPEN)

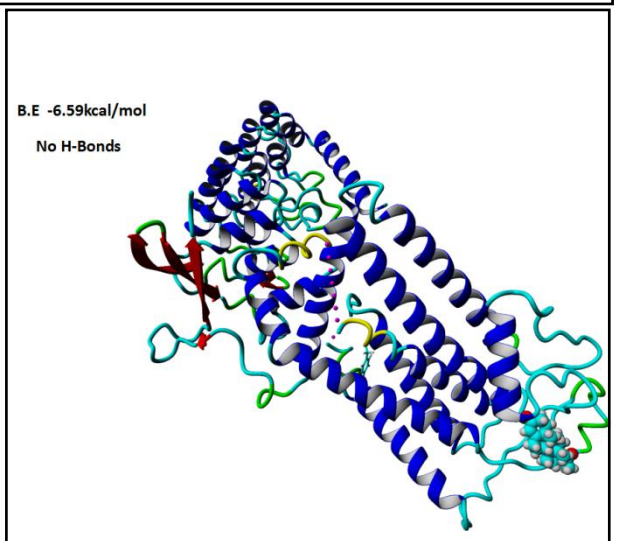
PROGESTERONE



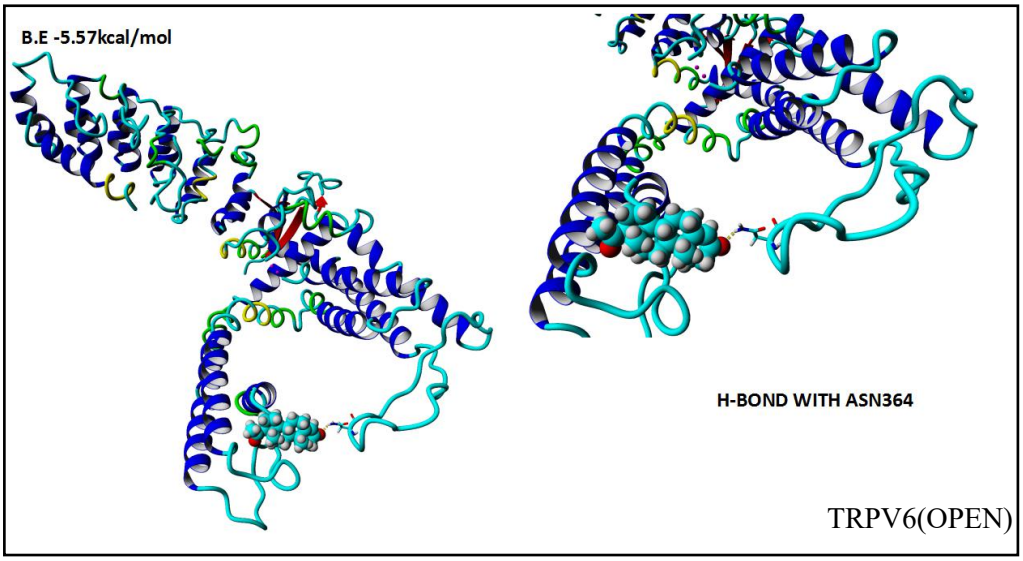
TRPV5(CLOSE



TRPV5(OPEN)

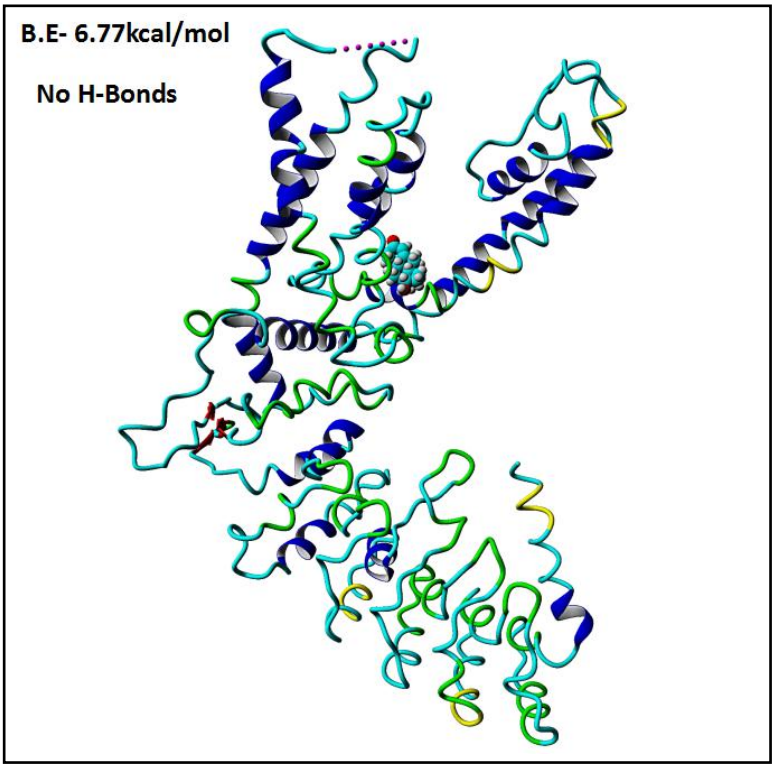


TRPV6(CLOSE)

