

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

INVESTIGATING THE POSSIBLE EFFECTS OF ATG9a-SIK2
INTERACTION ON AUTOPHAGY UNDER ER STRESS
CONDITIONS

GÜLCAN KARTAL
A THESIS SUBMITTED FOR THE DEGREE OF
MASTER OF SCIENCE
DEPARTMENT OF MOLECULAR BIOLOGY OF GENETICS

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PROF. DR. FERRUH ÖZCAN

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GEBZE TEKNİK ÜNİVERSİTESİ
FEN BİLİMLERİ ENSTİTÜSÜ

ATG9a-SIK2 ETKİLEŞİMİNİN OTOFAJİ
ÜZERİNDEKİ OLASI ETKİLERİNİN ER
STRES KOŞULLARI ALTINDA
ARAŞTIRILMASI

GÜLCAN KARTAL
YÜKSEK LİSANS TEZİ
MOLEKÜLER BİYOLOJİ VE GENETİK ANABİLİM DALI

DANIŞMANI
PROF. DR. FERRUH ÖZCAN

GEBZE
2021

GEBZE TEKNİK ÜNİVERSİTESİ	YÜKSEK LİSANS JÜRİ ONAY FORMU
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JÜRİ

ÜYE

(TEZ DANIŞMANI) : Prof. Dr. Ferruh ÖZCAN

ÜYE

: Dr. Öğr. Üyesi Can Murathan ERİŞTİ

ÜYE

: Doç. Dr. İbrahim YAMAN

ONAY

Gebze Teknik Üniversitesi Fen Bilimleri Enstitüsü Yönetim Kurulu'nun
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SUMMARY

Obesity is associated with various health problems including cardiovascular diseases, insulin resistance, type 2 diabetes, and some cancers. Despite massive global efforts, the molecular underpinnings of obesity and related metabolic diseases remain unknown. Low-grade inflammation has invariably been reported in these metabolically active tissues, including the CNS of obese individuals and animal models of obesity. Under persistent unmitigated ER stress conditions, two interconnected mechanisms, UPR and autophagy, appear to underpin the initiation of inflammatory pathways. SIK2 is a serine/threonine kinase that belongs to AMPK related kinase family and has crucial role in the regulation of major metabolic pathways including liver gluconeogenesis, lipogenesis, and ketogenesis in addition to protein homeostasis in response to hormonal induction and cellular stress conditions. SIK2 has been implicated in the upregulation of ERAD and autophagy by phosphorylating p97/VCP and HDAC4 respectively.

UPR pathways, ERAD, and autophagy act in concert to restore protein homeostasis under ER stress conditions. Unresolved chronic ER stress has been proposed to underlie various cellular pathologies including insulin and leptin resistances both in peripheral tissues and CNS leading to the development of obesity. ATG9a the only membrane-associated autophagy protein plays critical roles in the early autophagosome formation upon starvation-induced autophagy. Recently an autophagy-independent physiological role for ATG9a has been proposed in several studies. Here in this study, we identified a physical interaction between the only membrane protein of autophagy ATG9a and the hemostatic stress kinase SIK2 an upstream activator ERAD component VCP/p97, which presumably mediates a regulatory crosstalk between autophagy and the ERAD.

Key Words: ER Stress, SIK2-ATG9A, Autophagy, Obesity

ÖZET

Obezite kardiyovasküler hastalıklar, hipertansiyon, insülin direnci, tip 2 diyabet ve bazı kanserler türleride dahil olmak üzere birçok metabolik hastalıkla ilişkilendirilmektedir. Obeziteninin moleküler temelini anlamak, risk oluşturduğu birçok metabolik ve nörodejeneratif hastalığın ve hatta ilişkili bazı kanser türleri için yeni tedavilerin geliştirilmesi adına büyük önem taşımaktadır. Obez bireylerin merkezi sistemi ve obez hayvan modelleri dahil olmak üzere, metainflamasyonun varlığı metabolik olarak aktif dokularda birçok çalışmada rapor edilmiştir. Şiddeti azalmayan ER stres koşulları altında, birbirine bağlı olan UPR ve otofaji mekanizmalarının inflamasyon yollarının başlatılmasını desteklediği düşünülmektedir. SIK2, AMPK ile ilgili kinaz ailesine ait bir serin/treonin kinazdır ve karaciğer glukoneogenezi, lipogenez ve ketogenez dahil olmak üzere ana metabolik yolları düzenlemede ve hormonal ve hücrel stress koşullarına bağlı olarak protein homeostazı korumada önemli bir role sahiptir. SIK2, sırasıyla p97/VCP ve HDAC4'ü fosforilerek ERAD ve otofajinin yukarı regülasyonunu gerçekleştirmektedir.

ER stres yanıt yolları ERAD, UPR ve otofaji, protein homeostazını yeniden sağlamak için uyumlu bir şekilde hareket eder. Kronik ER stresin, insülin ve leptin direnci dahil olmak üzere çeşitli hücrel patolojilerin altında yattığı öne sürülmüştür. Öyleki MSS'nde kronik ER stress çözülemezse hücrel kayıplar ve obezite gelişimi gözlenebilir. ATG9A, membranla ilişkili tek otofaji proteindir ve besin yokluğu ile indüklenen otofajide, erken otofagozom oluşumunda kritik bir rol oynamaktadır.

Ayrıca, son zamanlarda, ATG9A için otofajiden bağımsız birçok fonksiyonel rolü rapor edilmiştir. Bu çalışmada, otofajinin tek membran protein ATG9a ile hemostatik stres kinaz proteini ve yukarı akış aktivatörü VCP/p97 (ERAD bileşeni) olan SIK2 arasındaki, muhtemelen otofaji ve ERAD arasındaki düzenleyici iletişimi sağlayabilen fiziksel etkileşimi tanımladık.

Anahtar Kelimeler: ER Stres, SIK2-ATG9a, otofaji, obezite

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LIST of ABBREVIATIONS and ACRONYMS

<u>Abbreviations and Acronyms</u>	<u>Explanations</u>
g	: Gram
G	: Gravity
M	: Molar
ml	: milliliter
mM	: Millimolar
nM	: Nanomolar
μM	: Micromolar
μg	: Microgram
ng	: Nanogram
U	: Unit
α	: Alpha
β	: Beta
δ	: Delta
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
ATG	: Autophagy related protein
BCA	: Bicinchoninic acid
BiP	: Binding immunoglobulin protein
BSA	: Bovine serum albumin
cAMP	: Cyclic AMP
C/EBP	: CCAAT-enhancer binding protein
CHOP	: C/EBP homologues protein
CNS	: Central nervous system
CREB	: CRE-binding protein
DMEM	: Dulbecco's modified eagle medium
DMSO	: Dimethyl sulfoxide

eIF2 α	: Eukaryotic translation initiation factor 2 alpha
ER	: Endoplasmic reticulum
ERAD	: Endoplasmic reticulum associated degradation
FBS	: Fetal bovine serum
IRE1 α	: Inositol requiring enzyme 1 alpha
JNK	: c-Jun N-terminal kinase
kDa	: Kilo Dalton
KD	: Kinase domain
KI	: Kinase inactive
LKB1	: Liver kinase B1
mRNA	: Messenger RNA
mTOR	: Mechanistic target of rapamycin
PBS	: Phosphate buffer saline
PCR	: Polymerase chain reaction
Pen/Str	: Penicillin / Streptomycin
PERK	: PKRK-like ER kinase
PFA	: Paraformaldehyde
PVDF	: Polyvinylidene difluoride
SIK2	: Salt inducible kinase 2
TBS	: Tris buffer saline
TGN	: Trans Golgi network
Tha	: Thapsigargin
TORC2	: Transducer of regulated CREB protein 2
TRAF2	: TNF receptor associated factor 2
UPR	: Unfolded protein response
VCP/p97	: Valosin containing protein/p97
WAT	: White adipose tissue
WT	: Wild type
XBP-1	: X-box binding protein
	:

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1. INTRODUCTION

1.1. Obesity

Obesity has become a global pandemic in recent decades. Despite increased awareness and intensive research efforts, the prevalence of obesity and associated metabolic diseases continues to rise global scale [1], [2]. Obesity has a significant impact on human health by constituting a risk factor for the development of variety of diseases such as Type 2 Diabetes, atherosclerosis, hypertension, neurodegeneration, and certain cancers [3]. In response to the growing burdens of obesity and related chronic diseases, many countries have implemented policies aimed at improving diet quality and sedentary lifestyle over the last decade. For the last decades research for the development of anti-obesity drugs focuses primarily on the pharmacological control of the specified hypothalamic neurons responsible for the energy homeostasis and feeding behaviors, as well as the metabolically active peripheral tissues including the liver and the adipose tissues [4], [5]. Low-grade inflammation, also known as meta-inflammation, has been observed in metabolically active tissues, including the central nervous system (CNS) of obese individuals and animal models of obesity [6]. Nonetheless, despite massive global efforts, the molecular underpinnings of metabolic inflammation are unknown. Under conditions of persistent unmitigated endoplasmic reticulum stress (ER), two interconnected mechanisms, unfolded protein response (UPR) and autophagy, appear to underpin the initiation of inflammatory pathways [7].

1.2. Endoplasmic Reticulum Stress

The endoplasmic reticulum (ER) is arranged in a dynamic membranous network and the major assembly site for the proper folding of almost all secretory and integral membrane proteins in eukaryotic cells. Protein folding machinery in the ER is a complex and error-prone process, as in the case of imbalance between a load of unfolded and misfolded proteins and the ER folding capacity, proteins can fail to fold into their correct native confirmation [8]. Impediments to ER folding capacity can cause accumulation and aggregation of unfolded or misfolded proteins in the ER lumen

a cellular condition collectively defined as ER stress [9]. ER stress is caused by physiological perturbations such as saturation of folding capacity, aberrant Ca^{2+} regulation, impaired protein glycosylation, viral infection, mutations in proteins [10]. Cells use different strategies to orchestrate the recovery of ER stress. The ER responds to the burden of unfolded protein in its lumen by activating intracellular signal transduction pathways, collectively termed the unfolded protein response (UPR). The role of UPR is monitoring conditions in the ER, sensing an insufficiency in the ER capacity, and restore ER homeostasis. UPR activation increases the ER folding capacity to match needs by upregulating the expression of folding related proteins [11], decreasing the flux of protein entering the ER by attenuation of protein synthesis [12], and an alternative mechanism for degrading proteins, autophagy [13]. When the cells undergo prolonged and unmitigated, ER stress cannot be restored and damaged cells are eliminated by apoptotic pathways which also triggers by the UPR system [14], [15].

Activation of UPR depends on a set of transmembrane ER-resident sensor proteins, including inositol-requiring transmembrane kinase/endonuclease 1 (IRE1 α), PKR-like ER kinase (PERK), and activating transcription factor 6 (ATF6) as indicated in Figure 1.1 [16]. Signaling in the UPR is initiated by those proteins which have the luminal domain that senses the stress and cytoplasmic effector domain that interact with the transcriptional or translational apparatus [17]. Under stress conditions, those transmembrane proteins become active by dissociation of ER chaperone immunoglobulin heavy chain binding protein (BiP) since BiP binds to unfolded proteins [18].

IRE1 α signaling governs the most phylogenetically conserved UPR signaling branch in eukaryotes [19]. As a type I transmembrane protein, IRE1 α contains an ER luminal amino-terminal domain, carboxy-terminal cytoplasmic kinase, and RNase domain and, a transmembrane region. In response to ER stress, IRE1 α oligomerizes, triggered by self-association of the ER luminal domain, and trans-autophosphorylates of the kinase domain which activates IRE1 α endoribonucleolytic activity [20]–[24]. IRE1 α is therefore a bifunctional enzyme, having both a protein kinase and a site-specific endoribonuclease that is regulated by its kinase module. Activation of the IRE1 α endoribonucleolytic domain cuts the precursor X-box-binding protein 1 (XBP-1) mRNA excising the 26 nucleotides which cause shifting in its reading frame [25]–[27]. The spliced mRNA is translated to the form of spliced XBP-1 (sXBP-1) encodes

an activator of UPR target genes. Thereby, sXBP-1 promotes increasing in ER protein folding capacity, and ER chaperones and components of ER-associated degradation (ERAD) machinery [27]–[31]. Several studies have also shown that IRE1 α has achieved additional functions to promote cell death and autophagy. Upon ER stress the kinase domain of IRE1 α binds to the TNF-receptor-associated factor 2 (TRAF2) and activates apoptosis signal-regulating kinase 1 (ASK1) which in turn leads to phosphorylation and activation of c-Jun N-terminal kinase (JNK) by upregulation of mitogen activating protein (MAP) kinase activation. [32]–[34]. In dominant-negative TRAF2 cells it has been observed that ER stress-activated autophagy is inhibited. Thus, IRE1 can regulate autophagic activity and apoptosis via interaction with TRAF2 and activation of autophagy-related gene Beclin1 with JNK mediated activation [35].

