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STUDYING THE ROLE OF SUMO IN THE INTERACTION BETWEEN HCN2 CHANNEL
AND ITS AUXILIARY SUBUNIT TRIP8b USING PULL-DOWN ASSAY

by

RAMYANI DE

Under the Direction of Deborah J. Baro, PhD

ABSTRACT

Hyperpolarization-activated cyclic nucleotide-gated (HCN) ion channels form an integral part of the plasma membrane, existing as tetramers and enclosing ion conducting pores. Ion channels are often regulated by auxiliary subunits and one such protein interacting with the HCN channel is TPR-containing Rab8b interacting protein (TRIP8b). TRIP8b is known to play a crucial role in controlling voltage dependence of HCN channel activation, channel trafficking and surface expression. Small Ubiquitin-like Modifier (SUMO) is a peptide that can be conjugated to a lysine residue on a target protein and modify protein- protein interactions. HCN2 channel can be SUMOylated and possible SUMOylation sites on TRIP8b exist. SUMOylation at these sites may strengthen or weaken the association between these proteins. In this study we use Pull-down assay technique to determine whether SUMO influences this interaction. By altering the baseline SUMOylation level, changes in the degree of HCN channel protein pulled down by TRIP8b is analyzed.

INDEX WORDS: HCN channel, TRIP8b, SUMO, Pull-down assay, Protein purification,
Protein-protein interaction

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RAMYANI DE

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of

Master of Science

in the College of Arts and Sciences

Georgia State University

2020

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by

RAMYANI DE

Committee Chair: Deborah J. Baro

Committee: Zehava Eichenbaum

Ritu Aneja

Geert de Vries

Electronic Version Approved:

Office of Graduate Services

College of Arts and Sciences

Georgia State University

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DEDICATION

I would like to dedicate my thesis to my family. My parents, Moumita and Sakti Taran have always encouraged me to pursue my passion for research. Their support has been invaluable. I could always count on them to stand by me in all my decisions. My husband, Arijit De has been supportive throughout this entire time. He is my biggest strength. I truly admire his patience and value his advice. My parents-in-law, Pronoti and Sadhan De who have always supported me in all my decisions and boosted my confidence with positivity.

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TABLE OF CONTENTS

ACKNOWLEDGEMENTS		V
LIST OF TABLES		VIII
LIST OF FIGURES		IX
1 INTRODUCTION		1
1.1 Review of Literature		5
1.1.1 Role of TRIP8b as an auxiliary subunit of HCN channel		5
1.1.2 SUMOylation modulates ion channel function and expression		8
1.1.3 Pull-down assay to study protein-protein interactions		11
1.2 Hypothesis		14
2 MATERIALS AND METHODS		18
2.1 Plasmids and Antibodies		18
2.2 Cloning		18
2.2.1 Plasmid preparation (pET51b+)		18
2.2.2 Insert preparation (TRIP8b)		19
2.2.3 Ligation of vector and insert		20
2.3 Protein Expression		20
2.4 Protein Purification		21
2.4.1 Purification using Strep-tag		21
2.4.2 Purification using His-tag		22

2.5	Cell Culture	22
2.6	Preparation of HCN2 cell lysate	23
2.7	Pull-Down Assay	23
2.8	Western Blot	24
3	RESULTS	27
3.1	TRIP8b is cloned into pET51b+ plasmid	27
3.2	TRIP8b is expressed and purified using dual tags.....	27
3.3	SUMOylation can be decreased without the addition of NEM.....	28
3.4	Level of HCN2 protein pulled down by TRIP8b decreases in absence of NEM ..	29
4	CONCLUSIONS	35
	REFERENCES	37

LIST OF TABLES

Table 2.1 Primary Antibodies..... 26

Table 2.2 PCR and Sequencing Primers..... 26

LIST OF FIGURES

Figure 1.1 Process of SUMOylation.	15
Figure 1.2 Pull-Down Assay	16
Figure 1.3 SUMO and SIM sites predicted by GPS-SUMO online tool highlighted in the crystal structure of proteins using PyMol.	17
Figure 3.1 Cloning TRIP8b(1a-4) into pET51b+ plasmid.....	31
Figure 3.2 Protein purification using dual tags.....	32
Figure 3.3 Alteration in HCN2 channel SUMOylation.	33
Figure 3.4 Pull-down assay using TRIP8b as bait and HCN2 lysate from two different treatment groups as prey.....	34

1 INTRODUCTION

Hyperpolarization-activated cyclic nucleotide-gated (HCN) channels are voltage-gated cation channels. In mammals, the different channel isoforms are encoded by 4 genes (HCN1-4) and are predominantly expressed in heart and central nervous system. In the nervous system the different types of this channel are diversely expressed. HCN1 is primarily found in the brainstem, hippocampus, spinal cord and dorsal root ganglion. HCN2 is expressed in all regions predominantly in thalamus and small nociceptive neurons. HCN3 has a low expression in the CNS whereas HCN4 has a high expression in the olfactory bulb and thalamic nuclei [1]. HCN channels can exist as either homotetramers or heterotetramers that are embedded in the plasma membrane and which encloses a pore through which ions can flow through. The structure of each subunit comprises of three distinct regions – an N-terminus, a trans-membrane core and a C-terminus. The trans-membrane core is made up of six trans-membrane segments (S1-S6) wherein S4 is the positively charged voltage sensor and S5 bound to S6 encloses the pore. A cyclic nucleotide-binding domain is present in the proximal end of the C-terminus whereas the distal end consists of a tri-peptide tail made up of three amino acids, SNL in case of HCN1,2,4 and ANM for HCN3 [2]. These channels normally get activated under membrane hyperpolarization and are constitutively active near resting membrane potential. They are permeable to both Na^+ and K^+ ions with a permeability ratio of around 1:5 and mediate an inward current known as hyperpolarization-activated current (I_h) [3]. Binding of 3'5'-cyclic adenosine monophosphate (cAMP) to CNBD stimulates channel opening by shifting the voltage of activation to a more depolarized membrane potential. Although cAMP is an important regulator of I_h , a distinct difference is observed between these currents in the heterologous system and in the native state. This can be attributed towards the expression of additional auxiliary subunits in the native

system that can bring about changes in channel biophysical properties [4]. Different channels vary in their rate of activation, HCN1 being fastest followed by HCN2 and HCN3 in the intermediate range and finally HCN4 which is the slowest [5]. HCN channels play an important role in neuronal excitability and responding to synaptic input. For instance, when HCN channels are activated, the channel conductance can reduce the membrane resistance inhibiting the effect of synaptic input and in turn decreasing the strength of post-synaptic potentials [6]. Conversely when I_h is inhibited there is an increase in input resistance that further strengthens the degree of EPSPs leading to hyper-excitability [7]. These channels also modulate synaptic plasticity and memory. The effect of cannabinoids on spatial memory formation has been found to be mediated by cannabinoid type1 receptor mediated HCN channel activation. I_h being a key regulator of dendritic excitability results in inhibition of long-term potentiation and spatial memory formation [8]. HCN channel involvement in various neurological disorders has been widely studied. Abnormal patterns of the channel activity have been associated with epilepsy and neuropathic pain. Loss of HCN channel expression and reduced I_h can lead to dysregulation in action potential firing triggering seizures [9], [10]. On the contrary blocking of HCN channels prevents both inflammatory and neuropathic pain [11]. HCN2 that has been identified as a factor contributing towards neuropathic pain might serve as a potential target for treating such conditions [10]. Auxiliary subunits play an important role in regulating ion channel expression and function. One such auxiliary subunit of the HCN channel is TPR-containing Rab8b interacting protein (TRIP8b).

TRIP8b was first identified in the brain primarily in the distal CA1 hippocampal and cortical pyramidal neurons [12]. The name is derived from its interaction with Rab8b crucial for vesicle trafficking. Apart from brain it is also expressed in the dorsal root ganglia and the testis.

The structure of TRIP8b has an N-terminus variable region comprising exons 1-4. Exons 5-16 remain constant and include a conserved region also known as TRIP8b core and a C-terminus composed of six tetratricopeptide repeats (TPRs) [13]. The N-terminus is capable of undergoing alternate splicing which generates the different isoforms of this protein. There are three distinct promoters 1a, 1b and 1c which drives the expression of the TRIP8b gene. Different combination of these exons gives rise to nine different isoforms of this protein in the brain. The most abundant isoform which is expressed in the brain is TRIP8b (1a-4) which comprises of almost 30-40% of the total protein mRNA. Out of the nine isoforms six have been studied widely whereas the other three are expressed in extremely low levels and their functions are yet to be determined [14]. The association of TRIP8b with the HCN channel has been defined as a bipartite interaction wherein the TRIP8b core binds to the CNBD of the channel and the TPR-domain binds to the extreme C-terminus tri-peptide sequence. A stoichiometry of 4:4 exists in a channel complex containing HCN bound to TRIP8b [15]. The C-linker region of the channel connects the CNBD to the transmembrane domain. Studies conducted using isoform TRIP8b (1b-2) reveals a stretch of 80 aa extending from residue 236-316 present in the core region that is mainly responsible for interaction with the C-linker/CNBD of the HCN1 channel [16]. A similar conserved region comprising of residues 223-303 was also identified in isoform TRIP8b (1a-4) [17], however recently through NMR experiments researchers have been able to isolate a stretch of 40 aa (residues 235-275) which is crucial for binding to the CNBD. This has been named TRIP8b_{nano} which has binding affinity similar to that of the core region. TRIP8b_{nano} binding site on the channel includes the C-helix of CNBD and the N-bundle loop that connects helix E' of C-linker to the helix A of CNBD [18]. X-ray crystallography experiments were performed in order to look into the structural conformation of the other binding site. There are two clusters of TPRs

each containing three TPR domains made up of two anti-parallel alpha-helices joined by a turn. In between these two sets lie a cleft in which the SNL-tripeptide of the channel fits in and form hydrogen bonds with the residues lying in this pocket [15]. TRIP8b has a significant role to play in homeostatic regulation of I_h . This is mostly attributed to its role towards channel trafficking to the plasma membrane. The function of this protein has been studied in neurological disorder such as epilepsy. Abnormal functioning and expression of HCN channels characterized by reduced I_h results in neuronal excitability resulting in seizures. Disruption in association of TRIP8b with the channel may be contributing toward such alterations in channel functioning [19]. Post-translational modifications play a crucial role in influencing protein-protein interactions. One such process that might affect the interaction of these two proteins is SUMOylation.

