

T.R.
GEBZE TECHNICAL UNIVERSITY
GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

**THE RECIPROCAL INTERACTION BETWEEN CHIP AND
SIK2 MAY FORM A REGULATORY SWITCH IN UNFOLDED
PROTEIN RESPONSE SIGNALING**

ŞEBNEM ÖZTÜRK

**A THESIS SUBMITTED FOR THE DEGREE OF
MASTER OF SCIENCE
DEPARTMENT OF MOLECULAR BIOLOGY AND GENETICS**

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T.C.
GEBZE TEKNİK ÜNİVERSİTESİ
FEN BİLİMLERİ ENSTİTÜSÜ

SIK2 VE CHIP'İN KARŞILIKLI
ETKİLEŞİMİ KATLANMAMIŞ PROTEİN
CEVABI SİNYAL İLETİMİNDE
DÜZENLEYİCİ BİR ANAHTAR GÖREVİ
GÖRÜYOR OLABİLİR

ŞEBNEM ÖZTÜRK
YÜKSEK LİSANS TEZİ
MOLEKÜLER BİYOLOJİ VE GENETİK ANABİLİM DALI

DANIŞMANI
PROF. DR. FERRUH ÖZCAN

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İMZA/MÜHÜR

SUMMARY

Obesity which affects more than 1.000.000.000 people worldwide continues to be one of the greatest threats to global health. Obesity is associated with other pandemics such as type 2 diabetes, non-alcoholic fatty liver disease, neurodegenerative disorders, and some cancer types. Obesity and its associated diseases are linked to chronic inflammation in the central nervous system (CNS) and metabolically active peripheral tissues. Inflammatory signaling disrupts insulin and leptin action in the respective tissues and leads to insulin and leptin resistance in obese individuals.

Endoplasmic reticulum (ER) stress and autophagy are implied in connecting impaired insulin signaling and inflammation in obesity and type 2 diabetes. Understanding the mechanisms that counteract ER stress may lead to correcting impaired insulin signaling and treat obesity and related metabolic and neurodegenerative diseases.

CHIP and SIK2 are two proteins which play key roles in restoring protein homeostasis in response to ER stress. SIK2 is a serine/threonine kinase involved in the regulation of various metabolic pathways and is also implied in the regulation of ERAD and autophagy. CHIP is a co-chaperone and an E3 ubiquitin ligase which takes part in ERAD and autophagy to counteract ER Stress.

In a previous phosphoproteomics based study our lab identified CHIP as a putative substrate for SIK2 implicating the latter in the regulation of either ubiquitin-proteasome system (UPS) or autophagy or both. In fact, our data is in line with the proposed roles of SIK2 in the upregulation of ERAD and autophagy pathways through phosphorylation of p97/VCP and HDAC4 under ER stress conditions respectively.

In this study the nature of the interaction between SIK2 and CHIP and its effect on CHIP interactome under ER stress has been investigated in HEK293T cells.

Key Words: ER Stress, ERAD, Ubiquitin-Proteasome System, Autophagy, CHIP, SIK2.

ÖZET

Dünya çapında bir milyardan fazla insanı etkileyen obezite en büyük global sağlık tehditlerinden biri olmaya devam etmektedir. Obezite tip 2 diyabet, alkole bağlı olmayan yağlı karaciğer hastalığı, nörodejeneratif hastalıklar ve bazı kanser tipleri gibi diğer pandemilerle ilintilidir. Obezite ve ilintili hastalıklar merkezi sinir sistemi ve periferik dokulardaki kronik enflamasyonla bağlantılıdır. Obez bireylerde enflamatuvar sinyalindeki artış ilgili dokularda insülin ve leptin iletim mekanizmalarını bozarak insülin ve leptin direncine neden olur.

Obezite ve tip 2 diyabette insülin iletimindeki bozulma ve enflamasyon arasındaki bağlantının endoplasmik retikulum (ER) stresi ve otofaji olduğu izlenimi oluşmaktadır. ER stresini gidermeye yönelik mekanizmaları anlamak bozulan insülin iletimini düzelterek obezite ve ilintili metabolik ve nörodejeneratif hastalıkların tedavi edilmesine katkıda bulunacaktır.

CHIP ve SIK2, ER stresine karşı protein homeostazını sağlamakta anahtar görevleri olan proteinlerdir. Çeşitli metabolik yolların düzenlenmesinde etkili bir serin/treonin kinaz olan SIK2, ERAD ve otofajinin protein homeostazını sağlayacak şekilde regülasyonunda rol oynar. CHIP ise ER stresi gidermek için tetiklenen bir dizi önlem olan katlanmamış protein cevabının ERAD ve otofaji kolunda görev alan bir ko-şaperon ve E3 ubiquitin ligazdır.

Laboratuvarımızda gerçekleştirilen bir fosfoproteomik çalışmada CHIP'in SIK2'nin olası bir substratı olduğunun saptanması SIK2'nin Ubikitin-Proteozom Sistemi (UPS), otofaji veya ikisini birden regüle ettiğini düşündürmüştür.

Bu çalışmada SIK2 ve CHIP'in etkileşiminin niteliği ve bunun ER stres altında CHIP interaktomuna etkisi HEK293T hücrelerinde araştırılmıştır.

Anahtar Kelimeler: ER Stresi, ERAD, Ubikitin-Proteozom Sistemi, Otofaji, E3 Ubikitin Ligaz CHIP, SIK2.

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LIST of ABBREVIATIONS and ACRONYMS

<u>Abbreviations and</u>	<u>: Explanations</u>
<u>Acronyms</u>	
α	: Alpha
β	: Beta
δ	: Delta
g	: Gram
G	: Gravity
M	: Molar
ml	: milliliter
mM	: Millimolar
nM	: Nanomolar
μ M	: Micromolar
μ g	: Microgram
ng	: Nanogram
U	: Unit
AMP	: Adenine monophosphate
AMPK	: Adenine monophosphate-activated protein kinase
ASK1	: Apoptosis signal-regulating kinase 1
ATF4	: Activating transcription factor 4
ATF6	: Activating transcription factor 4
BiP	: Binding immunoglobulin protein
CARMA1	: Caspase recruitment domain-containing protein
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CFTR	: Cystic fibrosis transmembrane conductance regulator
CHIP	: E3 ubiquitin-protein ligase CHIP
CHOP	: C/EBP homologues protein
DMEM	: Dulbecco's modified eagle medium
DMSO	: Dimethyl sulfoxide

DR5	: Tumor necrosis factor receptor superfamily member 10B
eIF2 α	: Eukaryotic translation initiation factor 2 alpha
ER	: Endoplasmic reticulum
ERAD	: Endoplasmic reticulum associated degradation
FBS	: Fetal bovine serum
FOXO1	: Forkhead box protein O1
GADD34	: Protein phosphatase 1 regulatory subunit 15A
HDAC6	: Histone deacetylase 6
HSC70	: Heat shock cognate 71 kDa protein
HSF1	: Heat shock factor protein 1
HSP70	: Heat shock protein 70
HSP90	: Heat shock protein 90
iNOS	: Inducible nitric oxide synthase
IR	: Insulin receptor
IRE1	: Inositol requiring enzyme 1
JNK	: c-Jun N-terminal kinase
kDa	: Kilo Dalton
KD	: Kinase domain
KI	: Kinase inactive
mRNA	: Messenger RNA
PBS	: Phosphate buffer saline
PERK	: PKRK-like ER kinase
PVDF	: Polyvinylidene difluoride
RIDD	: Regulated IRE1 dependent decay
SIK2	: Salt inducible kinase 2
TBS	: Tris buffer saline
TFEB	: Transcription factor EB
TH	: Thapsigargin
TRAF2	: TNF receptor associated factor 2
UPR	: Unfolded protein response
UPS	: Ubiquitin-proteasome system
VCP/p97	: Valosin containing protein/p97

WT : Wild type
XAF1 : X-linked inhibitor of apoptosis-associated factor-
1
XBP-1 : X-box binding protein



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1. INTRODUCTION

1.1. Obesity

According to World Health Organization data more than 1.000.000.000 people worldwide are afflicted by obesity which is a disease characterized by excessive accumulation of body fat [1]. Obesity is directly or indirectly associated with metabolic disorders such as type 2 diabetes, non-alcoholic fatty liver disease, cardiovascular diseases, and some cancers [2].

The etiology of obesity is linked to chronic low-grade inflammation in CNS and periphery which impairs adipose tissue homeostasis and cause insulin and leptin resistances in metabolic tissues and hypothalamus, adipose tissue malfunction and lipotoxicity [3]. Lipotoxicity which can be described as the harmful effects of high concentrations of fat to cells is associated with endoplasmic reticulum (ER) stress [4].

1.2. Endoplasmic Reticulum Associated Degradation (ERAD)

ER is a major organelle where synthesis, folding and maturation of secretory and transmembrane proteins, lipid biosynthesis and calcium ion storage take place [5]. Newly synthesized proteins are folded and co-translational and post translational modifications occur in the ER and only correctly folded proteins are transported to the Golgi apparatus [6], [7].

ER homeostasis is maintained by a quality control mechanism which involves molecular chaperone assisted correct folding, maturation, conformational maintenance, and degradation of proteins which are no longer required or unfolded/misfolded [8],[9]. Unfolded protein response (UPR), ER associated protein degradation (ERAD) and autophagy are components of ER quality control network [10], [11].

Maintaining ER homeostasis depends on rapid disposal of unfolded/misfolded proteins produced in the ER lumen through a process called ERAD which is an integral part of the ER quality control [12]. Substrate recognition by chaperone complex, substrate retrotranslocation across the ER membrane to the cytosol, polyubiquitination

of the substrate by E3 ligase and proteosomal degradation are primary steps of ERAD [13], [14]. Regulating the quantity of ER native proteins is also achieved by ERAD [15], [16].

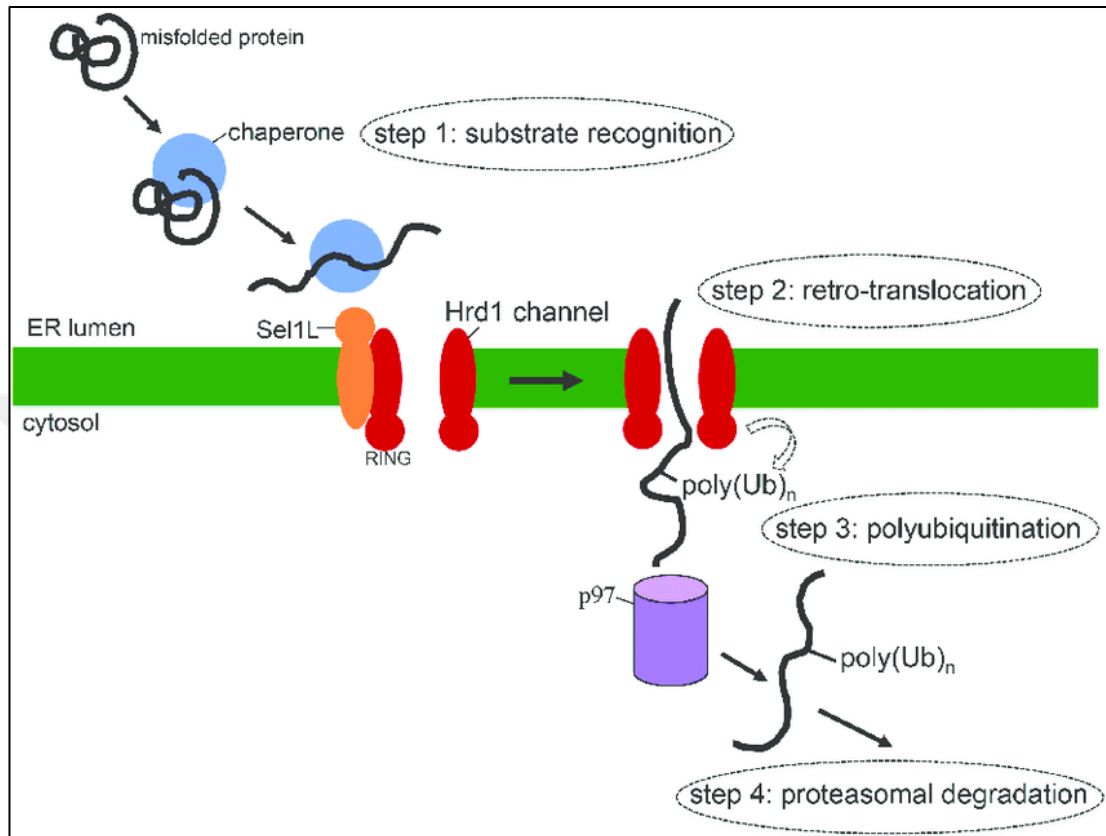


Figure 1.1: ERAD pathway.

Although most ERAD components reside on the ER membrane non-ER resident E3 ligases such as C-terminus of HSC70-interacting protein (CHIP) also participate in ERAD [17],[18].

Inhibition of ERAD leads to UPR but impairs UPR's ability to restore homeostasis and results in the activation of proapoptotic pathways instead [19].

1.3. Endoplasmic Reticulum (ER) Stress

When demand for protein synthesis exceeds ER folding capacity and misfolded/unfolded proteins exceed the capacity of ERAD, ER homeostasis is disturbed resulting in an accumulation of misfolded/unfolded proteins in the ER lumen

triggering ER stress [20] [21]. Insufficient or excessive nutrient availability, disturbances in the cellular calcium concentrations, viral infection and hypoxia are among the factors that cause ER stress [22] –[25].

1.3.1. The Unfolded Protein Response (UPR)

ER stress is counteracted by an evolutionarily conserved series of adaptive mechanisms called UPR to restore homeostasis in the ER lumen [26], [27].

Mammalian UPR is a complex process which is mediated by three transmembrane receptor proteins that act like ER stress sensors: PKR-like ER kinase (PERK), activating transcription factor 6 (ATF6) and inositol-requiring enzyme1 α (IRE1) [28]–[31]. Transmission of the stress signal by these stress sensors has a common initiation step which involves dissociation of ER chaperone BiP from their ER-luminal domains upon accumulation of misfolded/unfolded proteins [32], [33].