In response to ER stress PERK dimerizes and its cytosolic kinase domain trans-autophosphorylates. Activated PERK phosphorylates eukaryotic initiation factor 2 subunit alpha (eIF2 α). As a result of eIF2 α phosphorylation general protein synthesis and unfolded proteins load entering the ER are reduced by the decreasing of mRNA translation initiation [12]. Besides inhibitory effects of PERK on mRNA translation, PERK also selectively increases translation of activating transcription factor 4 (ATF4) that upregulates downstream of UPR target genes including transcription factor C/EBP homologous protein (CHOP) [36], [37]. The genes which are involved in apoptotic pathways are activated by CHOP. Thus, the PERK pathway tries to mediate a survival response however, upon prolonged ER stress, it switches into an apoptotic response [37].

Unlike IRE1 α and PERK, ATF6 is a type II transmembrane protein. ATF6 consists of carboxy-terminal stress sensing luminal domain and amino-terminal bZip transcription factor domain. Under the ER stress, ATF6 localizes from the ER to the Golgi apparatus. In Golgi amino terminus of ATF6 (ATF6-N) is released after proteolyzed by the site-1 and site-2 proteases (S1P and S2P) respectively. The liberated cytosolic fragment ATF6-N translocates to the nucleus and activates transcription of UPR target genes by binding to the ER stress response element [38], [39].

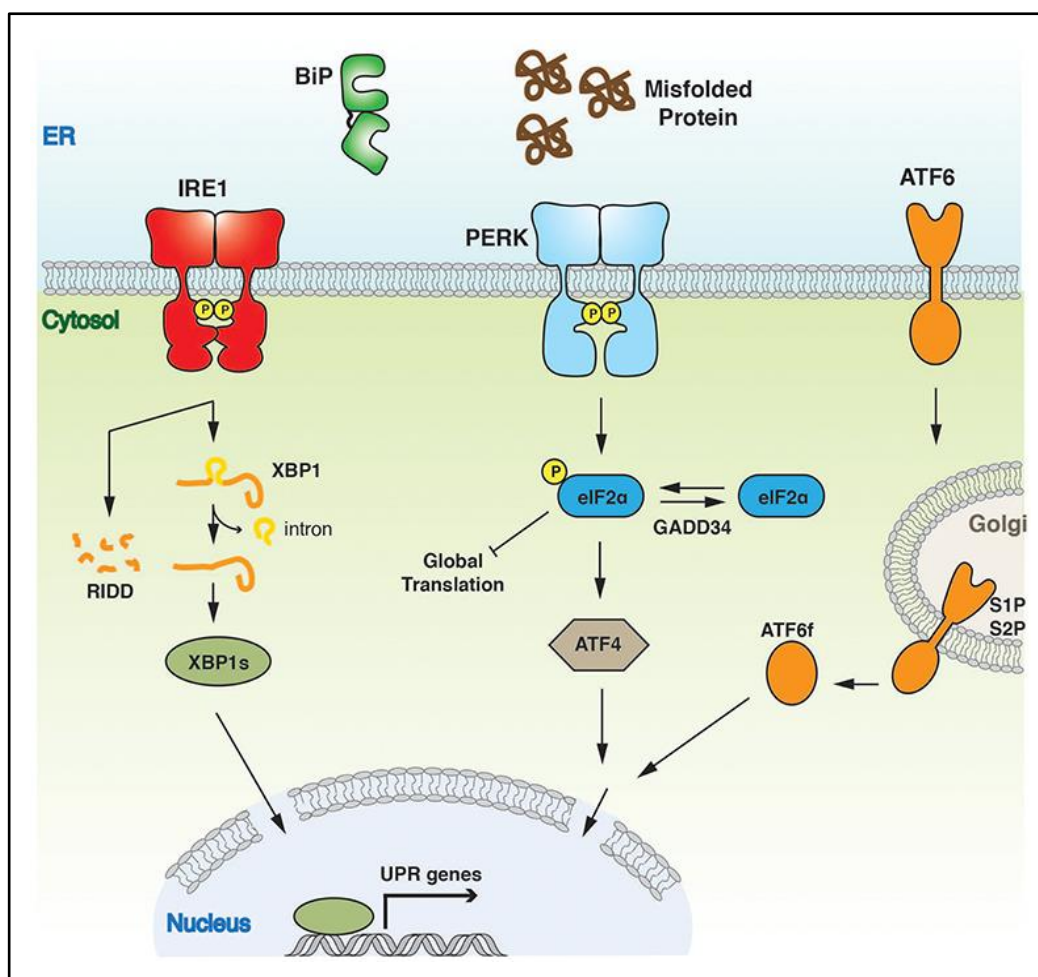


Figure 1.1: UPR sensors and ER stress signaling pathways.

1.3. Endoplasmic Reticulum Associated Degradation (ERAD)

In the cell when the folding capacity of the ER is inadequate or the environment of the ER is not optimal for the proper folding, misfolded or unfolded proteins are aggregated in the ER lumen. Accumulation of misfolded proteins causes ER stress which triggers UPR signaling as mentioned in previous sections. Those misfolded protein aggregates are cleared by ER-associated protein degradation (ERAD). Components of ERAD, such as chaperones, protein folding, and ubiquitination enzymes, are upregulated by the UPR [40]–[42].

ERAD controls ER and cellular homeostasis and involves the selection of terminally misfolded protein and translocate those proteins from the ER lumen to cytosol for the degradation by the proteasome [43]. However, some selection of

aggregates/misfolded proteins and lead them to the proteasome for disposal might be difficult. In this case, autophagy reduces those aggregates and tries to restore homeostasis [13], [44], [45].

Once the efficiency of ERAD is compromised and the proteasome-dependent pathway was saturated by the excess load of ubiquitylated proteins, ER stress-induced autophagic degradation is involved as a compensatory response. Several recent studies have shown that many components of ER stress-induced autophagic activity are regulated by the UPR branches [7], [46]–[48].

1.4. Autophagy

Autophagy is a highly conserved lysosome-dependent catabolic process and mediates cellular homeostasis. Autophagic activity is essential to protect cellular physiology in response to different forms of stress, such as nutrient starvation [49], [50], hypoxia [51], pathogen infection [52] and ER stress [45], [53]. Basal autophagy also has a pivotal role in cellular and organelle homeostasis. Therefore, abnormalities in the autophagic activity and/or pathway can lead to the pathogenesis of various metabolic and neurodegenerative diseases including cancer [54].

1.4.1. Mechanism of the Autophagy

Autophagy is a dynamic process that starts with the formation of the double membraned vesicles that is called an ‘autophagosome’. Autophagosomes are unique organelles that engulf part of the cytoplasm which can contain protein aggregates, long-lived proteins or pathogens, and dysfunctional organelle. Then this double membraned structure fuses with the lysosome which creates ‘autolysosome’ and the content of the autophagosome are degraded by the lysosomal enzymes that provide macromolecules to cells for reuse [54], [55]. There are three types of autophagy microautophagy, chaperone-mediated autophagy, and macroautophagy [56], [57]. Macroautophagy is most analyzed among the other two types of autophagy and frequently referred to as autophagy elsewhere in which cytoplasmic content or organelle is engulfed by autophagosome and then fuses with to lysosome to degrade to cargo. Autophagy has various operational steps from initiation and formation of

autophagosomes to elongation and fusion with lysosome or endolysosomal vesicles [58]. Both at physiological and cellular stresses conditions such as nutrient deprivation, a central nutritional and thereof energy sensor mechanistic (or mammalian) target of rapamycin (mTOR) kinase regulates autophagic activity through a set of over thirty autophagy-related protein (ATGs) proteins [59]. More than 30 ATG proteins are involved in maintaining autophagy. ATG proteins were firstly identified in yeast and have orthologous in mammalian cells [60]. These proteins are involved in the regulation of the autophagic process from the formation of autophagosome to autolysosome. In response to stress or any other metabolic stimuli, post-translational modification of ATG proteins contribute to the processing of autophagy as summarize in Figure 1.2 [61].

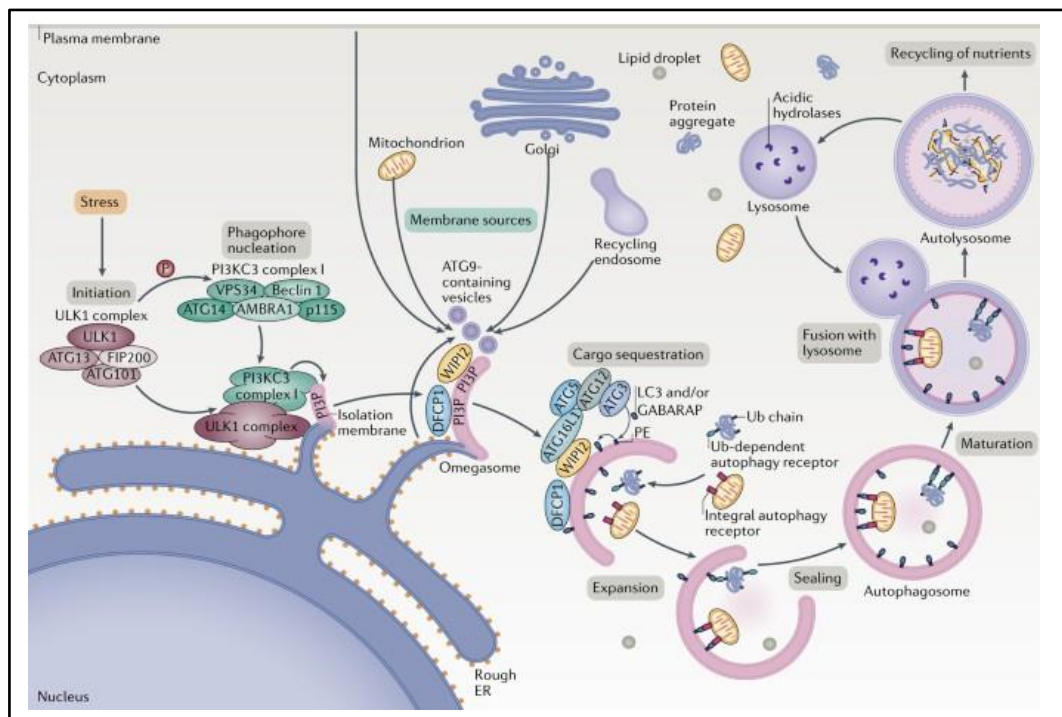


Figure 1.2: Overview of autophagy mechanism.

Nutrient deprivation in particularly depletion of amino acid and growth factors is the major inducer of autophagy in the organism and cultured cells. mTOR is a main serine/threonine kinase that plays a vital role in the nutrient-sensing pathway and cellular growth [62]. mTOR forms two specific signaling complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). mTORC1 mediates cellular growth and inhibits autophagy [63] whereas in the case of nutrient starvation mTORC1 is

inhibited and autophagy is induced to provide macromolecules and ATP in mammals and yeasts [64]. Pharmacological inhibition of mTOR (e.g., rapamycin) promotes autophagy induction under nutrient-rich conditions. One of the essential regulators of autophagy is TFEB (transcription factor of EB) which is negatively controlled by mTORC1. TFEB is the master regulator of cellular clearance, and controls lysosomal biogenesis and lipid catabolism upon starvation by directly regulating mTOR activity [65]. Reduced cellular energy level upregulates autophagy, through AMP protein kinase (AMPK) and serine/threonine liver kinase B1 (LKB1) pathway in response to glucose deprivation [66]. AMPK senses cellular energy levels to maintain homeostasis and is activated in response to ATP depletion, glucose starvation by the phosphorylation via LKB1. Autophagy can be activated by LKB1 through inhibition of mTORC1 via AMPK [67]. AMPK-activated autophagy can mediate direct phosphorylation of ULK1 and initiate autophagy in an mTOR-independent manner [68]. Moreover, various studies have also shown that autophagy is triggered by ER stress via UPR branches, IRE1 α , PERK, and ATF6 [69].

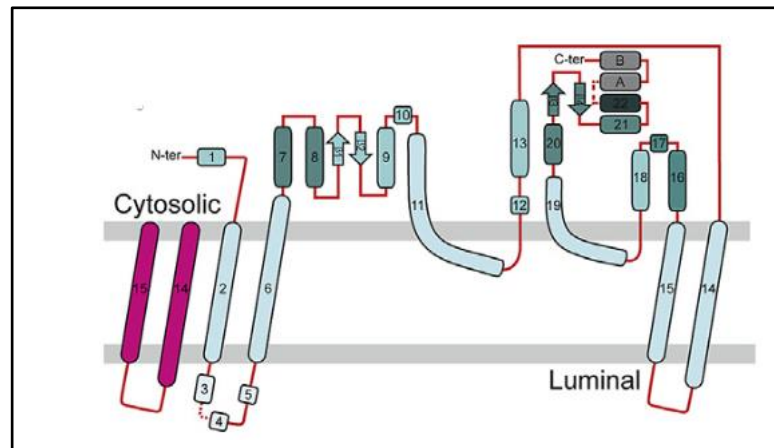


Figure 1.3: Schematic representation of ATG9a with α helices as rectangles and β strands indicated as filled arrows.