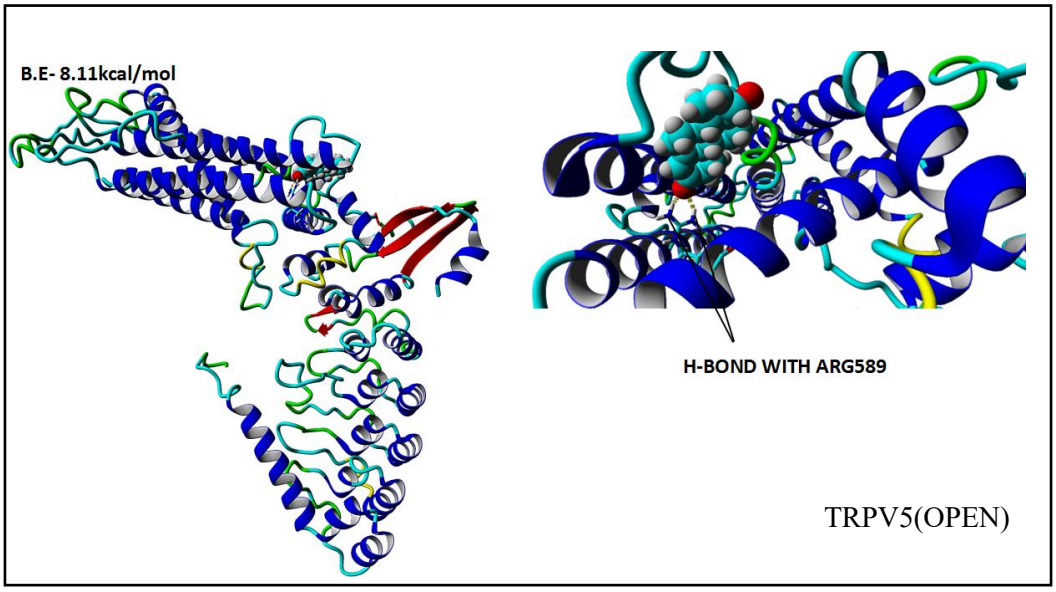


TRPV6(OPEN)

TESTOSTERONE



TRPV5(CLOSE)



TRPV5(OPEN)

No Hydrogen Bonding is found in case of both TRPV6 close and open conformation.

Figure 4.8: Various docking positions in TRPV5 and TRPV6 for both close and open conformations: Hydrogen bonds(Dotted yellow coloured) with their respective amino acids.

CHAPER 5

DISCUSSION

4.1 Phylogenetic Analysis of TRPV5 and TRPV6:

The Phylogenetic Tree of TRPV5 and TRPV6 is shown in Figure 4.1. From this, it was observed that both TRPV5 and TRPV6 are present in mammals, but only one of the two is present in birds and fishes, based on the current database. For example, only *trpv6* gene is present in Zebrafish and Tiger Pufferfish. This is not a single incident because the same is true for Chicken genome. Contrastingly, both TRPV5 and TRPV6 is present in chimpanzee, humans and mouse. As it is known that *trpv5* gene was generated by duplication of *trpv6* gene, from the tree, it is likely that gene duplication event occured during evolution of mammals from reptiles.

4.2 Evolution of TRPV5 and TRPV6 has almost similar pattern:

In this analysis, we have tested the conservation pattern of different domains, motifs and interacting sites, in both TRPV5 and TRPV6. For this, we have retrieved TRPV5 and TRPV6 sequences of different species, fragmented them into different domains, according to published literature, and prepared a boxplot. Figure 4.2 gives an important information that both TRPV5 and TRPV6 are neither a highly conserved protein like histone nor have evolved very early in the evolutionary history. This analysis reveals that the structurally and functionally important regions of both the proteins are conserved, though with different degree of variance. Among all the domains and motifs, TM4, Cytoplasmic loop 3 and TM5 has maximum degree of conservation, as compared to other regions. This throws light on the fact that, these domains are more or less important for channel function.

5.3.1 Amino acid conservation in lipid water interface:

The box plot in figure 4.3.1 represents evolutionary diverge in the 12 lipid water interfaces of both TRPV5 and TRPV6. From this, we found that there is a differential selection pressure on different lipid water interfaces. TM3N, TM4C and TM6C are found to be highly conserved in both the proteins, while additionally, TM5N is found to be conserved in TRPV6. Strikingly, other Lipid water interfaces of both the proteins shows some similarity in divergence pattern.

5.3.2 Amino acid conservation at the ends of Transmembrane helices facing the lipid water interface:

The presence of specific amino acids at the ends of lipid water interface were investigated with the aim of finding evolutionary significance. The lipid water interface amino acids on the inner side, i.e TM1N, TM2C, TM4C and TM6C (as in the figure 4.3.2) are found to be more conserved from duplication and evolution of *trpv5* from *trpv6* as compared to that on the outer face of the membrane protein. Moreover, Arginine, which is a snorkeling amino acid, is found to appear at many positions, in a conserved manner in both the proteins, which indicates its importance in the lipid water interface. In most of the places, Arginine is found to be fully conserved, as in TM2C, TM4C and TM5N of *trpv5* and in TM2N, TM2C and TM4C of *trpv6.* While Tyrosine is found to be selected over Phenylalanine and Asparagine at position TM1C of both *trpv5* and *trpv6.*

5.4.1 Conservation of Cholesterol binding motifs.

This analysis reveals information about which CRAC or CRAC like motifs are conserved in TRPV5 and TRPV6, throughout evolution. Figure 4.4.1 shows that TRPV5 and TRPV6 highly differs in divergence pattern of these motifs. TRPV6 shows conservation in four motifs, i.e. CRAC1, CARC4, CARC6 and CCM2. While TRPV5 shows conservation in only two such motifs, i.e. CARC1 and CARC3. This shows that, evolution of TRPV6 is more stable as compared to TRPV5, also shedding light on the fact that *trpv5* has arisen due to gene duplication event of *trpv6.*

5.4.2 Comparison of location of Cholesterol binding motifs and their evolution from TRPV6 to TRPV5:

From figure 4.4.2, we can compare the location and evolution of Cholesterol binding motifs in TRPV5 and TRPV6. The interchange and conversion of motifs among each other from *trpv6* to *trpv5,* at the same location can be clearly seen. The location of some motifs are totally conserved in the protein sequence, while some show a little shift.