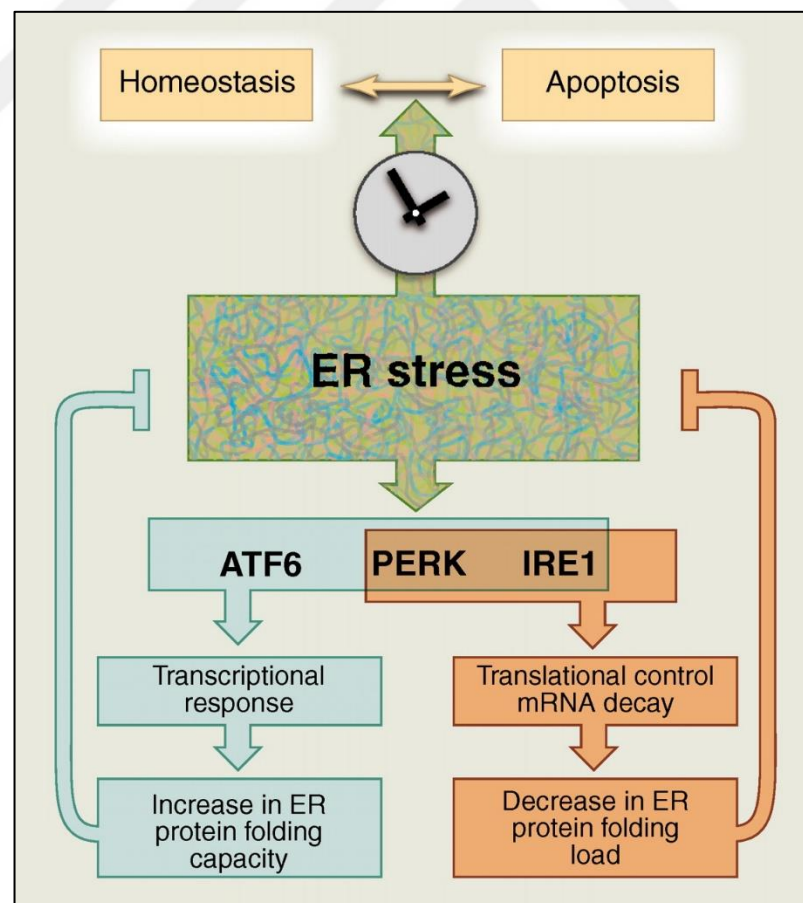


Figure 1.2: Simplified overview of ER stress, UPR and its possible outcomes.

1.3.2. Protein kinase R-like ER kinase (PERK)

PERK branch of the UPR progresses by activation of PERK upon dissociation of BiP via dimerization of its luminal domain and trans-autophosphorylation of its cytosolic protein serine/threonine kinase domain [34]. Once activated PERK phosphorylates the α -subunit of eukaryotic initiation factor 2 (eIF2 α) resulting in a reduction in global protein translation in order to relieve ER from additional new client protein-folding load [35]. Phosphorylation of eIF2 α by PERK also leads to the upregulation of ATF4 expression [36]. ATF4 enters the nucleus and activates the translation of C/EBP homology protein (CHOP) which is a transcription factor involved in the induction of apoptosis [6]. Thus PERK signaling pathway plays a protective role under low levels of stress whereas favors apoptosis under prolonged stress [21].

1.3.3. Activating Transcription Factor 6 (ATF6)

ER stress sensor ATF6 translocates to the Golgi after BiP dissociates from its luminal domain where two sequential cleavages free its N-terminal cytosolic portion ATF6 (ATF6N) [37], [38]. ATF6N which is a bZIP-class transcriptional activator enters the nucleus and directly binds to ER stress response element (ERSE) to upregulate protein folding ER chaperones, enzymes, and the X-box binding protein 1 (XBP1) mRNA [39], [40].

1.3.4. The Inositol-requiring Enzyme 1 (IRE1)

IRE1 branch of the UPR is the oldest and IRE1 is evolutionarily conserved among a diverse array of species [41]. Like PERK, IRE1 is a type I transmembrane glycoprotein with a stress sensing ER luminal dimerization domain and a cytosolic domain which has Serine/Threonine protein kinase and endoribonuclease (RNase) functions [17]. Recent studies suggest that direct binding of the ER luminal domain of IRE1 to accumulated unfolded proteins leads to its oligomerization [42], [43]. Oligomerization brings about juxtaposition of cytosolic kinase and RNase domains allowing trans-autophosphorylation which leads to the activation of the RNase domain

[44]. Activated RNase splices an intron from XBP1 mRNA causing a frame shift that generates an active transcription factor XBP1s [40], [45], [46]. XBP1s migrates into the nucleus and regulate genes that encode ER chaperones, components of ERAD and secretory pathways [47], [48]. Interestingly XBP1s is negatively regulated during the recovery phase of ER stress by XBP1u which is encoded by the unspliced form of XBP1 mRNA [49]. IRE1-XBP1 axis of the UPR favors survival by increasing the folding capacity of the ER and degradation capacity of ERAD. IRE1 can also mediate the decay of ER-targeted mRNAs in response to ER stress in a process called regulated IRE1-dependent decay (RIDD) which downregulates new protein client load [50], [51]. Interestingly RIDD can have opposing effects on cell fate. RIDD of death receptor 5 (DR5) inhibits apoptosis and has a prosurvival effect [52] whereas RIDD can also have proapoptotic effects [53], [54].

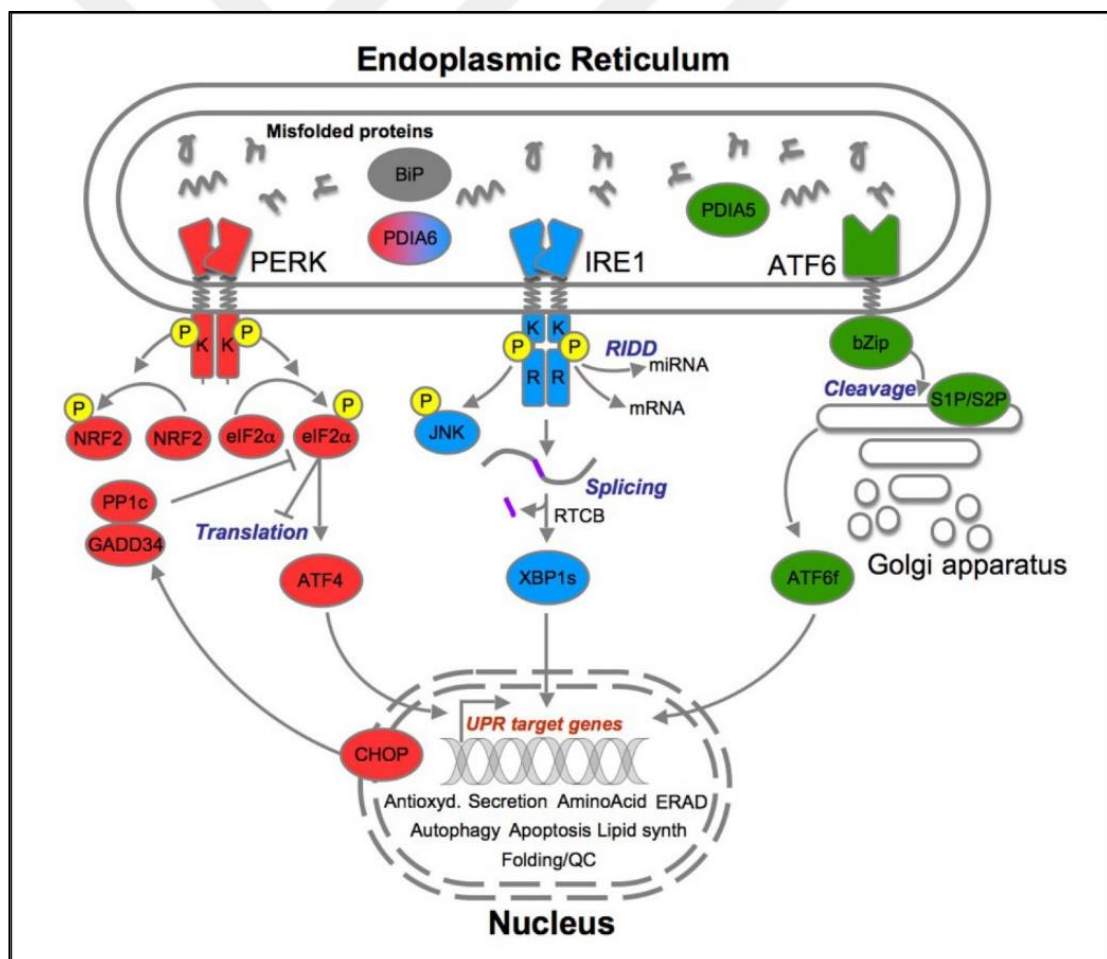


Figure 1.3: ER stress sensors.

1.3.5. ER Stress Induced-Apoptosis

Although UPR operates to resolve ER stress through reduction of new protein synthesis, facilitating protein folding and unfolded protein degradation, under prolonged ER stress UPR switches from prosurvival to proapoptotic mode [55]. Continuous ER stress leads to induction of CHOP through PERK/ eIF2 α /ATF4 pathway resulting in the upregulation of apoptotic genes which eventually leads to apoptosis [56]. Also, induction of GADD34 by CHOP mediates the activation of eIF2 α thus causing reversal of the reduction in global protein translation [57]. In addition continual activation of IRE1 due to persistent ER stress can promote apoptosis through induction of JNK pathway via TRAF2-ASK1 complex formation [58]-[60].

1.3.6. ER Stress and Autophagy

There are two main cellular degradation pathways in eukaryotes: ubiquitin-proteasome system and autophagosomal-lysosomal pathway [61], [62]. As mentioned above ERAD is an ER-specific degradation pathway that involves ubiquitin-proteasome system. But ERAD resistant proteins which cannot engage ERAD factors or which are prone to form aggregates fail to enter ERAD pathway [63]. When ERAD fails to restore homeostasis autophagy pathway may be initiated to degrade misfolded proteins and damaged ER (ER-phagy) [64], [65]. ER stress is among the various stimulants of autophagy [66]. UPR is capable of both inducing and suppressing autophagy. For example in the early phase of ER stress in tumor cells IRE1 mediated JNK activation induces autophagy [67] and spliced form of XBP1 induces autophagy via enhancing the transcription of Transcription factor EB (TFEB) [68]. Autophagy is normally a survival response to nutrient deprivation but due to persistent stress can also induce cell death [69]-[72].

1.4. Carboxyl Terminus Of HSC70-Interacting Protein (CHIP)

CHIP is an evolutionarily conserved 35 kDa cytosolic protein encoded by STUB1 gene in humans and plays a major role in protein quality control as a co-

chaperone with E3 and E4 ligase functions [73], [74]. It interacts with HSP90 and HSP70/HSC70 chaperone family of proteins through its N-terminal three tetratricopeptide domains. Subsequent coiled coil domain in the middle mediates dimerization. It has a C-terminal U-box domain which interacts with ubiquitin conjugating enzymes (E2) and has E3 and E4 ligase functionality which are responsible for substrate monoubiquitination and polyubiquitination respectively [75], [76]. The central and C-terminal regions are highly conserved in different species [74], [77].

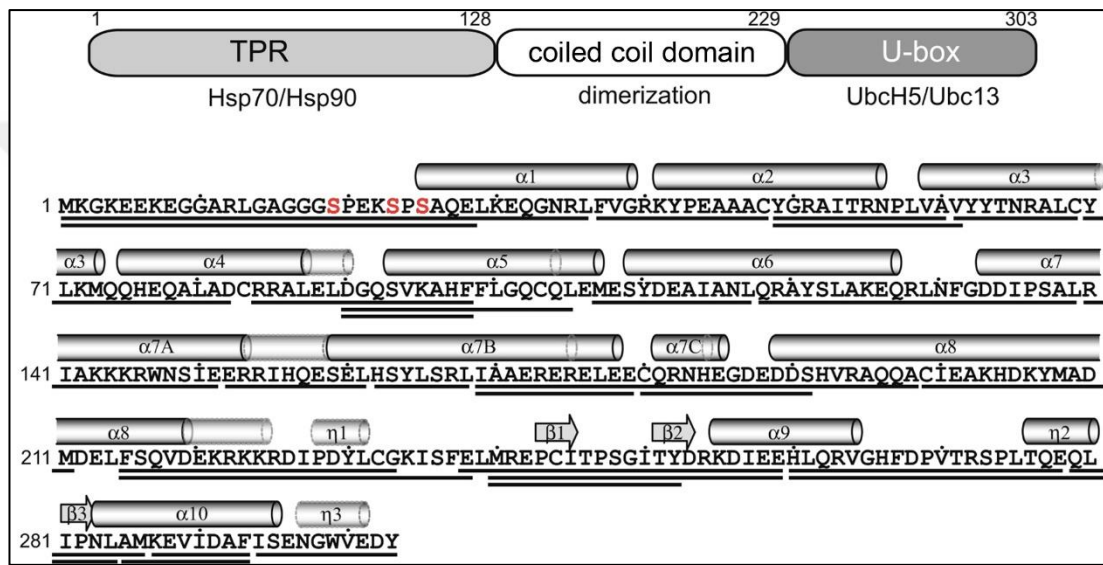


Figure 1.4: Domain structure and sequence of CHIP.

CHIP is one of the widely researched proteins among the large pool of E3 ligases due to the broad range of substrates which involve in multiple pathologies and important signaling pathways. CHIP is associated with neurodegenerative diseases such as Alzheimer's disease, Huntington's disease, spinocerebellar ataxia, Parkinson's disease, amyotrophic lateral sclerosis (ALS) and Lafora disease and presented as having a neuroprotective role. Besides neurodegenerative disorders CHIP's involvement in proteostasis, apoptosis, development, cell proliferation and aging makes it a promising therapeutic target [78].

CHIP interaction with its substrates can either be chaperone dependent or independent. Like a Swiss army knife CHIP can selectively target its substrates to distinct pathways depending on the nature of the lysine linkages of polyubiquitin chains. For example CHIP mediated K48- linked polyubiquitination targets its

substrates to proteosomal degradation [79], [80]. On the other hand K63-linked polyubiquitination by CHIP targets its substrates either to lysosomal degradation [81]-[85] or to function in signaling [86]. Also CHIP was reported to mediate K27-linked ubiquitination of CARMA1 promoting TCR-induced NF- κ B activation in T cells [87]. Another aspect of CHIP function is its role in aggresome pathway. CHIP mediated ubiquitination of iNOS either targets iNOS for proteosomal degradation or for aggresome sequestration when proteasome is overwhelmed [88]. One recent study suggests that inactive proteosomes which are polyubiquitinated by CHIP are transported to aggresomes by HDAC6/dynein motor either to be recovered or be degraded by autophagy [89].

CHIP is a key regulator of stress response not only by ubiquitination and subsequent degradation of unfolded/misfolded proteins but also by induction of HSP70 through activation of HSF1 during stress [90]. Upon stress relief CHIP ensures that the basal levels of HSP70 is restored through ubiquitination and proteosomal degradation of HSP70 [91]. Thus CHIP as well as taking part in stress response directly also functions in the recovery from stress response. In un-stressed conditions CHIP mediates ubiquitination and degradation of insulin receptor (IR) whose turnover promotes longevity through reducing insulin and insulin like growth factor1 signaling. Under stress conditions CHIP is more engaged in disposal of misfolded proteins which results in reduced IR turnover and aging [92]. Interestingly CHIP's involvement in quality control under stress conditions competes with its function of quantity control of its substrates.