1.4.2. Autophagy Related Protein9a (ATG9a)

Autophagy-related protein 9 among the other ATG proteins is the only multi membrane-spanning ATG protein. There are two identified ortholog of ATG9, ATG9a, and ATG9b. Expression of the ATG9a occurs all over the tissues (brain to the liver) and cells, and plays a pivotal role in autophagy [70]. On the other hand ATG9b

expression is mainly limited within the placenta and pituitary gland which indicates that ATG9b may have a function in embryogenesis [71]. Structurally, as indicated in Figure 1.3, ATG9a possesses six conserved transmembrane domains with both N- and C-terminal regions present on the cytoplasmic side of the membrane [72]. It has a wide intracellular distribution and can localize in several compartments. Under the nutrient-rich condition, ATG9a primarily localizes to the trans-Golgi network (TGN), recycling endosomes, early endosomes, and late endosomes. Upon autophagy induction, ULK1 phosphorylates ATG9a in the multiple sites and as a consequence of phosphorylation ATG9a dynamically translocates between these organelles [73], [74]–[76]. Although the role of ATG9a in autophagy has not been fully elucidated, it is known that ATG9a mediates lipid transfer from the ER membrane and ATG9a-containing vesicles is necessary for the elongation of the phagophore membrane [77]. ATG9a trafficking is also observed from the plasma membrane to recycling endosomes [76], [78], [79]. Brain-specific conditional ATG9a knockout mice results in axon-specific lesion [80]. In this vein, defective ATG9a trafficking impairs autophagy and causes neuroaxonal dystrophy [81], [82]. The level of ATG9a protein increases concomitant with the autophagy induction during neural differentiation [83]. Supporting this, ethanol treatment of the cells reduced ATG9a protein level and thereby inhibited autophagy leading to impaired neural differentiation [81]. It has also been shown that Beclin-2 has a role in the negative regulation of innate immune signaling and tumor development through its role in ATG9a-dependent autophagy [84]. The acetylated ATG9a also regulates induction of ER-specific autophagy [85], [86]. Taken together, we speculated that SIK2-mediated phosphorylation of ATG9a may be responsible for this acetylation by recruiting acetyl transferases to ATG9a.

Recent data points out novel autophagy-independent roles for ATG9a as the only membrane protein of the autophagy process. In a very recent publication, it has been reported that ATG9a protects cells from plasma membrane damage caused by exogenous and endogenous agents by cooperating with the ESCRT (endosomal sorting complexes required for transport) system, thereby protecting cells from necrotic death [87]. ATG9a is also implicated in the maintenance of actin cytoskeletal integrity both in the mammalian system and *Drosophila* [88], [89]. Furthermore, ATG9a was reported to enhance HIV-1 infectivity by interacting with the envelope glycoprotein of HIV-1 [90]. In primary cerebral cortical neurons, which were obtained from ATG9a knockout mouse brains, it has been observed that cells could not extend their axons straight and coiled

them in one place. On the other hand in the same study in primary cerebral cortical neurons from ATG7 knockout mouse brains (autophagy deficient) neurite extension was also impaired when ATG9a was knocked down, which demonstrated that neurite outgrowth regulated independently of autophagy [80]. Effect of ATG9a on the innate immune system has been also reported to limit double-strand DNA induced innate immune response by regulating translocation of stimulator of IFN genes (STING) and TANK-binding kinase 1 (TBK1) [91]. In another study with *Drosophila*, it has shown that ATG9a regulates c-Jun N-terminal kinase (JNK) activation and apoptotic cell death in response to oxidative stress by interacting with dTRAF2. [92].

1.5. Salt Inducible Kinases

The Salt Inducible Kinases (SIKs) belong to AMPK like kinase family of serine/threonine kinases. SIK1 was cloned from a high-salt diet-fed rat adrenal cortex followed by the identification of the two other isoforms SIK2 and SIK3 based on homology search of the genomic databases [93], [94]. SIKs have pivotal functions on energy homeostasis, regulation of metabolism, gene regulation, etc.... and have a wide range of expression in a large number of tissues. SIK1 is mainly expressed in the adrenal cortex, adipose and neuronal tissues [95]–[97], whereas SIK2 and SIK3 are primarily expressed in adipose, liver and neuronal tissues [98], [99].

SIKs have highly conserved amino kinase domain (KD) containing LKB1 phosphorylation site, sucrose non fermenting (SNF-1) homology (SNH) domain containing ubiquitin-associated (UBA) domain and variable length C-terminal domain containing multiple protein kinase A (PKA) phosphorylation site with as depicted in Figure 1.4 [100]. SIKs consists of an activation loop within the KD and like other AMPK-related kinases, they are phosphorylated by LKB1 on a highly preserved threonine residue within the activation loop. SIK1, SIK2, and SIK3 are phosphorylated at T182, T175, and T221, respectively and their catalytic activity depends on this phosphorylation. Beside this, LKB1 independent activation of SIK2 has been reported to occur especially under cellular stress conditions. In addition to KD, SIKs is phosphorylated at a C-terminal in particularly serine residues by PKA which depends on cellular cAMP level [101]–[104]. Thereby, members of the SIK family have a similar structure and play redundant and distinct roles in regulating distinct biological

processes ranging from cell survival, proliferation and differentiation to hormone synthesis and secretion all in association with organismal protein and energy homeostasis.

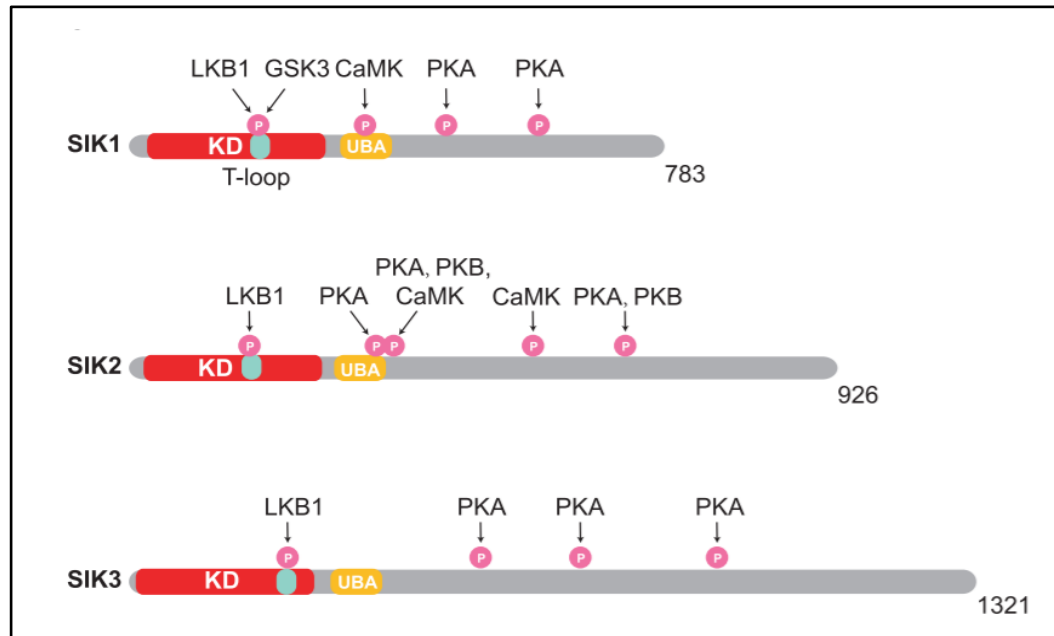


Figure 1.4: Isoforms of Salt Inducible Kinase

1.5.1. SIK2

SIK2 is excessively expressed in adipose tissue, liver, pancreas, and brain tissues. The activity and levels of SIK2 are elevated in the adipose tissue of obese mouse models. Prior studies indicate that SIK2 maintains cell homeostasis via controlling cAMP response element-binding protein (CREB)-mediated gene expression in response to stress and hormonal stimuli [105]. It involves different biological processes such as lipid metabolism, gluconeogenesis, ketogenesis, ERAD and autophagy. SIK2 has been shown to reduce hepatic adipogenesis by inhibiting phosphorylation of p300 / SREBp1c, an acetyltransferase that can activate carbohydrate- sensitive ChREBP, which is an important regulator of fatty acid synthesis and adipogenic genes (such as Fasn and Acc). In the same study, a decrease in SIK2 activity was also observed in the livers of ob / ob mice fed a high-fat diet [106]. In another study it has been reported that impaired SIK2 expression enhances increased adipogenic potential and insulin resistance in pre-adipocytes in a CRT2-

dependent manner [107], [108]. Class II histone deacetylases (HDACs) are another substrates of SIK2 and SIK2 mediated phosphorylation of HDAC stimulates development of skeletal muscles, regulatory T cells, leukemia and other pathological processes [109], [110]. SIK2 is also implicated in macrophage polarization via CRTC3 phosphorylation under cellular stress conditions [111]. SIK2 is also known to be the only AMPK member that interacts with Valosin Containing Protein (VCP)/p97 ATPase which in turn upregulate ERAD activity under physiological condition and ER stress. SIK2 and VCP/p97 localize in the ER membrane together and phosphorylation of the ATPase domain of VCP/p97 by SIK2 is essential for its ATPase activity. Thus, this cooperation regulates the degradation of ERAD substrates. Absence of functional SIK2 or its phosphorylation sites on VCP/p97 leads to defective ERAD degradation and disruption of ER homeostasis [112,] [113].

SIK2 is proposed to take critical role in the initiation of autophagy when the proteasomal degradation is either chemically or genetically disabled. However, limited data exists for the role of SIK2 in the autophagy process. In fact, in an earlier study p300/CBP-mediated acetylation of SIK2 caused sequestration of autophagosomes yet upon deacetylation with HDAC6 activity SIK2 activity is restored potentiating the fusion of autophagosomes with the lysosomes [114]. On the other hand, data for the anti-autophagic role for SIK2 were also demonstrated in cancer and normal cells warranting further studies for the exact role of SIK2 in autophagy process [115]. These studies indicated that function of SIK2 promotes triple negative breast cancer growth by restriction of autophagic flux while genetic or pharmacological inhibition of SIK2 increased autophagic flux in immortalized and tumor-derived cell lines [116].

ATG9a, the only transmembrane protein of autophagy was identified as a novel substrate for SIK2 in a LC-MS/MS based phospho-proteomics study in our laboratory. Beside, in an accompanying phosphopeptide based analysis of tryptic peptides revealed serine 735, 758, and 741 residues as potential phosphorylation sites for SIK2 in response to ER stress induction with thapsigargin a chemical inducer of ER stress [117]. Based on the literature and the phosphoproteomic data we decided to the investigate the physical interaction between ATG9a and SIK2 and its possible role in autophagy process.

2. MATERIALS

2.1. Mammalian Cell Lines

Human embryonic kidney cell lines HEK293T and HEK293FT were purchased from ABMGood (British Columbia, Canada).

2.2. Chemicals

Table 2.1: List of chemicals used in this study.

Name	Supplier
Acetic Acid	Merck, USA
Acrylamide: Bis-acrylamide (37.5:1, 40%)	Fisher Scientific, USA
Agarose	Sigma-Aldrich, USA
Ammonium Persulfate (APS)	Sigma-Aldrich, USA
Ampicillin	AppliChem, Germany
β -Mercaptoethanol	AppliChem, Germany
Blasticidine S Hydrochloride	Sigma-Aldrich, USA
Boric Acid	Sigma-Aldrich, USA
Bovine Serum Albumin (BSA)	AppliChem, Germany
Bromophenol Blue	AppliChem, Germany
ClearBand Re-Blot Stripping Solution	Eco-Tech, Turkey
DMSO	Sigma-Aldrich, USA
Dithiothreitol (DTT)	AppliChem, Germany
EDTA	AppliChem, Germany
Ethanol	Merck, USA
Glycerol	AppliChem, Germany
Glycine	AppliChem, Germany
Isopropanol	Merck, USA
Kanamycin	AppliChem, Germany
Methanol	Merck, USA

Table 2.1: Continue.