Small Ubiquitin-like Modifier (SUMO) is a peptide approximately 11 kDa in size that can be conjugated to a lysine residue on a target protein. Mammalian SUMO is encoded by four genes SUMO1-4. SUMO2 and SUMO3 are 97% identical differing from each other by only three N-terminal residues and are collectively known as SUMO2/3. They are approximately 50% similar to SUMO1. SUMO4 is only 87% identical to SUMO2 but it lacks the ability to form a mature protein which makes it unclear whether it can be added to target proteins or not [20]. SUMO is synthesized in an inactive form and hence needs to undergo cleavage by sentrin specific protease (SENP) which exposes the C-terminus di-glycine motif. The SUMO conjugation process involves several steps. After cleavage by SENP, the mature SUMO is transferred by SUMO activating enzyme or E1 to SUMO conjugating enzyme or E2 (also called Ubc9). E2 finally adds SUMO to a target lysine residue which may at times be facilitated by SUMO ligating enzyme or E3. The de-conjugation process involves SUMO getting cleaved from

target protein residue by SENP [21] (Figure 1.1). Thus, SENP has a dual function to play both in the process of SUMOylation and de-SUMOylation. SUMO is usually attached to a lysine residue within a consensus sequence ψ KxD/E, where ψ is a hydrophobic amino acid, K is lysine, x is another amino acid and D and E are aspartic acid and glutamic acid respectively [22]. Although many times the lysine residue may not be within a consensus sequence but it can still be SUMOylated. SUMO present on a target lysine residue serve as a docking site for SUMO-interacting motif (SIM) on another protein thus allowing them to form a complex [23]. Predicted SUMO conjugation sites and SIM domains are present on both TRIP8b and HCN2 as determined by GPS-SUMO online tool (Figure 1.3). A high probability SUMO site at K314 has been predicted for TRIP8b (1a-4) using SUMO Plot online tool. A previous study has shown that HCN2 channel is SUMOylated at sites K534 and K669. SUMOylation at K669 increases channel surface expression and I_h G_{max} [24]. Thus, SUMO may influence the interaction between HCN2 and TRIP8b. One of the widely used techniques to study protein-protein interaction is Pull-down assay. By manipulating the level of baseline SUMOylation of HCN2 channel protein, this technique has been utilized to study the interaction between these two proteins.

1.1 Review of Literature

1.1.1 *Role of TRIP8b as an auxiliary subunit of HCN channel*

TRIP8b association with the HCN channel has been studied extensively and researchers have been able to establish certain mechanisms by which this protein can alter the channel function and expression. As previously described nine different TRIP8b isoforms exist that display a great deal of variation when it comes to regulating this channel. Out of these nine isoforms, six of them have been studied in detail and they are 1a, 1a-2, 1a-2-4, 1a-4, 1b-2 and 1b-2-4. HCN1 current density was measured from *Xenopus* oocytes co-injected with the channel

cRNA along with each of these isoforms. TRIP8b (1b-2) was found to decrease I_h current density by 100-folds. Similar results were also observed with isoforms TRIP8b (1b-2-4 and 1a). On the other hand, isoforms TRIP8b (1a-2-4 and 1a-4) resulted in an approximately 6-fold increase in I_h current density. TRIP8b (1a-2) displayed no changes in the current density at all. When further studied it was seen that all the isoforms act in a similar fashion when it comes to channel gating i.e. they shift the voltage dependence of channel activation to more negative potentials. Also they slow down the rate of channel activation and speed up the process of deactivation upon hyperpolarization and depolarization respectively [14]. Therefore, the change in the current density corresponds to the change in channel surface expression. While isoforms under the control of promoter A, TRIP8b (1a) being an exception results in an upregulation of the channel surface expression, isoforms with promoter B reduces it [13]. One of the proposed mechanisms through which certain isoforms could be downregulating surface expression of the channels is by clathrin-mediated endocytosis. Exon 2 has a YXXL motif and when the tyrosine and leucine residues in this motif are mutated, TRIP8b (1b-2) was no longer able to reduce I_h . Interestingly the effect of TRIP8b (1a-2) which causes no change in current, could be explained in terms of opposing influences from exon 2 and exon 1a acting simultaneously. Adaptor protein -2 (AP-2) is made up of both adaptins α and β . A positive signal was detected when TRIP8b co-immunoprecipitated from brain was probed with antibodies against adaptins α and β suggesting that AP-2 associates with TRIP8b. Thus, it was thought that TRIP8b might be acting as a bridge recruiting AP-2 to the HCN channel and further initiating clathrin-mediated endocytosis which results in channel internalization as observed with certain specific isoforms [14].

Researchers have looked into each of the two interaction sites individually in order to establish their function through various mutation experiments. Binding of TRIP8b both at the CNBD and

C-terminus tri-peptide sequence of the channel influence the regulation of HCN current as well as surface expression. Interestingly it is the association with CNBD which is solely responsible for channel gating. When this was blocked, TRIP8b could no longer produce a hyperpolarizing shift in the activation of the channel. This change was not observed by blocking the association with the tri-peptide sequence thus establishing the significance of CNBD in channel gating [25]

Both TRIP8b and cAMP bind to the channel CNBD. While TRIP8b shifts channel opening to hyperpolarized membrane potentials, cAMP causes a shift in voltage of activation to more depolarized membrane potentials. It has been reported that TRIP8b reduces cAMP-dependent regulation of this channel, however the underlying mechanism is not very well understood. There are two existing models which support both a competitive and non-competitive mechanism. According to the competitive model, both TRIP8b and cAMP bind to the same site of the CNBD. The cAMP binding site at the CNBD has a highly conserved arginine residue (R-538 in HCN1 and R-591 in HCN2). It has been shown that mutating this amino acid to glutamate also blocks TRIP8b core binding to CNBD [25]. Similar results were also obtained in another study showing that one inhibits the binding of the other. Bankston et al. [26] based their study on whether TRIP8b can bring about a change in the active state of the channel while it is saturated with cAMP. DEER analysis results showed almost negligible effect thus confirming a partial overlap in their binding sites. The study concluded that TRIP8b must be acting as a competitive antagonist [26]. On the other hand, the non-competitive model describes the inhibition of conformational change of channel activation associated with cAMP in presence of TRIP8b. Binding of TRIP8b allosterically decreases the affinity of CNBD towards cAMP. Moreover, when the conserved arginine residue required for cAMP binding as previously described was mutated to alanine TRIP8b could still bind to CNBD suggesting that the arginine

residue after all may not be so specific for this protein binding [17]. Saponaro et al. [27] showed that with increase in cAMP concentration, less TRIP8b_{core} was able to bind to CNBD. Thus, TRIP8b preferentially binds to the cAMP free state of CNBD than cAMP bound state. TRIP8b is able to stabilize the cAMP free state of the channel CNBD thus reducing cAMP association with the channel. This is mediated by a conformational change in the N-terminal region of CNBD which results in decrease in cAMP affinity [27]. Although both the models have supporting data it might be interesting to see how the channel functions when it is used in its entirety. It is important to note that the two binding sites between HCN channel and TRIP8b also influence each other. In a recent study it has been established that the two sites show positive allosterism which is crucial for establishing specificity towards the channel while decreasing its affinity towards other non-HCN proteins. When the concentration of TRIP8b added to HCN1 tail was gradually increased in presence of CNBD, the apo-state of CNBD resulted in an increased affinity of the protein for the HCN1 tail over the cAMP bound conformation [28]. Thus, a collaborative functioning of both the sites could be inhibiting the effect of cAMP regulation of the channel.