CHIP is involved in ERAD pathway having a role in ubiquitination and retro translocation of misfolded proteins from ER lumen to cytosol for proteosomal degradation as can be exemplified by CHIP mediated degradation of immature CFTR [17]. CHIP mediated ubiquitination of IRE1 regulates IRE1/TRAF2/JNK signaling pathway under ER stress and CHIP knock down in HEK293 cells results in reduced autophagic flux [93]. CHIP also ubiquitinates TRAF2 in HEK293T, MDA-MB-231 and gastric cancer cells causing its degradation which further supports that CHIP has a role in IRE1/TRAF2 complex formation [94]-[97]. A recent study presents that X-linked inhibitor of apoptosis-associated factor-1 (XAF1) attenuates IRE1 phosphorylation via destabilizing CHIP [98].

Overexpression of XBP1s arm of the UPR in cardiomyocytes upregulates CHIP transcription which in turn mediates the ubiquitination and degradation of FoxO1 and reduces myocardial lipid accumulation [99], [100].

CHIP is involved in regulation of autophagy by specifically targeting inactive phosphorylated form of TFEB promoting its degradation thus increasing the probability of formation of active TFEB homodimers available for inducing transcription of autophagy related genes [101].

1.5. Salt Inducible Kinase 2 (SIK2)

Salt inducible kinase family of proteins were first discovered when SIK1 was found to be highly expressed in the adrenal cortex of mice in response to stress induced by a high salt diet [102]. Later two more SIK isoforms were discovered and named SIK2 (also known as QIK) and SIK3 (also known as QSK) respectively due to their structural similarity of catalytic domains to SIK1 [103], [104].

SIK2 contains 931 amino acids and has 3 domains: N terminal kinase, central SNF1 protein kinase homology (SNH) domain and C terminal phosphorylation domain which is enriched in PKA-dependent phosphorylation sites respectively [105].

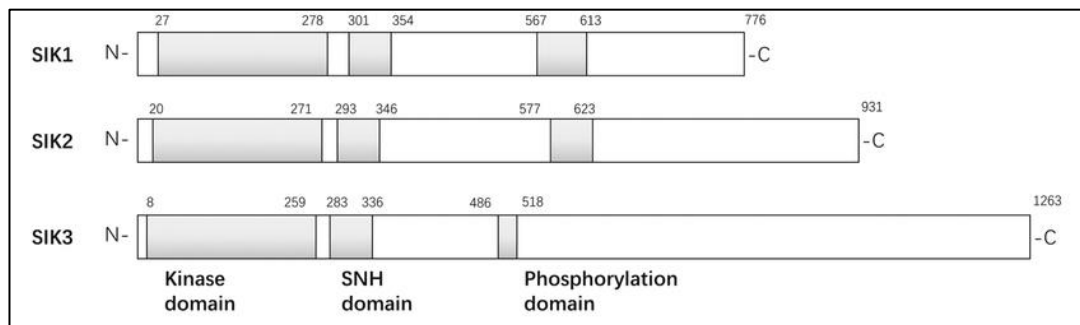


Figure 1.5: Structures of SIK family isoforms.

Although SIK2 is ubiquitous it is highly expressed in adipose and neural tissues [106]. In an early study it was found that SIK2 gene transcription is induced at a very early stage of adipocyte differentiation in 3T3-L1 preadipocytes. The same study also reported that SIK2 phosphorylates IRS1 at Ser⁷⁸⁹ (Ser⁷⁹⁴ in humans) suggesting that this inhibitory effect on insulin signaling might have a role in insulin resistance [104].

In β -cells SIK2 protein levels are increased in response to high glucose levels. SIK2 mediated phosphorylation induces the ubiquitination and proteosomal degradation of cyclin-dependent protein kinase 5 (CDK5) activator p35 which in turn results in deactivation of CDK5 and attenuation of phosphorylated state of voltage dependent Ca^{++} channel (VCDD). This results in increased Ca^{++} flux and insulin secretion [107].

SIK2 seems to have contradictory roles between healthy and cancer cells in lipid metabolism. In preadipocytes and adipocytes it was presented as a negative regulator of lipogenesis [108], [109] whereas in ovarian cancer cells it was shown to enhance fatty acid and cholesterol synthesis [110].

Various studies demonstrate SIK2's involvement in proteolytic pathways. Yang et al. showed that autophagosome-lysosome fusion is impaired in SIK2 knockdown cells resulting in the accumulation of autophagosomes [111]. Another study by the same group revealed that SIK2 co-localizes with p97/VCP which is an ATPase that functions in the retrotranslocation of proteins from the ER lumen and that phosphorylation of p97/VCP by SIK2 induces its ATPase activity and upregulates ERAD thus contributing to the ER homeostasis [112]. A recent study in adipocytes provides more evidence that SIK2 inhibition results in a reduction in autophagic flux and TFEB expression [113].

Obesity and type 2 diabetes are associated with ER stress. UPR is a multilayered and complex process involving the interplay between various regulators. Understanding the interactions between these regulatory proteins is important for designing therapeutic approaches for related diseases. But the pathways and components involved in ER stress and UPR has not yet been completely elucidated.

SIK2 and CHIP are two proteins which have regulatory key roles in metabolism and homeostasis. Previous studies in our laboratory demonstrated that SIK2 plays a regulatory role in maintaining proteostasis by taking part in ERAD and autophagy. In an LC-MS/MS based phosphoproteomics screen for the identification of ER resident SIK2 substrates our lab identified serine 19 residue in the N terminal region of CHIP as a major phosphorylation site for SIK2 [114]. Subsequent immunoprecipitation studies with overexpressed and endogenous CHIP and SIK2 confirmed the physical interaction between the two proteins under chemically induced ER stress conditions. In this study we provided substantial evidences for this interaction and possible outcomes in HEK293T cells under ER stress.

2. MATERIALS AND METHODS

2.1. Buffers and Solutions

The buffers and solutions and their contents are listed in Appendix B.

2.2. Primers, Molecular and Ligation-Independent Cloning and Site Directed Mutagenesis

2.2.1. Primers Used in This Study

Table 2.1 Primers Used in This Study.

Primer ID	Sequence (5'-3')	Application	RE sites
STUB1 1F	AAAAAGCTTATGAAGGGCAAGGAGGAGAAG	cloning	HindIII
STUB1 2R	AAAGGATCCGTAGTCCTCCACCCAGCCATT	cloning	BamHI
STUB1_EcoRI_R	ACACGAATTCAGCCACCATGAAGGGCAAGGAGGAG	cloning	EcoRI
STUB1_KpNI_F	TATGGTACCAGTAGTCCTCCACCCAGCC	cloning	KpNI
STUB1_S19AS23A_F	GCGCGCTCGGCGCCTTCTCGGGCGCTCCGCCGCCAGCG	SDM*	
STUB1_S19AS23A_R	CGCTGGCGGCGGAGCGCCCAGAAAGCGCCGAGCGCGC	SDM*	
STUB1H260Q_F	CCACACGCTGCAGCTGCTCCTCGATGT	SDM*	
STUB1H260Q_R	ACATCGAGGAGCAGCTGCAGCGTGTGG	SDM*	
STUB1_LIC_F	TACTTCCAATCCATGATGAAGGGCAAGGAGGAGAAG	LIC**	
STUB1_LIC_R	TATCCACCTTTACTGGTAGTCCTCCACCCAGCC	LIC**	

*SDM: Site-directed mutagenesis. **LIC: Ligation independent cloning.

For the design of cloning primers The Benchling R&D Cloud (Benchling) and of mutagenesis primers QuikChange Primer Design tool (Agilent Technologies) were used.

2.2.2. Molecular Cloning

2.2.2.1. Polymerase Chain Reaction (PCR)

PrimeStar HS DNA polymerase (Takara) was used to amplify DNA fragments to be sub-cloned and for SDM.

PCR reaction mix was prepared according to Table 2.2 and PCR conditions were set as described in Table 2.3 for subcloning.

Table 2.2: PCR reaction for PrimeStar HS DNA polymerase.

Component	Volume for 25 ul Reaction	Concentration
5X PrimeStar Buffer	5 μ l	1X
dNTP mix (2.5 mM each)	2 μ l	200 μ M each
Forward primer	0.5 μ l	2 μ M
Reverse primer	0.5 μ l	2 μ M
DNA template	variable	100 ng
HS DNA polymerase	0.25 μ l	1.25U/25 μ l
Nuclease-free water	Up to 25 μ l	

Table 2.3: PCR reaction conditions for PrimeStar HS.

Step	Temperature	Time	30 cycles
Initial Denaturation	98°C	30 sec	
Denaturation	98°C	10 sec	
Annealing	55°C	5 sec	
Extension	72°C	1 min/1000bp	
Final Extension	72°C	2 min	
Hold	4°C		

2.2.2.2. Restriction Enzyme Digestion of DNA

Restriction enzyme digestions of amplicon and vector backbone were performed in 20 µl reaction volumes according to fast digest enzyme (Thermo) manufacturer's instructions (approximately 30 minutes incubation at 37°C in appropriate buffer supplied by the manufacturer).

2.2.2.3. Agarose Gel Electrophoresis and Extraction of Digested DNA

Digested DNA alongside with 1 kb DNA ladder (ThermoFisher) was loaded and resolved on 1% agarose gels in 1X Tris-Acetate-EDTA (TAE) buffer containing 1X RedSafe (ChemBio) nucleic acid staining solution by agarose gel electrophoresis at 100V for 1 hour.

DNA bands were visualized by UV light box and excised with a sterile scalpel. The bands containing the digested amplicon and vector were transferred to 2 ml micro centrifuge tubes. DNA were extracted from the gels using NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel).

2.2.2.4. Ligation

Vector and inserts were combined in 1:5 and 1:7 molar ratios in 20 µl reaction volumes alongside with T4 DNA ligase (NEB) and T4 ligase buffer (NEB) for 30 minutes at room temperature and heat inactivated at 65°C for 10 minutes.

2.2.2.5. Transformation of *dH5α* Chemically Competent *E. coli*

10 µl of ligation mix or 50 ng plasmid is added to 50 µl of chemically competent cells in 2 ml micro centrifuge tubes which were thawed on ice. After incubation on ice for 30 minutes, the tubes are incubated on a water bath at 42°C for 1 min and transferred to ice for an additional 2 minutes. 500 µl LB media were added to the cells and the cells were incubated at 37°C for 1 hour in an orbital shaker at about 200 rpm.

After incubation 100 µl of the cell culture is spread on LB agar plates which contain the appropriate selection antibiotic and incubated overnight at 37°C in an inverted position.

2.2.2.6. Colony PCR

Taq polymerase (ABM) was used for colony PCR. To screen for positive colonies well developed colonies with smooth contours were selected with a sterile pipette from LB agar plates and mixed with the PCR mixture.

PCR products are loaded on 1% agarose gels alongside with appropriate DNA ladder, resolved and analyzed for the presence of positive colonies.

2.2.2.7. Plasmid Preparation

Plasmids were isolated from 5 ml cultures by DNA-Spin Mini Isolation kit (Intron) according to manufacturer's protocol before being verified by DNA sequencing.

For larger scale production the plasmids were isolated from 100 ml cultures with NucleoBond Xtra Midi kit (Macherey-Nagel) according to manufacturer's protocol.

Table 2.4: List of plasmids used in this study.

Plasmid	Origin	Vector Backbone
pcDNA4-HisC	Invitrogen, USA	
pCMV-3Tag-6	Agilent #240200	
HIS-SIK2-WT	(Kuser, 2011)	pcDNA4-HisC
HIS-SIK2-KI	(Kuser, 2011)	pcDNA4-HisC
FLAG-SIK2-WT	[115]	pCMV-3Tag-6
FLAG-SIK2-KI	[115]	pCMV-3Tag-6
FLAG-CHIP	[115]	pCMV-3xFLAG-7.1
FLAG-CHIP_S19A-S23A	This study	pCMV-3xFLAG-7.1

Table 2.4: Continued.

FLAG-CHIP_H260Q	This study	pCMV-3xFLAG-7.1
HIS-CHIP	This study	pNH-TrxT
HIS-CHIP_S19A-S23A	This study	pNH-TrxT
GFP-CHIP	This study	pEGFP-N2

2.2.3. Site Directed Mutagenesis (SDM)

2.2.3.1. Polymerase Chain Reaction (PCR)

Primestar HS DNA polymerase (Takara) was used for SDM.

PCR reaction mix was prepared according to Table 2.5 and PCR conditions were set as described in Table 2.3 for SDM.

Table 2.5: PCR reaction for SDM.

Component	Volume for 25 ul Reaction	Concentration
5X Primestar Buffer	5 μ l	1X
dNTP mix (2.5 mM each)	2 μ l	200 μ M each
Forward primer	0.5 μ l	2 μ M
Reverse primer	0.5 μ l	2 μ M
DNA template	variable	10 ng
HS DNA polymerase	0.25 μ l	1.25U/25 μ l
Nuclease-free water	Up to 25 μ l	

2.2.3.2. DPNI digestion of DNA

DPNI digestion of PCR product was performed in 30 μ l reaction volumes according to fast digest enzyme (Thermo) manufacturer's instructions (approximately 30 minutes incubation at 37°C in appropriate buffer supplied by the manufacturer).

After digestion the reaction mix was spun in SpeedVac vacuum concentrator for approximately 15 minutes to reduce the volume to 10 μ l.

5 μ l of concentrated mix was transformed as described in section 2.2.2.5 Transformation of dh5 α Chemically Competent E. coli with the only difference that SOC medium was used instead of LB medium. Colony PCR and plasmid preparations were performed as described in sections 2.2.2.6 Colony PCR and 2.2.2.7 Plasmid Preparation.

2.2.4. Ligation Independent Cloning (LIC)

2.2.4.1. Polymerase Chain Reaction (PCR)

Primestar HS DNA polymerase (Takara) was used for LIC. PCR reaction mix was prepared according to Table 2.6 and PCR conditions were set as described in Table 2.3.

Table 2.6: PCR reaction for LIC.

Component	Volume for 25 μ l Reaction	Concentration
5X Primestar Buffer	5 μ l	1X
dNTP mix (2.5 mM each)	2 μ l	200 μ M each
Forward primer	0.5 μ l	2 μ M
Reverse primer	0.5 μ l	2 μ M
DNA template	variable	50 ng
HS DNA polymerase	0.25 μ l	1.25U/25 μ l
Nuclease-free water	Up to 25 μ l	

2.2.4.2. Restriction Enzyme Digestion of DNA

BSAI digestion of bacterial expression vector pNH-TrxT (2 μ g) was performed in 20 μ l reaction volume according to fast digest enzyme (Thermo) manufacturer's instructions (approximately 30 minutes incubation at 37°C in appropriate buffer supplied by the manufacturer).

