

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

**INVESTIGATING THE ROLE OF SIK2 IN HYPOTHALAMIC
POMC LIKE NEURONS**

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

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PROF. DR. FERRUH ÖZCAN

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GEBZE TEKNİK ÜNİVERSİTESİ
FEN BİLİMLERİ ENSTİTÜSÜ

SIK2'NİN HİPOTALAMİK POMC BENZERİ
NÖRONLARDAKİ ROLÜNÜN
ARAŞTIRILMASI

AYŞE YAZICI
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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Gebze Teknik Üniversitesi Fen Bilimleri Enstitüsü Yönetim Kurulu'nun
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İMZA/MÜHÜR

ÖZET

Günümüzde obezite, yüksek kalorili diyet ve hareketsiz yaşam tarzını ile ilişkili epidemik bir sağlık sorunu olarak ele alınmaktadır. Obez bireylerde ve hayvan modellerinde, merkezi sinir sisteminde özellikle hipotalamusta, periferik metabolik dokulardan çok daha önce tespit edilebilen düşük dereceli metabolik inflamasyonun (meta-inflamasyon) obezite etiopatogenezinde önemli rol oynadığı düşünülmektedir. Enerji dengesinden sorumlu olan AgRP/POMC nöronlarındaki insülin ve leptin direncinin kaynağı olarak, ER stresi ve inflamasyon gösterilmektedir. Fakat bu süreçlerin altında yatan moleküler mekanizmalar halen tam olarak aydınlatılamamıştır. Merkezi sinir sistemlerinde fonksiyonel IRS2 ve IRS4 proteini susturulmuş farelerde, obezite ve azaltılmış enerji harcamasına bağlı olarak hiperglisemi ve insülin direnci geliştirmektedir. Bu durum, SIK2 proteini susturulmuş farelerde rapor edilen insülin direnci, besin alımındaki ve termogenezdeki azalma ile birlikte ele alındığında, SIK2 ve IRS4 arasında hipotalamik nöronlara özgün bir etkileşimin varlığını akla getirmektedir. Bu çalışmada, beslenme ve enerji dengesinde kritik öneme sahip POMC-benzeri nöronal hücre hattında SIK2-IRS4 ilişkisi irdelenmektedir. İlk defa bu çalışma ile daha önce grubumuz tarafından belirlenen IRE1-IRS4-SIK2 sinyal kompleksinde IRE1'in SIK2'yi fosforlayarak aktive ettiği gösterilmiştir. Bu çalışma aynı zamanda SIK2'nin hücresel enerji durumuna bağlı olarak aktivasyonunu sağlayan ve uzun süredir araştırmalara konu olan LKB1-bağımsız SIK2 aktivasyonunun IRE1 tarafından gerçekleştirildiğini gösteren ilk yayındır. Buna ek olarak IRE1-IRS4-SIK2 sinyal kompleksinin muhtemel diğer üyelerini belirlemek amacıyla yürüttüğümüz immün-çöktürme bağlantılı kütle spektrometresi (IP-MS) çalışmalarında AP3 (ve diğer izoformları) gibi vesiküler transport ile ilişkili proteinler ortaya çıkarılmıştır. SIK2 tarafından ER stresine bağlı olarak düzenlendiğini gösterdiğimiz IRE1-IRS4 sinyal yolağındaki proteinlerin, ileride obezite, Alzheimer, ALS ve PD gibi metabolik ve nörodejeneratif hastalıkların tedavisinde farmakolojik hedefler olabileceğini öngörmekteyiz.

Anahtar Kelimeler: Obezite, ER Stres, Hipotalamus, Enerji Dengesi, SIK2, Metainflamasyon.

SUMMARY

Obesity is seen as a global epidemic as an inevitable consequence of a high-calorie diet and sedentary lifestyle. Metainflammation observed continuously in the central nervous system and peripheral metabolic tissues of obese individuals and animal models are believed to be one of the most important factors linked to obesity etiopathogenesis. Although ER stress and inflammation are attributed as the source of insulin and leptin resistance in AgRP/POMC neurons responsible for energy homeostasis, the molecular mechanism underlying these processes has not been fully elucidated. IRS2 and IRS4 knockout mice in their central nervous system develop hyperglycemia and insulin resistance in association with obesity and reduced energy expenditure. This highlights the possibility of a neuron-based interaction between SIK2 and IRS4 when considering together with insulin resistance and reduced food intake in SIK2 deficient mice. In this study, the interaction between SIK2 and IRS4 in the POMC-like neuronal cells, which is critical in nutrition and energy balance, is examined. For the first time with this study, it was shown that IRE1 activates SIK2 by phosphorylating it within SIK2-IRE1-IRS4 signaling complex. In addition, with immunoprecipitation-coupled mass spectrometry (IP-MS) studies that we conducted to identify other possible members of this signaling complex, proteins associated with vesicular transport such as AP3 were revealed. We anticipate that proteins in this signaling complex, which we have shown to be regulated by SIK2 due to ER stress, may be pharmacological targets in the future treatment of metabolic and neurodegenerative diseases such as obesity, Alzheimer's, ALS and PD.

Key Words: Obesity, ER Stress, Hypothalamus, Energy Balance, SIK2, Metainflammation.

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LIST of ABBREVIATIONS and ACRONYMS

<u>Abbreviations</u>		<u>Explanations</u>
<u>and Acronyms</u>		
°C	:	Centrigrade degree
G	:	Gravity
M	:	Meter
M	:	Molar
mM	:	Milimolar
uM	:	Micromolar
Ml	:	Mililiter
Ng	:	Nanogram
U	:	Unit
A	:	Alpha
β	:	Beta
Δ	:	Delta
AgRP	:	Agouti-related protein
ALS	:	Amyotrophic lateral sclerosis
AMP	:	Adenosine monophosphate
AMPK	:	Adenine monophosphate-activated protein kinase
ARC	:	Arcuate nucleus
ATF4	:	Activating transcription factor 4
ATF6	:	Activation transcription factor 6
BAT	:	Brown adipose tissue
BCA	:	Bicinchoninic acid
BCL2	:	B-cell lymphoma 2
BSA	:	Bovine serum albumin
Ca	:	Calcium
cAMP	:	Cyclic AMP
CHOP	:	C/EBP homologous protein
cDNA	:	Complementary deoxyribonucleic acid
CRTC2	:	CREB regulated transcription coactivator 2
DMEM	:	Dulbecco's modified eagle's medium
eIF2α	:	Eukaryotic translation initiation factor 2

ER	:	Endoplasmic reticulum
ERAD	:	Endoplasmic reticulum-associated protein degradation
FBS	:	Fetal bovine serum
FOXO	:	Members of the class O of forkhead box transcription factors
Hsp	:	Heat shock protein
IKK β	:	Inhibitor of nuclear factor kappa-B kinase subunit beta
IP	:	Immunoprecipitation
IRE1	:	Inositol-requiring enzyme 1
IRS4	:	Insulin receptor substrate 4
LB	:	Luria-Bertani
mRNA	:	Messenger ribonucleic acid
NaCl	:	Sodium chloride
NF-kB	:	Nuclear factor kappa-B
PAGE	:	Polyacrylamide gel electrophoresis
PD	:	Parkinson's disease
PCR	:	Polymerase chain reaction
PERK	:	PKR-like ER kinase
POMC	:	Pro-opiomelanocortin
PVDF	:	Polyvinilidin difluoride
RNA	:	Ribonucleic acid
RT-PCR	:	Reverse transcriptase-polymerase chain reaction
SDS	:	Sodium dodecyl sulphate
SIK2	:	Salt-inducible kinase 2
T _m	:	Melting temperature
TRAF2	:	TNF receptor-associated factor 2
UPR	:	Unfolded protein response
WHO	:	World Health Organization

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1. INTRODUCTION

1.1. Obesity and Energy Balance

Obesity has been considered a worldwide epidemic. According to the World Health Organization (WHO), 650 million adults obese were in 2019 [1]. Obesity is also considered as a risk factor for various diseases including metabolic syndrome, type 2 diabetes, neurodegeneration, cardiovascular diseases, and cancer [2], [3] which threaten human health and longevity.