1.1.2 SUMOylation modulates ion channel function and expression

Among several post-translational modifications, SUMOylation plays a crucial role in controlling neuronal activity such as neuronal excitability and transmission of synapses. First identified to be associated with Ran-GAP1, SUMO is involved in processes such as DNA repair and transcription [29]. Over the years the non-nuclear functions of SUMOylation have been studied one of which involves the regulation of ion channel. A great deal of the plasma membrane is made up of voltage-gated ion channels and SUMO contributes towards altering the channel function and surface expression. SUMOylation of Nav 1.2 channels in rat cerebellar

granule neurons has been shown to increase I_{Na} under hypoxic conditions [30]. SUMO1 gets added to the K38 residue in Nav1.2 channel. SUMOylation increased the peak current by almost 76% under hypoxia thus triggering membrane depolarization and generating action potential. Drugs targeting this pathway might be useful in preventing hypoxia induced cellular damage [30]. Several potassium channels have also been found to be regulated by SUMO. In the hippocampal neurons Kv2.1 potassium channels are SUMOylated at the neuronal cell surface which shifts the voltage of half activation to more depolarized potentials thus suppressing the excitability of these neurons [31]. SUMOylation of Kv4.2 potassium channel by SUMO2/3 results in a 22% decrease in mean I_A G_{max} while increasing the channel surface expression by approximately 70%. This was found to be mediated by two distinct lysine residues – K437 and K579. Through site-directed mutagenesis it was found that each of these residues have distinct role to play in controlling the channel activity when SUMOylated. SUMOylation at K437 was responsible for increasing the channel surface expression whereas K579 mediated reduction in I_A G_{max} [32]. SUMO also regulates the K2P1 potassium leak channels by silencing them [33]. Thus, it is interesting to see how SUMOylation of different residues can contribute towards the overall functioning of the channel. Calcium is another important signaling molecule that regulates membrane potential and its level is maintained predominantly by voltage-gated calcium channels present on the plasma membrane. P/Q type current is mediated by Cav2.1 calcium channels which are primarily found in the pre-synaptic terminals in the CNS [34]. $\alpha 1A$ subunit of the Cav2.1 channel when co-expressed with SUMO1 in HEK293 cells results in a decrease in the wild-type channel current density [35]. In case of HCN2 channels, these channels have been found to be SUMOylated. HCN2 channel immunoprecipitated from mouse brain gave a positive signal when probed with both SUMO1 and SUMO2/3 antibodies suggesting that these channels

are SUMOylated in vivo. In a heterologous system, increasing the channel SUMOylation by 50% produced around 77% increase in $I_h G_{max}$ along with 70% increase in channel surface expression. However, voltage dependence of channel activation remained unaltered. The key lysine residue identified which was responsible for these changes is K669. Mutating this site abolished increase in both $I_h G_{max}$ and channel surface expression observed with increased SUMOylation [24].

The role of SUMOylation in protein-protein interaction has been studied over the years and one of the primary domains through which SUMO works is through a SIM. Proteins possessing these SIMs are capable of forming multiple protein complexes. A typical SIM consists of a hydrophobic core surrounded by negatively charged amino acids on either side ([V/I]-x-[V/I]-[V/I]) [22]. Researchers have looked into the mechanisms by which SUMO can interact with SIM. One of the studies describe SUMO1 forming a groove in between the α -helix and β -strand into which SIM fits in via an elongated structure. SUMO/SIM interaction enables multiple protein-protein interaction and plays a critical role in various functions. HIV integrase is a key enzyme in incorporating viral DNA into host genome which requires them to bind to certain co-factors such as LEDGF/p75 which is facilitated by this mechanism [36]. With respect to ion channels, both the channel and its respective auxiliary subunit can be modified by SUMO and they also possess SIMs. Collapsin response mediator protein 2 (CRMP2) is an auxiliary subunit of voltage-gated sodium channel NaV1.7 that is capable of undergoing SUMOylation. Mutating the putative SUMOylation site (K374) on CRMP2 resulted in a decrease in the current density which was found to be a downstream effect of reduced cell surface channel expression [37]. Hence, it can be implied that SUMO mediates interaction between these two proteins which is lost upon mutation. CRMP2 SUMOylation also plays a role in calcium signaling via CaV2.2

N-type voltage-gated calcium channel. De-SUMOylation of CRMP2 by SENPs results in an increased calcium influx suggesting the importance of SUMO in enabling association of CRMP2 with the channel and subsequently altering its activity [38]. Alternatively binding of auxiliary subunits also determine the level of ion channel SUMOylation. In the case of KCNQ1 channels in cardiac myocytes where SUMOylation of the channel was found to be dependent on its auxiliary subunit KCNE1 which causes a depolarizing shift in the voltage of half-activation of the channel [39]. The HCN channel has 4 primary auxiliary subunits in the nervous system – TRIP8b, Filamin A, S-SCAM and MINT2 [10]. Like TRIP8b, Filamin A has also been found to regulate HCN1 channels in hippocampal neurons decreasing their surface expression and subsequently downregulating I_h [40]. S-SCAM and MINT2 are synaptic scaffolding proteins that has been shown to bind to HCN2 channels [4], [41]. A recent study has demonstrated that phosphorylation of a specific TRIP8b residue enhances the binding of this protein to the HCN channel which in turn influences channel localization and gating [42]. In a similar manner, residue K314 on TRIP8b which can be SUMOylated is located very close to the interaction site between TRIP8b core and the channel CNBD. SUMO bound to both TRIP8b and HCN2 channel along with the SIMs might influence the interaction between these two proteins that in turn might be responsible for altering channel function and surface expression.

1.1.3 Pull-down assay to study protein-protein interactions

Proteins are capable of associating with other proteins or molecules and form enormous complexes that participate in a wide variety of cellular functions. Around 80% of proteins that have been identified do not operate alone but are found in such complexes [43]. Most of the functions such as cell cycle, vesicle trafficking can be explained in terms of such interactions and hence it is crucial to identify the interacting partners in a protein complex. The actual physical

interaction between two proteins can be studied using several in vitro techniques such as Pull-down assay, Two-hybrid assay, Surface plasmon resonance spectroscopy, Forster resonance energy transfer (FRET) [44]. Pull-down assay is one of the oldest techniques that have been used widely to study protein-protein interactions. Proteins used in this method can be either cell lysates or purified proteins. Several protein expression systems can be utilized such as bacterial [45], yeast [46], insect [47] or mammalian [48] expression systems. The choice of an expression system greatly depends on the biochemical and biological properties of the target protein [49]. Target genes cloned into a suitable vector can be transformed or transfected into a suitable host system to allow protein expression to take place. After this the recombinant protein needs to be purified. The most common form of purification technique is affinity chromatography which utilizes a specific affinity tag that can bind to a ligand or resin in a column. Plasmids consisting of various tags such as His, GST, HA-tags are available that facilitate such purification process. Alternatively, the tags can also be cloned into the plasmid along with the target gene which subsequently gets expressed during translation. Certain protein-protein interactions can be implicated by post-translational modifications (PTM) such as phosphorylation, acetylation or SUMOylation and a great deal of research focuses on the specific role of such PTMs in the binding process [50]. While some might favor the interactions others might act in an inhibitory fashion. Pull-down assay serves to be a useful tool in studying the roles of such PTMs. This technique is very similar to co-immunoprecipitation (Co-IP) however while Co-IP uses immobilized antibodies to bind to protein complexes, pull-down assay uses an immobilized protein to capture other interacting partners [51].

The basic principle of pull-down assay is based on the concept of a bait and prey protein binding to each other very similar to the lock and key mechanism. The bait protein usually has

the affinity tag that not only helps with the purification process but also enables its immobilization to an affinity ligand in a column. The bait and prey proteins are incubated for a period of time in order to allow for them to interact. Washing the column eliminates all the non-interacting partners thus minimizing any contamination in the final solution. The final step is elution of the bound protein complex which is performed using a buffer containing a competing molecule that will bind to the resin and displace the protein complex. If a different type of purification technique is employed such as ion-exchange or size-exclusion chromatography, corresponding buffer solutions with altered ionic strengths can be used for elution. The eluted proteins can be further resolved on a SDS-PAGE gel and visualized by gel staining techniques like coomassie blue staining or using antibodies against the protein of interest and detecting it by Western Blot [51] (Figure 1.2). One of the main application of this technique is to study direct or predicted interaction between proteins in a complex. In this respect the binding affinity between the two proteins is extremely important and is represented in terms of dissociation constant or K_d [52]. This is measured by keeping the concentration of bait protein constant while gradually increasing the concentration of prey protein till the saturation level has been reached. The amount of prey pulled down can be used to derive a binding curve. K_d will be the concentration of the prey protein when 50% of bait protein is bound to prey and can be derived from the fit sigmoidal curve [53]. The smaller the value of K_d the stronger is the affinity between the two proteins and vice versa. Another useful application of this technique is to measure changes in amount of prey pulled down by bait. In cases where a third molecule such as another protein or a PTM might be influencing the interaction between the bait and the prey protein, the overall stability of the complex may get altered. Here this technique can serve to be useful and can be used to determine such variations in protein interactions in a semi-quantitative manner. The

altered binding affinity might be due to a specific domain or a particular interaction site in the entire protein which can be identified using truncated version of the protein. Changes in the quantity of protein pulled down can be attributed to the missing domain or association and hence its function can be established in such protein-protein interactions.

1.2 Hypothesis

It is a well-known fact that SUMO plays a role in protein-protein interactions. Therefore, the overarching hypothesis in this study is that SUMO might influence the interaction between HCN2 channel and its auxiliary subunit TRIP8b (1a-4). This is tested using pull-down assay technique where the amount of TRIP8b and HCN2 lysate used are kept constant. However, HCN2 channel SUMOylation is manipulated while preparing the cell lysate and the two different HCN2 lysates are used as prey in the different experimental groups. The specific aim is to determine whether SUMO is influencing the binding of the two proteins by analyzing changes in the amount of HCN2 pulled down by TRIP8b in the different treatment groups.