The position of CARC motif in N-terminal cytoplasmic region of *trpv6* is also maintained in *trpv5.* Similar is the case with CRAC motif in TM1-TM2 loop and CARC motif in C-terminal domain of *trpv6.* While CCM motif in TM2, TM3 and TM5 and CARC motif near TM5 of *trpv6* is seen to be little shifted in *trpv5.*

CARC motif in TM1 region of *trpv6* shows transition to CCM motif in *trpv5.* While, CARC motif in TM2-TM3 loop and TM4-TM5 loop shows partial change to CRAC motif in *trpv5.*

5.5 Homology Modeling:

From figure 4.5, we can clearly see the extent of similarity between TRPV5 and TRPV6 structures, in both closed and open conformation. These structures were basically used for docking study.

5.6 *In silico* Docking(Global) of Cholesterol and its derivatives:

Figure 4.7 clearly shows that in case of cholesterol and its derivatives, the average binding energy is high with the closed conformation of both TRPV5 and TRPV6. While, Estradiol shows a striking difference in the average binding energy, which is higher with the open conformation of TRPV6, as compared to the close conformation.

5.7 *In silico* Docking(Local) of Cholesterol and its derivatives:

Figure 4.8 shows some of the selected docking positions of cholesterol and its derivatives, in the TM4-TM5 loop of TRPV5 and TRPV6. These were selected as they showed strong bonding (Hydrogen Bonds) with the protein. Cholesterol showed no hydrogen bonding with TRPV5, while Estradiol and Testosterone showed no H-bonds with TRPV6. We cannot conclude anything from this, but only the confirmation, that these small molecules binds to that specific loop region strongly, just like PIP_2.

CHAPER 6

CONCLUSION

6.1 SUMMARY:

From the work presented above, it can be concluded that TRPV6 is more conserved as compared to TRPV5. This also confirms the fact that, *trpv5* is duplicated from *trpv6* during the course of evolution, and thus shows evolutionary differences. This in silico analysis also throws light on the possibility of binding of cholesterol and its derivatives to TRPV5 and TRPV6, as they show really high binding energies (some value exceeding -8kcal/mol) with both of them.

6.2 FUTURE WORK:

The binding of cholesterol and its derivatives to both TRPV5 and TRPV6 needs to be performed experimentally to confirm whether they have a role in regulation and gating mechanism of these ion channels. This may then help to solve some problems associated with Calcium homeostasis and also bone mineralization.

REFERENCES

1.David Clapham, Loren Runnels. The TRP ion channel family. Article in *Nature reviews Neuroscience* · July 2001

2.Beech DJ, Bahnasi YM, Dedman AM, Al Shawaf E. *TRPC channel lipid specificity and mechanisms of lipid regulation*. Cell Calcium 2009.

3. Kim AY, Tang ZX, Liu Q, Patel KN, Maag D, Geng YX, et al. Pirt, a phosphoinositide-binding protein, functions as a regulatory subunit of TRPV1. *Cell* 2008; 133:475-85

4.Liu B, Hui K, Qin F. Thermodynamics of heat activation of single capsaicin ion channels VR1. *Biophys J* 2003; 85:2988-3006.

5.Parnas M, Katz B, Lev S, Tzarfaty V, Dadon D, Gordon-Shaag A, et al. Membrane lipid modulations remove divalent open channel block from TRP-like and NMDA channels. *J Neurosci* 2009; 29:2371-83.

6.Parnas M, Peters M, Minke B. *Linoleic acid inhibits TRP channels with intrinsic voltage sensitivity: Implications on the mechanism of linoleic acid action*. Channels (Austin) 2009; 3:164-6

7.Riske KA, Dobereiner HG. Diacylglycerol-rich domain formation in giant stearoyl-oleoyl phosphatidylcholine vesicles driven by phospholipase C activity. *Biophys J* 2003; 85:2351-62.

8.Formigli L, Sassoli C, Squecco R, Bini F, Martinesi M, Chellini F, et al. Regulation of transient receptor potential canonical channel 1 (TRPC1) by sphingosine 1-phosphate in C2C12 myoblasts and its relevance for a role of mechanotransduction in skeletal muscle differentiation. *J Cell Sci* 2009; 122:1322-33.

8.Yogi A, Callera GE, Tostes R, Touyz RM. Bradykinin regulates calpain and proinflammatory signaling through TRPM7-sensitive pathways in vascular smooth muscle cells. *Am J Physiol Regul Integr Comp Physiol* 2009; 296:201-7.

9.Pani B, Ong HL, Liu X, Rauser K, Ambudkar IS, Singh BB. Lipid rafts determine clustering of STIM1 in endoplasmic reticulum-plasma membrane junctions and regulation of store-operated Ca2+ entry (SOCE). *J Biol Chem* 2008; 283:17333-40

10.Pani B, Ong HL, Brazer SC, Liu X, Rauser K, Singh BB, et al. *Activation of TRPC1 by STIM1 in ER-PM microdomains involves release of the channel from its scaffold caveolin-1*. Proc Natl Acad Sci USA 2009; 106:20087-92.

11.Nilius B, Owsianik G, Voets T. Transient receptor potential channels meet phosphoinositides. *EMBO J* 2008; 27:2809-16.

12. Chou T, Jaric MV, Siggia ED. Electrostatics of lipid bilayer bending. *Biophys J* 1997; 72:2042-55.

13.Gkika D, Prevarskaya N. Molecular mechanisms of TRP regulation in tumor growth and metastasis. *Biochim Biophys Acta* 2009; 1793:953-8.