A delicate balance between food intake and energy expenditure ensures energy homeostasis thereby body weight control. Different brain areas including dorsal raphe nucleus [4], amygdala [5], the paraventricular nucleus of the hypothalamus (PVN) [6], [7], and specified neurons of the arcuate nucleus (ARC) [8] collectively are involved in energy balance. The peripheral hormones, leptin, insulin, and ghrelin; nutrients, glucose and free fatty acids act differently on these hypothalamic anorexigenic; POMC/ CART, and orexigenic; NPY/ AgRP neurons [9]. In a fasting state, the secretion of agouti-related protein (AgRP) and neuropeptide Y (NPY) neuropeptides increases food intake whereas in feeding state the secretion of proopiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcript (CART) neuropeptides decreases food intake and body weight [9], [10].

Leptin produced by adipose tissue is one of the key factors regulating energy homeostasis in proportion to total body fat mass. Leptin binding to its receptor activates the Janus kinase JAK-STAT3 axis. The phosphorylated signal transducer and activator of transcription 3 (STAT3) promotes the expression of POMC [11] while inhibiting the expression of AgRP [12] by binding to their cognate promoters. Insulin is secreted from pancreatic β cells in response to blood glucose level in a short term. Insulin, like leptin, produces anorexigenic effects by concomitantly promoting POMC activation and suppressing AgRP neurons. Binding of insulin to its cognate receptor recruits insulin receptor substrate (IRS) to stimulate phosphoinositide 3-kinase (PI3K)- protein kinase B (AKT) pathway [13]. The activated AKT regulates many proteins and transcription factors including FOXO [14], mTOR [15], [16], and, AMP-activated protein kinase (AMPK) [16], [17] to regulate metabolic pathways and protein homeostasis. Upon phosphorylation, FOXO which is an upstream inhibitor of POMC

expression is exported from the nucleus thereby relieving the negative effects on POMC expression. On the other hand, the nuclear export of FOXO in AgRP neurons allows STAT3 binding to *agrp* promoter thus obstructing AgRP expression [14]. Hence, PI3K-FOXO signalling cascade is an intersection point for leptin and insulin action on appetite [15]. Moreover, Akt mediated phosphorylation of intracellular energy and nutrient sensor mTOR negatively regulates feeding as opposed to AMPK signal [16].

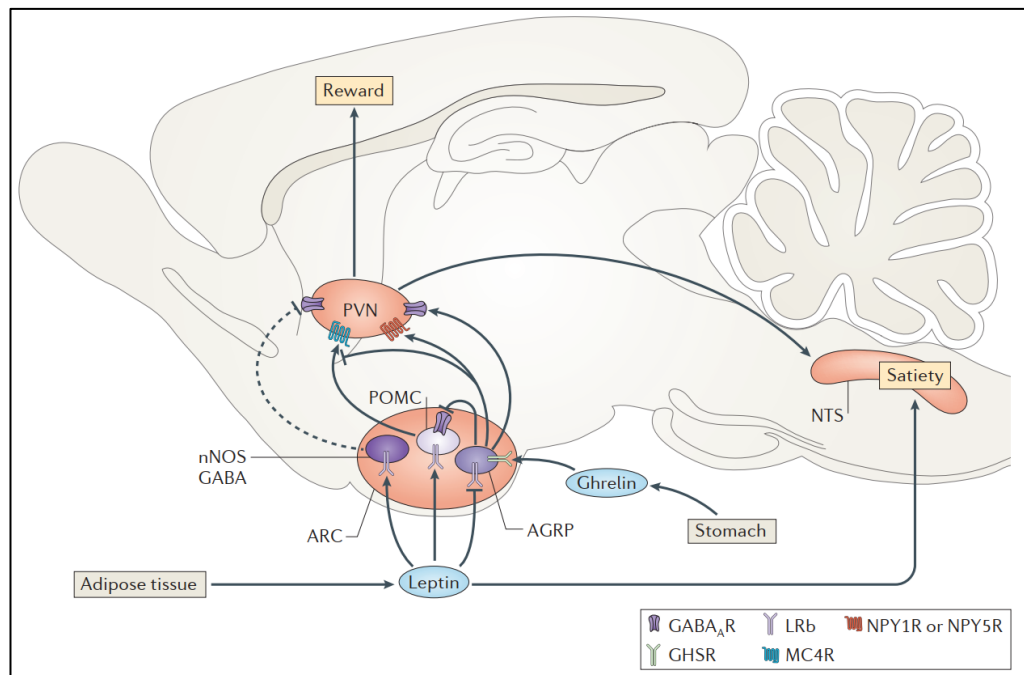


Figure 1.1: The neuronal circuits involving in normal energy balance. Abbreviations: GABA_AR, type A GABA receptor; GHSR, growth hormone secretagogue receptor (ghrelin receptor); LRb, leptin receptor; NPY1R, NPY receptor type 1.

The studies have shown that both leptin and insulin positively stimulate (depolarize) POMC neurons while inhibiting counter acting AgRP neurons. Ghrelin, a secreted gastric hormone, stimulates AgRP neurons leading to increased food intake [11], [12]. Indeed, the activated AgRP neurons inhibit POMC neurons by releasing gamma-aminobutyric acid (GABA). In parallel, NPY and GABA released by AgRP neurons act on innervating PVN neurons to increase food intake and appetite [18]. Contrarily, the stimulation of POMC neurons by leptin or insulin stimulate the secretion of α -melanocyte stimulating hormone (α -MSH) which targets melanocortin receptor 4 (MC4R) expressing PVN neurons to decrease food intake and increase

energy expenditure thereby regulates feeding behavior [19]. The neuronal circuits involved in normal energy balance are schematized as in Figure 1.1 [20]. Previous studies showed that the absence of MC4R is linked to development of obesity and hyperphagia, hyperinsulinemia, and hyperglycemia in mice [21].

1.2. Endoplasmic Reticulum (ER) Stress

The endoplasmic reticulum (ER) is a membranous subcellular organelle where protein synthesis, degradation, folding, assembly and secretion; lipid and cholesterol biosynthesis; calcium storage, and post-translational modifications take place. The imbalance between protein load in ER lumen and folding capacity of the ER triggers the accumulation of misfolded protein in the lumen causing “ER stress” and related compensatory mechanisms called unfolded protein response (UPR). UPR system supports the cell to restore ER homeostasis by up-regulating foldases and chaperons, while decreasing protein translation, inducing ER membrane synthesis to increase ER volume, and degrading of the misfolded protein through ER-associated degradation (ERAD) [22] and ER-associated autophagic pathways [23]. The UPR has three ER-localized transmembrane sensors and transducers including protein kinase R (PKR)-like ER kinase (PERK; also known as the eukaryotic translation initiation factor 2 α kinase 3) [24], inositol-requiring enzyme 1 α (IRE1 α) [25], and activating transcription factor 6 (ATF6) [26] to maintain ER homeostasis otherwise induce apoptosis. In the absence of ER stress, the sensor proteins are bound to ER chaperone binding immunoglobulin protein (BiP, also called GRP78). When the ER stress is triggered by any physiological or pathological perturbation such as nutrient deprivation, redox status, and ER calcium levels, BiP dissociates from the luminal domain of the sensor proteins, thus activates IRE1 α , PERK and ATF6 [25].

IRE1 harbors both kinase and endoribonuclease activities in its cytosolic compartment. Upon ER stress IRE1 α monomers on the ER membrane are believed to undergo either dimerization or oligomerization associated with the activation of its X-box binding protein 1 (XBP1) splicing or IRE1-dependent mRNA decay (RIDD) activities or TNF receptor-associated factor 2 (TRAF2) / c-Jun N-terminal kinase (JNK) pathways to set off survival or intrinsic death pathways. The autophosphorylation of IRE1 α induces its endoribonuclease activity towards XBP1

mRNA causing excision of the 26 nt introns leading to a shift in its reading frame for the expression of transcription factor XBP1s (spliced) [27]. XBP1s regulates the UPR target genes including ER chaperones, ER biogenesis, and components of ERAD machinery [28], thus contributing to protein homeostasis. In addition, IRE1 leads to degradation of the ER localized mRNA through its RIDD activity [29], in order to decrease the protein load on ER and regulating apoptotic pathways respectively [30]. The activated IRE1 α recruits adaptor protein TRAF2 and mediates autophagy and apoptotic pathways through JNK. Furthermore, TRAF2 mediated activation of JNK is implicated in phosphorylation of insulin receptor substrate 1(IRS1), resulting in perturbation insulin-mediated signal cascade [30], [31].