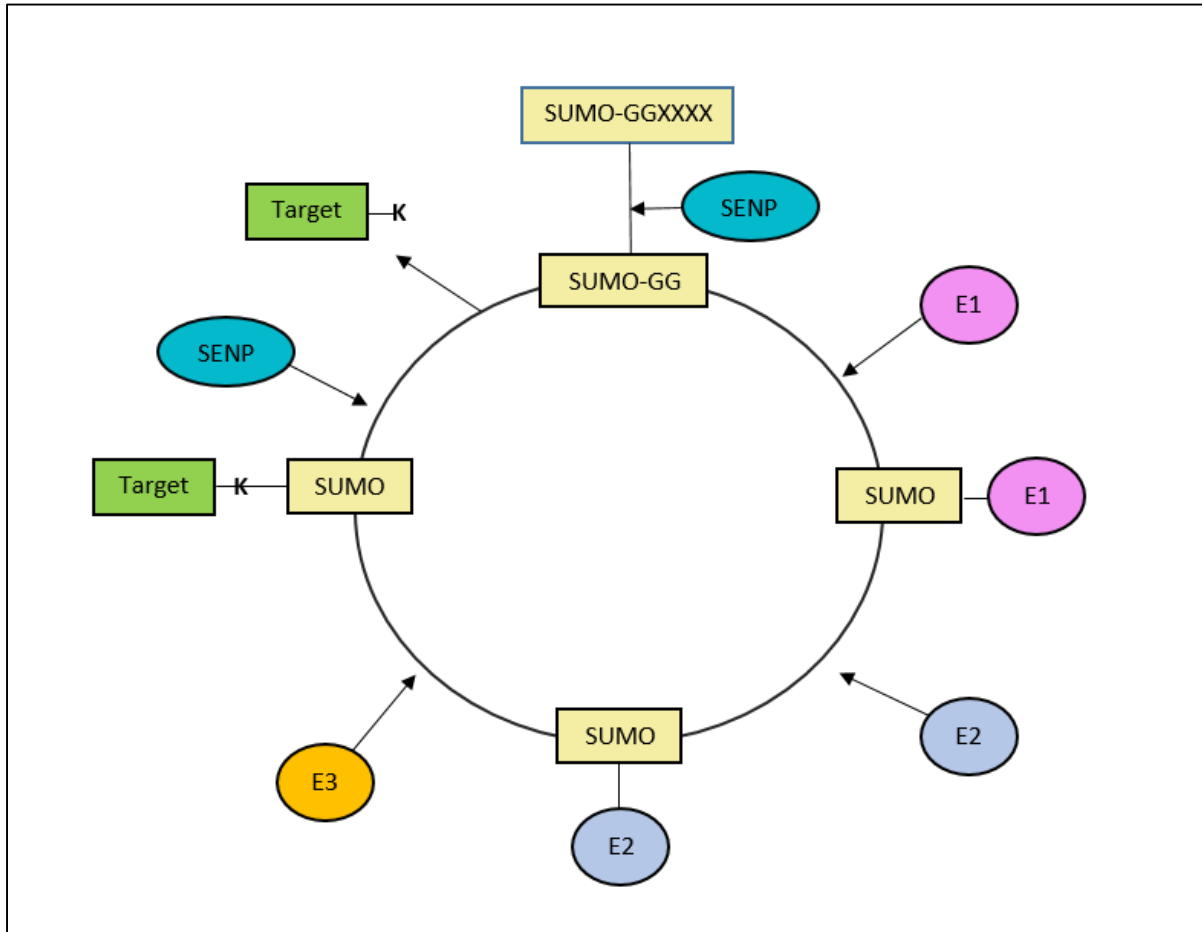


Figure 1.1 Process of SUMOylation.

The SUMO precursor protein is first cleaved by SENP to expose the C-terminal di-glycine motif. Next the mature SUMO becomes bound to SUMO Activating Enzyme or E1. E1 then passes SUMO on to SUMO conjugating enzyme or E2 (also known as Ubc9). Finally, SUMO gets added to the lysine residue on a target protein, which may be facilitated by SUMO ligating enzyme or E3. De-conjugation is initiated by SENP that removes SUMO from the target protein.

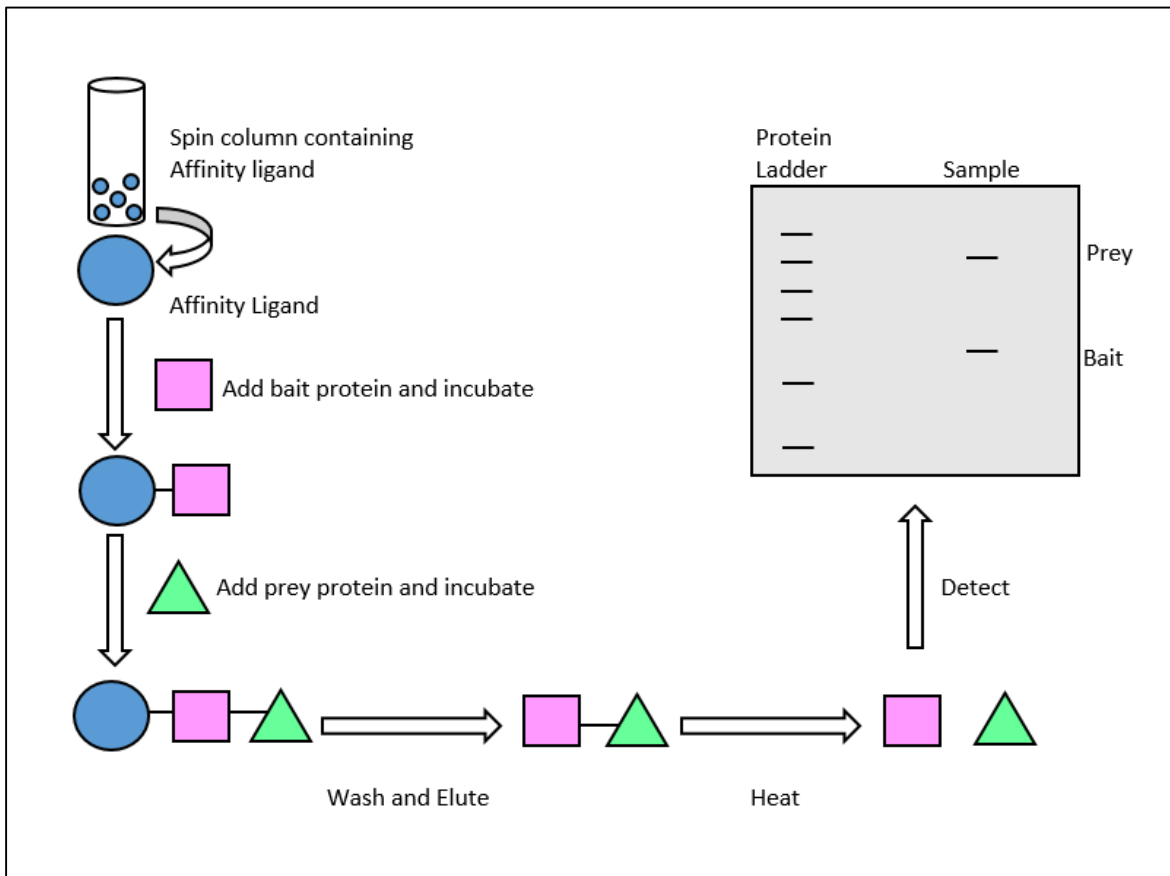


Figure 1.2 Pull-Down Assay.

A suitable affinity ligand is taken in a spin column and equilibrated with appropriate buffer. Next the bait protein is added and incubated to facilitate its binding to the affinity ligand. Following this the prey protein is added and allowed to incubate with the bait protein in order to enable their binding. The column is then washed a few times and finally the bait and prey protein complex is eluted. The complex is mixed with a suitable gel loading buffer and briefly heated in order break the bond between the two proteins before they are loaded on a SDS-PAGE gel. The proteins can be detected by a suitable method such as Western Blot.

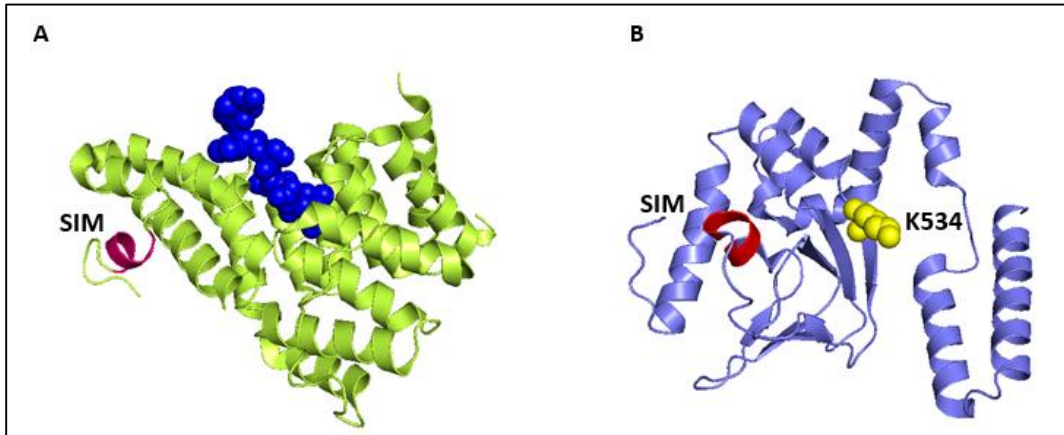


Figure 1.3 SUMO and SIM sites predicted by GPS-SUMO online tool highlighted in the crystal structure of proteins using PyMol.

A) Cartoon representation of mouse TRIP8b C-terminus TPR-domain (green) binding to SNL tripeptide at the C-terminus of HCN2 channel (blue). Predicted SIM on TRIP8b is highlighted in magenta (ref: PDB file 4EQF). B) Cartoon structure of mouse HCN2 CNBD domain with predicted SIM (highlighted in red) and SUMO site K534 which has been represented in yellow (ref: PDB file 5KHJ).