N, N, N', N'-tetramethyl ethylenediamine (TEMED)	AppliChem, Germany
N, N'-Methylenebisacrylamide	Sigma-Aldrich, USA
NP-40	AppliChem, Germany
Paraformaldehyde	Sigma-Aldrich, USA
Phosphate Saline Buffer (PBS)	Lonza, Switzerland
Polyethyleneimine (PEI)	Sigma-Aldrich, USA
Puromycin Dihydrochloride	SantaCruz, USA
Sodium Chloride (NaCl)	Fisher Scientific, USA
Sodium Deoxycholate	Sigma-Aldrich, USA
Sodium Dodecyl Sulfate (SDS)	AppliChem, Germany
Sodium Fluoride	Merck, USA
Sodium Hydroxide	Sigma-Aldrich, USA
Thapsigargin	Sigma-Aldrich, USA
Tris-Base	AppliChem, Germany
Triton X-100	AppliChem, Germany
Tween 20	Sigma-Aldrich, USA
WesternBright ECL	Advansta, USA

2.1. Buffers and Solutions

Table 2.2: Solutions and buffers used in this study.

Solutions/Buffers	Contents
Ampicillin stock	100 mg/ml in distilled water Sterilized by filtration 100 µg/ml (working concentration)
Kanamycin stock	50mg/ml in distilled water Sterilized by filtration 50µg/ml (working concentration)
Blocking Solution for western blot	5% non-fat milk powder in 1X TBS-T

Table 2.2: Continue.

Luria-Bertani medium (LB) (Caisson, LBP01)	25 g in 1L distilled water Sterilized by autoclave
LB Agar (Caisson, LBP02)	40 g in 1L distilled water Sterilized by autoclave
RIPA Lysis Buffer	150 mM NaCl 50 mM Tris-HCl (pH 7.4) 1% NP-40 0,5% Sodium deoxycholate 0,1% SDS 1 mM PMSF 5 ug/ml Aprotinin 5 ug/ml Leupeptin 5 mM Sodium Fluoride 1 mM Sodium orthovanadate (Na ₃ VO ₄)
IP Lysis Buffer	145 mM NaCl 20 mM Tris-HCl (pH 8.0) 2 mM EDTA 0.2% NP40 1 mM PMSF 5 ug/ml Aprotinin 5 ug/ml Leupeptin 5 mM Sodium Fluoride 1 mM Sodium orthovanadate (Na ₃ VO ₄)
Mounting Media	90% (v/v) Glycerol 20 mM Tris-HCl pH 8.0
20X Tris-Acetic acid EDTA (TAE)	96,8 g Tris base 7,44 g EDTA 22,84 ml Glacial Acetic Acid distilled water up to 1L
10X TE Buffer	100mM Tris-HCl 10mM EDTA, pH 7,4

Table 2.2: Continue.

5X TBE Buffer	54 g Tris-base 27,5 Boric acid 20 ml EDTA pH 8.0 distilled water up to 1 L
10X SDS Running Buffer	30.3 g Tris base 144 g Glycine 10 g SDS distilled water up to 1 L
10X Transfer Buffer	30.3 g Tris base 144 g Glycine distilled water up to 1 L
1X Transfer Buffer	100 ml 10X Transfer Buffer 200 ml Methanol distilled water up to 1 L
1X Tris Buffer Saline with Tween-20 (TBS-T)	20 ml Tris-HCl (1M, pH 7.4) 37.5 ml NaCl (4M) 10 ml Tween-20 (10%) distilled water up to 1 L
4X SDS-PAGE Loading Dye	200 mM Tris-HCl pH 6.8 8% SDS 40% Glycerol 4% β -mercaptoethanol 50 mM EDTA 0,08% Bromophenol Blue
4% (w/v) Paraformaldehyde (PFA)	10 g PFA 1x PBS up to 250 ml, pH 7.4 adjusted by adding 1 M NaOH

2.4. Plasmids, Primers and Antibodies

Table 2.3: List of plasmids used in this study.

Construct Name	Origin	Backbone
Empty pCMV-3tag-6	Agilent #240200	
Empty pLenti-III-HA	ABM #LV022	
Empty lentiCRISPR v2	Addgene #52961	
Empty psPAX2	Addgene #12260	
Empty pCMV-VSV-G	Addgene #8454	
ATG9a-RFP	Addgene #60609	pMXs-puro
ATG9a-gRNA-A	This study	lentiCRISPR v2
ATG9a-gRNA-B	This study	lentiCRISPR v2
ATG9a-gRNA-C	This study	lentiCRISPR v2
ATG9a-gRNA-D	This study	lentiCRISPR v2
ATG9a-FLAG	This study	pCMV-3tag-6
SIK2-FLAG	[117]	pCMV-3tag-6
IRE1-FLAG	[117]	pCMV-3xFLAG-7.1
SIK2 WT-HA	Boğaziçi University	pLenti-III-HA
SIK2 KI-HA	Boğaziçi University	pLenti-III-HA
IRE1 WT -HA	Boğaziçi University	pLenti-III-HA
IRE1 KI -HA	Boğaziçi University	pLenti-III-HA
CD3δ-GFP	[117]	pEGFP-N2
IRE1-sYFP	[117]	sYFP-N3

Table 2.4: List of primers used in this study.

Primer Name	Sequence	Application
ATG9a_gRNA_1A_1	CACCGTGTTGGTGCACGTCGCCGAG	Cloning
ATG9a_gRNA_1A_2	AAACCTCGGCGACGTGCACCAACAC	Cloning
ATG9a_gRNA_1B_1	CACCGCACTGAATACCAGCGCCTAG	Cloning
ATG9a_gRNA_1B_2	AAACCTAGGCGCTGGTATTCAGTGC	Cloning
ATG9a_gRNA_1C_1	CACGTGCCCTTCCGTATTGCACG	Cloning
ATG9a_gRNA_1C_2	AAACCGTGCAATACGGAAGGGCAC	Cloning
ATG9a_gRNA_1D_1	CACCGTGTTGGTGCACGTCGCCGAG	Cloning
ATG9a_gRNA_1D_2	AAACCTCGGCGACGTGCACCAACAC	Cloning
1F_HindIII_ATG9a	GAGACCAAGCTTATGGCGCAGTTTGA CAC	Cloning
5R_SalI_ATG9a	GAGGTCGACTACCTTGTGCACCTGAG GGG	Cloning

Table 2.5: List of antibodies used in this study.

Antibody	Host	Dilution	Supplier	kDa
ATG9a	Rabbit	1:1000	CST #13509	100-110
SIK2	Rabbit	1:1000	CST #6919	130
pSIK2	Rabbit	1:1000	Invitrogen #PA5-64607	~180
IRE1 α	Rabbit	1:1000	CST #3294	130
FLAG	Rabbit	1:1000	CST #2368	
p62	Rabbit	1:1000	Sigma #p0067	62
TRAF2	Rabbit	1:1000	CST #4724	53
CHIP	Rabbit	1:1000	CST #2080	32
LC3B	Rabbit	1:1000	Sigma #L7543	16-18
pJNK	Rabbit	1:1000	CST #4668	46-54
β -actin	Rabbit	1:3000	CST #4970	43
GAPDH	Rabbit	1:3000	CST #2118	37

3. METHODS

3.1. Molecular Cloning

3.1.1. Polymerase Chain Reaction (PCR)

Taq polymerases (ABM, G009) and PrimeStar HS DNA polymerase (Takara, R010A) were used to get constructs for overexpression. PrimeStar HS DNA polymerase was used to amplify DNA fragments to be cloned, while Taq polymerase was used in the colony PCR reaction for verification of the cloned constructs. The PCR reaction mixes were prepared as in Table 3.1 and Table 3.2, respectively. The PCR reaction conditions for both DNA polymerases are demonstrated in Table 3.3.

3.1.2. Restriction Enzyme Digestion of DNA

Restriction enzyme digestion of the empty vector plasmid and the amplicon was performed according to the manufacturer's recommendations. Shortly, a 20 µl reaction mix with appropriate enzyme was incubated at 37°C for 30 minutes using Fast Digest (Thermo, USA). After the restriction reaction, DNA samples were loaded in the 1% Agarose gel and run at 100 Volt constant voltage for 60 minutes. The success of the restriction reaction was checked by visualizing the agarose gel under UV light using ChemiDoc XRS+ Imaging System (Biorad, USA). DNA samples were excised from agarose gel using NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel, (MN)) according to the manufacturer's protocol.

Table 3.1: PCR reaction for Taq DNA polymerase.

Component	Volume for 25ul Reaction	Final Concentration
10X PCR Buffer	2,5ul	1X
dNTP mix (10 mM each)	0,5ul	200uM each
Forward primer	0,5ul	0,2uM
Reverse primer	0,5ul	0,2uM
Template	Variable or picked colony	50ng
Taq DNA poly.	0,25ul	1,25U/25 ul
Nuclease-free water	up to 25ul	

Table 3.2: PCR reaction for PrimeStar HS DNA polymerase.

Component	Volume for 25ul Reaction	Final Concentration
5X PrimeSTAR Buffer	5ul	1X
dNTP mix (2,5 mM each)	2ul	200uM each
Forward primer	0,5ul	0,2uM
Reverse primer	0,5ul	0,2uM
Template	variable	50ng
PrimeSTAR HS DNA poly.	0,25ul	1,25U/25 ul
Nuclease-free water	up to 25ul	

Table 3.3: PCR reaction conditions for PrimeStar HS and Taq DNA polymerase.

	PrimeStar HS		Taq		
Step	Temperature	Time	Temperature	Time	Cycle
Initial Denaturation	98°C	30 sec	95°C	3 min	1
Denaturation	98°C	10 sec	95°C	30 sec	35
Annealing	55°C	5 sec	57°C	30 sec	
Extension	72°C	1 min per 1kb	72°C	1 min per 1kb	
Final Extension	72°C	2 min	72°C	5 min	1
Hold	4°C	∞	4°C	∞	

3.1.3. Ligation

T4 DNA Ligase (NEB, M0203S) was used for ligation of the digested and linearized plasmid DNA and inserts according to the manufacturer's instruction. For each 20 μ l of the reaction mix, 50 ng digested vector and inserts were mixed with a 1:1 or 1:3 or 1:5 molar ratio. The ligation reaction was carried out at room temperature (25°C) for 2 hours without heat inactivation. The bacterial transformation was performed after the ligation reaction was completed.

3.1.4. Transformation of Chemically Competent DH5 α

Half of the ligation product or 50 ng plasmid were added into 50 μ l chemically competent DH5 α bacteria for bacterial transformation. The vial was incubated on ice for 30 min, then at 42°C heat shock was applied for 1 minute. After that, the vial was incubated on ice for 2 minutes. 750 μ l LB medium was added onto the transformation product and incubated for 1 hour at 37°C with vigorous shaking (~250rpm). After incubation, the transformation product was centrifuged for 5 min at 3000rpm. The pellet was dissolved within 100 μ l LB medium and the whole cell suspension was spread on appropriate antibiotic-containing LB-agar plates, and cells were grown overnight at 37°C.

3.1.5. Positive Clone Screening by Colony PCR

For positive clone screening, colony PCR was performed using Taq DNA polymerase as mentioned in section 3.1.1. PCR reaction mix preparation and conditions are given in Tables 3.1 and 3.3. Shortly, each colony was picked from an agar plate with a pipette or toothpick then mixed with a PCR reaction mix. PCR products were checked by running in 1% agarose gel at 100 volts for 45-60 minutes. Positives clones that contain amplicon of interest were purified.

3.1.6. Plasmid Isolation

Plasmid isolations were carried out with a DNA-spin Mini kit (Intron, 17069) in order to use particularly for the transfection of the mammalian cells, plasmids were isolated with NucleoBond Xtra Midi kit (MN, 740410). The quality of the plasmids was controlled by agarose gel electrophoresis. Constructs were verified by sequencing.

3.2. Cells Culture Methods

3.2.1. Cells and Culture Conditions

HEK293T and HEK293FT cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco) containing 10% Fetal Bovine Serum and 1% Penicillin/Streptomycin and 4.5 g/l glucose (high glucose) and maintained under 5% CO₂ condition at 37°C. Subculturing of the cells was performed before confluency reaching 90% by using 0,25% trypsin/EDTA (Gibco). Cells stored within the grown medium containing 5% (v/v) Dimethyl Sulfoxide (DMSO) at nitrogen vapor phase.

3.2.2. Transient Transfection of Cells

Polyethyleneimine (PEI) (PolyScience, 23966) was used for transfection of the HEK293T and HEK293FT cell lines. Cells were seeded on an appropriate cell-culture dish the day before transfection to obtain 60-70% confluency after overnight maintenance. The next day, an appropriate amount of plasmid DNA and PEI were mixed with a 1:3 (v: v) ratio within 150mM 1X TE buffer containing 150mM NaCl, and the mixture was incubated at room temperature for 10 minutes. The mixture was applied over the cell culture medium dropwise. In order to dispense the mixture, cell culture dish was gently agitated. After overnight (16 hours) incubation media were renewed adequate amount of fresh medium.