The cells could decide that prolonged ER stress is enough to overwhelm adaptive survival response by alternating the UPR outputs which bias UPR's cell fate away from adaptation to destructive cell death (terminal UPR). According to previously reported studies, terminal UPR-mediated apoptosis and secretory malfunctions are centrally involved in the pathogenesis of degenerative diseases such as diabetes [32] and neurodegeneration [33]. Additionally, the improper adaptive UPR triggers survival of cell tumors such as multiple myeloma [34]. The master regulator of the UPR and a bifunctional enzyme, IRE1 α could sense the unfolded protein through an N-terminal endoplasmic reticulum luminal domain, its monomers dimerize and oligomerize in the plane of the ER membrane. These oligomerization enables juxtaposition on its cytosolic kinase domain, allowing trans-autophosphorylation. In turn, this event conformationally activates IRE1 α 's RNase activity and causes XBP1s-mediated expression of UPR target genes [27]. Indeed, IRE1 α promotes adaptation to survival via the XBP1s axis. However, upon chronic -prolonged-ER stress, the hyperactivation of IRE1 α 's RNase extends its substrate specificity to endonucleolytic cleave of many ER-localized mRNA (RIDD activity) beside XBP1 splicing [29]. Under these chronic state, the precursors of apoptosis-inhibitory microRNAs are also cleaved by IRE1 α 's RNase [35].

Some small molecules provide the possibility of distinct allosteric control of IRE1 α 's kinase activity. APY29 binds to ATP-binding site to inhibit kinase activity as well as activate RNase-mediated XBP1 mRNA splicing even in the absence of ER stress. Compound 3 could inhibit the Kinase/RNase activity through the same ATP-binding site, even under ER stress [36]. KIRA6, another small molecule which is ATP-competitive, IRE1 α Kinase-inhibiting/RNase attenuator, allosterically blocks IRE1 α 's

RNase activity via breaking oligomerization. It was reported that KIRA6 enhances survival under acute ER stress, also with systemic administration, leads to preserved β -cells, improved glucose tolerance in Akita diabetic mice [37]. Additionally, the covalent modification-based RNase inhibitors, STF-083010, or 4 μ 8C34 allow kinase activity. STF-083010-mediated cytotoxic effect on human multiple myeloma promised that the IRE1-XBP1 axis is in terms of the anti-cancerogenic target [38]. On the other hand, the IRE1-XBP1 axis has been studied in the context of energy balance in hypothalamic POMC neurons. HFD fed POMC-specific XBP1 knockout (KO) mice tend to eat more without any significant body weight alteration via the compensatory effect of increased energy expenditure [110]. POMC-specific IRE1 depletion causes increased energy expenditure and resistance to HFD-induced adiposity, which is explainable with increased expression of POMC processing enzymes, prohormone convertase 2 (PC2), carboxypeptidase E (CPE), and peptidylglycine- α -amidating monooxygenase (PAM) promoting elevated α -MSH production [39]. ER stress induction or XBP1 deficiency in neurons causes hyperphagia with severe hypothalamic leptin resistance via UPR activation [40], however, XBP1-overexpressed POMC neurons results in increased energy expenditure thus resistance to obesity [41] via the possible positive effect on ER biogenesis.

PERK has a cytosolic kinase domain which phosphorylates eukaryotic initiation factor 2 subunit α (eIF2 α) under the ER stress condition [24] leading to global translational attenuation, however, selectively induces translation of the transcription factor ATF4 [42]. ATF4 upregulates its target genes related to redox control, autophagy, and protein folding and synthesis. The phosphorylated eIF2 α is dephosphorylated by growth arrest and DNA damage-inducible protein (GADD34) which itself is an ER stress-inducible gene acting in a negative feedback loop. Upon early ER stress, UPR-activated IRE1 induces the accumulation of XBP1s. Additionally, PERK-mediated phosphorylation of eIF2 α protects XBP1s from rapid mRNA turnover by inhibiting its translation. Whereas with prolonged ER stress, GADD34 mediates the dephosphorylation of eIF2 α and translational recovery of XBP1. The accumulated XBP1s also stimulates its own transcription (unspliced XBP1), and the loss of translational inhibition causes rapid XBP1s mRNA turnover. These findings manifested that the accumulation of the unspliced XBP1 neutralized the effects of the IRE1-mediated XBP1s cues, by supporting the negative feedback loop [46]. Also, in the case of the prolonged activation of UPR, ATF4 also engages

apoptotic pathways by inducing the expression of C/EBP homologous protein (CHOP) which down-regulates anti-apoptotic regulator BCL-2 family protein members [43].

ATF6 has a stress-sensing luminal domain and a cytoplasmic basic leucine zipper domain which acts as a transcription factor. Upon ER stress, ATF6 translocates from ER membrane to Golgi apparatus where it is processed to form an active DNA-binding domain (ATF6f) via sequential cleavage by the serine site 1 (S1P) and site 2 (S2P) proteases [44]. After ATF6f penetrates the nucleus, modulates the expression of its target genes involved in ERAD and UPR, also including BiP [45]. The activated ATF6 induces XBP1 expression thus contributing to the IRE1-XBP1s axis in adaptive UPR response [46]. On the other hand, ATF6-CHOP and caspase 12 pathway is previously reported in addition to ATF4-induced CHOP expression regarding ER stress-induced apoptosis [47]. It has been shown the NF kappa B activation by ATF6 which is suggested that ATF6-Akt-NF kappaB can contribute insulin resistance by up-regulating protein tyrosine phosphatase 1B (PTP1B) and suppressor of cytokine signaling 3 (SOCS3) [48].

The activation of UPR has been reported in the hypothalamic neurons during fasting/feeding state [41]. According to this study, the increased expression of XBP1s targeted genes involved in ER protein translocation, Golgi trafficking, besides activating transcription factor 4 (ATF4) and ATF6 has been seen on the AgRP neurons of fasted animals. Stimulation of expression of AgRP-specific ERAD-related transcripts has been also reported with fasting. However, refeeding of fasted mice activates the UPR in POMC neurons which are manifested by the increase of XBP1s, ATF4, and ATF6 expression [49]. Moreover, the link between autophagy and UPR has been interrogated regarding metabolic dysregulation in hypothalamic neurons related to obesity. ATF4 KO mice on both POMC and AgRP neurons result in similar resistance to diet-induced weight gain via negatively regulation of autophagy related 5 (ATG5) protein. While deletion of both ATG5 and ATF4 cause HFD-induced weight gain, the absence of negative effect of ATF4 on ATG5 induces autophagy and increased α -MSH secretion thus protecting from obesity [50], [51].

2.1.1. Hypothalamic ER Stress in Obesity

ER stress and the UPR have a crucial role in metabolic dysfunction in obesity pathogenesis. According to various genetic and pharmacologic studies, chemical ER stress inducers, such as thapsigargin and tunicamycin, cause the impaired leptin signaling in the hypothalamus through elevating the expression of the negative regulators (SOCS3 and PTP1B) of leptin receptor signalling cascade (Figure 1.2) [52], [53]. In HFD induced obese rodents, elevated hypothalamic IRE1 and PERK phosphorylation, CHOP and BiP expression, and XBP1 splicing have been observed [54]. Increased PERK and eIF2 α phosphorylation were restricted to ARC nucleus but not PVN in response to diet-induced obesity (DIO). Despite the prevalent leptin resistance in their hypothalamus treatment of DIO rodents with chemical chaperones 4-phenylbutyric acid (4-PBA) or tauroursodeoxycholic acid (TUDCA) restored the ER stress-induced hyperphagia and body weight [52]. In another study has been shown that saturated fatty acid, palmitate leads ER stress by activating toll-like receptor 4 (TLR4) (Figure 1.2) [55]. Neuronal absence of TLR4 adapter protein MyD88 confers protective effects against HFD-induced obesity and leptin resistance in mice [56]. The previous results concerning energy balance suggest that the impairment of ER homeostasis triggers decreased energy expenditure and oxygen consumption while increasing food intake, thus tend to gain weight, obesity, and other associated comorbidities.