2.2.4.3. Agarose Gel Electrophoresis and Extraction of Digested DNA

Digested vector and PCR product alongside with 1 kb DNA ladder (ThermoFisher) was loaded and resolved on 1% agarose gels in 1X Tris-Acetate-EDTA (TAE) buffer containing 1X RedSafe (ChemBio) nucleic acid staining solution by agarose gel electrophoresis at 100V for 1 hour.

DNA bands were visualized by UV light box and excised with a sterile scalpel. The bands containing the amplicon and digested vector were transferred to 2 ml micro centrifuge tubes. DNA were extracted from the gels using NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel).

2.2.4.4. Overhang Creation And Annealing

Overhangs were synthesized with T4 DNA ligase (NEB) the reaction was set up as described in Table 2.7.

Table 2.7: Overhang reaction for T4 DNA polymerase.

Component	Volume for 40 ul Amplicon overhang Reaction	Volume for 40 ul Vector overhang Reaction
10X 2.1 Neb Buffer	4 µl	4 µl
dCTP (100mM)	1 µl	-
dGTP (100mM)	-	1 µl
DTT (100mM)	2 µl	2 µl
Extracted PCR product (10-50ng)	variable	-
Extracted vector digest (up to 2000ng)	-	variable
T4 DNA polymerase	1 µl	1 µl
Nuclease-free water	Up to 40 µl	Up to 40 µl

The reaction conditions were 30 min. incubation at 25°C followed by 20 min. incubation at 75°C.

After the reaction the concentrations of vector and insert was determined by NanoDrop. 50 ng vector was mixed with insert in 1:3 and 1:7 molar ratios in 5 µl reaction volumes and incubated for 10 minutes at room temperature for annealing and inactivated by the addition of 0.5 µl of 25mM EDTA.

The total of the annealing mixture was transformed immediately after annealing reaction as described in section 2.2.2.5 Transformation of dH5α Chemically Competent E. coli with differences that SOC medium and sucrose agar were used instead of LB medium and LB agar respectively. Colony PCR and plasmid preparations were performed as described in sections 2.2.2.6 Colony PCR and 2.2.2.7 Plasmid Preparation.

2.3. Protein Purification

The bacterial expression vectors for CHIP WT and S19AS23A prepared in the previous section were transformed into E. coli BL21(DE) cells. A single colony was selected from the plate and pre-inoculated in 5 ml LB agar with Kanamycin on a shaker at 37°C for 16 hours. 500 µl of pre-inoculum was used to inoculate 50 ml LB broth with Kanamycin and incubated on a shaker at 37°C for 4 hours. The culture was induced with 0.12 mM isopropyl-β-D-thiogalactopyranoside (Sigma) at 37 °C for 4 h. The culture was centrifuged at 6000g 4°C for 15 min. The pellet was resuspended in 20mM Imidazole binding buffer which was prepared according to His Spin Trap (Sigma) purification kit manual, incubated on ice for 30 min. and sonicated on ice. The suspension was cleared by centrifugation at 16000rpm 4°C for 15 min. The supernatant was passed through His Spin Trap columns according to the manufacturers instructions and eluted with 200mM imidazole containing elution buffer. The eluents were condensed with VivaSpin centrifugal concentration columns (Vivaproducts) according to manufacturer's instructions. The purity of the proteins was checked by resolving the eluents on SDS-PAGE and staining the gels with 0.1% Coomassie brilliant blue G-250 (Merck).

Purified CHIP is shown in Appendix C.

2.4. Cell Culture

2.4.1. Growth and Maintenance

HEK293T (ABM Good) cells were maintained in DMEM high glucose (Gibco) supplied with %10 FBS (Gibco) and 1% Penicillin-Streptomycin solution (Lonza). The cells were incubated at 37°C and 5% CO₂.

The cells were passaged before they reached 90% confluency.

2.4.2. Transient Transfection of Cultured Cells

HEK293T cells were seeded in appropriate plastic culture dishes one day before transfection to obtain 60-70% confluency on the day of transfection. Appropriate amount of vector is diluted in 1X TE buffer with 150 mM NaCl. 1 µg of vector for 1 ml of culture and 3:1 ratio of Polyethyleneimine (PEI) (PolyScience) to vector were used for transfection. After the vector-PEI mixture was incubated for 10 minutes it was applied to the cell culture drop by drop and mixed by gentle swirling. Transfection medium was replaced with fresh growth medium after 8 hours.

2.4.3. Administration of Treatments to Cells

48 hours after transfection vehicle (DMSO) or one of the following agents were added to the culture medium at time points indicated in the results section:

Thapsigargin (Santa-Cruz) 1 µM, Hydroxychloroquine (Apexbio), Epoxomicin (Apexbio)/VR23 (Selleckchem).

2.4.4. Preparation of The Cell Lysates

After the cells are washed with 1X ice cold PBS, an appropriate volume of IP lysis buffer (0.2% NP40, 150 mM NaCl, 20 mM Tris-HCl (pH:8.0), 2 mM), 2 µg/ml aprotinin, 10 µM leupeptin, 1 mM PMSF, 1 mM NaF, 1 mM Na₃VO₄) was added to the cells. The cells were scraped on ice and transferred to 1.5 ml microcentrifuge tubes

(mct). After passing the lysates through syringes with 21-gauge needle 5 times the cells were incubated for 30 minutes on ice. After incubation the cells were pelleted at 14000g for 15 minutes at 4°C. The supernatant was then collected in clean micro centrifuge tubes and stored at 20°C.

Protein concentrations were determined with Bradford Reagent (EcoTech) according to the manufacturer's protocol.

2.4.5. Immunoprecipitation

Pierce magnetic agarose beads (Thermo Scientific) or anti-FLAG-M2 agarose beads (Sigma) were used to immunoprecipitate Flag tagged CHIP.

Briefly 500 µl of lysate containing 1 mg of total protein was incubated with 5 µl anti-Flag antibody overnight at 4°C on a rotator. 40 µl of magnetic agarose beads were washed twice with 560 µl and 500 µl IP lysis buffer respectively. The lysate from overnight incubation was added to the beads and incubated on a rotator at RT for 1 hour. After incubation the tube was placed on magnetic rack and the lysate is removed from the beads. The beads were either stored in -80°C until on bead digestion and preparation for LC-MS/MS or were washed thrice with IP lysis buffer and suspended in 20 µl IP lysis buffer and Laemmli buffer and processed for Western Blotting as described in the next section. %2 of preimmunoprecipitation lysates were used as input control.

anti-FLAG-M2 agarose beads were washed three times with 500 µl of IP lysis buffer and incubated overnight with 500µl of lysate containing 1 mg of total protein. After incubation, the lysates were centrifuged at 4°C for 3 min at 500 g and washed three times with 500 µl of IP-lysis buffer. After the last wash the immunoprecipitants were suspended in 50 µl of IP lysis buffer with 1X Laemmli Buffer and processed for Western Blotting as described in the next section. %2 of preimmunoprecipitation lysates were used as input control.

2.4.6. SDS-PAGE and Western Blot

10% Separating and 5% stacking polyacrylamide gels were cast according to Table 2.8.

Protein samples are mixed with 4X Laemmli Buffer (Thermo) bringing the final concentration of buffer to 1X. Samples were boiled at 95°C for 5 min. And loaded on to gel alongside the protein ladder (Intron) in a Mini-Protean Tetra System (Bio-Rad) tank and ran at 80V alongside the stacking gel and 120V alongside the separating gel respectively.

Table 2.8 SDS-PAGE Gel Contents.

	10% Separating	5% Stacking
1,5M Tris-HCl (pH 8.8)	2.6 ml	-
1M Tris-HCl (pH 6.8)	-	750 µl
SDS 10% (w/v)	100 µl	60 µl
Acrylamide: Bisacrylamide (37.5:1, 40%)	2.5 ml	600 µl
APS 10% (w/v)	100 µl	30 µl
TEMED	10 µl	6 µl
dH2O	4.7 ml	4.55 ml

After the run is complete the proteins were transferred onto PVDF membranes (Thermo) with Trans-Blot Turbo Transfer System (BioRad) for 30 minutes.

After the transfer the membranes were blocked with 5% non-fat dried milk in TBS-T buffer for 1 hour. The membranes were washed 3 times for 5 min. with TBS-T buffer before being incubated with appropriate primer antibodies overnight at 4°C with gentle agitation. After incubation the membranes were washed 3 times for 5 min. with TBS-T buffer and incubated with secondary antibodies for 2 hours at 4°C with gentle agitation. After incubation the membranes were washed 3 times for 5 min. with TBS-T buffer. WesternBright ECL solution (Advansta) were applied to the membranes and chemiluminescence was visualized and captured with ChemiDoc XRS+ (Biorad). ImageJ software was used for the analysis of the bands.

2.5. Liquid Chromatography with tandem mass spectrometry (LC-MS/MS) Analysis

The immunoprecipitates stored on bead from section 2.5.4 were handed to Gebze Technical University Proteomics Laboratory for label-free LC-MS/MS analysis. The raw data was analyzed by Proteome Discoverer and MaxQuant software for label free quantification and identification of phosphosites. Functional enrichment analysis were performed using Perseus, FunRich, g:GOST [137] and Interactivenn [138] applications.



3. RESULTS

3.1. The Cross Check Between SIK2 And CHIP May Determine Their Precise Level And/Or Activities

In an organelle specific sub-phosphoproteomics study our lab identified a few novel SIK2 substrates localized on and in the vicinity of the endoplasmic reticulum. Relative quantification of the peak data unraveled an increase in the level of N terminal region of CHIP peptide phosphorylated at its three consecutive serine residues in response to SIK2 overexpression compared to control which included inactive SIK2. The comparative analysis of the respective phosphopeptide abundances pointed out the significance of two serine residues at S19 and S23 as the prominent sites for SIK2 phosphorylation. Earlier studies reported serine 19 (S20 in mouse) as a phosphorylation site used by other kinases as well [115], [116]. The authenticity of these phosphorylation sites was further corroborated on the basis of their reliance on the SIK2 phosphorylation motif by using GPS v.3.0 software which is a kinase specific phosphosite prediction tool. To get further insight into the biological relevance of these SIK2 phosphorylation sites our laboratory created a truncated version of CHIP by the deletion of the first 23 amino acids from the N terminal region (Δ N23). Former PhD student Şeker T. demonstrated the physical interactions between CHIP and SIK2 by co-immunoprecipitation studies and pointed out the significance of N terminal CHIP in this interaction. Consistent with his data I noticed a significant decrease in SIK2 protein level when both CHIP and SIK2 were overexpressed in HEK293T cells. This decrease was realized without respect to ER stress induction and inhibition of proteosomal degradation indicating the possible involvement of lysosomal degradation.

Based on the data presented we hypothesized that a putative CHIP mediated downregulation of SIK2 may introduce additional control over protein degradation through ERAD or autophagy or both. Indeed, it is equally important to assess the possible outcomes of SIK2 mediated phosphorylation of CHIP forming a highly regulative check point in protein homeostasis by bringing together an E3 ligase and a stress kinase in a switch like negative feed back loop.

In my project I started with the confirmation of interaction between SIK2 and CHIP (data not shown). Next, I decided to find out whether the degradation of SIK2 observed in CHIP overexpressing cells was mediated by UPS or autophagy pathway or both. To test this hypothesis, I conducted immunoprecipitation studies from the HEK293T cells overexpressing CHIP alone or with SIK2 (Figure 3.1). CHIP mediated induction of SIK2 degradation cannot be restored by blocking the autophagy flux with chloroquine. However, CHIP protein level which was dramatically reduced upon SIK2 overexpression increased back to almost two third of its level that we reported in the absence of SIK2 overexpression suggesting that autophagy may contribute to CHIP degradation (Figure 3.1, lanes 5 and 6). Although we do not know how SIK2 promotes CHIP degradation by autophagy pathway it is plausible to suggest that SIK2 mediated phosphorylation of CHIP may recruit other E3 ligases to trigger its own degradation. A previous study by Sakamaki, J. et. al. reported that SIK2 mediated phosphorylation of CDK5 activator p35 sets off ubiquitination and subsequent degradation of p35 [107] suggesting that SIK2 may produce a similar effect by phosphorylating CHIP on its N terminal region. CHIP is known to work in coordination with other E3 ligases such as Parkin and capable of auto-ubiquitination [117], [118].

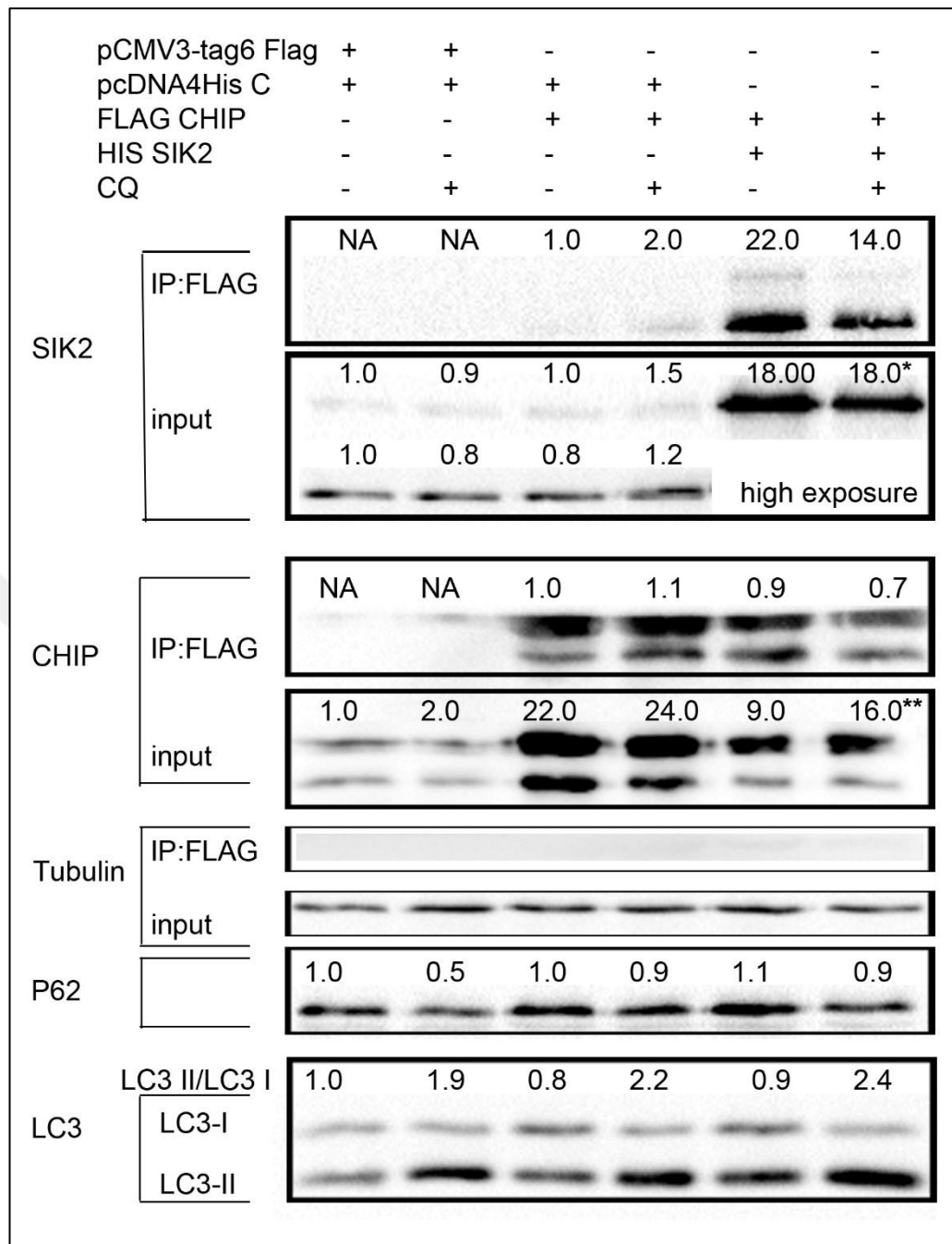


Figure 3.1: The effect of SIK2 overexpression on autophagy mediated CHIP degradation. HEK293T cells were co-transfected with Flag-CHIP WT/ Flag-empty backbone and HIS-SIK2 WT/ HIS-empty backbone. 48 hours after transfection the cells were treated with either 200 μ M chloroquine diphosphate (CQ) or DMSO for 4 hours. The lysates were immunoprecipitated with anti-flag agarose beads.