3.2.3. Treatments on the HEK293T Cells

For the thapsigargin treatment, 1,250,000 HEK293T cells were seeded into 60mm cell culture dishes. The following day, thapsigargin (1 μ M, final concentration) or vehicle buffer as a control (mostly DMSO) were added into cell culture media. Then, the cells were harvested in the indicated time points for further analysis. Furthermore, for other assays, HEK293T cells were treated with 1 μ M thapsigargin and 10 μ M Mg132 after 42-48 hours transfection, independently.

3.2.4. Lentiviral Particle Production

Lentiviral particles were generated to get stable cell lines. The lentiviral vector containing the puromycin resistance gene as a selectable marker was used to generate particles (Figure 3.1A). HEK293FT cells ($\sim 8 \times 10^6$) were seeded to 100 mm cell culture dish (TPP) the day before transfection of lentiviral vectors to obtain 90% confluency. The next day, cells were reached 90% confluency were transfected as mentioned in section 3.2.2. In order to pack lentiviral expression vectors (pLenti-III-HA, SIK2 WT-HA, SIK2 KI-HA, IRE1 WT-HA, IRE1 KI-HA), psPAX2 and pCMV-VSV-G plasmids were used. After overnight (16 hours) incubation media were renewed adequate amount of fresh medium and cells were allowed to produce lentiviral particles for 24 hours. End of the 24 hours, cell media containing viral particles was collected into 15 ml tubes and fresh media added onto cells and cultured for another 24 hours for viral production. The next day, cell media was collected and mixed with the first one. Collected virus containing media was centrifuged at 500 g for 3 minutes and then the supernatant was filtered by using a 0.45 μ m filter syringe. Lentiviral particles were divided into 2 ml tubes and stored at -80 °C.

3.2.5. Transduction and Stable Cell Line Generation

The day before the transduction 600,000 HEK293T cells were seeded on one well of the 6 well cell culture plate. The next day, cell media was discarded and prewarmed lentiviral particles were added onto cells with the help of polybrene (8

$\mu\text{m/mL}$, final concentration). The medium was replaced with a fresh growth medium after overnight cultivation.

After 48 hours, cells were selected with puromycin ($2 \mu\text{l/mL}$, final concentration) which was determined by killing curve. The cell media changed every 3 days and cells were maintained as described in section 3.2.1. Puromycin selection was carried out for 7 days and continued up to 14 days with half of the puromycin concentration. Thus, cells were survived under puromycin treatment were generated stable cell lines. End of the selection procedure cell lysate was harvested, and expression level was confirmed with Western Blotting.

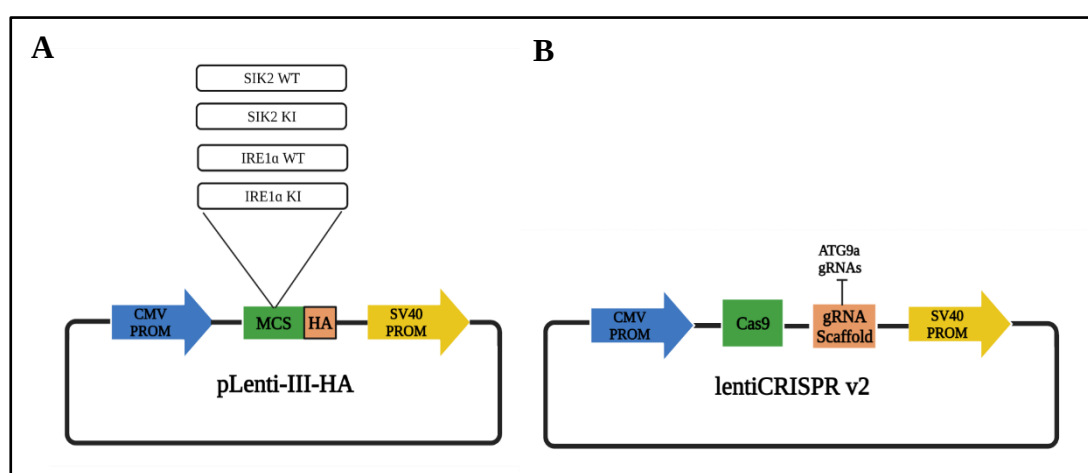


Figure 3.1: Demonstration of the pLenti-III-HA (A) and lentiCRISPR v2 vectors.

3.3. CRISPR Editing of ATG9a

Using Benchling software, the single guide RNAs (sgRNAs) were designed in accordance with Dr. Zhang Lab's instructions [118] by using Benchling software. The Zhang Lab GeCKO pooled library amplification protocol [119] was used to synthesize primers and clone into the lentiCRISPR v2 vector (Figure 3.1B). Four ATG9a-targeting sgRNA oligos (Table 2.4) were synthesized and tested to reduce the possibility of nonspecific targeting. HEK293T cells were transduced with the viral particles prepared in the same cell line as described in section 3.2.4 and 3.3.5. The sgRNA with the highest knockout efficiency as determined by Western Blot assay. Then Addgene's limiting dilution protocol was performed to obtain single colonies in

96 well plates. Once single colony was formed, cells were allowed to grow back to 70-80% confluency before transferring them sequentially to 10 cm cell culture dishes. Finally, cells derived from several distinct colonies were checked for the expression level of ATG9a by performing Western Blot.

3.4. SDS-PAGE Gel Electrophoresis and Western Blotting

3.4.1. Cell Lysis, Protein Extraction and Protein Quantification

Protein lysates were harvested from HEK293T and HEK293FT cells by using RIPA lysis buffer. Cells were washed with 1x PBS and the surface of the culture dish scraped with lysis buffer. The cell lysate was incubated on ice for 10 minutes, then centrifuged 15 minutes at 14000 rpm at 4°C. The supernatant was transferred to new 1.5 ml tube and protein lysates were stored at -20°C.

For the determination of the protein concentration Bicinchoninic acid (BCA) assay kit (Thermo Scientific, 23225) was used according to the manufacturer's instruction. A standard curve was obtained by using bovine serum albumin (BSA) dilutions ranging from 0.125 to 2 mg/ml. The concentration of the protein sample was calculated from the standard curve.

3.4.2. SDS-PAGE and Western Blot

Separating and stacking polyacrylamide gels were prepared as described in Table 3.4. Mini-Protean Tetra System (Bio-Rad) was used to cast, run the SDS-PAGE gels. The samples were prepared according to the result of the BCA assay within 4x SDS loading dye (1x, final concentration). Then, protein samples were boiled at 95°C for 5 minutes to denature proteins. After that, samples were loaded into gels. Loading volume was calculated according to the BCA assay. Gels were run at 80 Volt, once proteins passed the separating gel, voltage adjusted to 120 volts.

After the run was completed, Mini-Transblot Turbo (Bio-Rad) was used to transfer proteins to the PVDF membranes (Merck, IPVH00010). Following the transfer, the membrane was blocked with 5% non-fat milk powder within the 1x TBS-

T by shaking for 1 hour at room temperature. After incubation PVDF membranes were washed three times with 1x TBS-T for 4 minutes. Then, membranes were incubated with the specific primary antibodies which were prepared within 5% BSA containing TBS-T. Primary antibody incubation was carried out overnight at 4°C. The next day, primary antibodies were collected, and membranes were washed three times with 1x TBS-T for 4 minutes. Membranes were incubated with secondary HRP-conjugated antibody at 4°C for 2 hours on the shaker. After secondary antibody incubation membranes were washed again as mentioned. Immunoblot images were developed by adding WesternBright ECL solution (Advansta, K-12045-D20) and were visualized with ChemiDoc XRS+ (Biorad). ImageJ software was used for the analysis of the immunoblot images and housekeeping proteins were used for normalization.

Table 3.4: Preparation of SDS-PAGE gels.

	Separating (8%)	Separating (10%)	Separating (12%)	Stacking (5%)
1,5M Tris-HCl (pH 8.8)	2,6 ml	2,6 ml	2,6 ml	-
1M Tris-HCl (pH 6.8)	-	-	-	750 µl
SDS 10% (w/v)	100 µl	100 µl	100 µl	60 µl
Acrylamide: Bis-acrylamide (37.5:1, 40%)	2 ml	2,5 ml	3 ml	600 µl
APS 10% (w/v)	100 µl	100 µl	100 µl	30 µl
TEMED	10 µl	10 µl	10 µl	6 µl
dH ₂ O	5,2 ml	4,7 ml	4,2 ml	4,55 ml
Total volume	10 ml	10 ml	10 ml	6 ml

3.5. Co-Immunoprecipitation (Co-IP)

3.5.1. Lysate Preparation

After 48 hours of transfection HEK293T cells were treated with 1 μ M thapsigargin or DMSO at different time points. After harvesting cell lysate with, 500 μ l IP lysis buffer the lysate suspension was passed through an insulin-syringes 5 times. Then, the lysate was incubated on ice for 30 minutes after that samples were centrifuged at 14.000 rpm for 15 minutes at 4°C. The supernatant was stored at -20°C.

The total protein concentration in the lysates was quantified by using the BCA Assay kit as described in section 3.3.1. 10% of the sample was separated to use as input control for Western Blotting. The samples were pre-cleared by using the samples containing 1 mg protein were pre-cleared by using protein A/G plus agarose beads (Santa-Cruz, Sc-2003) to avoid non-specific protein-bead interaction. Before use, A/G plus agarose beads were equilibrated by washing with 300 μ l IP lysis buffer and centrifuged at 1000 g for 5 minutes 3 times. After the third wash, 1 mg protein lysate was pre-cleared with 20 μ l of protein A/G plus agarose beads by incubating the mixture for 30 minutes at 4°C with agitation. After that, the protein-bead mixture was centrifuged at 1000 g for 5 min at 4°C. The pre-cleared supernatants were transferred into fresh 1,5 ml microtubes and IP protocol was carried out.

3.5.2. Immunoprecipitation Using anti-FLAG M2 Affinity Agarose Bead

Anti-FLAG M2 agarose beads (Sigma, A2220) were used to immunoprecipitate FLAG-tagged recombinant protein. Firstly, for each sample 40 μ l FLAG-agarose beads were equilibrated by washing with 500 μ l IP lysis buffer and centrifuged at 5000 g for 1 minute 3 times. 1 mg of the pre-cleared lysates were added to the equilibrated FLAG-agarose bead and the mixture were incubated for 16 hours at 4°C with agitation. End of the incubation the protein-bead mixture was centrifuged at 1000 g for 5 minutes at 4°C. The supernatant was discarded, and the bead-protein complex was washed with 500 μ l IP lysis buffer containing protease and phosphatase inhibitors, at 1000 g for 5 minutes at 4°C 3 times. After the third wash, the bead-protein complex was resuspended with 30 μ l IP lysis buffer containing 1x SDS loading buffer and boiled at

95°C for 5 minutes. The whole IP samples were used for Western Blotting to detect the proteins which thought to be immunoprecipitated. Western Blot was performed as described in section 3.3.2.

3.6. Confocal Imaging

225,000 of HEK293T cells were grown in a 12-well plate containing sterilized microscope cover glass (circular, 20 mm). After overnight cultivation cells were transfected with fluorescent-tagged protein expressing vectors as described in section 3.2.2. Following the 42-46 hours post-transfection cells were treated with thapsigargin Mg132. After the determined time period, cell media was discarded and washed with 1x PBS three times. Then cells were fixed on a coverslip with 4% PFA and incubated for 10 minutes at room temperature. After washing three times with 1x PBS coverslip was inverted and placed on a slide that has mounting media. Prepared coverslips were visualized with Zeiss LSM 880 Confocal Laser Microscopy. ImageJ software was used for measuring integrated density and the overlap coefficients [120].

3.7. Statistical Analysis

Statistical significance of the differences was calculated by one or two-way ANOVA using GraphPad Prism 8 Software.

4. RESULTS

4.1. SIK2 Regulates IRE1 α Signalosome

We started with the characterization of SIK2 in HEK293T cells by defining the SIK2 activation profile in response to chemical ER stressor, thapsigargin. First, the level and activity of SIK2 were detected in response to treatment of cells with thapsigargin by using anti-SIK2 and pSIK2 antibodies respectively the latter of which recognizes the activation loop phosphorylation at Thr175 (human). Treatment of the cells with thapsigargin promoted a transient peak of 2-2.5-fold increase in pSIK2 level at around 30 minutes of induction which follows a similar activation profile reported earlier in retinal Muller glia cells [121]. pSIK2 level peaked at 30 min of ER stress induction started to decline gradually to return pre-induction level within 3 hours and remained unchanged for another three hours (Figure 4.1A). We monitored the pSIK2 level for an extended period of time up to 24 hours after ER stress induction although no changes were detected in the level of SIK2 activity compared to control cells demonstrating that SIK2 activity but not the level was responsive to ER stress induction in a spatiotemporal manner (data not shown). We then sought to understand if stress induced local SIK2 activity has anything to do with the proposed role of SIK2 in autophagy [114].