Recent studies have shown that post-translational processing of the neuropeptides, including POMC, play a crucial role in the pathogenesis of obesity. In HFD-induced obese rodent, dysregulation of POMC processing in the related neurons leads to decrease in the level of orexigenic neuropeptide α -MSH through the accumulation of POMC precursors in the ER [52]. Although, there is no alteration of POMC mRNA level, the decrease of PC2, which catalyzes the conversion of adrenocorticotropin (ACTH) into α -MSH, contributes to development of obesity [57]. The treatment of these animals with α -MSH could restore glucose metabolism and decrease excess adiposity [58]. Although, the chemical chaperones 4-PBA and TUDCA could restore the impaired POMC processing, they do not significantly alter the level of hypothalamic NPY [52]. The ERAD components suppressor-enhancer of Lin-like 1 (Sel1L) and hydroxymethylglutaryl (HMG)-CoA reductase degradation

protein (Hrd1) targets POMC for proteasomal degradation, thereby providing quality control and proper trafficking of POMC. Consistently, POMC specific Sel1L deficiency causes accumulation of POMC associated with the impaired leptin sensitivity and hyperphagia in POMC Sel1L^{-/-} mice [58]. On the other hand, the treatment with salubrinal which a selective phosphatase inhibitor cause the activation of specifically PERK pathway via inhibiting dephosphorylation of p-eIF2 α and this PERK activation increases PC2 protein level. These findings has been suggested that the PERK–eIF2 α arm of UPR can rescue the ER stress-induced negative effect on PC2 expression [59]. The mechanisms of interruption of neuropeptide maturation in response to diet induced hypothalamic ER stress in POMC neurons is depicted in Figure 1.2 [60].

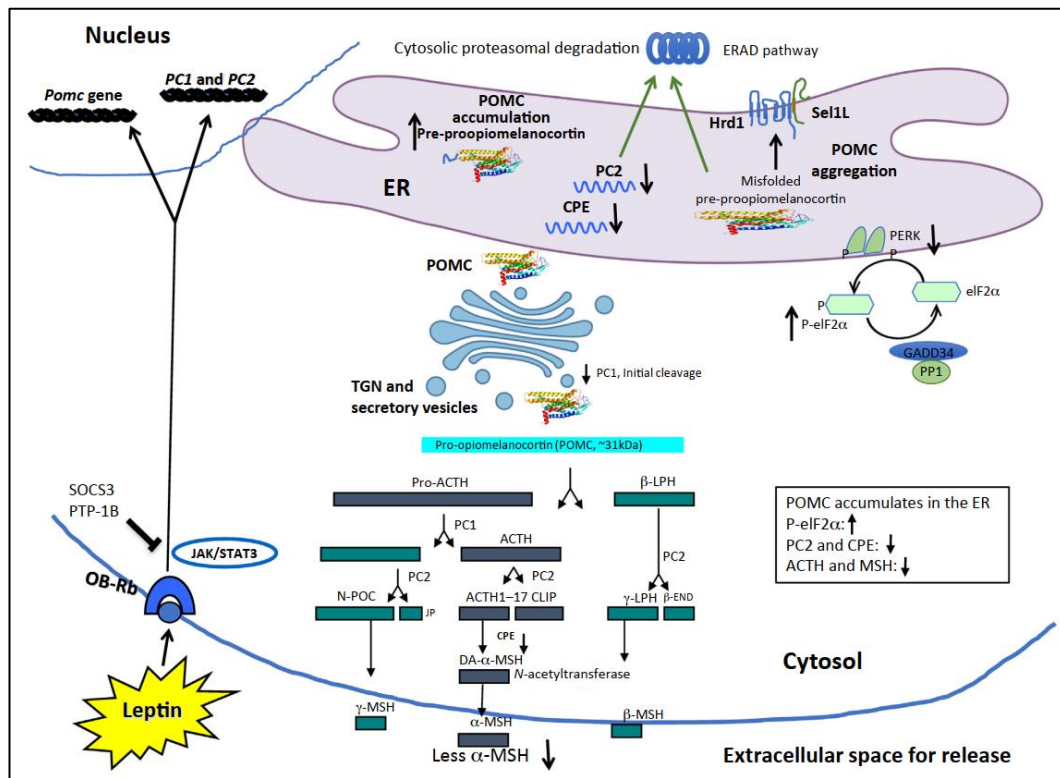


Figure 1.2: The hypothalamic ER stress-mediated dysregulations in POMC neuron upon HFD-induced.

2.1.2. Hypothalamic ER Stress-Induced Inflammation in Obesity

Obesity is commonly accompanied by acute low-grade inflammation in the central nervous system (CNS), and in metabolically active peripheral organs including adipose tissue, liver, and pancreas. ER stress and inflammation form a positive feedback loop to fuel a vicious cycle underlying pathogenesis of various metabolic immune and neurodegenerative diseases such as obesity, Alzheimer, Huntington and many more. ER stress-induced inflammation in the peripheral metabolic tissues and hypothalamic neurons is linked to leptin and insulin signaling abnormalities [53]. ER stress-induced activation of IKK β –NF- κ B in hypothalamic neurons increases SOCS3 expression linking proinflammatory gene expression to inhibition of insulin and leptin signaling pathways [61]. HFD consumption and saturated fatty acid infusion activates inflammatory cascades. The intracerebroventricular palmitate infusion or HFD-feeding (independent of high calorie diet) activates a marker of inflammatory signaling, IKK β and triggers hypothalamic insulin resistance [55], [62], [63]. However, high calorie diet fed-induced microglia activation is also demonstrated in the ARC. Inflammatory mediators such as interleukin (IL)-1 α , tumor necrosis factor (TNF) and complement component 1q (C1q) secreting from astrocytes could be induced by this microglia activation [64]. The suppression of neuroinflammation serves metabolic improvements including protection from diet-induced obesity and increased leptin's anorectic action through central and peripheral administration of a JNK2/3-specific inhibitor [65], hypothalamus-specific deletion of IKK β [61].

Hypothalamic inflammation emerges within 24 hours of HFD feeding [62] much earlier than the appearance of inflammation and related insulin resistance in peripheral metabolic tissues observed several weeks after HFD [66]. Saturated fatty acid mediated TLR4 activation triggers IRE1-XBP1 and downstream proinflammatory cytokines in macrophages [67]. Macrophages-specific IRE1 α deletion reversed the HFD-induced obesity and related metabolic phenotype [68].