14.Diogenes A, Patwardhan AM, Jeske NA, Ruparel NB, Goffin V, Akopian AN, et al. Prolactin modulates TRPV1 in female rat trigeminal sensory neurons. *J Neurosci* 2006; 26:8126-36

15. Plant TD, Zollner C, Kepura F, Mousa SS, Eichhorst J, Schaefer M, et al. *Endothelin potentiates TRPV1 via ETA receptor-mediated activation of protein kinase C*. Mol Pain 2007; 3:35.

16.Zhang H, Cang CL, Kawasaki Y, Liang LL, Zhang YQ, Ji RR, et al. Neurokinin-1 receptor enhances TRPV1 activity in primary sensory neurons via PKCepsilon: a novel pathway for heat hyperalgesia. *J Neurosci* 2007; 27:12067-77.

17.Hellwig N, Albrecht N, Harteneck C, Schultz G, Schaefer M. Homo- and heteromeric assembly of TRPV channel subunits. *J Cell Sci* 2005; 118:917-28.

18.Chandan Goswami, Md, Shahidul Islam. *TRP Channels*. Channels 4:2, 1-12; March/April 2010; © 2010 Landes Bioscience.

19.Bernd Nilius, Veit Flockerzi *Editors*. *Mammalian Transient Receptor Potential(TRP) Cation Channels*, Volume 1, Springer.

20. Vennekens R, Hoenderop JG, Prenen J, Stuiver M, Willems PH, Droogmans G, Nilius B, Bindels RJ (2000) Permeation and gating properties of the novel epithelial Ca2+ channel. *J Biol Chem* 275:3963–3969

21. J.G.J.Hoenderop, T.Voets1 , S.Hoefs, F.Weidema, J.Prenen1 , B.Nilius1 and R.J.M.Bindels2.Homo- and heterotetrameric architecture of the epithelial Ca2+ channels TRPV5 and TRPV6. *The EMBO Journal* Vol. 22 No. 4 pp. 776±785, 2003

22. Peng JB, Chen XZ, Berger UV, Vassilev PM, Brown EM, Hediger MA (2000a) A rat kidneyspecific calcium transporter in the distal nephron. *J Biol Chem* 275:28186–28194

23. Peng JB, Chen XZ, Berger UV, Weremowicz S, Morton CC, Vassilev PM, Brown EM, Hediger MA (2000b) Human calcium transport protein CaT1. *Biochem Biophys Res Commun* 278:326–332

24. Hoenderop JG, Bindels RJ, Klaushofer K, van Leeuwen JP (2013) The vitamin D analog ZK191784 normalizes decreased bone matrix mineralization in mice lacking the calcium channel TRPV5. *J Cell Physiol* 228:402–407

25. Chamoux E, Bisson M, Payet MD, Roux S (2010) TRPV-5 mediates a receptor activator of NF-kappaB (RANK) ligand-induced increase in cytosolic Ca2+ in human osteoclasts and down-regulates bone resorption. *J Biol Chem* 285:25354–25362

26. Suzuki Y, Kovacs CS, Takanaga H, Peng JB, Landowski CP, Hediger MA (2008) Calcium channel TRPV6 is involved in murine maternal-fetal calcium transport. *J Bone Miner Res* 23:1249– 1256

27. Loh NY, Bentley L, Dimke H, Verkaart S, Tammaro P, Gorvin CM, Stechman MJ, Ahmad BN, Hannan FM, Piret SE, Evans H, Bellantuono I, Hough TA, Fraser WD, Hoenderop JG, Ashcroft FM, Brown SD, Bindels RJ, Cox RD, Thakker RV (2013) Autosomal dominant hypercalciuria in a mouse model due to a mutation of the epithelial calcium channel, TRPV5. *PLoS One* 8:e55412

28. Van Cromphaut SJ, Dewerchin M, Hoenderop JG, Stockmans I, Van HE, Kato S, Bindels RJ, Collen D, Carmeliet P, Bouillon R, Carmeliet G (2001) Duodenal calcium absorption in vitamin D receptor-knockout mice: functional and molecular aspects. *Proc Natl Acad Sci USA* 98:13324–13329

29. Irnaten M, Blanchard-Gutton N, Praetorius J, Harvey BJ (2009) Rapid effects of 17beta-estradiol on TRPV5 epithelial Ca2+ channels in rat renal cells. *Steroids* 74:642–649

30. Hoenderop JG, van Leeuwen JP, van der Eerden BC, Kersten FF, van der Kemp AW, Merillat AM, Waarsing JH, Rossier BC, Vallon V, Hummler E, Bindels RJ (2003c) Renal Ca2+ wasting, hyperabsorption, and reduced bone thickness in mice lacking TRPV5. *J Clin Invest* 112:1906– 1914

31. Suzuki Y, Kovacs CS, Takanaga H, Peng JB, Landowski CP, Hediger MA (2008a) Calcium channel TRPV6 is involved in murine maternal-fetal calcium transport. *J Bone Miner Res* 23:1249–1256

32. van der Eerden BC, Weissgerber P, Fratzl-Zelman N, Olausson J, Hoenderop JG, SchreudersKoedam M, Eijken M, Roschger P, de Vries TJ, Chiba H, Klaushofer K, Flockerzi V, Bindels RJ, Freichel M, van Leeuwen JP (2012) The transient receptor potential channel TRPV6 is dynamically expressed in bone cells but is not crucial for bone mineralization in mice. *J Cell Physiol* 227:1951–1959

33. Bianco SD, Peng JB, Takanaga H, Suzuki Y, Crescenzi A, Kos CH, Zhuang L, Freeman MR, Gouveia CH, Wu J, Luo H, Mauro T, Brown EM, Hediger MA (2007) Marked disturbance of calcium homeostasis in mice with targeted disruption of the Trpv6 calcium channel gene. *J Bone Miner Res* 22(2):274–285