To address this question, we constructed stable HEK293T cell lines expressing IRE1 WT, IRE1 KI, SIK2 WT, and SIK2 KI using an integrated lentiviral mammalian expression system. Lentiviral particles containing the gene of interest were used to infect HEK293T cells in the presence of polybrene. Antibiotic selection was initiated after 48–72-hour post-transduction to generate pools stably expressing the gene of interest. Control group expressing HA tag only were used for the normalizations of the overexpressed genes with Western Blot. After a few subculturing and subsequent propagation of stable cell lines. Detection of stably expressed IRE1 α WT, IRE1 α KI, SIK2 WT, and SIK2 KI genes in HEK293T cells with Western studies demonstrated on average an order of magnitude higher protein levels compared to their endogenous counterparts in control cells (Figure 4.1B).

In order to use for the further functional assay, we generated ATG9a knockout HEK293T cell line (Figure 4.1C).

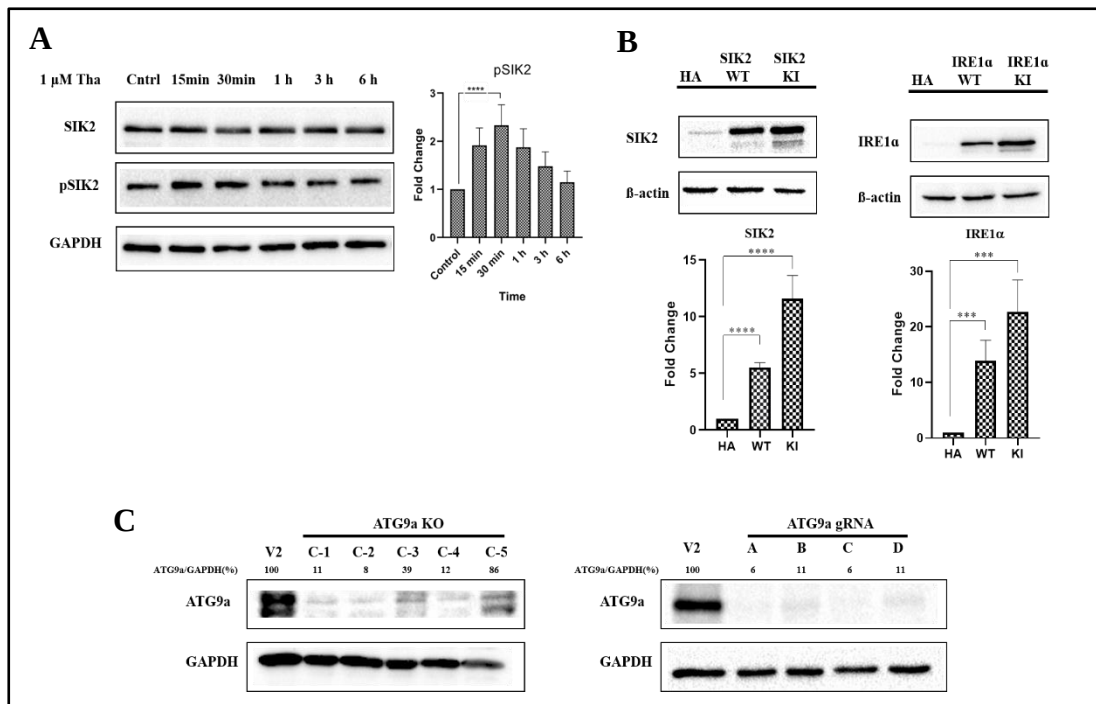


Figure 4.1: SIK2 activation profile of Hek293T cell upon 1 μ M thapsigargin (Tha) treatment for indicated time period (15 min to 6 h) (A). Confirmation of stable protein expression level of IRE1 α WT, IRE1 α KI, SIK2 WT and SIK2 KI in HEK293T cell line (B). Generation and confirmation of ATG9a knock-out (KO) HEK293T cell line (C). p-Values based on one-way ANOVA; ****<0,0001, ***<0,001.

4.2. Identification of IRE1 α -ATG9a-SIK2 Signalingosome

Based on our earlier identification of ATG9a as a novel SIK2 substrate in an LC-MS/MS based phosphoproteomics study conducted at Yale proteomics facility [117]. We decided on further investigating the biological significance of this interaction. Therefore, we started off with the co-immunoprecipitation studies (co-IPs) to validate physical interaction between ATG9a and SIK2. HEK293T cells transiently transfected both with Flag-SIK2 and ATG9a-RFP or Flag-SIK2 alone were used in co IPs with anti-flag antibody. Western Blot analyses of the resulting immunoprecipitants confirmed the authenticity of the interaction between ATG9a and SIK2 and the interaction appeared to be more pronounced when both proteins were overexpressed (Figure 4.2A). Intriguingly, chemically induced ER stress with thapsigargin (1 μ M) downregulated the SIK2 interaction with ATG9a to a significant extent suggesting an

unprecedented biological role for ATG9a presumably downstream or parallel to IRE1 α signaling.

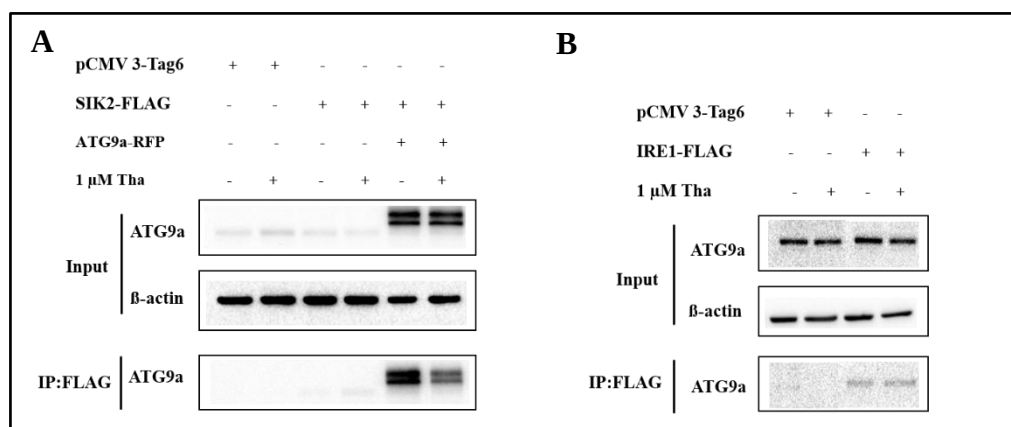


Figure 4.2: Co-immunoprecipitation of SIK2-FLAG either with endogenous ATG9a or ATG9a-RFP (A) and of IRE1 α -FLAG with endogenous ATG9a (B) upon 1 μ M Tha treatment for 6 hours in HEK293T cells. Co-IPs were carried out with anti-FLAG agarose bead and immunoprecipitants were analyzed by Western Blot using antibodies of interest.

SIK2 has been implicated in the upregulation of autophagosome formation, autophagosome lysosome fusion [114] and ERAD [113] by interacting with phosphorylating HDAC and VCP/p97 translocase respectively. Based on the apparent link between SIK2 and its regulatory role in ER based homeostatic processes we were intrigued by the idea that SIK2 by interacting with ATG9a master regulator of autophagosome formation and the only transmembrane protein of autophagy process may mediate crosstalk between the IRE1 α signalosome and autophagy process thereby apoptosis [92]. In order to test this hypothesis, we decided to explore the possibility that ER localized ATG9a may engage in IRE1 α signalosome directly or indirectly by interacting with IRE1 α or its two well characterized downstream effectors TRAF2 and CHIP respectively. Although in this study we did not address the possible involvement of other components of the IRE1 α signalosome we saved this idea for future considerations with LC-MS/MS based explorative interactome studies. First, HEK293T cells overexpressing flag tagged IRE1 α were used in a series of co-IP studies with anti-flag antibody to see if IRE1 α is in complex with ATG9a on the ER membrane. Surprisingly, in our first co-IP study the interaction between IRE1 α and the endogenous ATG9a was observed on Western Blot. It should be note that our

results do not necessarily exclude the possibility that this interaction is mediated by other proteins that may or may not include the known components of the IRE1 α signalosome (Figure 4.2B and 4.3).

On the other hand, we were able to show ATG9a SIK2 interaction which appeared to be inversely proportional to ER stress induction as depicted in Western Blot (Figure 4.2 and 4.3). However, the ER stress responsiveness of this interaction disappeared when we co immunoprecipitated the ATG9a with anti-flag antibody to flag tagged SIK2 warranting further investigation of the dynamic nature of this interaction. As expected further co-immunoprecipitations with anti-flag antibody identified the interaction between FLAG-ATG9a and the two other IRE1 α signaling components, CHIP and TRAF2. It is interesting to note that ER stress decreases the physical interaction between ATG9a and IRE1 α which is further corroborated and consistent with the concomitant decreases both in TRAF2 and CHIP interaction with ATG9a (Figure 4.3A). Intriguingly, the observed affects are totally reversed by overexpression of SIK2 suggesting that it may be the key enzyme responsible for the regulation of crosstalk between UPR and Autophagy via IRE1 α and ATG9a respectively on the ER membrane (Figure 4.3B).

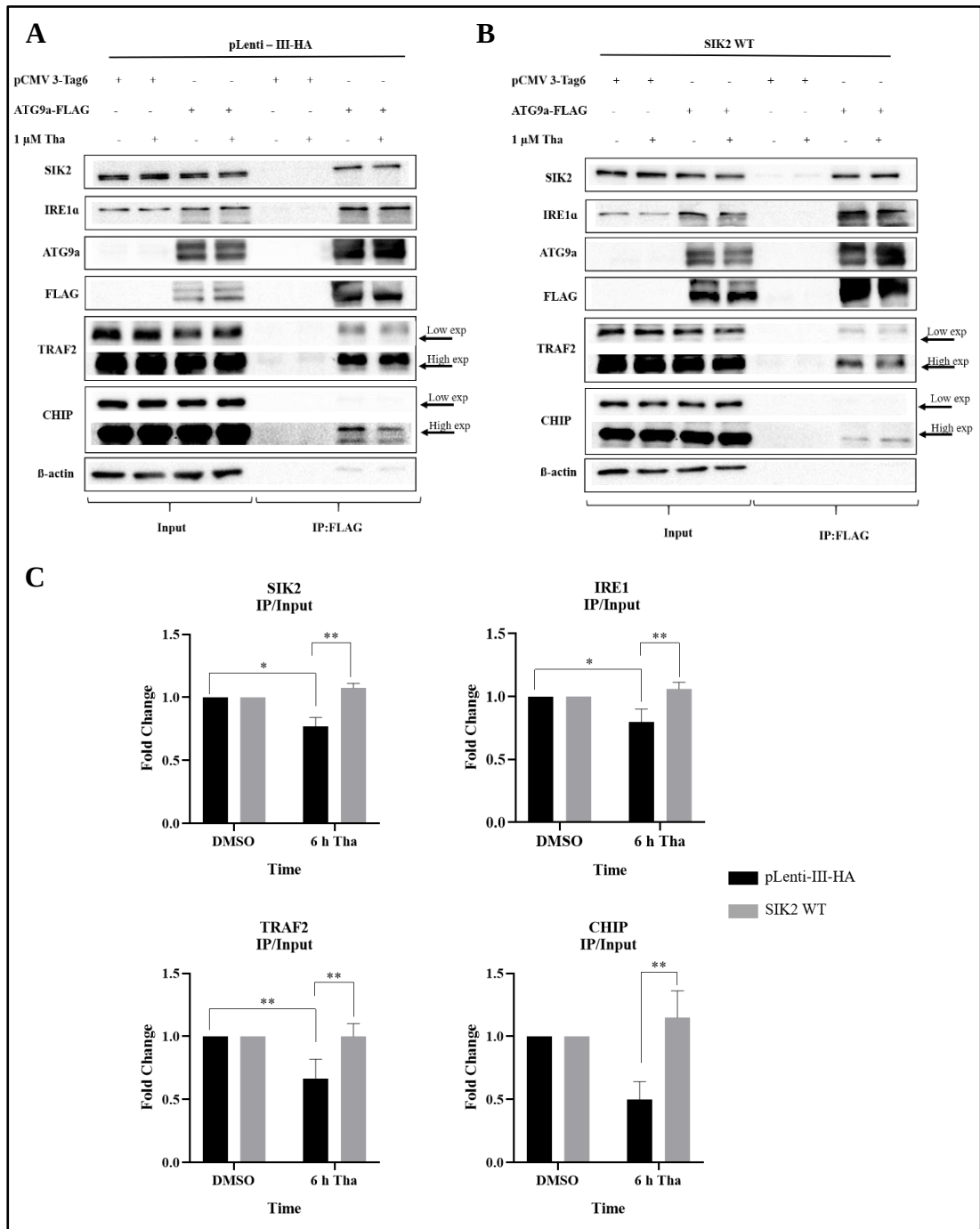


Figure 4.3: Stress induced and SIK2 dependent changes in ATG9a interactome. HEK293T cells stably over expressing pLenti-III-HA as a control (A) or the wild type SIK2 (B) were transfected with Flag-ATG9a for the anti-flag IPs. Cells were treated with 1 μ M Tha for 6 hours and lysates were immunoprecipitated with the anti-Flag agarose beads and then analysed for the presence of SIK2, IRE1 α and its downstream effectors TRAF2 and CHIP using Western Blot. p-Values based on two-way ANOVA; **<0,01, *<0,05.