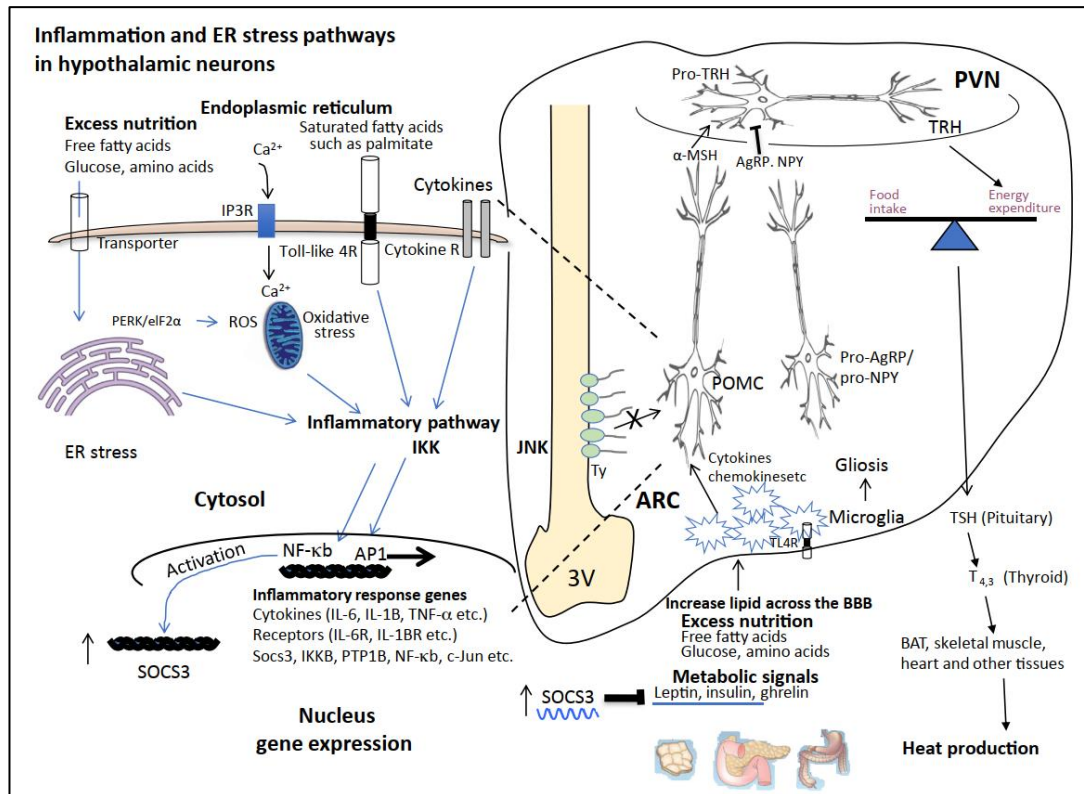


Figure 1.3. Overall view of ER stress-coupled hypothalamic inflammation in obesity. Abbreviations: ARC, arcuate nucleus; BAT, brown adipose tissue; BBB, blood–brain barrier; PVN, paraventricular nucleus; TRH, thyrotropin releasing hormone; TSH, thyrotropin stimulating hormone; Ty, tanycytes; 3V, third ventricle.

ER stress-coupled hypothalamic inflammation in obese models, is demonstrated in Figure 1.3 [60]. Neuronal and microglial inflammation in hypothalamus abrogate the feeding circuitry leading to the development of obesity. Like ER stress, central inflammation has distinct effects, such as acute neuroinflammation contributes to the hypothalamic anorexic action, but chronic central inflammation activation results in increased weight gain and leptin/insulin resistance, and presumably obesity.

1.3. Salt Inducible Kinase Family and SIK2

Salt inducible kinases (SIKs) belong to the AMP-activated protein kinase family which comprises 14 members. The first known SIKs member, SIK1 was first cloned from the adrenal cortex on high salt diet-induced rodents [69]. In mammals, SIK1 is highly expressed in the brain, testes, adrenal glands, and skeletal muscle, but, in a small proportion in the liver, heart, and adipose tissue [70]. Although, SIK3 is ubiquitously expressed [71], SIK2 predominantly expressed in brain, liver and adipose tissues [72]. Accumulating evidence points out critical roles for SIK2 both in metabolism and maintenance of cellular proteostasis by fine tuning lipogenic, gluconeogenic, ubiquitin proteasome system (UPS), and autophagy pathways respectively under cellular stress conditions and presumably in response to hormonal induction [70]. SIK2 phosphorylates its targets on serine or threonine residues in LX(RKH)(S/T)XSXCXXL motif [72]- [74]. In corroboration with the SIK2's regulatory role in metabolic pathways, SIKs were also implicated in the phosphorylation dependent inhibition of class II histone deacetylases (HDAC4, 5, 7) causing inactivation of FOXO1 [75]. In a recent study ER stress-induced steatosis in obesity and types 2 diabetes was shown to be blunted by the overexpression of phosphorylation-defective HDAC5 [76]. It is proposed that HDACs-SIKs axis could integrate ER stress and fasting glucagon to hepatic steatosis which is commonly triggered by impaired fatty acid oxidation [75]- [77].

SIK2 modulates both lipogenesis and gluconeogenesis in response to hormonal induction or cellular stress conditions [77], [78]. With the increment of circulating insulin during refeeding, SIK2 phosphorylates and inactivates CREB regulated transcription coactivator 2 (CRTC2, also called TORC2) which activates gluconeogenic genes *Pepck* and *G6Pase* in mouse liver and primary hepatocytes [76]. Similarly, SIK2 decreases hepatic lipogenesis via inhibitory phosphorylation of p300 required for the expression of lipogenic genes [77]. Furthermore, SIK2 level and activity increases in white adipose tissue (WAT) of leptin receptor deficient *db/db* diabetic mice. SIK2 phosphorylated IRS1 at Ser789 suggesting that SIK2 may negatively regulate insulin signaling pathway related to insulin resistance and type 2 diabetes [70]. Contrary to adipose tissue, lowered SIK2 activity was reported in the livers of leptin deficient *ob/ob* and HFD-fed mice [78]. Also, SIK2 KO mice displayed

hyperglycemia and higher blood glucose levels which were associated with impaired glucose and concomitantly insulin tolerance [72]. In addition to its metabolic engagements SIK2 has been recently connected with protein homeostasis due to its positive regulatory roles in ERAD and autophagy pathways. SIK2-mediated phosphorylation of valosin-containing protein (VCP)/p97, an ATPase translocase involved in ERAD of misfolded proteins, upregulates ATPase activity of this enzyme to alleviate the ER stress [79]. SIK2 increases autophagy flux by enhancing autophagosome lysosome fusion which appears to be dependent on its reversible acetylation-deacetylation cycle mediated by HDACs presumably in autophagosomes. While p300/CBP-mediated Lys-53 acetylation of SIK2 halts its kinase activity, however, HDAC6-induced deacetylation of SIK2 restores its kinase activity [80].

Recent data demonstrate that PKA-mediated phosphorylation of SIK2 is requisite in proper adaptive thermogenesis induced by the sympathetic inputs. In this study sympathetic neuron system (SNS)-mediated activation of brown adipocytes was shown to increase cAMP level under cold exposure leading to protein kinase A (PKA) mediated inhibition of SIK2. This inhibition promoted Ucp1 expression and ultimately adaptive thermogenesis in BAT tissue. In corroboration with these findings SIK2 KO mice display enhanced adaptive thermogenesis via up-regulation of Ucp1 expression [81].

1.4. Insulin Receptor Substrates (IRSs) and IRS4

Insulin receptor substrates IRS1, IRS2, IRS3 and IRS4, are principal targets for activated tyrosine kinase receptors (RTKs), including the insulin and insulin-like growth factor-1 receptors (IGF1) [82]. IRSs have a key conjunctive role in both insulin and leptin signalling cascades. In addition to insulin and IGF1 receptors, IRSs mediates signaling downstream of various cytokine receptors including leptin, IL-4, IL-9, and IL-13 [83]- [85] and growth hormone receptors [86]. IRSs have common structural domains including an NH2-terminal pleckstrin homology (PH) domain followed by a phosphotyrosine binding (PTB) domain and a carboxy-terminal tail. The carboxy-terminal region contains many Try and Ser/Thr phosphorylation motifs which can bind and activate src Homology2 (SH2) domain containing downstream effector proteins such as PI3K under insulin receptor (IR) acting like an adaptor protein. The IRSs and