34. Hoenderop JG, Dardenne O, van Abel M, van der Kemp AW, van Os CH, St Arnaud R, Bindels RJ (2002) Modulation of renal Ca2+ transport protein genes by dietary Ca2+ and 1,25- dihydroxyvitamin D3 in 25-hydroxyvitamin D3-1alpha-hydroxylase knockout mice. *FASEB J* 16:1398–1406

35. de Groot T, Lee K, Langeslag M, Xi Q, Jalink K, Bindels RJ, Hoenderop JG (2009) Parathyroid hormone activates TRPV5 via PKA-dependent phosphorylation. *J Am Soc Nephrol* 20:1693– 1704

36. Cha SK, Wu T, Huang CL (2008b) Protein kinase C inhibits caveolae-mediated endocytosis of TRPV5. *Am J Physiol Renal Physiol* 294:F1212–F1221

37. Irnaten M, Blanchard-Gutton N, Praetorius J, Harvey BJ (2009) Rapid effects of 17beta-estradiol on TRPV5 epithelial Ca2+ channels in rat renal cells. *Steroids* 74:642–649

38. Hsu YJ, Dimke H, Schoeber JP, Hsu SC, Lin SH, Chu P, Hoenderop JG, Bindels RJ (2010) Testosterone increases urinary calcium excretion and inhibits expression of renal calcium transport proteins. *Kidney Int* 77:601–608

39. Hofmeister MV, Fenton RA, Praetorius J (2009) Fluorescence isolation of mouse late distal convoluted tubules and connecting tubules: effects of vasopressin and vitamin D3 on Ca2+ signaling. *Am J Physiol Renal Physiol* 296:F194–F203

40. Suzuki Y, Landowski CP, Hediger MA (2008c) Mechanisms and regulation of epithelial Ca2+ absorption in health and disease. *Annu Rev Physiol* 70:257–271

41. Pike JW, Zella LA, Meyer MB, Fretz JA, Kim S (2007) Molecular actions of 1,25-dihydroxyvitamin D3 on genes involved in calcium homeostasis. *J Bone Miner Res* 22 (Suppl 2):V16–V19

42. van Abel M, Hoenderop JG, van der Kemp AW, van Leeuwen JP, Bindels RJ (2003) Regulation of the epithelial Ca2+ channels in small intestine as studied by quantitative mRNA detection. *Am J Physiol Gastrointest Liver Physiol* 285:G78–G85

43. Ajibade DV, Dhawan P, Fechner AJ, Meyer MB, Pike JW, Christakos S (2010) Evidence for a role of prolactin in calcium homeostasis: regulation of intestinal transient receptor potential vanilloid type 6, intestinal calcium absorption, and the 25-hydroxyvitamin D(3) 1alpha hydroxylase gene by prolactin. *Endocrinology* 151(7):2974–2984

44. Peng JB, Zhuang L, Berger UV, Adam RM, Williams BJ, Brown EM, Hediger MA, Freeman MR (2001) CaT1 expression correlates with tumor grade in prostate cancer. *Biochem Biophys Res Commun* 282(3):729–734

45. Bolanz KA, Hediger MA, Landowski CP (2008) The role of TRPV6 in breast carcinogenesis. *Mol Cancer Ther* 7(2):271–279

46. Bond H, Dilworth MR, Baker B, Cowley E, Requena Jimenez A, Boyd RD, Husain SM, Ward BS, Sibley CP, Glazier JD (2008) Increased maternofetal calcium flux in parathyroid hormonerelated protein-null mice. *J Physiol* 586(7):2015–2025

47. R Gaudet (2008) TRP channels entering the structural era. *J Physiol* 586.15 (2008) pp 3565-3575

48. Bond H, Dilworth MR, Baker B, Cowley E, Requena Jimenez A, Boyd RD, Husain SM, Ward BS, Sibley CP, Glazier JD (2008) Increased maternofetal calcium flux in parathyroid hormonerelated protein-null mice. *J Physiol* 586(7):2015–2025

49. Wheeler DL, Barrett T, Benson DA, Bryant SH, Canese K.(2007) *Database resources of the National Center for Biotechnology Information*. Nucleic Acids Res 35 (Database issue): pp D5-12

50. Edgar RC (2004b) MUSCLE: *multiple sequence alignment with high accuracy and high throughput*. Nucleic Acids Res 32: 1792-1797

51. Flicek P, Aken BL, Ballester B, Beal K, Bragin E, et al. (2010) Ensembl's 10th year. Nucleic Acids Res 38(Database issue): D557-62

52. Hubbard TJ, Aken BL, Ayling S, Ballester B, Beal K, et al. (2009) Ensembl 2009. Nucleic Acids Res 37: D690-697

53. Jones D.T., Taylor W.R., and Thornton J.M. (1992) The rapid generation of mutation data matrices from protein sequences. *Computer Application in the Biosciences 8: 275-282*

54. Kumar S., Stecher G., and Tamura K. (2016)., MEGA7 Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. *Molecular Biology and Evolution 33: 1870-1874*

55. Vanoevelan J., Janssens A., Huitema L.F.A., and Merker S.S. TRPV5/V6 is vital for epithelial calcium uptake and bone formation. *The FASEB Journal* 25(9):3197.

56. Picazo-Juarez G., Romero-Suarez S., Nieto-Posadas A., Llorente I., Jara-Oseguera A., Briggs M., et al. (2011). Identification of a binding motif in the S5 helix that confers cholesterol-sensitivity to TRPV1. *J. Biol. Chem.* 286, 24966–24976 10.1074/jbc.M111.237537

57. *Protter: interactive protein feature visualization and integration with experimental proteomic data*. Omasits U, Ahrens CH, M ller S, Wollscheid B. Bioinformatics. 2014 Mar 15;30(6):884-6. doi: 10.1093/bioinformatics/btt607.