3.2. The Effects Of SIK2 On CHIP Stability As A Function Of ER Stress Duration

SIK2's kinase activity is proposed to be governed by constitutively active LKB-1 kinase and presumably by cellular stress conditions by an unidentified kinase. To test whether ER stress and thereby SIK2 activity have any effect on its interaction with CHIP, we overexpressed CHIP along with SIK2 or SIK2 Kinase dead (KI) mutant or empty vector constructs in HEK293 cells. The cells were treated with ER stress inducer Thapsigargin (TH) or DMSO for indicated time periods (Figure 3.2).

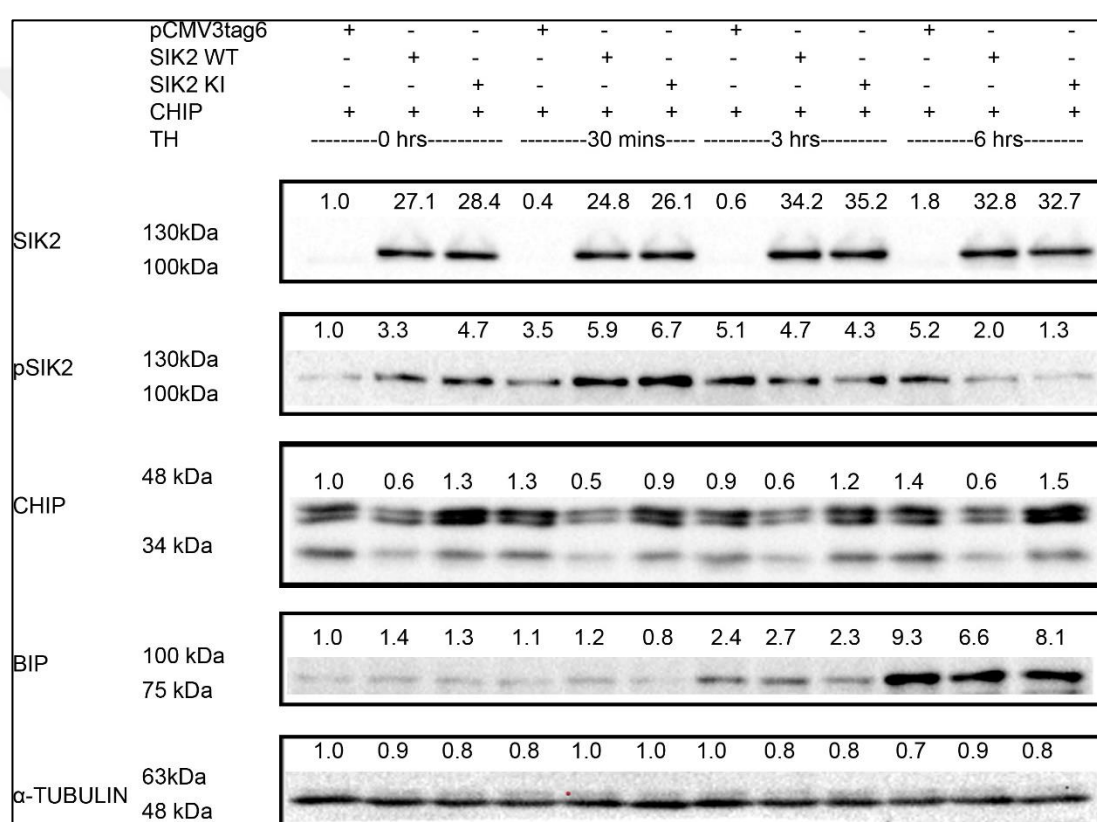


Figure 3.2: The effect of ER stress on CHIP destabilization by SIK2. CHIP overexpressing cells were transfected with either one of SIK2 WT, SIK2 KI or empty vector. 48 hours after transfection the cells were treated with either 1 μ M TH or DMSO for indicated time periods. pSIK2 recognizes the activation loop phosphorylation at Thr175 (human).

pSIK2 levels peaked at 30 minutes of TH treatment and gradually decreased as expected. CHIP protein levels seemed to drop 1-fold upon SIK2 WT overexpression without regard to the presence of ER stress. SIK2 KI overexpression on the other hand

reversed SIK2's degradative activity suggesting that SIK2 kinase activity is needed for CHIP degradation.

To test whether SIK2 mediated CHIP degradation is dependent on its phosphorylation of CHIP at S19 and/or S23 residues, a mutant CHIP having S19A-S23A non-phosphorylatable form was constructed via site directed mutagenesis. The cells overexpressing either CHIP or CHIP S19A-S23A were transfected with either SIK2 WT, SIK2 KI or empty vector and treated with chemical ER stress inducer TH for up to 6 hours (Figure 3.3).

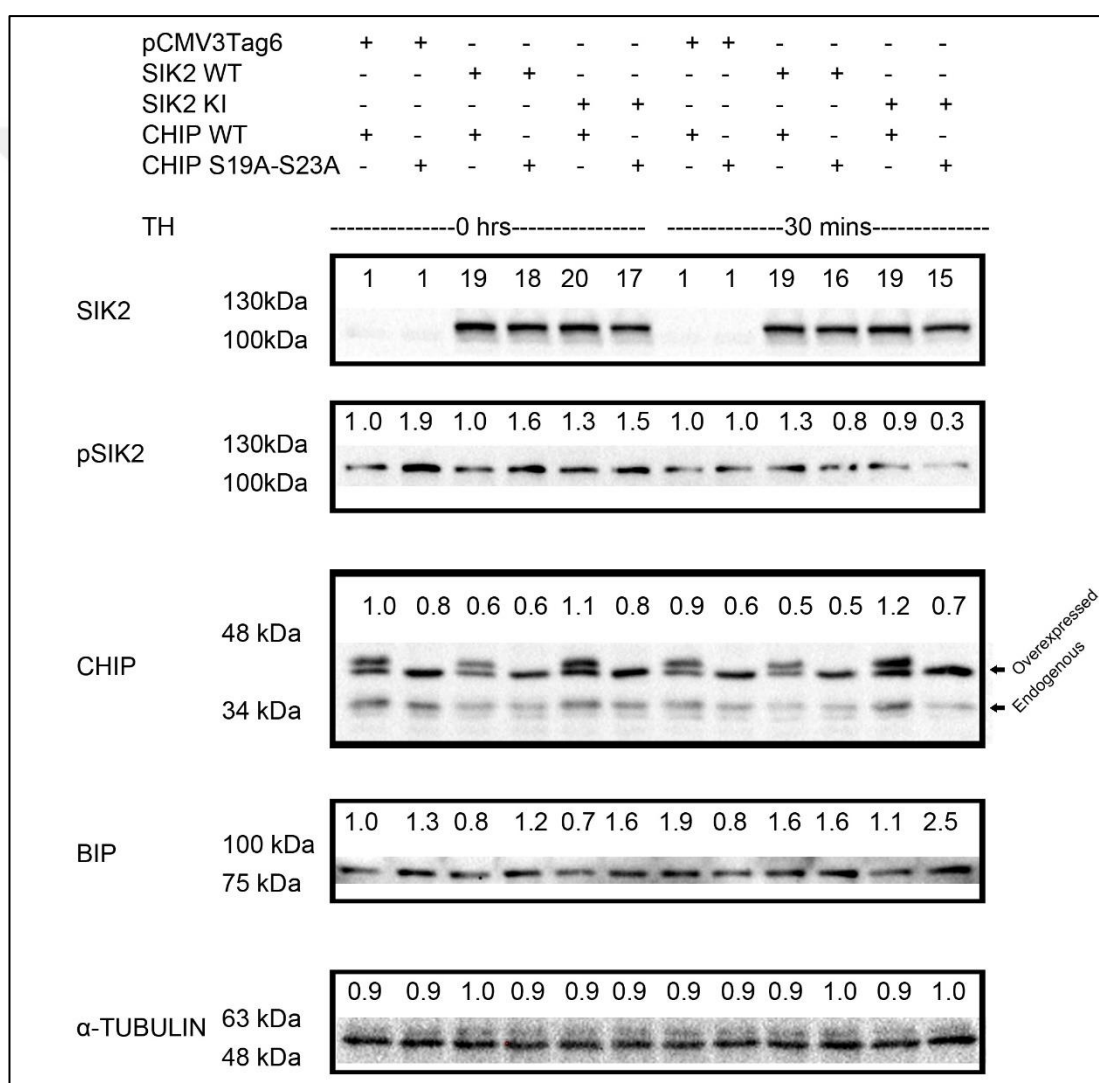


Figure 3.3: Dependence of SIK2 mediated CHIP degradation on its phosphorylation of CHIP at S19 and/or S23 residues. The cells overexpressing either CHIP WT or CHIP S19A-S23A were transfected with either one of SIK2 WT, SIK2 KI and empty vector. 48 hours after transfection the cells were treated with either 1 μ M TH or DMSO for indicated time periods.

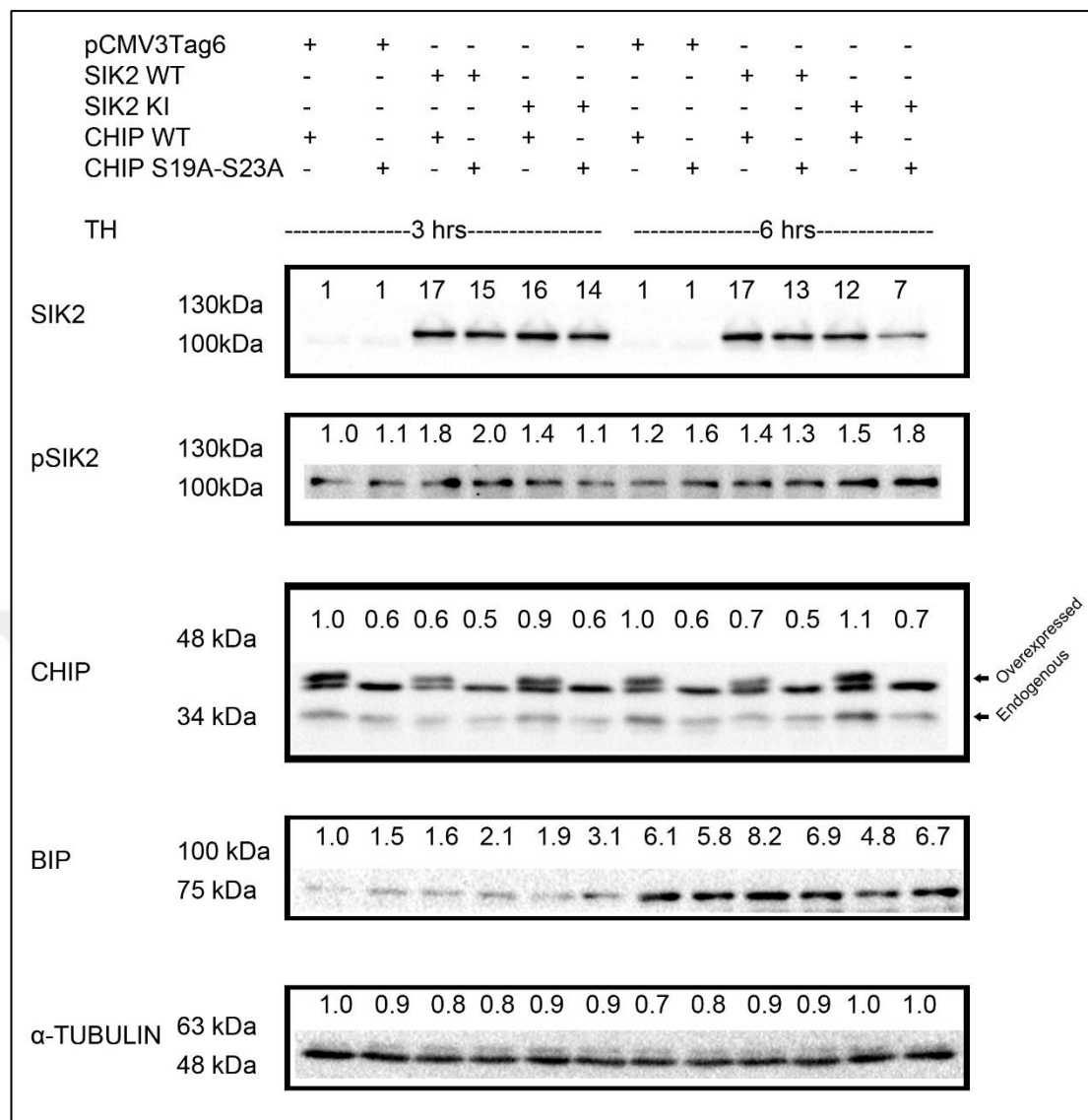


Figure 3.3: Continued. The cells overexpressing either CHIP WT or CHIP S19A-S23A were transfected with either one of SIK2 WT, SIK2 KI and empty vector. 48 hours after transfection the cells were treated with either 1 μ M TH or DMSO for indicated time periods.

The top band of CHIP WT which is absent in CHIP S19AS23A seems to be caused by a shift in mobility due to phosphorylation.