Due to the earlier reports on SIK2 involvements both in the upregulation of Autophagy and ERAD processes we sought to evaluate the effect of SIK2 on the newly identified IRE1 α -ATG9a signaling complex which theoretically reside at the crossroads of these processes by overexpressing its wild type SIK2 WT and kinase inactive SIK2 KI dominant negative forms in HEK293T cells. In order to test this hypothesis cells were treated with thapsigargin for 30 min to represent maximal SIK2 activity preceding the co-IPs as described before probing the dynamic nature of IRE1 α signalosome (IRE1 α -ATG9a-TRAF2-CHIP). In parallel we also looked at the level of pJNK for assessing the activation status of TRAF2-ASK-JNK axis downstream of IRE1 α (Figure 4.4). Beside the two autophagy reporter proteins LC3B/II and p62 were also monitored by immunoblotting to assess the effect of SIK2 on IRE1 α and/or ATG9a mediated autophagy process (Figure 4.4A).

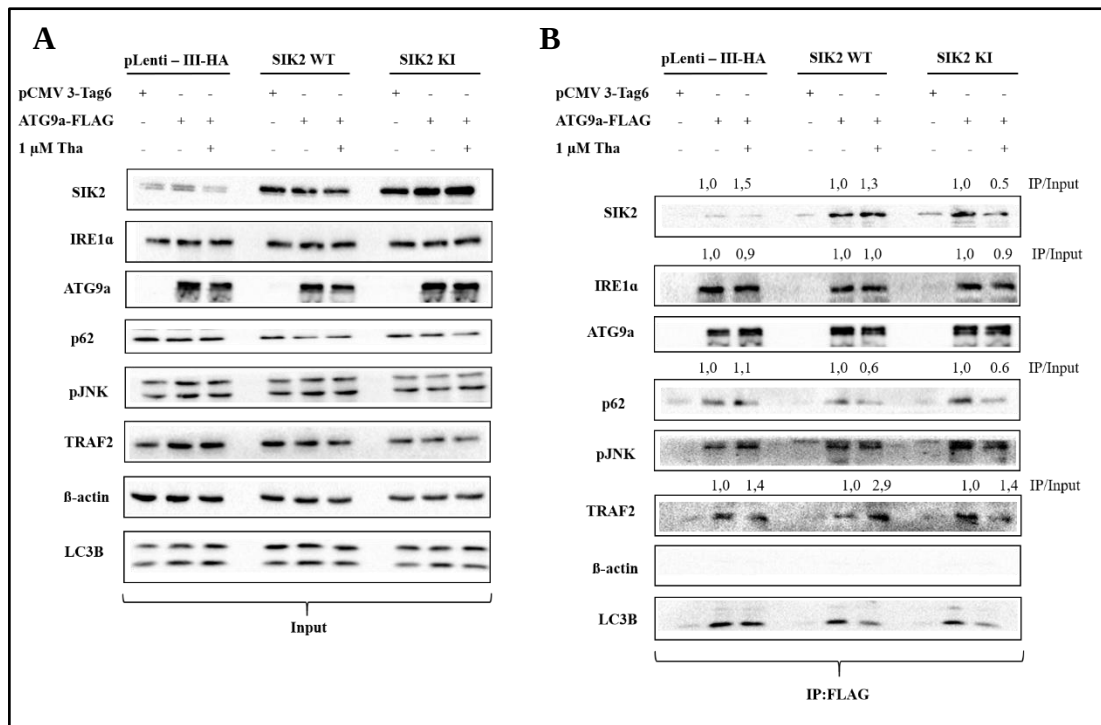


Figure 4.4: ATG9a interacts with IRE1 α signalosome. HEK293T cells stably overexpressing either SIK2 wild-type (SIK2 WT) or its kinase inactive form SIK2 (SIK2 KI) were transiently transfected with Flag-ATG9a or its vector backbone as control and subjected to ER stress agent thapsigargin for 30 minutes and lysates were immunoprecipitated with the anti-FLAG agarose beads and analyzed by Western Blot using antibodies specific to IRE1 α signaling components and the autophagy markers LC3 and p62.

Western Blot analysis indicated that neither wild-type nor kinase-inactive form of SIK2 has any effect on ATG9a- IRE1 α complex formation. The most intriguing finding of these co-IP studies was the robust interaction detected between ATG9a and TRAF2 in the presence of SIK2 activity under ER stress conditions suggesting a direct interaction between the two molecules presumably regulated by SIK2. We noticed that SIK2 over expression reduced the autophagy flux at 30 min of ER stress induction which is inconsistent with the earlier reports indicating positive role for SIK2 in autophagy flux. However, the absence of data pertinent to later time points as reported by others preclude the comparison between these studies. Therefore, for the assessment of more accurate and time dependent effect of the SIK2 on autophagy process we need to monitor autophagy for longer period times consistent with the other reports. The interaction between ATG9a and SIK2 is enhanced in the presence of ER stress yet downregulated when kinase inactive SIK2 KI form is overexpressed (Figure 4.4B). This appears to be in line with the SIK2 kinase activity dependent interaction between ATG9a and TRAF2 (Figure 4.4B).

To further investigate SIK2 dependent interaction between ATG9a and IRE1 α we performed fluorescent-based co-localization assays on SIK2 WT and SIK KI stable HEK293T cell lines transiently transfected for the co-expression of ATG9a-RFP and IRE1 α -sYFP (Figure 4.5.). Although, the co-localization of ATG9a and IRE1 α on the ER membrane remained relatively unchanged in response to acute ER stress induction (30 minutes) without respect to presence of SIK2 we noticed a stress dependent downregulation of this co-localization under prolonged stress conditions (6 hours) yet the affect is less pronounced when SIK2 was also supplied independent of its kinase activity (Figure 4.5).

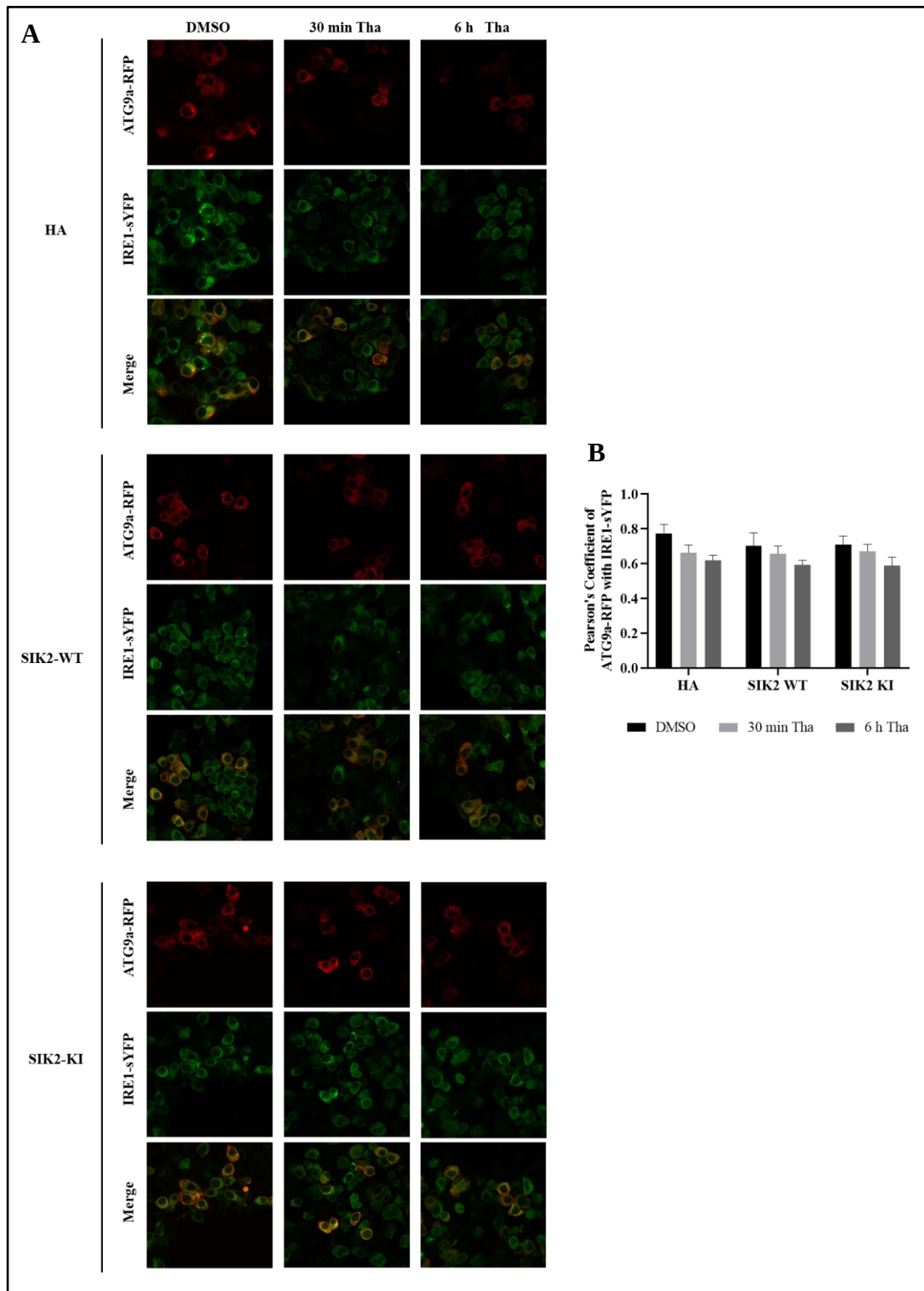


Figure 4.5: The effect of SIK2 on ATG9a and IRE1a co-localization. Cells were treated with Thapsigargin for 30 minutes and 6 hours. Control cells received DMSO. Analysis of the colocalization were performed with ImageJ software. The images of at least 45-65 cells were processed for each condition. Error bars represent mean with standard deviation (Mean with SD).

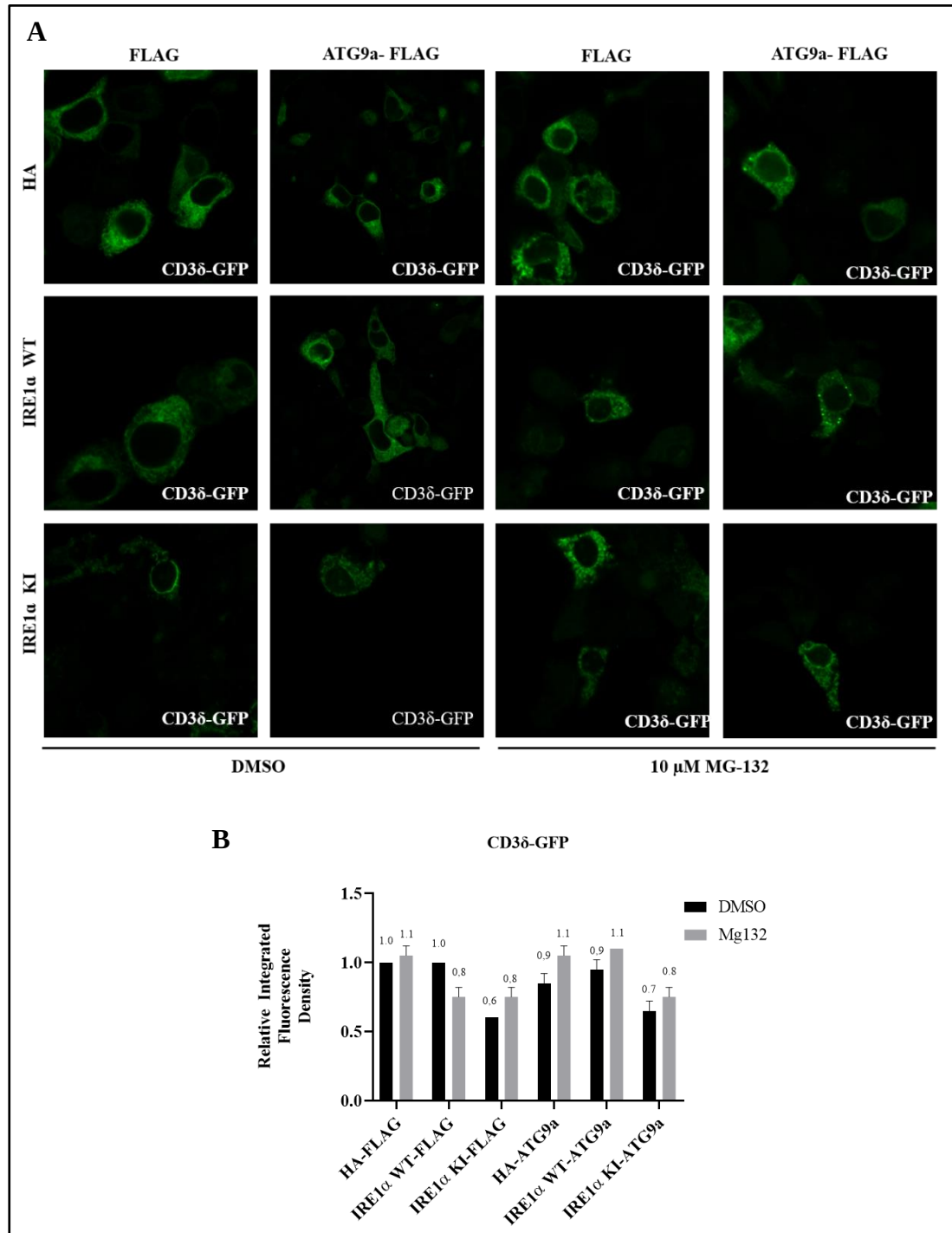


Figure 4.6: Effects of ATG9a and IRE1α on CD3δ-GFP fluorescence in the absence or presence of proteasome inhibitor Mg132 (10 μ M). HEK293T-HA, HEK293T-IRE1α WT and HEK293T-IRE1α KI cells were transiently transfected with CD3δ-GFP construct. After 48 h transfection cells were treated with 10 μ M Mg132 for 6 hours. DMSO was applied to control groups as a vehicle. The images of at least 25-40 cells were processed for each conditions Error bars represent mean with standard deviation (Mean with SD).