PI3K interaction leads to activation of AKT to mediate cellular insulin action [87]. IRSs have been implicated in insulin resistance, hyperglycemia and type 2 diabetes [70], [88], [89]. The absence of both IRS1 and IRS2 in the liver [90], [91] or IRS2 alone in the brain leads to development of obesity, insulin resistance and hyperglycemia. On the other hand, in this respect elimination of neither IRS3 nor IRS4 caused any alteration in the organismal phenotype [92]- [96]. Although IRS4 appears to be dispensable for adipocyte differentiation, the IRS1 KO-mediated defect in adipocyte differentiation could be compensated by IRS4 [96]. Unlike IRS1 and IRS2, which is ubiquitously expressed throughout the rat forebrain, IRS4 displays a discrete expression profile primarily mapped to the ventral hypothalamus and arcuate nucleus in healthy animals [97]- [99]. It was previously reported that both leptin receptor and the negative regulatory proteins in leptin signaling, SOCSs (SOCS2, 6, and 7) are identified as IRS4 interactors in embryonic mouse hypothalamic cell line N38 [83]. IRS4-null mice (also, lacking IRS2 only in CNS) displayed severe obesity, decreased energy expenditure, and insulin resistance. However, the body weight or fed blood glucose level of IRS4-null mice (also, lacking IRS2 only in leptin receptor (LepR) expressing neurons) is indistinguishable from mice only lacking IRS2 in LepR-neurons. Therefore, IRS2 and IRS4 synergically converge in non-LepR expressing neurons to regulate the energy homeostasis and glucose metabolism [100]. In a recent study IRS4-expressing cells wherein and adjacent to PVN and projecting to hindbrain regions were shown to play critical role in sustaining energy homeostasis. The Cre-dependent activation of IRS4-expressing PVN neurons results in attenuated feeding, increased energy expenditure and total activity in the absence of food. Unlike from IRS4 KO mice, the permanent inactivation of IRS4 expressing PVN neurons caused an elevated fat percentage and tends to increase body weight via modulating feeding behavior [7]. However, despite the critical role of IRS4 bearing PVN neurons in energy homeostasis and metabolism the molecular engagements of IRS4 in these neurons remain unidentified.

According to an organelle-based phosphoproteomics study in our lab, IRS4 was identified as a novel ER-resident substrate for SIK2 [111]. As previously mentioned that SIK2 mediated phosphorylation of IRS1 may be responsible for the insulin resistance observed in some metabolic tissues including white adipose tissue (WAT) [70]. We hypothesized that observed metabolic phenotype of mice upon inactivation of IRS4 expressing hypothalamic neurons might be at least partially accounted by its

interaction partners in these specified neurons. We propose that SIK2 being an upstream kinase for IRS4 in hypothalamic neurons might be partially responsible for the metabolic phenotype observed in SIK2 KO mice. To test our this hypothesis, we investigated the effects of SIK2 on IRE1 signaling pathway through newly identified IRE1-IRS4 complex in POMC like neurons under chemically induced ER stress conditions Taken together with the identification of novel IRE1-IRS4-SIK2 signaling complex we hypothesized that IRE1 could be the long-sought elusive upstream activator kinase for the SIK2. Here we provided data for the characterization of SIK2-IRS4 interaction in mHypoA cells and further propagated this interaction to include evolutionary the most conserved branch of UPR pathways, IRE1 under ER stress conditions.

2. MATERIALS

2.1. Mammalian Cell Lines

Adult mouse hypothalamus cell line, mHypoA A-2/30 (mHypoA) and human embryonic kidney cell line, HEK293T (293T) were purchased from Caderlane and ABMGood (British Columbia, Canada), respectively.

2.2. Chemicals

Table 2.1: List of used chemicals 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
APY29	TOCRIS, USA
B-Mercaptoethanol	AppliChem, Germany
Boric Acid	Sigma-Aldrich, USA
Bovine Insulin	CELL, USA
Bovine Serum Albumin (BSA)	AppliChem, Germany
Bromophenol Blue	AppliChem, Germany
Calcium chloride dehydrate	AppliChem, Germany
DMSO	Sigma-Aldrich, USA
EDTA	AppliChem, Germany
Ethanol	Merck, USA
Glycerol	AppliChem, Germany
Glycine	Applchem, Germany
HEPES	Sigma-Aldrich, USA
Iodoacetamide	Sigma-Aldrich, USA
Isopropanol	Merck, USA
Kanamycin	AppliChem, Germany
KIRA6	Merck, USA
Magnesium Chloride	Sigma-Aldrich, USA
Methanol	Emsure, Germany

Table 2.1: Continue.

N, N, N', N'-tetramethylethylenediamine (TEMED)	AppliChem, Germany
N,N'-Methylenebisacrylamide	Sigma-Aldrich, USA
NP-40	AppliChem, Germany
Paraformaldehyde	Sigma-Aldrich, USA
PEI	PolyScience, USA
Phosphate Saline Buffer (PBS)	Lonza, Switzerland
Recombinant Mouse Leptin	R&D, 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
STF-083010	Merck, USA
Thapsigargin	Sigma-Aldrich, USA
Tris-Base	AppliChem, Germany
Triton X-100	AppliChem, Germany
Trypsin	Fisher Scientific, USA
Tween 20	Sigma-Aldrich, USA
Ultrapure DTT	Invitrogen, USA

2.3. Buffers and Solutions

Table 2.2: Solutions and buffers used in this study.

Solutions/Buffers	Contents/Suppliers
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
Ampicillin stock	100 mg/ml in distilled water 100 µg/ml (working concentration)
Kanamycin stock	50mg/ml in distilled water 50µg/ml (working concentration)

Table 2.2: Continue.

20X Tris-Acetic acid EDTA (TAE)	96,8 g Tris base 7,44 g EDTA 22,84 ml Glacial Acetic Acid distilled water upto 1L
10X TE Buffer	100mM Tris{HCl 10mM EDTA, pH 7,4
5X TBE Buffer	54 g Tris-base 27,5 Boric acid 20 ml EDTA pH 8.0 distilled water upto 1L
10X SDS Running Buffer	30.3 g Tris base 144 g Glycine 10 g SDS distilled water upto 1 L
10X Transfer Buffer	30.3 g Tris base 144 g Glycine distilled water upto 1 L
1X Transfer Buffer	100 ml 10X Transfer Buffer 200 ml Methanol 700 ml distilled water
1X Tris Bufferd 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 upto 1 L
Blocking Solution for western blot	5% non-fat milk powder in 1X TBS-T
4X SDS-PAGE Loading Dye	200mM Tris-HCl pH 6.8 8% SDS 40% Glycerol 4% β -mercaptoethanol 50mM EDTA 0,08% Bromophenol Blue

Table 2.2: Continue.

IP Lysis Buffer	145mM NaCl 20mM Tris-HCl (pH 8.0) 2mM EDTA 0.2% NP40 1X Protease Inhibitor Cocktail 1X Phosphatase Inhibitor Cocktail
RIPA Lysis Buffer	150mM NaCl 50mM Tris-HCl (pH 7.4) 1% NP-40 0,5% Sodium deoxycholate 0,1% SDS 1X Protease Inhibitor Cocktail 1X Phosphatase Inhibitor Cocktail
IP-Mass Lysis Buffer	0,5% NP-40 50mM HEPES pH 7.6 150mM KCl 2mM EDTA 0,5mM DTT 1X Protease Inhibitor Cocktail 1X Phosphatase Inhibitor Cocktail
2X HBS Buffer pH 7.13	274mM NaCl 9,5mM KCl 15mM Glucose 42mM HEPES 1,4mM Na ₂ HPO ₄
Mounting Media	90% (v/v) Glycerol within 20mM Tris-HCl pH 8.0
4% (w/v) Paraformaldehyde (PFA)	10 g PFA Dissolved within 250ml 1X PBS Adjusted pH 7.4 by adding 1M NaOH

2.4. Plasmids and Antibodies

Table 2.3: List of used plasmids in this study.

Construct Name	Origin	Backbone
Empty pCMV-3tag-6	Agilent #240200	
mIRS4-FLAG	This study	pCMV-3tag-6
hIRS4-FLAG	University of Dundee, MRC-PPU Reagents #DU36186	pcDNA5-FRT-TO- FLAG
hIRE1-FLAG	(Seker, 2017) [111]	pCMV-3xFLAG-7.1
hIRS4-mCherry	(Seker, 2017) [111]	mCherry2-N1
hIRE1-sYFP	(Seker, 2017) [111]	sYFP-N3

Table 2.4: List of used antibodies in this study.