2 MATERIALS AND METHODS

2.1 Plasmids and Antibodies

TRIP8b(1a-4) cloned into pGH19 oocyte expression plasmid, was generously provided by Dr. Bina Santoro and Dr. Steven Siegelbaum (Columbia University). Bacterial expression plasmid pET51b+ (Novagen #71553-3) was used for protein expression. All antibodies are listed in Table 2.1.

2.2 Cloning

2.2.1 Plasmid preparation (pET51b+)

0.5 µl of pET51b+ plasmid was added to 50 µl of XL1-Blue competent cells (Agilent). The contents were mixed by gently flicking the tube followed by incubation on ice for 30 min. The cells were heat shocked for 45 s at 42°C and cooled on ice for 2 min. 200 µl of NZY broth was then added to the tube and the reaction was incubated at 37°C for 30 min. The cells were then plated on NZY plates containing 100 µg/ml ampicillin (Amp) and incubated at 37°C overnight. A single colony was used to inoculate NZY broth containing 100 µg/ml Amp and incubated at 37°C, shaking at 220 rpm, overnight. Plasmid was isolated using Qiagen Plasmid Mini Kit according to manufacturer's instructions. Restriction digestion was used to cleave pET51b+ plasmid at two restriction sites using AatII and NotI restriction enzymes (New England BioLabs) respectively in a sequential digestion. The first reaction was carried out using 10 µg of DNA, AatII, NotI, 10X Cut smart buffer in a 100 µl reaction. AatII was added first and the reaction was incubated at 37°C for 1 h followed by heat inactivation at 80°C for 20 min. Next NotI was added to the same reaction and incubated again at 37°C for 1 h followed by heat inactivation at 65°C for 20 min. The DNA was purified using QIAquick PCR Purification Kit according to manufacturer's instructions. 6 µg of cut plasmid from the previous step was further

digested using the same restriction enzymes only this time together at 37°C for 3 h. The final cut vector was dephosphorylated using Quick Dephosphorylation Kit (New England BioLabs) followed by which the DNA was run on 1% agarose gel at 90 V for 1 h and 30 min. The DNA fragment was excised from gel and the cut plasmid was isolated using QIA Quick gel extraction kit (Qiagen) according to manufacturer's instructions. The final concentration was determined using nanodrop.

2.2.2 Insert preparation (TRIP8b)

PCR reaction was used to add the AatII and NotI restriction sites to TRIP8b. The primers were designed to add the restriction sites, His-tag at the N-terminus of TRIP8b and a stop codon at the end of C-terminus (primers listed in Table 2.2). PCR reaction was set up using around 400 ng of DNA template in a 50 µl reaction containing 50X Advantage2 polymerase (Takara), 10X Advantage2 PCR Buffer (Takara), 50X dNTP mix and 10µM primers. The cycling conditions were as follows: 1 X 95°C 1 min; 1 X 95°C 30 s, 42°C 1 min, 68°C 2 min; 1 X 95°C 30 s, 44°C 1 min, 68°C 2 min; 1 X 95°C 30 s, 46°C 1 min, 68°C 2 min; 1 X 95°C 30 s, 48°C 1 min, 68°C 2 min; 21 X 95°C 30 s, 68° 2 min; 1 X 68°C 5 min. After verifying the correct band size, four additional PCR reactions were set up. The PCR products were then pooled from five reactions and the DNA was precipitated using 1 µl of glycogen, 1/10 volume of sodium acetate pH 5.0 and 3 volumes of ethyl alcohol. After centrifugation the pellet was re-suspended in 30 µl of water. 10 µg of TRIP8b DNA was used to set up a single restriction digestion reaction with AatII and NotI restriction enzymes, the cut DNA was isolated from 1% agarose gel and the concentration was determined as previously described.

2.2.3 Ligation of vector and insert

Ligation was performed using equal molarity of pET51b+ (vector) and TRIP8b (insert). A 10 µl ligation reaction was set up using appropriate volumes of vector and insert along with T4 DNA Ligase (Thermo Scientific), T4 Ligase buffer (Thermo Scientific) and polyethylglycol at 14°C overnight. Different vector:insert ratios were used – 1:1, 1:3 and 3:1 along with two controls – vector only without ligase and vector only with ligase. The following day 1 µl of reaction from each group was transformed into 50 µl of competent cells and plated. Individual colonies were picked from the experimental groups and grown in 50 µl of NZY broth with 100 µg/ml Amp at 37°C for 1 h. 1 µl of this was used to set up 15 µl PCR reactions for individual colonies using Taq polymerase (Takara), buffer, dNTPs, forward primer complementary to the vector and reverse primer complementary to the insert. The cycling conditions were as follows: 1 X 95°C 1 min; 30 X 95°C 30 s, 45°C 1 min, 68°C 1 min; 1 X 68°C 5 min. The PCR product was ran on 1% agarose gel and the positive colonies were identified by determining the correct band size. The positive samples were grown overnight in NZY broth and 100 µg/ml Amp followed by which plasmid was isolated using Qiagen Plasmid Mini Kit. Finally, the isolated plasmids were sequenced using suitable primers (Table 2.2) at the Georgia state University Cell Protein and DNA Core facilities. The sequences were analyzed using Larsgene software (DNASar).

2.3 Protein Expression

TRIP8b cloned into pET51b+ plasmid was transformed into 20 µl BL21DE3 competent cells (Millipore Sigma) according to manufacturer's instructions. A pre-culture was started by inoculating a single colony into 6 ml NZY broth containing 100 µg/ml Amp and growing it at 37°C, shaking at 250 rpm for 8 h. After 8 h, 800 µl of the culture was added to 250 ml NZY broth containing 100 µg/ml Amp and was incubated at 37°C, shaking at 250 rpm overnight for

expansion to take place. 100 ml of overnight culture was next added to two separate flasks containing 500 ml NZY broth, 100 µg/ml Amp and 500 µM isopropyl β-D-1 thiogalactopyranoside (Sigma) each. The flasks were incubated at 37°C, shaking at 250 rpm for 5 h for induction to take place. The culture was then taken in 250 ml sorvall bottles and pelleted at 10,000 x g for 10 min at 4°C followed by which the pellet was re-suspended in Buffer W (IBA, 100 mM Tris/HCl pH 8.0, 150 mM NaCl, 1 mM EDTA). This was sonicated on ice at 15% amplitude using a 10 s 'on' and 30 s 'off' protocol for 70 cycles using Fisher Scientific Sonic Dismembrator Model 500. Sonicate was then centrifuged at 16,000 x g for 10 min at 4°C. The supernatant was transferred to a fresh tube and re-centrifuged at 16,000 x g for another 10 min. The cleared lysate was further used for protein purification.

2.4 Protein Purification

2.4.1 Purification using Strep-tag

The cleared lysate was purified using ready-to-use column with 1 ml high capacity Strep-Tactin resin (IBA). The column was first equilibrated with 2 ml Buffer W followed by addition of protein lysate and allowing it to flow down by gravity. The column was then washed five times with 1 ml Buffer W. The protein bound to the column was eluted in different fractions and collected in 1.5 ml tubes using Buffer E (IBA, 100 mM Tris/HCl pH 8.0, 150 mM NaCl, 1 mM EDTA, 2.5 mM desthiobiotin) - 0.6 column volume (CV), 1.6 CV and 0.8 CV. The concentration of protein was determined using BCA Assay (Thermo Scientific Pierce BCA Kit) according to manufacturer's instructions. 20 µl of sample from each elution was run on a SDS-PAGE gel overnight at 25 V. The gel was first fixed using a solution containing 25 ml methanol, 5 ml acetic acid and 20 ml water for 1 h with gentle shaking. The gel was next stained using a solution containing 50 mg Coomassie Blue R-250, 25 ml methanol, 5 ml acetic acid and 20 ml water for

20 min on shaker. De-staining was performed using a solution containing 20 ml methanol, 5 ml acetic acid and 25 ml water with gentle shaking. This was repeated three times till the gel was clear and the protein bands were distinctly visible.

2.4.2 Purification using His-tag

500 μ l of Ni-NTA resin (Qiagen) was equilibrated with 1 ml Lysis buffer (50 mM NaH_2PO_4 pH 8.0, 300 mM NaCl, 10 mM imidazole) in a 1.5 ml tube. 1 mg of Strep-purified protein was mixed with equal volume of Lysis buffer and incubated with the beads shaking at 4°C for 1 h. The solution was then carefully passed through a 25 ml empty column (BioRad). The resin was washed six times with 500 μ l Wash buffer (50 mM NaH_2PO_4 pH 8.0, 300 mM NaCl, 20 mM imidazole). The protein was finally eluted using 500 μ l of Elution buffer (50 mM NaH_2PO_4 pH 8.0, 300 mM NaCl, 250 mM imidazole) in eight different fractions. 20 μ l of each of the fractions were run on a SDS-PAGE gel overnight and further stained with coomassie blue dye to visualize the protein bands. The fractions in which the bands were visible were pooled together and de-salting was performed using PD-10 desalting column (GE Healthcare) and buffer was exchanged for 20 mM Tris/HCl pH 7.5 and 250 mM NaCl. The final protein concentration was determined using BCA assay and the protein was once again run on a SDS-PAGE gel overnight and stained with coomassie blue dye to verify the correct size protein band.