In lanes 1 and 2 the expression level of wild type CHIP is higher than that of the mutant CHIP. Taking this into account when comparing lanes 3 and 4, 9 and 10, 15 and 16, 21 and 22 it can be said that the decrease in CHIP WT levels due to overexpression of SIK2 WT is considerably more pronounced than those of CHIP S19A-S23A. Also, in the cases where SIK2 KI is overexpressed as in lanes 5 and 6, 11 and 12, 17 and 18, 23 and 24 both CHIP WT and mutant levels are closed to those of control. These results suggest that SIK2 mediated serine 19 and 23 phosphorylation

of CHIP is a degradative signal presumably recruiting another E3 ligase to CHIP or may be inducing auto ubiquitination.

3.3. E3 Ligase Activity Of CHIP Is Required For SIK2 Destabilization

To unravel the requirement of CHIP's E3 ligase activity in SIK2-CHIP interaction, we generated H260Q mutant of CHIP which is defective for binding to E2 enzymes by site directed mutagenesis. [119].

The cells overexpressing SIK2 WT were transfected with either CHIP WT or CHIP H260Q or empty vector backbone. The cells were treated with proteasome inhibitor MG132 to see the effects of proteasome inhibition on CHIP mediated SIK2 degradation (Figure 3.4). In the cells co-overexpressing CHIP H260Q and SIK2 the level of the latter was restored and even exceeded the level when co-expressed with wild type ligase active CHIP without respect to proteasome inhibition albeit to a lesser extent. This affect may be observed due to upregulation of autophagy which is contingent upon proteasome inhibition (Lanes 3 and 6). Mutant CHIP overexpression seemed to cause some elevation in SIK2 levels compared to control (Lanes 1 and 3) suggesting that CHIP H260Q compete with the wild type CHIP and act as a dominant negative form.

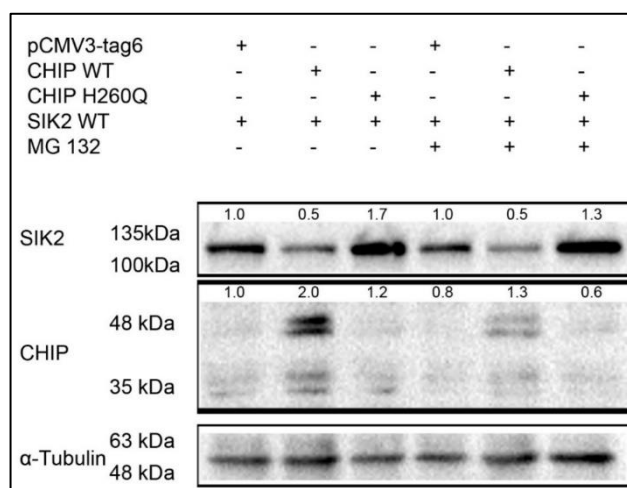


Figure 3.4: E3 Ligase Activity of Chip Is Required For SIK2 Destabilization. SIK2 WT overexpressing cells were transfected with either CHIP WT or CHIP H260Q or empty vector backbone. 48 hours after transfection the cells were treated with 10 μ M MG132 or DMSO for 4 hours.

3.4. SIK2 Modulates CHIP Interactome Under ER Stress Conditions

To evaluate the hypothesis that SIK2 modulates the interactome profile of CHIP under ER stress conditions, cells stably expressing either wild type SIK2 or its kinase dead form SIK2 KI were transiently transfected with CHIP WT or CHIP S19A-S23A to provide the co-overexpression of all combinations of the two proteins both in the presence and absence of ER stress. After treatment of the cells with thapsigargin, lysates were obtained for the subsequent immunoprecipitations with the tag antibody. Before the execution of the IP protocol overexpression of both proteins were confirmed by immunoblotting with specific antibodies (Figure 3.5).

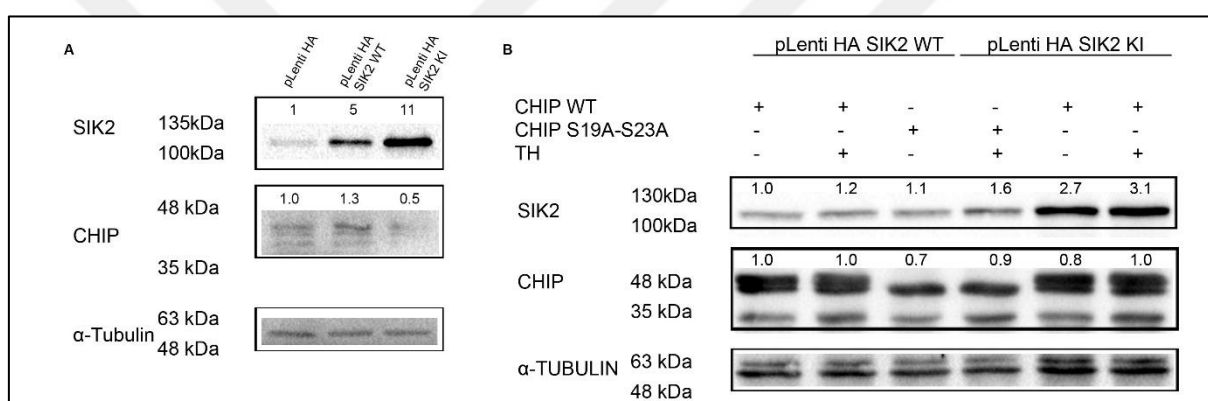


Figure 3.5: Confirmation of A) stable expression of SIK2 WT and SIK2 KI in and B) transient overexpression of CHIP in HEK293T cells treated with 1 μ M DMSO or 1 μ M thapsigargin for 6 hours. Note that no phosphorylated CHIP detected in lanes 3 and 4.

The IPs were analyzed by LC-MS/MS with an Orbitrap Elite mass spectrometer. When the raw files were analyzed for phosphorylation, it was confirmed that CHIP was indeed phosphorylated at sites 19 or 23. However, no significant change in phosphorylation levels on these sites were detected in cells overexpressing SIK2 KI compared to control. Another protein of interest that was found in the phospho-interactome was Insulin receptor-related protein (INSRR) (Appendix D).

Gene ontology (GO) analysis of differentially interacting CHIP interactors under unstressed or ER stressed conditions were performed as summarized in Table 3.1.

Table 3.1: Comparison chart for GO analysis of CHIP interactors in the given conditions.

	FLAG CHIP WT	FLAG CHIP S19/23A
HA SIK2 WT	1) TH - vs TH+	3) TH - vs TH+
HA SIK2 KI	2) TH - vs TH+	

The GO terms associated with the proteins that differentially interact with CHIP in HEK293T cells co-overexpressing CHIP and SIK2 under ER stressed conditions (Figure 3.6) included the functions that is required to restore ER stress: Protein folding (e.g. 'De novo' protein folding), chaperone activity (ATP-dependent protein folding chaperone), translation (Cytosolic ribosome), protein degradation (COP9 signalosome), alternative splicing (Spliceosomal complex), translation of molecular chaperons (Cytosolic ribosome) are among these functions and processes.

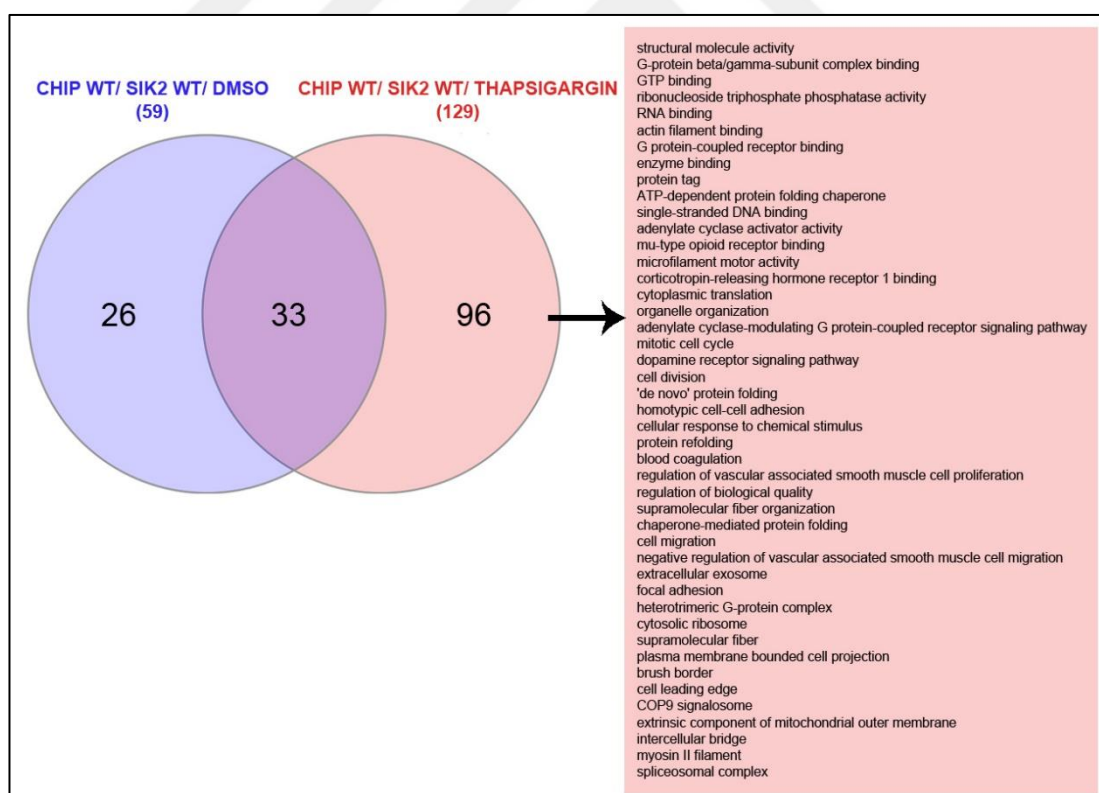


Figure 3.6: ER Stress Specific Changes in Functional Annotation of Proteins In CHIP Interactome Of Samples Which co-express CHIP and SIK2.

The biological processes associated with the proteins that differentially interact with CHIP in HEK293T cells overexpressing CHIP and kinase dead SIK2 KI under ER stressed conditions (Figure 3.7) are related to the structure and organization of DNA, structure and organization of cytoskeleton, translation, hormone signaling and ion export across membranes, cellular organization, and transport. The GO terms associated with the interactome common to both unstressed and stressed conditions in the absence of SIK2's kinase activity (Appendix C) include ER stress associated proteins which suggest that lack of SIK2 activity result in a condition akin to ER stress.

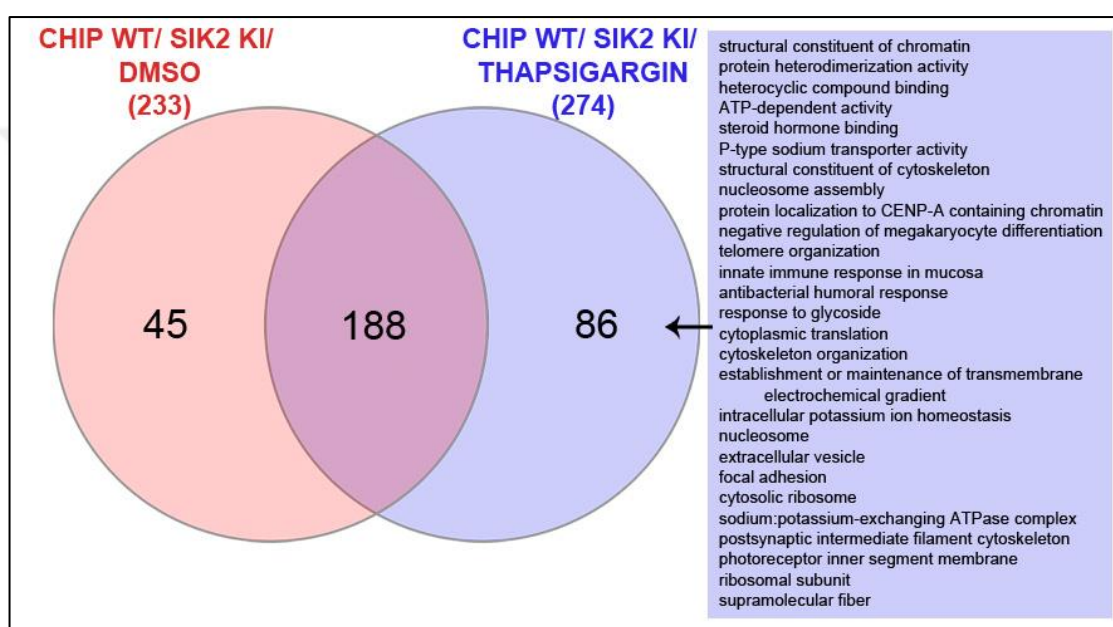


Figure 3.7: ER Stress Specific Changes in CHIP Interactome Of Samples Which Co-Overexpress CHIP WT And SIK2 KI.

There are quite a few proteins that differentially interact with CHIP in HEK293T cells co-expressing CHIP S19A/S23A and SIK2 WT under ER stress conditions (Figure 3.8). The GO terms associated with these proteins seem to be related to chromatin structure and translation initiation.

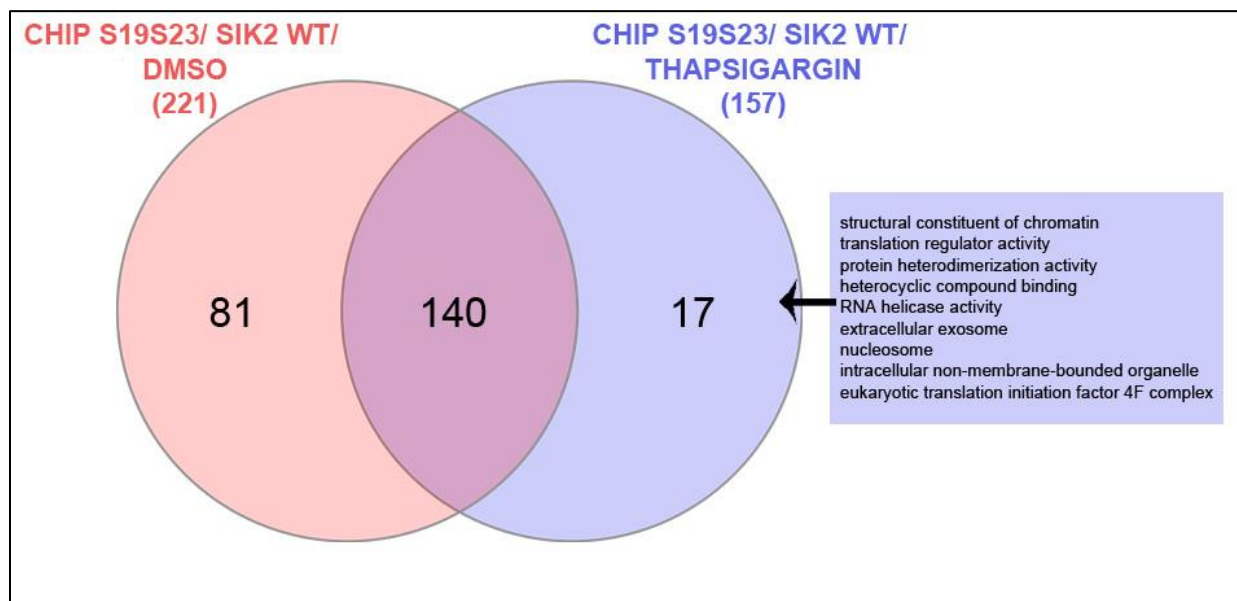


Figure 3.8: ER Stress Specific Changes in CHIP Interactome Of Samples Which Co-Overexpress CHIP S19AS23A And SIK2 WT.