4.3. The Effects of ATG9a-IRE1 α Interaction on Autophagy

Although the recent structural and biochemical studies provided unprecedented molecular insight into the concerted action of ATG9a and ATG2 in lipid transfer from ER to nascent nucleating autophagosomes the complex ATG9a interactome studies repeatedly emerged in the interactome databases and in our lab indicates more elaborate functional engagements of ATG9a in or out of autophagy initiation. Here we investigated the autophagy mediated clearance of GFP tagged ERAD substrate CD3 δ a subunit of T cell receptor by chemically interfering with the 26S proteasome to avoid possible confounding affect contributed by ERAD. Consistent with the Western studies ATG9a overexpression enhanced the clearance of Cd3 δ both in the absence and presence of ERAD albeit to a greater extend in the former. Furthermore, the level of Cd3 δ remained relatively the same without respect to accompanying ERAD indicating a significant role for autophagy in the clearance of a well-defined ERAD substrate. Although this affect appears to be blunted by co expressing IRE1 α our study warrants further investigations in the presence of more specific proteasome inhibitors.

5. DISCUSSION

Obesity is referred to as a global pandemic caused by pathological increase of body fat content. Obesity considered by itself as a metabolic chronic disease has been firmly associated with the development of various metabolic and neurodegenerative diseases and some cancers [3]. Low grade inflammation or metainflammation has been invariably reported in these metabolically active tissues including the central nervous system CNS of obese individuals and animal models [122]. However, despite the enormous global effort the molecular underpinnings of the metabolic inflammation in peripheral and CNS tissues have yet to be fully elucidated. Two interrelated mechanisms, UPR and autophagy appear to underlie the initiation of inflammatory pathways under persistent unmitigated ER stress conditions in these aforementioned tissues [123]. Intriguingly, here in this study we identified a novel molecular interface between autophagy and Ubiquitin Proteasome System (UPS) mediated by the only membrane protein of autophagy ATG9a and the hemostatic stress kinase SIK2 respectively [7], [113], [114].

SIK2 is a serine/threonine kinase implicated in the regulation of various metabolic pathways such as lipogenesis, gluconeogenesis, and ketogenesis. SIK2 is known to regulate insulin secretion from pancreatic beta cells under physiological conditions. SIK2 may play critical role in cell survival, proliferation, and differentiation of various cell types [124]. It also plays regulatory roles in maintaining protein homeostasis by upregulating ERAD and autophagy pathways under cellular physiological and cellular stress conditions. The level and the activity of SIK2 was reported to be increased in the white adipose tissue (WAT) of obese mouse models. In a LC-MS/MS based phosphoproteomics study our group identified ATG9a as a novel substrate for SIK2. Phosphopeptide analysis of tryptic peptides indicated serine 735, 758, and 741 residues as potential phosphorylation sites for SIK2 under the ER stress conditions [117].

ATG9a the only transmembrane protein of over 30 ATGs primarily involved in autophagosome formation by transferring lipids from the source membranes to the nascent autophagosomes with the aid of ATG2 [125]. ATG9a a six-pass transmembrane protein with its N- and C- terminals facing the cytoplasmic side of the membrane form trimer in the ER membrane. ATG9A interactome studies unveiled

that it interacts with other proteins primarily via its cytoplasmic part [72], [126], [127]. Intriguingly, phosphoproteomics data revealed putative SIK2 phosphorylation sites at residues S735, S758 and S741 within the C terminal part of ATG9a overlapping with the binding sites of other interactors [72], [92], [128]. In addition to ER, ATG9a also localizes to the trans-Golgi network, recycling endosomes, early endosomes, and late endosomes in nutrient-rich conditions, but upon autophagy induction ATG9a relocates between these sites. Recent studies reported autophagy independent roles for ATG9a including inflammation, secretion, and axon maintenance. Based on the phosphoproteomics data along with the SIK2's involvement in the upregulation of both autophagy and the ERAD processes, we decided to investigate the possible interaction between SIK2 and ATG9a with IP studies in cells chemically induced or not for the ER stress.

Before doing that we initially checked the temporal SIK2 activation profile in HEK293T cells in response to ER stress induction with thapsigargin. The phospho specific antibody against the activation loop Thr175 was used to detect phosphorylation status of SIK2 during six hours of treatment with THA. ER stress resulted in a transient 2-2.5-fold increase in pSIK2 levels peaking at around 30 min of THA treatment. pSIK2 returned to a basal level within 3 hours of ER stress induction and stayed the same for another 3 hours (Figure 4.1A). Next we interrogated if stress-induced transient SIK2 activity had anything to do with role of SIK2 in autophagy initiation or progression as we pointed out earlier [114], [115].

The interaction between ATG9a and SIK2 was confirmed by Western Blot analysis of immunoprecipitants from cells transiently overexpressing both FLAG-SIK2 and ATG9a-RFP or FLAG-SIK2 alone after 6 hours of thapsigargin treatment (Figure 4.2A). It is proposed that SIK2 plays important regulatory roles in proteostasis presumably by acting on autophagy and ERAD processes. Yet, the exact role of SIK2 in autophagy is not fully elucidated. Therefore, we were intrigued by the idea that SIK2 may be the long sought mediator of crosstalk between the ERAD and Autophagy pathways by bringing together IRE1 α branch of the UPRosome and the ERAD machinery through ATG9a the only membrane proteins of autophagy [92], [114]–[116]. In order to verify this hypothesis, co-IPs were performed with anti-flag antibody from HEK293T cells transiently transfected with FLAG-IRE1 α and treated or not with thapsigargin for six hours. In Western Blots we detected the interaction between the endogenous ATG9a, and flag tagged IRE1 α independent of ER stress induction (Figure

4.2B). Then we asked whether this interaction is direct or indirectly mediated by TRAF2 or CHIP which are the two well defined downstream effectors of IRE1 α . As expected, both TRAF2 and CHIP were co-immunoprecipitated with FLAG tagged ATG9a suggesting possible involvement of these two E3 ligases in IRE1-ATG9a complex formation. Surprisingly, further co-IPs identified a slightly reduced interaction between ATG9a and IRE1 α in response to ER stress concomitant with a corresponding decline in TRAF2 and CHIP levels indicating a functional co-existence among these signaling components (Figure 4.3A).

Despite the ER stress mediated downregulation of this signaling complex we identified a counteracting positive regulation mediated by SIK2 (Figure 4.3B). Based on the data obtained in this study and others we proposed a mechanistic model in which SIK2 may act as a molecular switch to toggle between ERAD and autophagy processes presumably by phosphorylating VCP/p97 and ATG9a respectively to maintain proteostasis under ER stress conditions. However, if the ER stress persists this molecular switch may promote apoptotic pathways downstream of IRE1 α . Taken together, our data suggests that both hormone (physiological) and stress (ER stress) responsive kinase SIK2 could be the key regulatory enzyme that mediate cross talk or act as a switch between UPR and autophagy pathways.

Western Blot analysis of co-IPs revealed that wild type or kinase inactive form does not have any noteworthy effect on formation of IRE1 α -ATG9a complex (Figure 4.4). We also performed co-localization assay to further investigate SIK2 dependent IRE1 α -ATG9a interaction. HEK293T cells were stably overexpressed with SIK2-WT and SIK2 KI, respectively and were transiently co-transfected with both ATG9a-RFP and IRE1 α -sYFP and confocal analysis carried out. Neither overexpression of SIK2-WT nor SIK2 KI form has any significant effect on co-localization of ATG9a and IRE1 α complex on the ER membrane in response to 30 minutes acute ER stress induction (Figure 4.5) which stand by with the results of co-IPs (Figure 4.4). The authenticity of IRE1 α complex was also confirmed by probing the signaling components of IRE1 α complex, TRAF2 and we also checked level of the pJNK to assess the activation status of TRAF2-ASK-JNK axis which are known to be downstream of IRE1 α . (Figure 4.4). Interestingly, in contrast to steady state interaction profile of IRE1 α with ATG9a, under ER stress conditions, a robust interaction between ATG9a and TRAF2 was detected in the presence of SIK2 activity while this interaction was significantly reduced as a result of overexpression of kinase inactive SIK2

suggesting that a direct interaction between the two molecules might be regulated by SIK2 as a stress kinase. Earlier study which performed in *Drosophila* revealed that interaction between *Drosophila* TRAF2 (dTRAF2) and ATG9a which stated that interaction regulates the JNK signaling pathway in response to oxidative stress. In the same study, the role of ATG9a regulating JNK signaling was also demonstrated in mammalian cells [92]. Taken together, SIK2 activity might be able to regulate dynamic nature of the IRE1 α /TRAF2/JNK arm of ER stress via the ATG9a (Figure 4.4B) Besides that we also noticed that overexpression of SIK2 reduced the autophagy flux at 30 min of ER stress induction by checking two autophagy reporter proteins LC3-I/II and p62 (Figure 4.4A) which is inconsistent with the earlier reports indicating that SIK2 positively regulates autophagosome and lysosomes fusion via its kinase activity [114], [116].

These co-IP results provided impetus to investigate the autophagy dependent clearance of CD3 δ in HEK293T cells IRE1 α or its kinase inactive form overexpressed with ATG9a in the absence/presence of proteasome inhibitor Mg132. It is well-known that IRE1 α upregulates ERAD components via XBP-1 in response to ER stress induction [40]–[42]. The degradation of ERAD substrate CD3 δ was monitored both in the absence and the presence of proteasome inhibitor MG132 in the cells overexpressing ATG9a. Overexpression of ATG9a alone appears to enhance the destruction of the CD3 δ . The level of CD3 δ remained relatively unchanged despite the presence of MG132 suggesting the inclusion of autophagy process in the clearance of CD3 δ . Surprisingly, in the absence of IRE1 α kinase activity CD3 δ clearance was more noticeable even when ERAD activity was inhibited, and the effect was independent of ATG9a (Figure 4.6). The kinase activity of IRE1 α might have impact on its interaction with ATG9a. Further investigations are needed to unravel the biological significance of ATG9a-IRE1 α interaction for the precise allocation of CD3 δ clearance by ERAD and autophagy processes respectively.

As a result of this study, we confirmed the physical interaction between ATG9a and SIK2 using co-IPs and fluorescent based co-localization studies providing further support for the SIK2 mediated phosphorylation of ATG9a as we reported earlier in a LC-MS/MS based phosphoproteomics study. When we consider the crosstalk between the autophagy and UPS/ERAD molecular details are not fully understood, therefore, in the future, further studies should consider revealing details of ATG9a-IRE1 α interaction and function of IRE1 α -ATG9a-SIK2 signalosome.

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BIOGRAPHY

Gülcan Kartal graduated from Kenan Evren Anatolian High School. She earned her bachelor's degree in Molecular Biology and Genetics from Istanbul University, Istanbul, Turkey, in 2015. She joined Gebze Technical University, Graduate School of Natural and Applied Sciences, Department of Molecular Biology and Genetics, in September 2017 and graduated in 2021.