2.5 Cell Culture

A HEK293 cell line stably expressing GFP-HCN2 fusion protein (HEK-HCN2) was cultured at 37°C and 5% CO_2 in EMEM media supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin, and 500 μ g/ml geneticin (Gibco). The cells were cultured in 100mm plates in order to prepare HCN2 cell lysates.

2.6 Preparation of HCN2 cell lysate

Plates containing HEK-HCN2 cells were washed twice with ice cold 1X PBS. Followed by this cells were lysed for 30 min on ice using RIPA buffer (1% NP40, 50 mM Tris/HCl pH 7.4, 150 mM NaCl, 0.1% SDS, 0.5% DOC, 2 mM EDTA, 1:100 protease inhibitor cocktail) with and without 20 mM N-ethylmaleimide (NEM). Cells were scraped from the plates using cell scraper and transferred into sterile 1.5 ml centrifuge tubes. The cells were centrifuged at 14,000 rpm for 15 min to pellet the cell debris. The supernatant was carefully transferred to a fresh sterile tube. BCA assay was performed to determine protein concentration.

2.7 Pull-Down Assay

HCN2 cell lysates prepared using with and without NEM were used in three sets of pull-downs for each group. All centrifugations were performed at 1000 x g for 1 min. 50 μ l of Ni-NTA resin was taken into spin columns (Pierce spin columns Thermo Scientific) and equilibrated with 100 μ l of Equilibration buffer (20 mM Tris/HCl pH 7.5, 250 mM NaCl, 10 mM imidazole). The columns were plugged in, 7 μ g of TRIP8b (bait) was made up to 200 μ l volume using Equilibration buffer and added to each one of them. The tubes were incubated at 4°C on shaker for 1 h followed by 10 min on ice for the resin to settle down. The plugs were then removed, the columns were centrifuged and the flow-through was collected which was marked as the unbound bait protein. The columns were plugged back. 300 μ g of HCN2 lysates (prey) was made up to 200 μ l volume using Equilibration buffer and added to the respective tubes. They were once again incubated at 4°C on shaker for 1 h followed by 10 min on ice. The plugs were then removed, the columns were centrifuged and the flow-through was collected which was marked as the unbound prey protein. The resin was washed six times using 100 μ l Wash Buffer (20mM Tris/HCl pH 7.5, 250 mM NaCl, 20 mM imidazole) but only the first wash was saved.

The column was plugged again and 100 μ l of Elution Buffer (20 mM Tris/HCl pH 7.5, 250 mM NaCl, 500 mM imidazole) was added and the column was incubated on ice for 10 min followed by centrifugation and collection of protein in a fresh tube. The eluted fraction was re-loaded into the column and the step was repeated in order to maximize the amount of protein eluted. Next 20 μ l of eluted protein from each pull-down was mixed with 6.7 μ l of 4X Lemmli Lysis Buffer (62.5 mM Tris/HCl pH 6.8, 2% SDS, 10% glycerol, 0.01% bromophenol blue, 5% β -mercaptoethanol) in small PCR tubes. The tubes were heated to 100°C in a thermocycler in order to break the bond between bait and prey proteins. 20 μ l of each of the sample was the loaded into SDS-PAGE gel and run overnight at 25 V.

2.8 Western Blot

Protein samples were transferred to a PVDF membrane for 3 h using a semi-dry electroblotting system. The membrane was then air dried for 30 min at room temperature followed by blocking with 5% non-fat dry milk in 1X TBS (50 mM Tris/HCl pH 7.4, 150 mM NaCl) shaking at room temperature for 1 h. The membrane was next washed once with 1X TTBS (TBS and 0.1% Tween 20). Primary antibodies were diluted in 1% non-fat dry milk in 1X TTBS as described in Table 1 and the membrane was added to this and incubated at 4°C shaking overnight. The next day membrane was washed three times for 5 min each with 1X TTBS and then incubated with appropriate alkaline conjugated secondary antibody diluted in 1% non-fat dry milk in 1X TTBS at room temperature for 2 h. After the incubation the membrane was washed three times 10 min each with 1X TTBS. The membrane was next placed on a cling wrap and treated with Alkaline Phosphatase substrate (BioRad) for 5 min and then it was exposed to film for different time durations. The chemiluminiscent signals were captured using a film developer (Kodak X-Omat 2000A imager). In some cases, the membrane was stripped and re-

probed using a different primary antibody. The membrane was washed with Mild Stripping Buffer (200 mM Glycine, 0.1% SDS, 1% Tween 20, 50 mM KCl, $\text{Mg}(\text{CH}_3\text{COO})_2$, pH 2.2) twice for 10 min each, 1X PBS twice for 10 min each and 1X TTBS twice for 5 min each. AP substrate was applied again for 5 min and the membrane was exposed to film for 10 min and developed to ensure that the original chemiluminiscent signal was gone. Image J was used to measure optical densities for individual bands of interest as previously described [24].

Table 2.1 Primary Antibodies

Antigen	Verified By	Species, manufacturer and catalog number	Concentration used
HCN2	Specificity verified by the company, WB analysis showing HCN2 from rat brain membranes.	rabbit, alomone, apc 030	WB: 1:1,000
SUMO 2/3	Specificity verified by company, WB analysis recognizes 15kDa SUMO2 band and 18kDa SUMO3 band	rabbit, abcam, ab3742	WB: 1:1,000

Table 2.2 PCR and Sequencing Primers

Primer Name	Primer Sequence (5'-3')
PCR	
mTRIP8b for	TATGACGTCTCATCACCATCACCATCACATGTACCAGGGACAC
mTRIP8b 1a-4 rev	TATGCGGCCGCTACTAAGGATCCAAATTGAAAGCTCTCAG
Sequencing	
T7	TAATACGACTCACTATAGGG
mTRIP8b 1a-4 F1	AGGGCCAGAGACATCATCCTTAG
mTRIP8b F2	TTGCAGCCCAACAACCTGAAG
mTRIP8b 1a-4 F3	TGGAAGCTGCCACCAAAAATG
T7 term rev	GCTAGTTATTGCTCAGCGG
mTRIP8b1a-4 R1	AACAGAACTCCCAGACCTGTCTGC
mTRIP8b1a-4 R2	TTCAAATGCTCCAGGCCAGTC
mTRIP8b1a-4 R3	TAAGGATGATGTCTCTGGCCCTC

3 RESULTS

3.1 TRIP8b is cloned into pET51b+ plasmid

The pET51b+ plasmid utilizes bacteriophage T7 promoter to drive transcription and further translation of target genes. It is a highly efficient system used for cloning and recombinant protein expression in *E. coli* that serves as the host cell, providing T7 RNA polymerase required for this process. TRIP8b was successfully cloned into the pET51b+ plasmid and a positive clone was obtained (Figure 3.1B). The expected band size of 1100 bp corresponds to that of the positive clone. The plasmid already had an N-terminal Strep-tag II and the sequencing results along with the alignment report (Figure 3.1C) confirm successful addition of the His-tag at the same end. As TRIP8b interaction with HCN2 mostly happens at the C-terminal end, keeping the tags at N-terminus was crucial to mediate un-interrupted binding. Hence, the C-terminal His-tag in the plasmid was blocked by adding two stop codons at the end of TRIP8b nucleotide sequence in order to prevent its translation.

3.2 TRIP8b is expressed and purified using dual tags

The BL21DE3 cells used as host in our experiment has the T7 RNA polymerase gene under the control of lacUV5 promoter, which can be induced with IPTG. TRIP8b cloned into the pET51b+ vector was successfully transformed into these *E. coli* cells which when induced with IPTG resulted in protein expression. The utilization of dual tag was employed in order to derive highly pure TRIP8b recombinant protein, which was further used in downstream assay. The tags enable efficient protein purification by affinity chromatography technique. In this process the protein first binds to the stationary phase within a column, which separates it from the unwanted components. At the final step the protein can be successfully eluted. The protein tags generally bind to metal matrices and can be retained within a column. The Strep-tag II consists of 8 amino

acids and binds to strep-tactin (an engineered version of streptavidin) with a binding affinity of $1\mu\text{M}$. TRIP8b protein possessing this tag bound efficiently to the column after which it was eluted using desthiobiotin in a competitive manner. Most of the protein was eluted in fraction E2 as represented by the dark blue band at 70 kDa when stained with coomassie dye (Figure 3.2A). The protein solution was next subjected to a second round of purification utilizing the His-tag. This tag has an affinity for the nickel metal ion and hence Ni-NTA resin was used. The protein was finally eluted using imidazole and was detected by coomassie staining (Figure 3.2B). To retrieve maximum amount of protein out of the column multiple elution steps were performed which also meant a high concentration of imidazole in the final solution. Hence, a de-salting step was necessary which was performed using PD-10 column in order to get rid of the excess imidazole. This column works on the principle of size exclusion chromatography. Salts like imidazole enter the resin beads and hence their flow rate is slowed down whereas proteins enter the void volume of the column and flow faster thus getting effectively separated from the salts. The final de-salted TRIP8b protein band with a size of 70 kDa was visualized on coomassie stained SDS-PAGE gel (Figure 3.2C).