Taken together the effect of SIK2 on CHIP interactome depending on the ER stressed conditions can be seen in Figure 3.9 where SIK KI overexpressing cell lysates under both unstressed and stressed conditions share more common CHIP interactors with SIK2 WT overexpressing cell lysates under stressed conditions which suggests that lack of SIK2’s kinase activity results in a condition similar to ER stress.

The lack of SIK kinase activity shifted the CHIP interactome towards proteins which involve in mitotic processes, organization of DNA, regulation of gene expression, organization of cellular structure, maintenance of cellular homeostasis and cellular communication.

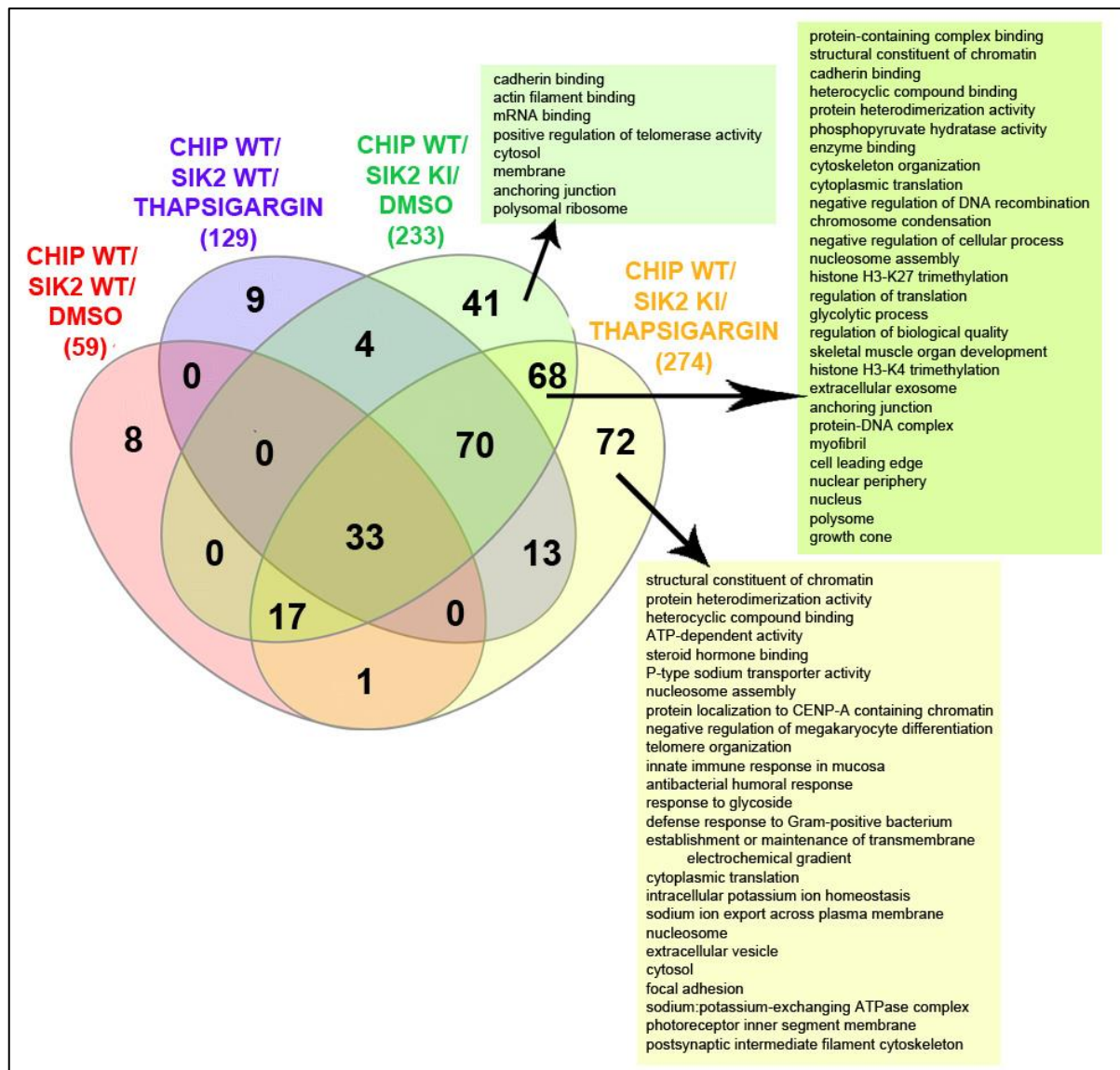


Figure 3.9: The effect of SIK2's kinase activity on CHIP interactome under unstressed and ER stressed conditions.

The comparison of the interactomes of CHIP WT and CHIP S19A/S23A under both unstressed and stressed conditions are shown in Figure 3.10. CHIP S19A/S23A interactomes under both conditions share more common CHIP interactors with CHIP WT overexpressing cell lysates under stressed conditions.

4. DISCUSSION

Caloric excess and a sedentary lifestyle promote obesity which is often characterized by increased adiposity and abnormal insulin and leptin actions in CNS and periphery. The risk factors associated with obesity include type 2 diabetes, non-alcoholic fatty liver syndrome, cardiovascular diseases, neurodegenerative diseases, and some cancers [120]. The Pathology of obesity and related diseases are linked to inflammation stimulated by the release of pro-inflammatory cytokines such as IL-1 β , IL-6, and TNF- α in response to excessive micronutrients by adipose tissue [121].

Being the major compartment in the cells where synthesis, folding, maturation and trafficking of secretory and transmembrane proteins take place endoplasmic reticulum seems to be the organelle which integrates disturbances responsible for obesity related pathologies [5].

Disturbance of ER homeostasis due to accumulation of nascent unfolded proteins cause ER stress which induces the activation of UPR to resolve the stress and restore homeostasis [26]. Although UPR is an adaptive mechanism prolonged ER stress results in a switch towards induction of inflammatory pathways by mechanisms which have not been fully elucidated. Identifying those mechanisms would lead to development of effective therapeutic approaches [122].

CHIP is an E3 ubiquitin ligase which functions at the intersection of complex interactions between components of the UPR. CHIP is a component of the ERAD pathway and involved in ubiquitination and retro translocation of misfolded proteins from ER lumen to the cytosol [17]. CHIP mediated ubiquitination of IRE1 may upregulate IRE1/TRAF2/JNK signaling pathway under ER stress conditions towards enhancing autophagy [93].

SIK2 is a serine/threonine kinase which has a key role in metabolic pathways and protein homeostasis. SIK2 enhances insulin secretion by pancreatic β cells via causing the inactivation of CDK5 through phosphorylating and leading to the degradation of CDK5 enhancer p38 [107]. Through phosphorylation of VCP which is a part of the ER translocon complex, SIK2 enhances ERAD [112]. SIK2 is also involved in autophagy pathway and functions in the autophagosome-lysosome fusion [111] which points SIK2 as an important component for the regulation of proteostasis.

However, despite the multitude of related pathways regulated by SIK2 the underlying molecular mechanisms and its components has been elusive. For this reason, our lab performed an LC-MS/MS based phosphoproteomics screen and identified various substrates of SIK2 which underwent phosphorylation in response to ER stress induction. Not surprisingly we detected CHIP as one of the major substrates of SIK2 in ER stress induced HEK293 cells by presumably phosphorylating serine 19. We hypothesized a reciprocal functional interaction between SIK2 and CHIP and generating a molecular switch made up of dual enzymes in which CHIP may ubiquitinate SIK2 to downregulate its autophagic activity while the phosphorylation of CHIP by SIK2 may either cause its degradation or alter its interactions with its substrates or change the subcellular localization.

IP-MS based analysis of CHIP interactome under unstressed and ER stressed conditions did not detect SIK2 among the proteins interacting with CHIP. However, among the potential CHIP interactors identified in this study YWHAE, CORO1C, HSPA2, TPM2, YES1, CALD1 are known SIK2 interactors.

Also, INSRR which was detected in the phospho enrichment study in the absence of SIK2's kinase activity might be of interest considering the involvement of SIK2 and INSRR with IRS1.

Among the proteins that differentially interacted with CHIP which were found in SIK2 KI samples under both unstressed and ER stressed conditions is YWHAE (also known as 14-3-3 epsilon) of interest. Salt inducible kinases are known to interact with 14-3-3 proteins [123] and SIK2 was found to be interacting with YWHAE in previous screens [124].

Another interesting potential novel interactor of CHIP under ER stress is SNAP23. SNAP23 has been implicated in insulin-stimulated glucose uptake [125] and GLUT4 translocation to the plasma membrane [126]. SNAP23 was also implicated in regulation of autophagy and apoptosis by controlling BAX protein levels and activation through an ATG9 dependent manner [127]. Interestingly SIK2 has been shown to positively regulate GLUT4 levels and glucose uptake in adipocytes [128]. This novel potential interaction of CHIP with SNAP23 may be related to SIK2-CHIP interaction.

The MS data shows that lack of SIK2's kinase activity in both unstressed and stressed conditions result in a CHIP interactome which is comparable to that obtained with SIK2 overexpression under ER stress which is consistent with SIK2's protective

effect against ER stress [129]. A similar effect is observed with CHIP S19A/S23A interactome under both unstressed and ER stressed conditions suggesting that CHIP phosphorylation is important to remediate ER stress.

In this study, although we could not provide sufficient information on the degradation of SIK2 by CHIP we did provide substantial evidence for the degradation of CHIP by SIK2 (Figures 3.1,3.2,3.3). We suggest that SIK2 achieve this destabilization through utilizing another E3 ligase such as PJA2 [107]. CHIP and SIK2 are two key regulatory proteins whose functions cross in various pathways important in regulating metabolism including insulin signaling, cell cycle, ERAD and autophagy. This study shed some light into the overly complex nature of their relationship.



5. FUTURE PROSPECTS

AMPK-related kinases which include the SIK family are the only kinases that contain a ubiquitin associated domain (UBA) in humans [130]. In other proteins UBA domains form mono or polyubiquitin binding motifs yet none of the SIKs-UBA domains has been shown to be interacting with ubiquitin oligomers [131]. This makes SIK2's interaction with E3 ubiquitin ligase CHIP more intriguing.

Chaperone mediated autophagy (CMA) is a specialized form of autophagy where HSC70 which is in complex with co-chaperones including CHIP recognizes a sequence called KFERQ-like motif on the target protein. Upon binding the target protein is translocated to a special type of lysosome which contains LAMP2A receptors on the surface. Chaperone complex dock onto that receptor inducing the multimerization of LAMP2A creating a translocon complex. Another HSC70 which resides on the luminal side of the lysosome assists the unfolding and entry of the substrate protein into the lysosomal lumen for degradation. [132]. The substrate protein must have a KFERQ-like motif to be recognized and be targeted for CMA. The motif is flexible and there is an online tool called KFERQ-like motif finder which checks protein sequences for KFERQ-like motifs [133]. When SIK2 sequence was inputted, the tool found 5 candidate motifs (Figure 5.1).

motifs in the selected proteins:								
entry	status	protein_names	gene_names	length	motif	motif_start	motif_type	ubi.inactivated
Q9H0K1	reviewed	Serine/threonine-protein kinase SIK2 (EC 2.7.11.1) (Qin-induced kinase) (Salt-inducible kinase 2) (SIK-2) (Serine/threonine-protein kinase SNF1-like kinase 2)	SIK2 KIAA0781 QIK SNF1LK2	926	KIIDK	49	acetyl. act.	TRUE
Q9H0K1	reviewed	Serine/threonine-protein kinase SIK2 (EC 2.7.11.1) (Qin-induced kinase) (Salt-inducible kinase 2) (SIK-2) (Serine/threonine-protein kinase SNF1-like kinase 2)	SIK2 KIAA0781 QIK SNF1LK2	926	IKLYQ	80	phos. act.	TRUE
Q9H0K1	reviewed	Serine/threonine-protein kinase SIK2 (EC 2.7.11.1) (Qin-induced kinase) (Salt-inducible kinase 2) (SIK-2) (Serine/threonine-protein kinase SNF1-like kinase 2)	SIK2 KIAA0781 QIK SNF1LK2	926	QRVLE	227	canonical	FALSE
Q9H0K1	reviewed	Serine/threonine-protein kinase SIK2 (EC 2.7.11.1) (Qin-induced kinase) (Salt-inducible kinase 2) (SIK-2) (Serine/threonine-protein kinase SNF1-like kinase 2)	SIK2 KIAA0781 QIK SNF1LK2	926	VERLK	334	acetyl. act.	TRUE
Q9H0K1	reviewed	Serine/threonine-protein kinase SIK2 (EC 2.7.11.1) (Qin-induced kinase) (Salt-inducible kinase 2) (SIK-2) (Serine/threonine-protein kinase SNF1-like kinase 2)	SIK2 KIAA0781 QIK SNF1LK2	926	QKREV	568	canonical	TRUE

Figure 5.1: KFERQ-like motif finder survey results. There is a possibility that SIK2-CHIP interaction results from their participation in the CMA process.

Our preliminary data demonstrates that CHIP overexpression is coincident with the significant decrease in SIK2 level. However, overexpression of CHIP and SIK2 in

the presence of autophagy inhibitor CQ did not support CHIP mediated clearance of SIK2 via autophagy. In fact, although E3 ligase activity of CHIP was required for SIK2 destabilization we were not able to detect SIK2 ubiquitination in CHIP immunoprecipitation studies (data not shown). Macroautophagy referred to as autophagy in this study requires the ubiquitination of target substrates [134]. Therefore, ubiquitin antibody based western studies conducted in our study do not appear to be conclusive about the SIK2 ubiquitination by CHIP. Although it has not been exemplified yet SIK2 UBA domain may still be active in recognizing ubiquitylated substrates thus should be further tested in different ubiquitin chains [131]. However, the observed downregulation of SIK2 protein levels by an E3 ligase may still be mediated through chaperone mediated autophagy (CMA) therefore should be assessed by mutational sequential disruption of KFERQ-like motifs found in SIK2. CMA does not require the ubiquitination of the target protein [132]. Repeating the co-overexpression of CHIP and SIK2 experiment in the presence of a lysosome specific inhibitor such as leupeptin with ammonium chloride (NH₄Cl) can provide new insight into SIK2 degradation via CMA [135]. The last and not the least is the possibility that CHIP and SIK2 interaction may be indirect and mediated through HSP70 which is a chaperone with key functions in both CMA and chaperone-assisted selective autophagy (CASA), through constructing a CHIP mutant which cannot bind chaperones can be informative about the nature of this interaction [136].