58. Crooks GE, Hon G, Chandonia JM, Brenner SE. WebLogo: A sequence logo generator, *Genome Research*, 14:1188-1190, (2004)

59. *One-way ANOVA followed by Dunnett's multiple comparisons test was performed using GraphPad Prism version 7.00 for Windows, GraphPad Software*, La Jolla California USA, www.graphpad.com

60. Nijenhuis T, Hoenderop J.G.J, Bindels R.J.M. TRPV5 and TRPV6 in Ca^{2+} (re)absorption : regulating Ca^{2+} entry at the gate. *Pflugers Arch - Eur J Physiol* (2005) 451 : 181-192

61. Björkholm P, Ernst A.M, Hacke M, Wieland F, Brügger B, Heijne G.V. Identification of novel sphingolipid-binding motifs in mammalian membrane proteins. *Biochimica et Biophysica Acta* 1838 (2014) 2066–2070

62. Lomize M.A., Pogozheva I,D, Joo H., Mosberg H.I., Lomize A.L. OPM database and PPM web server: resources for positioning of proteins in membranes. Nucleic Acids Res., 2012, 40(Database issue):D370-6

APPENDIX

Table A: Accession no. and sources of TRPV5 sequences used for this analysis

Sr. No.	ACCESSION NO.	ORGANISM NAME
		AMPHIBIANS
1.	NP_001186854.1	WESTERN CLAWED FROG
2.	AFS63976.1	AFRICAN CLAWED FROG
		AVES
3.	A0A093GC36	DOWNY WOODPECKER
4.	OWK63958.1	BENGALESE FINCH
5.	A0A093L431	NORTHERN FULMAR
6.	XP_005506369.2	ROCK DOVE
7.	A0A091VVN4	HOATZIN
8.	XP_015501037.1	THE GREAT TIT
9.	A0A091QJH5	NORTHERN CARMINE BEE EATER
10.	XP_008633638.1	AMERICAN CROW
11.	XP_023795149.1	EURASIAN BLUE TIT
12.	A0A091J8X6	LITTLE EGRET
13.	A0A093RTN2	GOLDEN COLLARED MANAKIN
14.	A0A091H291	COMMON CUCKOO
15.	A0A093N985	ADELEI PENGUIN
16.	A0A091S486	KEA
17.	A0A091SSV8	DALMATIAN PELICAN
18.	A0A093Q457	GREAT CORMORANT
19.	A0A087R5U6	EMPEROR PENGUIN
20.	R0K1V0	MALLARD
21.	A0A091I8N5	ANNAS HUMMINGBIRD
22.	A0A091Q7E2	CUCULUS DISCOLOR

23.	A0A091KV81	SPECKLED MOUSEBIRD
24.	A0A094KHN5	GREAT CRESTED GREBE
		REPTILES
25.	XP_019404056.1	SALTWATER CROCODILE
26.	XP_003226604.1	CAROLINA ANOLE
27.	J3S9T0	EASTERN DIAMONDBACK RATTLESNAKE
		FISH
28.	JAP01830.1	BLACKSTRIPE LIVEBEARER
29.	XP_005798837.2	SOUTRHERH PLATYFISH
30.	XP_022056826.1	SPINY CHROMIS DAMSELFISH
31.	XP_012730583.2	MUMMICHOG
32.	XP_020783631.1	BLUE SPOTTED MUDSKIPPER
33.	XP_019724545.1	TIGER TAIL SEAHORSE
34.	XP_018591254.1	ASIAN AROWANA
35.	ADV32915.1	ZEBRAFISH
36.	XP_005732007.1	HAPLOCHROMINE TYPE CICHLID
37.	JAR69603.1	ATLANTIC KILLIFISH
		RODENTS
38.	NP_001007573.1	HOUSE MOUSE
39.	NP_446239.2	BROWN RAT
40.	XP_005326661.1	THIRTEEN-LINED GROUND SQUIRREL
41.	XP_021509810.1	MONGOLIAN JIRD
42.	XP_005083139.1	GOLDEN HAMSTER
43.	XP_008826832.1	BLIND MOLE RAT
44.	XP_003504418.1	CHINESE HAMSTER
45.	XP_005363891.1	PRAIRIE VOLE
46.	XP_012877136.1	ORDS KANGAROO RAT
47.	XP_004661133.1	LESSER EGYPTIAN JERBOA
48.	XP_015349417.1	APLINE MARMOT
49.	XP_023568692.1	COMMON DEGU
		MAMMALS
50.	NP_062815.3	HUMAN

51.	NP_001076126.1	COMMON RABBIT
53.	NP_001157428.1	HORSE
54.	XP_003791601.1	NORTHERN GREATER GALAGO
55.	XP_023106420.1	DOMESTIC CAT
56.	XP_023040702.1	UGANDAN RED COLOBUS
57.	XP_022434851.1	WHITE WHALE
58.	XP_013975308.1	DOMESTIC DOG
59.	XP_003896799.1	OLIVEBABOON
60.	XP_008065942.1	PHILLIPPINE TARSIER
61.	XP_021548583.1	HAWAIIAN MONK SEAL
62.	XP_012302887.1	NANCY MA'S NIGHT MONKEY
63.	XP_003360142.4	WILD BOAR
64.	XP_020853689.1	KOALA
65.	XP_012623534.1	GREY MOUSE LEMUR
66.	XP_019815160.1	HUMPED CATTLE
67.	XP_011229262.2	GIANT PANDA
68.	XP_019570929.1	CHINESE ROFOUS HORSESHOE BAT
69.	XP_019487855.1	GREAT ROUNDLEAF BAT
70.	XP_018886143.1	WESTERN LOWLAND GORILLA
71.	XP_017831188.1	COMMON MARMOSET
72.	XP_017902334.1	DOMESTIC GOAT
73.	XP_017521821.1	SUNDA PANGOLIN
74.	XP_017366818.1	WHITE HEADED CAPUCHIN
75.	XP_016813796.1	COMMON CHIMPANZEE
76.	XP_016050928.1	NATAL LONG FINGERED BAT
77.	XP_007522411.1	EUROPEAN HEDGEHOG
78.	XP_016012685.1	EGYPTIAN FRUIT BAT
79.	XP_006910521.1	BLACK FRUIT BAT
80.	XP_006768053.1	DAVID'S MYOTIS
81.	XP_010802929.1	CATTLE
82.	XP_005551083.1	CRAB EATING MACAQUE
83.	XP_006210153.1	ALPACA