3.3 SUMOylation can be decreased without the addition of NEM

N-Ethylmaleimide (NEM) is an organic compound that is used to prevent de-SUMOylation of proteins which they do so by inhibiting the SENP activity. It is used in RIPA buffer while preparing HCN2 lysate so that HCN2 channel proteins remain SUMOylated. When the lysis buffer is made without NEM, high molecular weight SUMO conjugates are lost. In our experiment HCN2 lysates were prepared both in presence and absence of NEM. The lysates from both groups were run on SDS-PAGE gel and analyzed using Western Blot experiments. The blot was initially probed with anti-SUMO2/3 antibody and the SUMO signal was detected by

chemiluminescence (Figure 3.3A). The OD for the HCN2 doublet was measured using Image J. The blot was next stripped and re-probed with anti-HCN2 antibody and again the HCN2 signal was detected and the OD was measured (Figure 3.3B). The GFP-HCN2 channel in the HEK cells was observed as a doublet consisting of two individual bands between 150 and 250 kDa. This represents two distinct forms of the channel. This was previously described in a study as the slower migrating band being N-glycosylated whereas the faster migrating band has simple or no glycosylation [24]. The fraction of SUMOylated channel was calculated by dividing the OD of SUMO signal by the OD of HCN2 signal. By using this semi-quantitative method, it was seen that the fraction of channel SUMOylation reduced by approximately 6-fold without the addition of NEM (Figure 3.3C). The alteration of baseline HCN2 SUMOylation in absence of NEM might indicate an increased SENP activity in the SUMO de-conjugation process.

3.4 Level of HCN2 protein pulled down by TRIP8b decreases in absence of NEM

TRIP8b acting as the bait protein was immobilized onto the nickel resin. HCN2 lysate was used as the prey protein in this experiment. Although the interaction between these two proteins predominantly occurs at the C-terminus, the channel was used in its entirety in order to see if SUMO mediates their binding. HCN2 lysate from each treatment group i.e. with and without NEM was used in three sets of pull-down experiments. The eluted bait and prey protein was run on a SDS-PAGE gel and analyzed using Western Blot. The blot was probed with anti-HCN2 antibody and the HCN2 signal was detected by chemiluminescence (Figure 3.4A). OD was measured using gel analysis feature of Image J. Values obtained for area under curve for each HCN2 doublet was used to calculate the average area of three bands from the individual treatment groups, which has been plotted in the graph (Figure 3.4B). The average area under curve measured for HCN2 signal obtained using lysate prepared without NEM is around 24%

less than lysate prepared with NEM. This might indicate the influence of SUMO in promoting a stronger association and stability between TRIP8b and HCN2. Stabilizing the interaction between these two proteins may have resulted in a higher amount of HCN2 to be pulled down by TRIP8b when NEM was added during preparation of the lysate.

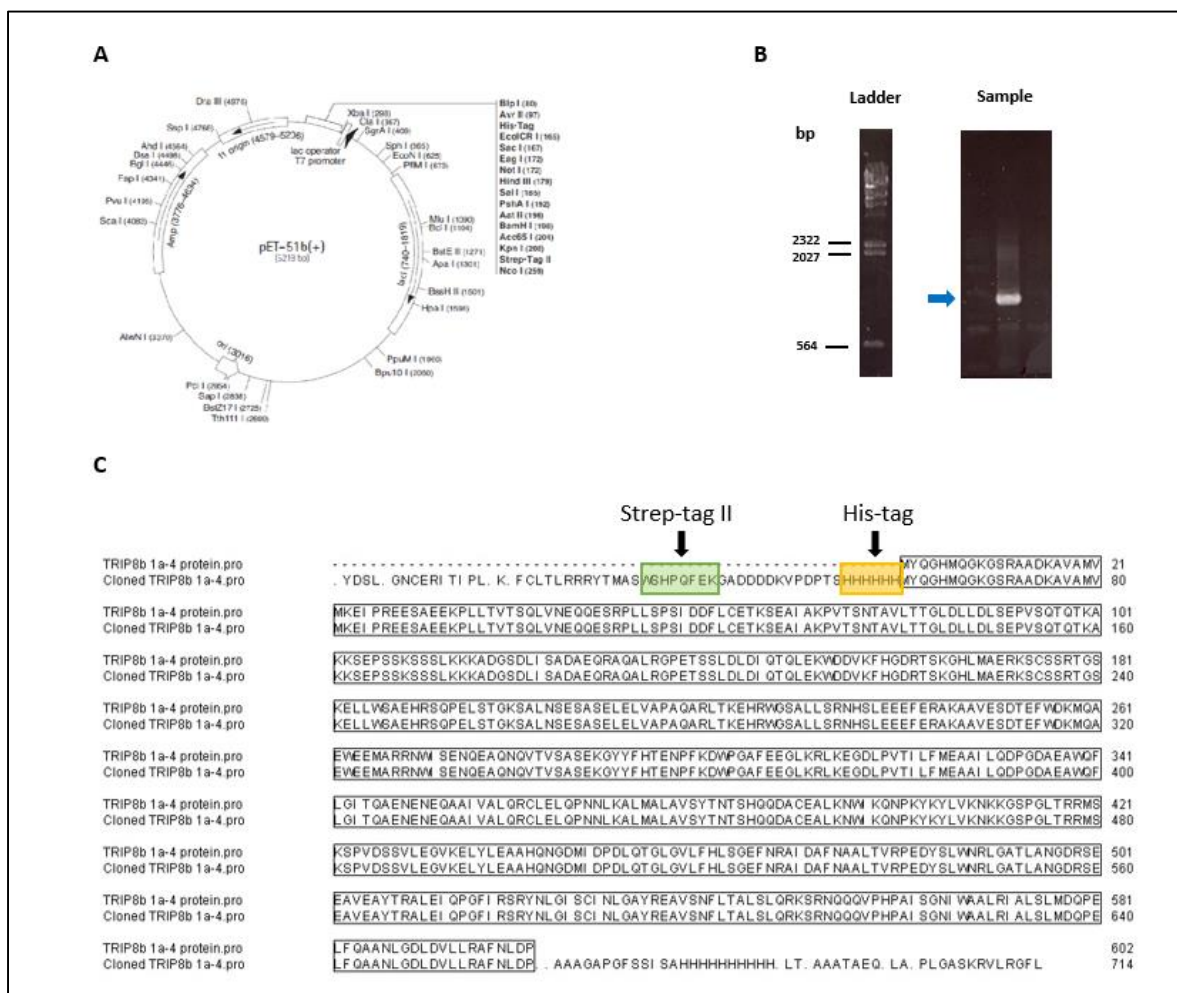


Figure 3.1 Cloning TRIP8b(1a-4) into pET51b+ plasmid.

A) pET51b+ plasmid map showing the different restriction sites. TRIP8b was cloned in between AatII and NotI sites. B) Positive clone (blue arrow) identified when pick-colony PCR was performed and the DNA was run on agarose gel. The expected band size was approximately 1100bp, which complements with that of the positive clone. C) Alignment report of the cloned TRIP8b with that of the original protein sequence (Accession# AEH94554.1) was generated using Meg Align feature of DNASTar. The tags on the insert are highlighted – Strep-tag II (green) and His-tag (orange).

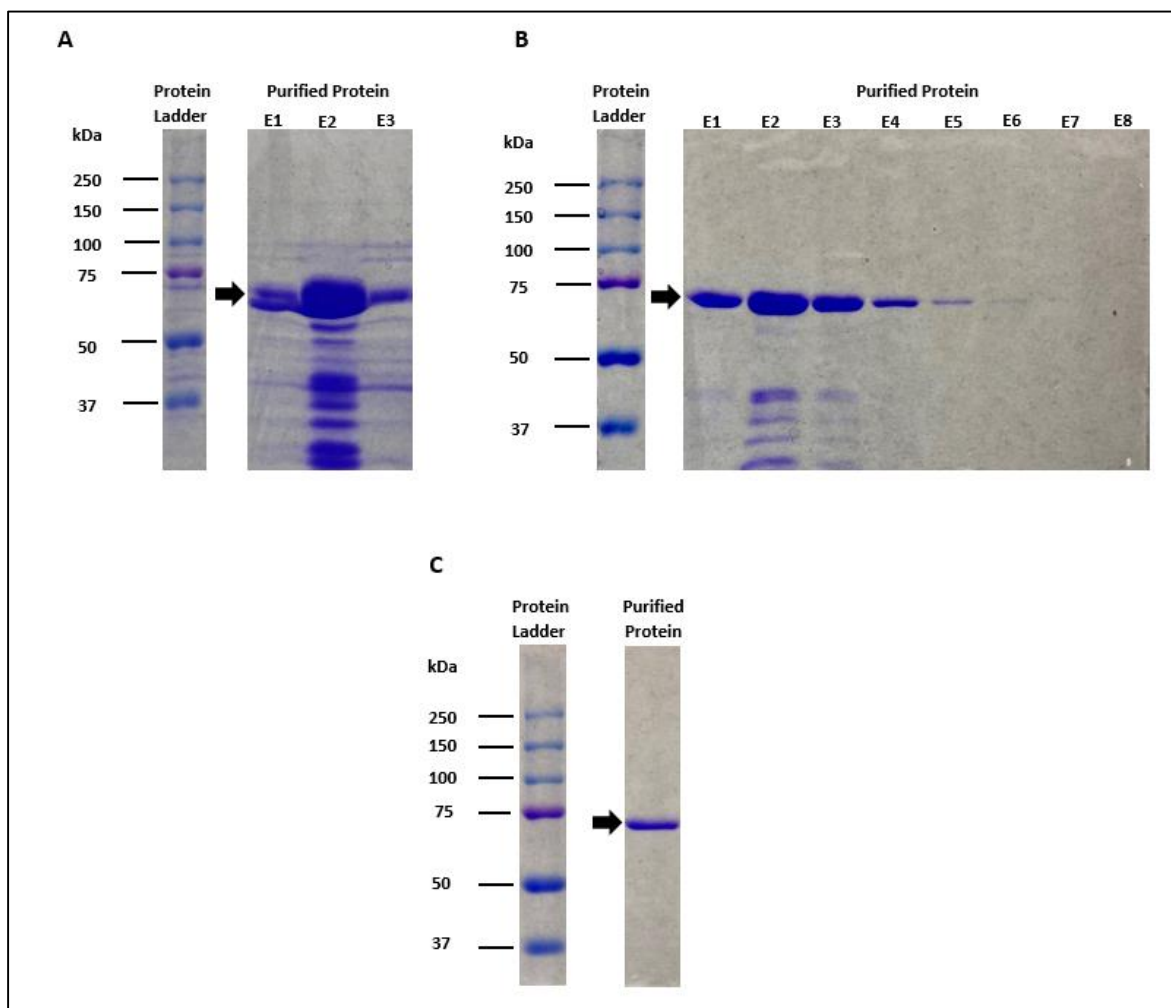


Figure 3.2 Protein purification using dual tags.