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BIOGRAPHY

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APPENDICES

Appendix A : Antibodies Used In This Study

Table A 1.1: Table Of Antibodies Used In This Study.

Antibody	Host	Dilution	Supplier
CHIP	Rabbit	1:1000	CST #2080
SIK2	Rabbit	1:1000	CST #6919
pSIK2	Rabbit	1:1000	Invitrogen #PA5-64607
α -TUBULIN	Rabbit	1:3000	CST #2144
FLAG	Rabbit	1:1000	CST #2368
β -ACTIN	Rabbit	1:1000	CST #4970
P62	Rabbit	1:1000	Sigma #p0067
LC3B	Rabbit	1:1000	Sigma #L7543
BiP	Rabbit	1:1000	CST #3177

Appendix B: Buffers And Solutions Used In This Study

Table B 1.1: Table Of Buffers And Solutions Used In This Study.

Buffer /Solution	:	Content
10X Tris EDTA (TE) Buffer pH 7.4	:	0.1 M Tris-HCl 0.01 M EDTA
1X Lysis Buffer for IP	:	145mM NaCl 20mM Tris-HCl (pH 8.0) 2mM EDTA %0.2 NP40
1X Lysis Buffer for IP/Mass	:	150mM KCl 50mM HEPES (pH 7.6) 2mM EDTA 0.5mM DTT %0.5 NP40

Table B 1.1: continued.

20X Tris-Acetic acid EDTA (TAE) Buffer	:	96,8 g Tris base 7,44 g EDTA 22,84 ml Glacial Acetic Acid Final volume is brought to 1 L with distilled water.
1.5 M Tris-HCl, pH 8.8	:	18.15 g of Tris base 80 ml Distilled water pH is adjusted to 8.8 with 6N HCl. Final volume is brought to 100 mL with distilled water.
1 M Tris-HCl pH 6.8	:	12.1 g of Tris base 80 ml Distilled water pH is adjusted to 6.8 with 6N HCl. Final volume is brought to 100 mL with distilled water.
10X SDS Running Buffer	:	30.3 g Tris base 144 g Glycine 10 g SDS Final volume is brought to 1L with distilled water.
10X Transfer Buffer	:	30.3 g Tris base 144 g Glycine Final volume is brought to 1L with distilled water.

Table B 1.1: continued.

1X Transfer Buffer	:	100 ml 10X Transfer Buffer 200 ml Methanol Final volume is brought to 1L with distilled water.
1X Tris Buffered Saline (TBS)	:	20 ml Tris-HCl (1M, pH 7.4) 37.5 ml NaCl (4M) Final volume is brought to 1L with distilled water.
1X Tris Buffered Saline with Tween-20 (TBS-T)	:	20 ml Tris-HCl (1M, pH 7.4) 37.5 ml NaCl (4M) 10 ml Tween-20 (10%) Final volume is brought to 1L with distilled water.
Blocking Buffer for western blot	:	5% non-fat dried milk in 1X TBS-T.
Luria-Bertani medium (LB)	:	25 g LB Medium (Caisson, LBP01) is dissolved in 1L distilled water and sterilized by autoclaving.
SOC medium	:	2 g tryptone, 0.5 g yeast extract, 0.05 g NaCl is dissolved in 80 ml dH ₂ O and pH is adjusted to 7.0 with NaOH. The volume is brought to 100 ml and sterilized by autoclaving. 20mM sterile glucose, 10mM sterile MgCl ₂ , 10mM sterile MgSO ₄ are added just before use.

Table B 1.1: continued.

LB Agar	:	40 g LB agar (Caisson, LBP02) is dissolved in 1L distilled water and sterilized by autoclaving.
LB + Sucrose Agar	:	40 g LB agar (Caisson, LBP02) is dissolved in 750 ml distilled water. 50 g sucrose is dissolved in 250 ml distilled water. The solutions are sterilized by autoclaving separately and mixed when they are cool enough to be poured into petri dishes.

Appendix C: Purified CHIP

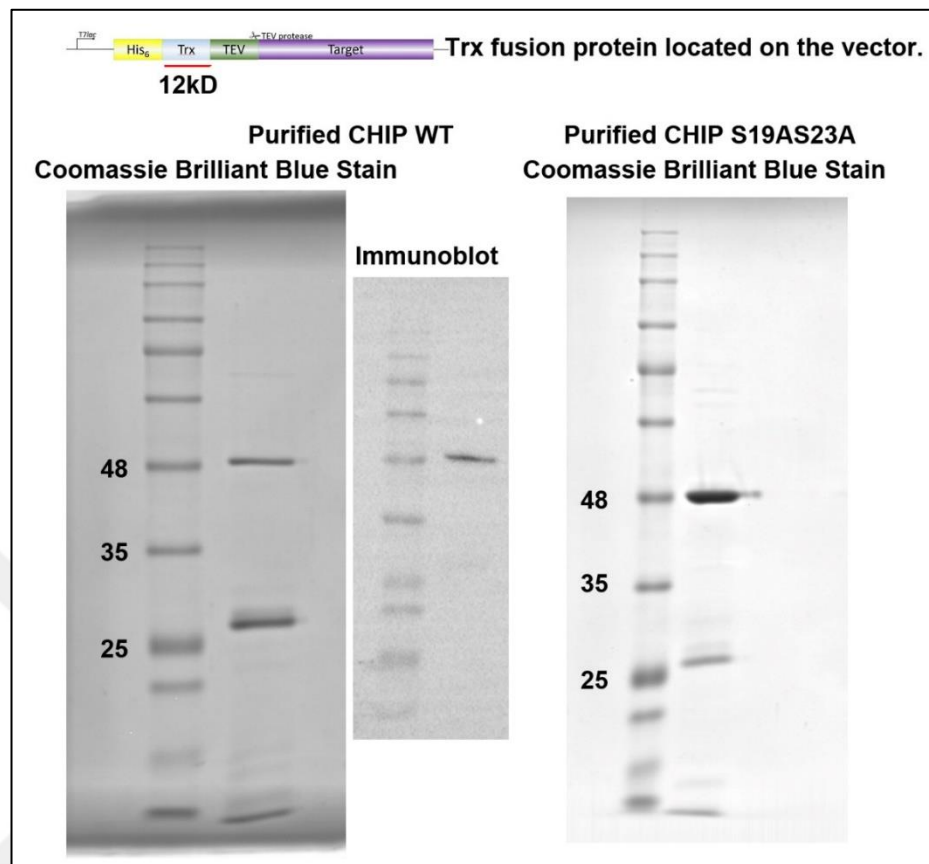


Figure C1.1: Purified CHIP WT and S19AS23A can be seen at 48 kDa. The upward shift in the bands from 34 kDa are caused by fusion proteins.

Appendix D: Overview Of LC-MS/MS Data

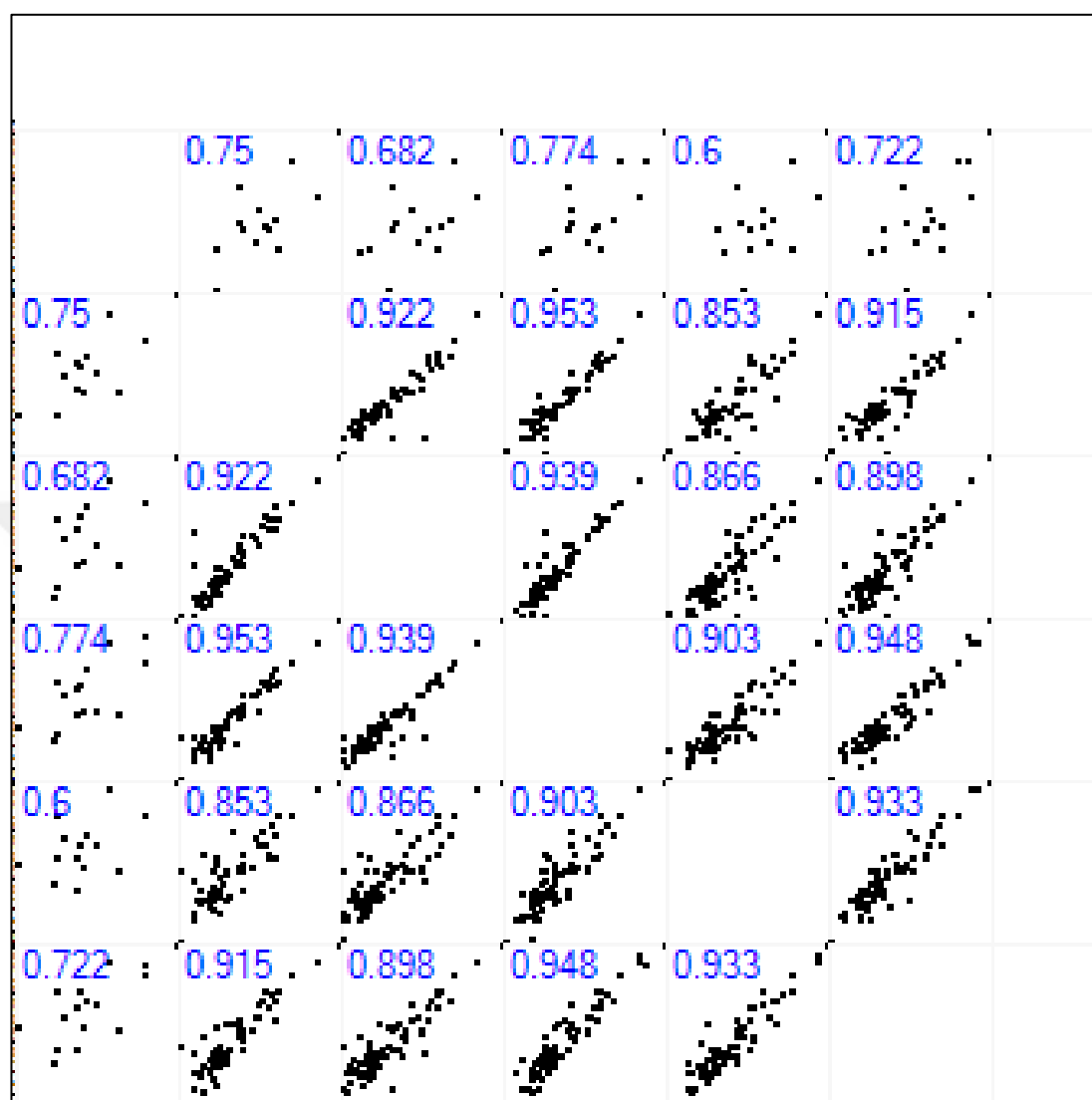


Figure D1.1: Correlational relationship of protein abundances between samples. On x-axis from top to bottom CHIP WT SIK2 WT DMSO, CHIP WT SIK2 WT TH TH, CHIP MT SIK2 WT DMSO, CHIP MT SIK2 WT TH, CHIP WT SIK2 KI DMSO, CHIP WT SIK2 KI TH versus each sample on the y-axis.

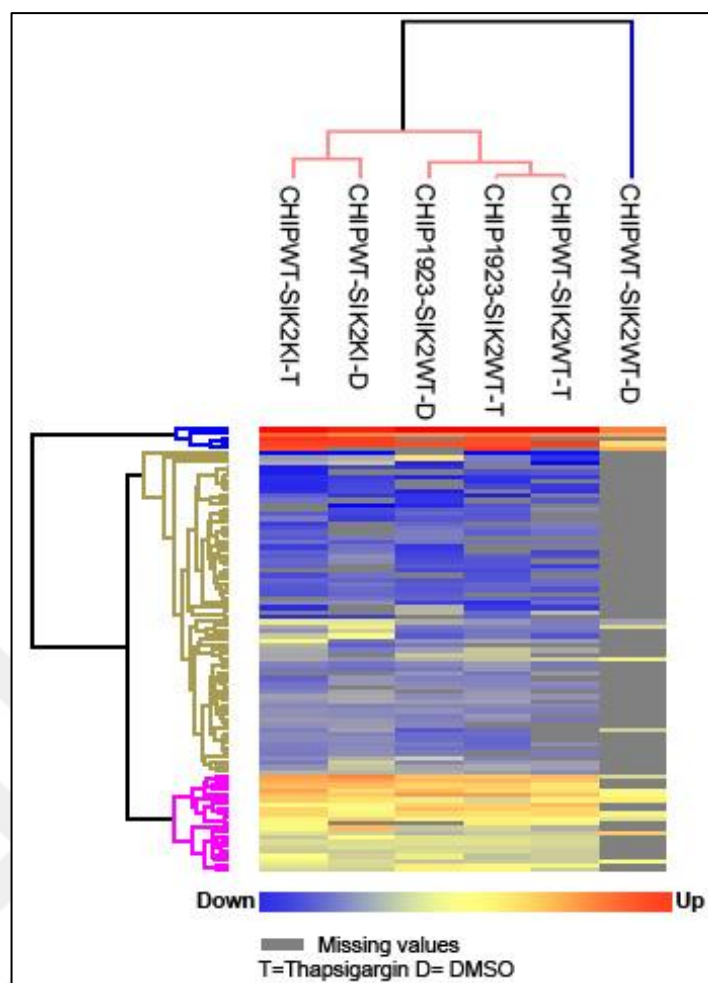


Figure D1.2: Relative protein abundances of proteins identified in the CHIP interactome.

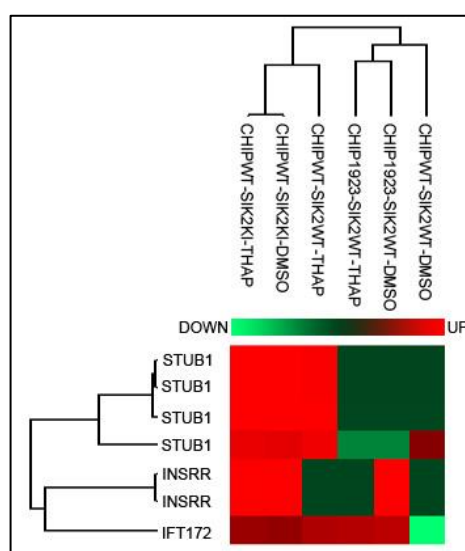


Figure D1.3: Relative protein abundances of phosphoproteins identified in the CHIP interactome.