84.	XP_014990601.1	RHESUS MACAQUE
85.	XP_014940212.1	CHEETAH
86.	XP_014700695.1	WILD ASS
87.	XP_005609461.1	HORSE
88.	XP_005862844.1	BRANDTS BAT
89.	XP_014415353.1	WILD BACTRIAN CAMEL
90.	XP_005900978.1	WILD YAK
91.	XP_006094797.1	LITTLE BROWN BAT
92.	XP_003820606.1	PYGMY CHIMPANZEE
93.	XP_004781475.1	FERRET
94.	XP_004608389.1	COMMON SHREW
95.	XP_004594396.1	AMERICAN PIKA
96.	XP_004694386.1	STAR NOSED MOLE
97.	XP_012520549.1	COQUERELS SIFAKA
98.	XP_004414628.1	WALRUS
99.	XP_004272164.1	KILLER WHALE
100.	XP_003270895.1	NORTHERN WHITE CHEEKED GIBBON
101.	XP_011912892.1	SOOTY MANGABEY
102.	XP_011721864.1	SOUTHERN PIG TAILED MACAQUE
104.	XP_011845254.1	DRILL
105.	XP_011796739.1	ANGOLA COLOBUS
106.	XP_011376392.1	LARGE FLYING FOX
107.	XP_010955337.1	BACTRIAN CAMEL
108.	XP_010853340.1	PLAIN'S BISON
109.	XP_003929845.2	BLACK CAPPED SQUIRREL MONKEY
110.	XP_010377685.1	GOLDEN SNUB NOSED MONKEY
111.	XP_002818624.1	SUMATRAN ORANGUTAN
112.	XP_008695537.1	POLAR BEAR
113.	XP_008585013.1	SUNDA FLYING LEMUR
114.	XP_008536714.1	PRZEWALSKIS HORSE
115.	XP_008152191.1	BIG BROWN BAT
116.	XP_007981426.1	GREEN MONKEY

117.	XP_007466701.1	BAIJI
118.	XP_007184750.1	NORTH PACIFIC MINKE WHALE
119.	XP_006051604.1	WATER BUFFALO
120.	XP_007121687.1	SPERM WHALE
121.	XP_006861333.1	CAPE GOLDEN MOLE
122.	XP_006887306.1	CAPE ELEPHANT SHREW
123.	XP_006743620.1	WEDDELL SEAL
124.	XP_005981916.1	TIBETAN ANTELOPE

Table B: <u>Accession no. and sources of TRPV6 sequences used for this analysis</u>

SR. NO.	ACCESSION NO.	ORGANISM NAME
		AMPHIBIANS
1.	XP_002944196.3	WESTERN CLAWED FROG
2.	AFS63976.1	AFRICAN CLAWED FROG
		AVES
3.	XP_004938199.1	RED JUNGLEFOWL
4.	XP_009996599.1	CHIMNEY SWIFT
5.	XP_009897936.1	DOWNY WOODPECKER
6.	XP_009470079.1	CRESTED IBIS
7.	XP_008936450.1	NORTHERN CARMINE BEE EATER
8.	F1NAX8	CHICKEN
9.	A0A094LS06	CHUCK WILLS WIDOW
10.	A0A01V4JAC1	BAND TAILED PEGION
11.	A0A0Q3X8P2	BLUE FRONTED AMAZON PARROT
12.	A0A091UPA2	WHITE TAILED TROPIC BIRD
13.	A0A093D7W3	RED CRESTED TURACO
14.	A0A093HVD9	SOUTH AFRICAN OSTRICH
15.	A0A0A0A222	KILLDEER
16.	A0A099Z0A4	WHITE THROATED TINAMOU

17.	A0A093HFX4	RED THROATED DIVER
18.	A0A093EMD5	BARN OWL
19.	A0A091LJM7	TURKEY VULTURE
20.	A0A091MAL5	RED LEGGED SERIEMA
21.	A0A091SCR0	BROWN ROATELO
		REPTILES
22.	XP_019383237.1	GHARIAL
23.	KYO37499.1	AMERICAN ALLIGATOR
24.	XP_006145141.1	CHINESE TREE SHREW
25.	XP_007068670.1	GREEN SEA TURTLE
26.	V8P2P1	KING COBRA
27.	A0A1W7RC65	SOUTHERN COPPERHEAD
		FISH
28.	NP_001001849.1	ZEBRAFISH
29.	NP_001027938.1	TIGER PUFFER
30.	AHH40275.1	CHANNEL CATFISH
31.	I3K4X4	NILE TILAPIA
32.	H3CS94	SPOTTED GREEN PUFFERFISH
33.	A0A0F8AK85	LARGE YELLOW CROAKER
		RODENTS
34.	NP_071858.3	HOUSE MOUSE
35.	NP_446138.1	BROWN RAT
36.	XP_021582903.1	THIRTEEN LINED GROUND SQUIRREL
37.	XP_021509815.1	MONGOLIAN GERBIL
38.	XP_004860572.1	NAKED MOLE RAT
39.	XP_005083140.1	GOLDEN HAMSTER
40.	XP_021020675.1	RYUKYU MOUSE
41.	XP_021046227.1	GAIRDNER'S SHREWMOUSE
42.	XP_010606001.1	DAMARALAND MOLE RAT
43.	XP_008826831.1	BLIND MOLE RAT
44.	XP_003504432.1	CHINESE HAMSTER
45.	XP_005405444.1	LONG TAILED CHINCHILLA