Antibody	Host	Dilution	Supplier (cat.no.)	kDa
SIK2	Rabbit	1:1000	CST #6919	130
pSIK2	Rabbit	1:1000	Invitrogen #PA5-64607	~130
IRE1 α	Rabbit	1:1000	CST #3294	130
pIRE1 α	Rabbit	1:1000	Invitrogen #PA1-16927	~130
BiP	Rabbit	1:1000	CST #3177	78
TRAF2	Rabbit	1:1000	CST #4724	53
IRS4	Goat	1:500	Invitrogen #PA5-37926	~160
FLAG	Rabbit	1:1000	CST #2368	
GAPDH	Rabbit	1:1000	CST #2118	37
β -Actin	Rabbit	1:1000	CST #4970	43

3. METHODS

3.1. Molecular Cloning

PrimeStar HS (Takara, #R010A)) and Taq polymerases (Abm, #G009) were used for cloning purposes. While PrimeStar HS DNA polymerase which has high fidelity and efficiency was used for the amplification of DNA fragments to be cloned, Taq DNA polymerase was routinely used in the colony PCR reaction for checking 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. The PCR reactions were performed by using MJ Mini Thermal Cycler (Bio-Rad).

The empty vector plasmid and insert digestion with an appropriate restriction enzyme was performed according to the manufacturer's recommendations. Then, DNA samples were loaded in the 1% Agarose gel and run at 100 Volt constant voltage for 45-60 minutes. Gels were visualized under UV light using the Azure Gel Imaging System (Azure Biosystems). DNA samples were extracted from agarose using NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel, (MN)).

Digested and linearized plasmid DNA and inserts were ligated using T4 DNA Ligase (NEB, #M0203S) according to the manufacturer's recommendations. 50ng digested vector and inserts with 1:1 or 1:3 or 1:5 molar ratio were exist within each 20ul reaction volume. The ligation reaction was performed at room temperature for 2 hours without heat-inactivation. After ligation, 50% of ligation mix and 50 ng plasmid (for non-insert control) were added into 50ul competent DH5 α bacteria for bacterial transformation. After incubation on ice for 30 min, the vial was exposed to the heat shock in 42°C for 1 min, then immediately placed on ice for 2 min. 750ul LB medium was added onto bacteria and incubated for 1 hour at 37°C with moderately shaking (200-250rpm). After incubation, the whole suspension was centrifuged for 5 min at 3000rpm, and the pellet was dissolved within 50-100ul LB and spread on appropriate antibiotic-containing LB-agar plates, and cells were grown overnight at 37°C.

Table 3.1: PCR reaction mix for PrimeStar HS DNA polymerase.

Component	for 25ul Reaction	Final Concentration
Nuclease-free water	up to 25ul	
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

Table 3.2: PCR reaction mix for Taq DNA polymerase.

Component	Volume for 25ul Reaction	Final Concentration
Nuclease-free water	up to 25ul	
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

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	30-35
Anneling	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	∞	

For positive clone screening, colony PCR was performed using Taq DNA polymerase as described in the manufacturer's protocol and the PCR reaction mix preparation and conditions given in Table 3.2 and 3.3. After then, plasmid purifications were carried out using a DNA-spin Mini kit (Intron, #17069) or NucleoBond Xtra Midi kit (MN, #740410) to be also used in especially transfection of the mammalian cells which is required endotoxin-free transfection grade yield. Finally, after sequencing, verified clones were used for further assays.

3.2. Cells and Culture Conditions

mHypoA and HEK293T cells were cultured within *Dulbecco's Modified Eagle Medium* (DMEM) (Gibco) containing 10% Fetal Bovine Serum and 1% Penicillin/Streptomycin and 4.5 g/l glucose (high-glucose) in 5% CO₂-supplemented incubator at 37°C. Cells were passaged before 90% confluency by using 0,05% (for mHypoA) or 0,25% (for 293T) trypsin/EDTA (Gibco) and stored within the growth medium containing 10% (for mHypoA) or 5% (for 293T) (v/v) Dimethyl Sulfoxide (DMSO) at nitrogen vapor phase.

3.3. Transfection of mHypoA and 293T Cells

Traditional CaPO₄ transfection protocol was optimized for mHypoA. 500.000 cells were seeded on a 100mm cell-culture dish (TPP) the day before transfection thus cells reached 60-70% confluency after overnight cultivation. Before preparation of transfection mixture, media on the cells were replaced with fresh growth medium, since acidic pH in the environment negatively affected the transfection efficiency. For 100mm dish, 16ug plasmid DNA and 50ul 2M CaCl₂ were mixed, and the total volume was completed up to 400ul with sterile distilled water. Then, 400ul 2X HBS buffer was added into the mixture, however, 50ul (1/8 of to be used total buffer volume) 2X HBS added and vortexed for 7 sec in each time, thereby this step would be repeated 8 times. After then, it was allowed to precipitate to sit undisturbed for minutes. The solution was dropped evenly over the cell culture medium on the plate. And it was gently agitated the dish to distribute the precipitates evenly over the cells on the plate.

After overnight (16 hours) incubation, media were replaced with fresh growth medium.

In IP-MS assay, 293T cells were transfected using polyethyleneimine (PEI) (PolyScience, 23966). 8×10^6 cells were seeded on a 150mm cell-culture dish the day before transfection thus cells reached 60-70% confluency after overnight cultivation. At the transfection day, 20ug plasmid DNA and PEI were mixed with 1:3 (v:v) ratio within 1.250 ul 150mM 1X TE buffer containing 150mM NaCl. The mixture was dropped evenly over the cell culture medium on the plate. And it was gently agitated the dish to distribute the precipitates evenly over the cells on the plate. After overnight (16 hours) incubation, media were replaced with fresh growth medium.

3.4. Treatments on the Mammalian Cells

In characterization assay, 500.000 mHypoA cells were seeded into 60mm cell-culture dishes. The next day, either thapsigargin (100nM final concentration, [52]) or leptin (100nM final concentration, [101]) or insulin (100nM final concentration, [102]) or vehicle buffer (mostly PBS or DMSO) as a control were added into the media of the cells. Then, the cells were harvested in the indicated time points. Thapsigargin was routinely used as a chemical ER stress inducer agent causing calcium depletion in the ER by inhibiting sarco/endoplasmic reticulum Ca^{2+} transport ATPase (SERCA).

In IRE1-inhibitors assay, 500.000 mHypoA cells seeded into 60mm cell-culture dishes the day before, were treated with either APY29 or KIRA6 or STF-083010 or vehicle solution at the various concentration for 2 hours. And then, 100nM thapsigargin was added into the media of the cells. After additional incubation for 15 minutes, the cells were harvested.

Also, at the post-transfection 42-48 hours, both mHypoA and 293T cells were treated with 100nM and 1uM [111] (final concentraion) thapsigargin, respectively.

3.5. Co-Immunoprecipitation (Co-IP)

3.5.1. Lysate Preparation and Protein Quantification

Post-transfection 42-48 hours cells were treated with thapsigargin or vehicle for time course. After aspiration, 500 μ l IP lysis buffer was added into cells, and lysates were harvested by using a scraper. The suspension was passed through an insulin-syringes 6-7 times and incubated on ice for 30 minutes. Then, samples were centrifuged at 14,000 rpm for 15 minutes at 4°C, thereby the samples were clarified by removing the lysates from cell debris. The total protein concentration in the lysates was quantified by using BCA Assay kit (Thermo Scientific, #23225) according to the manufacturer's recommendations. Bicinchoninic Acid (BCA) assay based on a standard curve obtained with bovine serum albumin (BSA) dilutions varying from 0,125 to 2mg/ml. After the separation of 5-10% of the lysate to be an input control for western blotting, the samples were pre-cleared by using 20 μ l of protein A/G plus agarose (Santa-Cruz, #sc-2003) to decrease non-specific protein-bead interaction. Before using, the beads were equilibrated by washing with 300 μ l IP lysis buffer and centrifuged at 1000 g for 5 minutes 3 times. The bead-lysate mixture was incubated for 30 min at 4°C and collected the pellet after centrifugation at 1000 g for 5 min at 4°C. The pre-cleared supernatants were transferred into fresh 1,5 ml Eppendorf tubes to be continued for IP protocol.