TRIP8b was purified using in a two-step purification process. A) Coomassie blue stained SDS-PAGE gel showing protein purified using Strep-tag II on Strep-tactin column. Protein was eluted in three fractions (E1 to E3). The 70 kDa band represents *TRIP8b* (black arrow). B) Coomassie blue stained SDS-PAGE gel showing protein from previous purification step further purified using His-tag on Ni-NTA column. Protein was eluted in eight fractions (E1 to E8). The 70 kDa band corresponds to *TRIP8b* (black arrow). C) Coomassie blue stained SDS-PAGE gel showing His-purified protein passed through PD-10 de-salting column. The 70 kDa band represents *TRIP8b* (black arrow).

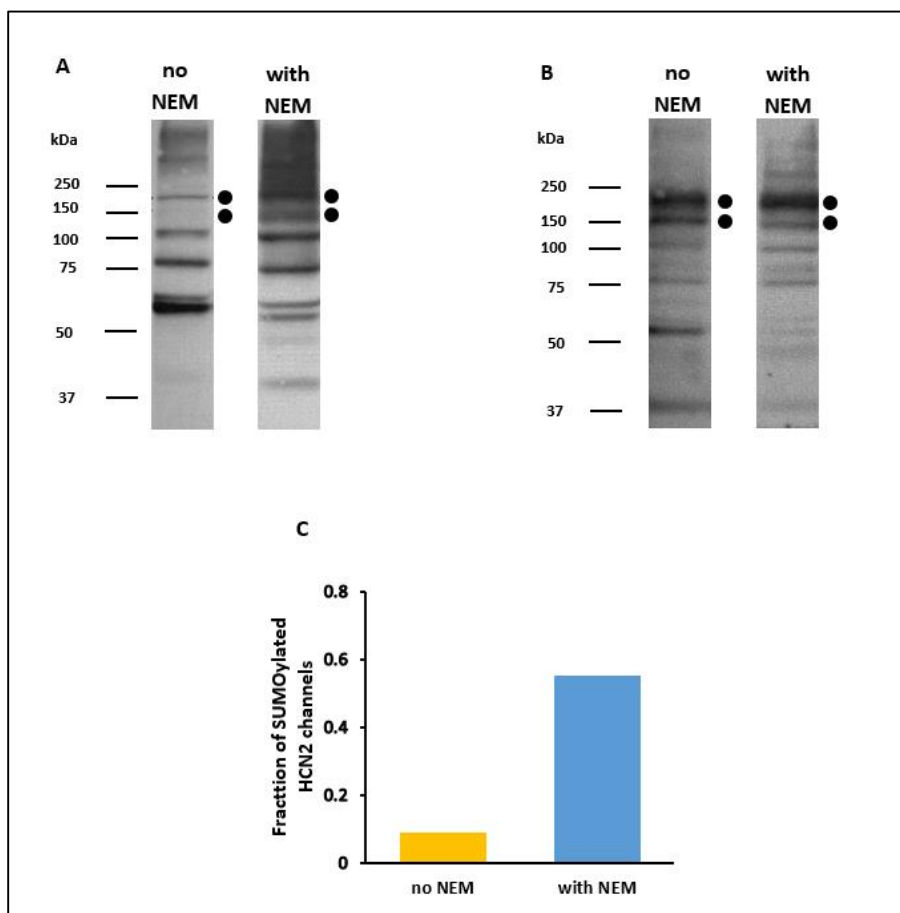


Figure 3.3 Alteration in HCN2 channel SUMOylation.

HCN2 lysate was prepared using RIPA lysis buffer with and without NEM. Western blots containing the lysates from the two treatments were first probed for SUMO2/3 and then stripped and re-probed for HCN2. A) Representative blots showing HCN2 doublet (black circles) when probed with anti-SUMO2/3 antibody. B) The blots were stripped and re-probed with anti-HCN2 antibody. Representative blots showing HCN2 doublet (black circles). C) The fraction of SUMOylated HCN2 is represented for each treatment group. This was calculated by using $\text{SUMO doublet OD} \div \text{HCN2 doublet OD}$.

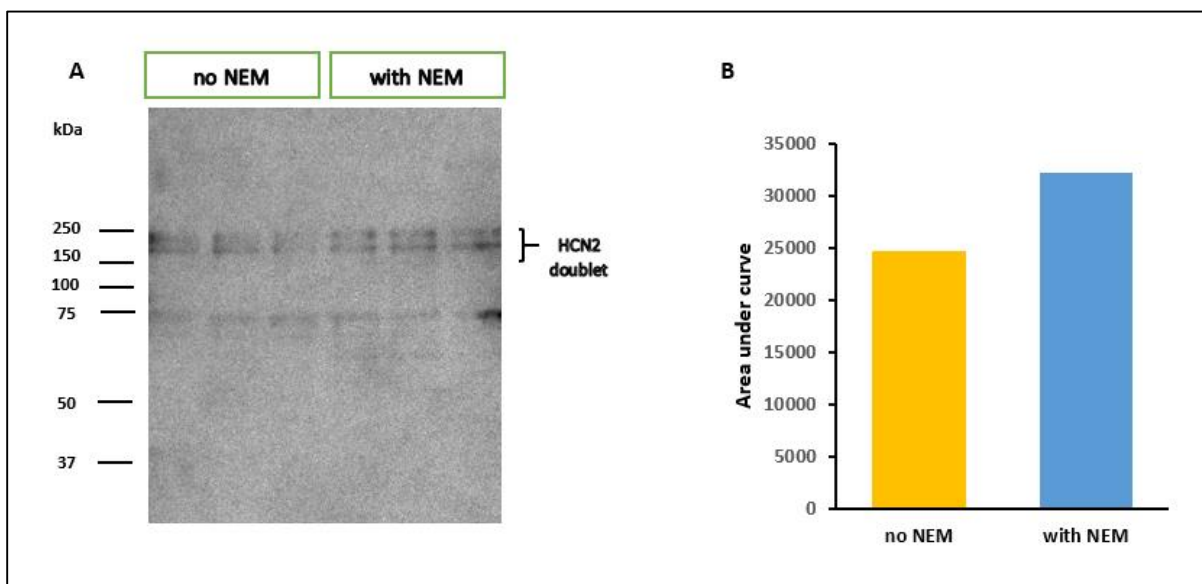


Figure 3.4 Pull-down assay using *TRIP8b* as bait and *HCN2* lysate from two different treatment groups as prey.

The eluted protein was resolved using SDS-PAGE and Western Blot was performed. A)

Representative blots showing *HCN2* channel (labelled *HCN2* doublet) when probed with anti-*HCN2* antibody. *HCN2* doublet was observed in both the treatment groups. B)

The area under curve of the individual bands was measured using Image J Gel Analysis Feature. The average area of the three individual bands under each treatment group was calculated and plotted in the graph.

4 CONCLUSIONS

The main purpose of this study was to see if SUMO alters the interaction between HCN2 channel protein and its auxiliary subunit TRIP8b. Recombinant protein expression was successfully carried out for TRIP8b. The dual tag purification process ensured the purity of the bait protein and minimized the presence of any non-specific protein interacting with the HCN channel. Preparing HCN2 lysate without using NEM effectively reduces the amount of baseline SUMOylation. However, performing immunoprecipitation (IP) using an antibody against the HCN2 channel and running the IP product on the gel would have given a more accurate measurement of the actual difference in degree of SUMOylated channel between the two treatment groups. Another way to reduce baseline SUMOylation would be by using siRNA against Ubc9 and ensure its complete knockdown. Without the SUMO conjugating enzyme, the level of SUMOylation can be greatly reduced. In our study HCN2 channel protein was successfully pulled down by TRIP8b reinforcing their interaction in vitro. Our results indicate that there is a 24% decrease in the amount of HCN2 pulled down when baseline SUMOylation was reduced. However, the experiment was performed only once and hence repeating the process by keeping the parameters same is necessary to get statistical significant data. Another important set of treatment would be to increase SUMOylation by transfecting HEK-HCN2 stable line with SUMO and Ubc9. Over-expressing SUMO might result in a significant increase in the amount of HCN2 pulled down by TRIP8b. As previously described TRIP8b also has possible SUMOylation sites and SUMO can be added to this protein after the purification step. Using SUMOylated bait and prey protein together might further enable us to study the role of SUMO in this protein-protein interaction. Taking this one step further, site-directed mutagenesis can be used to mutate

the putative SUMOylation sites on HCN2 and TRIP8b in order to identify the exact residue which contribute towards such changes.

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