46.	XP_005363892.1	PRAIRIE VOLE
48.	XP_003475354.1	GUINEA PIG
49.	XP_012877135.1	ORD'S KANGAROO RAT
50.	XP_004661210.1	LESSER EGYPTIAN JERBOA
51.	XP_004629087.1	COMMON DEGU
		MAMMALS
53.	OWK06246.1	EUROPEAN RED DEER
54.	BAH87024.1	WILD BOAR
55.	NP_061116.5	HUMAN
56.	NP_001193118.1	CATTLE
57.	AAY34564.1	COMMON RABBIT
58.	XP_003791600.1	NORTHERN GREATER GALAGO
59.	XP_003771733.3	TASMANIAN DEVIL
60.	XP_019681505.1	CATTLE
61.	XP_023040701.1	UGANDAN RED COLOBUS
62.	XP_539861.4	DOMESTIC DOG
63.	XP_009202289.2	OLIVE BABOON
64.	XP_008065941.1	PHILLIPPINE TARSIER
65.	XP_021548629.1	HAWAIIAN MONK SEAL
66.	XP_012302868.1	NANCY MA'S NIGHT MONKEY
67.	XP_020853716.1	KOALA
68.	XP_020746707.1	WHITE TAILED DEER
69.	XP_012623532.1	GRAY MOUSE LEMUR
70.	XP_019814773.1	ZEBU
71.	XP_002924207.1	GIANT PANDA
72.	XP_019487854.1	GREAT ROUNDLEAF BAT
73.	XP_019308565.1	LEOPARD
74.	XP_004046421.1	WESTERN LOWLAND GORILLA
75.	XP_017831053.1	COMMON MARMOSET
76.	XP_017902336.1	GOAT
77.	XP_017722308.1	BLACK SNUB NOSED MONKEY
78.	XP_017366826.1	CEBUS CAPUCINUS IMITATOR

79.	XP_009452668.1	COMMON CHIMPANZEE
80.	XP_001362648.1	GRAY SHORT TAILED OPOSSUM
81.	XP_016050926.1	NATAL LONG FINGERED BAT
82.	XP_007522368.1	EUROPEAN HEDGEHOG
83.	XP_016012686.1	EGYPTIAN FRUIT BAT
84.	XP_006997204.1	DEER MOUSE
85.	XP_006768054.1	DAVID'S MYOTIS
86.	XP_015349418.1	ALPINE MARMOT
87.	XP_005551080.1	CRAB EATING MACAQUE
88.	XP_006210152.1	ALPACA
89.	XP_014990599.1	RHESUS MACAQUE
90.	XP_004008178.1	SHEEP
91.	XP_014700694.1	WILD ASS
92.	JAN97297.1	HORSE
93.	XP_005862841.1	BRANDT'S BAT
94.	XP_006185432.1	WILD BACTRIAN CAMEL
95.	XP_005900975.1	WILD YAK
96.	XP_008972252.1	PYGMY CHIMPANZEE
97.	XP_004781478.1	FERRET
98.	XP_004594397.1	AMERICAN PIKA
99.	XP_004694460.1	STAR NOSED MOLE
100.	XP_012520550.1	COQUEREL'S SIFAKA
101.	XP_004414627.1	WALRUS
102.	XP_004272165.1	KILLER WHALE
104.	XP_004455695.1	NINE BANDED ARMADILLO
105.	XP_003270894.1	NORTHERN WHITE CHEEKED GIBBON
106.	XP_011912870.1	SOOTY MANGABEY
107.	XP_011721872.1	SOUTHERN PIG TAILED MACAQUE
108.	XP_011845253.1	DRILL
109.	XP_011796736.1	ANGOLA COLOBUS
110.	XP_011376393.1	LARGE FLYING FOX
111.	XP_010955234.1	BACTRIAN CAMEL

112.	XP_010997560.1	ARABIAN CAMEL
113.	XP_010853296.1	PLAIN'S BISON
114.	XP_003929844.1	BLACK CAPPED SQUIRREL MONKEY
115.	XP_010377684.1	GOLDEN SNUB NOSED MONKEY
116.	XP_002818622.2	SUMATRAN ORANGUTAN
117.	XP_008695538.1	POLAR BEAR
118.	XP_008585016.1	SUNDA FLYING LEMUR
119.	XP_008536721.1	PRZEWALSKI'S HORSE
120.	XP_008152192.1	BIG BROWN BAT
121.	XP_007981425.1	GREEN MONKEY
122.	XP_007954789.1	ANTBEAR
123.	XP_007466700.1	BAIJI
124.	XP_007184754.1	NORTH PACIFIC MINKE WHALE
125.	XP_006051607.1	WATER BUFFALO
126.	XP_007121691.1	SPERM WHALE
127.	XP_006861332.1	CAPE GOLDEN MOLE
128.	XP_006887305.1	CAPE ELEPHANT SHREW
129.	XP_006743614.1	WEDDELL SEAL
130.	XP_005981915.1	TIBETAN ANTELOPE
131.	JAB08699.1	COMMON MARMOSET