3.5.2. Immunoprecipitation Using Specific Antibody and anti-FLAG M2 Affinity Agarose Bead

The ratio of 1:50 (v:v) anti-SIK2 antibody (CST) was added into the pre-cleared lysates including 500 μ g total protein. The antibody-lysate mixture was pre-incubated for 1 hour at 4°C on a rotator before adding 20 μ l of Protein G plus agarose (Santa-Cruz, #sc2002). If FLAG-agarose beads (Sigma, #A2220) was used to immunoprecipitate flag-tagged recombinant protein, the beads were equilibrated by washing with 500 μ l IP lysis buffer and centrifuged at 5000 g for 1 minutes 3 times. Then, 1 mg of the pre-cleared lysates were mixed with 40 μ l of the equilibrated anti-FLAG-M2 affinity agarose beads. After these steps, IP samples were incubated overnight at 4°C on the rotator. SIK2 and Flag IP samples containing antibody-bead-

protein complexes were centrifuged and washed three times with 500 ul IP lysis buffer at 1000 g for 5 min and 5000 g 1 min at 4°C, respectively. Finally, after the last washing, IP samples were dissolved with 30 ul IP lysis buffer containing 1X SDS loading dye and heated at 95°C for 5 min to denaturation. For western blotting, the whole IP sample volume was loaded into the gel to detect the specific immunoprecipitated proteins. The pre-immunoprecipitation lysates containing 20 ug total protein were used as input control.

3.6. Immunoprecipitation-coupled Mass Spectrometry

3.6.1. On-bead Trypsin Digestion

Following the previously described IP protocol, after the last IP-mass lysis buffer washing step, the beads additionally washed two times on ice with 1 ml of ice-cold 20 mM Tris-HCl pH 8.0 containing 2 mM CaCl₂ without any protease inhibitors. Then, 10% of the mix was taken for western blotting. The remaining samples centrifuged at 5000 g for 1 min and removed the last drop of liquid with a pipette. After this step, the beads were stored at -20°C and continued later. The liquid-free beads were resuspended within 9 ul of mM Tris-HCl pH 8.0. In order to reduction of samples, 0,4 ul of the 100 mM DTT (Invitrogen) was added and incubated at room temperature for 30 minutes with moderate agitation. In order to alkylation 0,6 ul of 100 mM iodoacetamide (Sigma) and incubated at room temperature for 10 minutes with moderate agitation and protected from light. Then, 500 ng trypsin (Pierce) was added and incubated at 37°C on the rotator for 12 hours. After first tryptic digestion, the samples were centrifuged at 5000 g for 1 min and transferred the supernatant to a fresh Eppendorf tube. An additional 500 ng trypsin within 5 ul of 20 mM Tris-HCl pH 8.0 was added and incubated at 37°C for 4 hours with gentle agitation. In order to stop the digestion, formic acid (Fisher Scientific) to a final concentration of 2% (v:v) was added into the samples.

3.6.2. LC-MS/MS Analysis

The digested peptides were loaded onto a reverse-phase column on a nanoflow ultra-high performance liquid chromatography (nano-UHPLC) instrument (Ultimate 3000 RSLCnano, Thermo Fisher Scientific) coupled on-line to an LTQ Orbitrap Velos mass spectrometer with a nano-electrospray ion (ESI) source (Thermo Fisher Scientific). The peptides were mixed with a sampler buffer containing 5% acetonitrile and 0,1% formic acid and separated with a linear gradient of 5–55% buffer B (80% acetonitrile, 20% water in 0.1% formic acid) at a flow rate of 300 nL/min. Each sample was analyzed by LC-MS/MS three times. The data were acquired using the following parameters. Unassigned precursor ion charge states, as well as 4+ and above-charged species, were excluded from fragmentation. The isolation window was set to 1,5 Da and fragmented with normalized collision energies of 35. The activation time was set to 10 ms. Data were acquired using Xcalibur software (Thermo). Raw MS data were analyzed using Proteome Discoverer 2.2 software (Thermo). The initial maximum allowed mass deviation was set to 10 ppm for precursor ions and 0,8 Da for fragment ion peaks. The trypsin was set for enzyme specificity. Methionine oxidation, and N-terminal acetylation were set as variable modifications, while cysteine carbamidomethylation as a fixed modification. GO-terms and pathway analysis were performed by using the functional annotation tool DAVID 6.8 [103]. Significant enriched pathways and terms were filtered according to the Benjamini having p-values (<0.1). The schematic representation was created with InteractiVenn [104] software.

3.7. RNA Isolation and Quantitative PCR

Total RNA isolation (MN NucleoSpin RNA purification kit), cDNA synthesis (Thermo maxima first strand cDNA synthesis kit) and real-time qPCR (Promega GoTaq qPCR Master Mix) were performed according to the manufacturer's protocol. The isolated RNA was loaded onto %1 agarose gel and run within 0,5X TBE buffer at 80 Volt constant voltage for 20-30 minutes for quality control. Two independent experiments and three technical repeats were performed using the primers indicated in Table 2.4. The $2^{(-\Delta\Delta Ct)}$ formulation was used when calculating the relative fold differences.

3.8. Confocal Imaging

120.000 mHypoA cells were seeded on each well of the 6-well cell-culture dish carrying sterilized glass coverslips with a diameter of 22x22 mm and a thickness of 0.18 mm, the day before transfection thus cells reached 60-70% confluency after overnight cultivation. Using fluorescent-tagged protein-expressing plasmids the optimized CaPO₄ transfection was performed as previously described in section 3.3. After ER stress induction for indicated time intervals, the post-transfection 48 hours cells was aspirated using 1 ml of 1X PBS for three times. In order to fixate the cells, 1 ml of 4% PFA solution was added onto the aspirated cells, then, washed with 1 ml of 1X PBS for 3 times. After then, removed coverslips were placed onto glass slides containing 8 ul mounting media with downward direction. After waiting 15-20 minutes for the coverslips to settle well, the surround of the coverslips was sealed using transparent nail polish. Prepared samples were observed under T-PMT Hal 100 Confocal Laser Microscopy (Zeiss LSM).

3.9. Western Blotting

Mini-Protean Tetra System and Mini-Transblot Turbo (Bio-Rad) were used for cast, run and transfer the SDS-PAGE gels. Routinely used 10% resolving and 2,5% stacking gel were prepared as discribed below Table 3.4.

Table 3.4. Component of the prepared SDS-PAGE gels.

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

The samples within 1X SDS loading dye were heated at 95°C for 5 minutes. Samples were loaded and run at 80 Volt. After the run was completed, proteins were transferred onto PVDF membranes (Merck, #IPVH00010) and then membrane blocking was carried out with 5% non-fat dried milk within the 1X TBS-T by shaking for 1 hour at room temperature. After washing three times with 1X TBS-T for 4 min, membranes were incubated with the specific antibodies in the 5% BSA within 1X TBS-T overnight in a cold room on the shaker. After incubation, membranes were treated within 5% BSA solution containing secondary HRP-conjugated antibody for 2 hours in at 4°C on the shaker. The immunoblots were visualized with ChemiDoc XRS+ (Biorad) by using western blotting luminol detection kits, LumiGo ECL (CST, #7003) and WesternBright Sirius (Advansta, #K12043).

3.10. Statistical Analysis

Data for each experiment were obtained from three independent experiments performed in triplicate. Statistical significance of the differences was calculated by two-way ANOVA using GraphPad Prism 5 Software (USA).

4. RESULTS

4.1. Characterization of POMC Like mHypoA Cells

First, we needed to verify the POMC and AgRP like properties of our in vitro cell model based on the recently characterized mouse adult hypothalamic cell line mHypoA. These neurons were reported to be heterogenous in nature demonstrating characteristics features of both POMC and AgRP neurons [105]. Since these counteracting hypothalamic arcuate nucleus neurons bear significant heterogeneity unraveled by recent single cell transcriptomic studies, we decided to focus firstly on POMC like neurons for the characterization of newly identified SIK2-IRS4 complex in association with IRE1. Towards this goal, first we checked out the expression levels of all SIK (1-3), IRS (1-4), and IRE1 isoforms (α and β) along with the critical neuropeptides POMC and AgRP by using quantitative PCR under normal and chemically induced ER stress conditions (Figure 4.1).

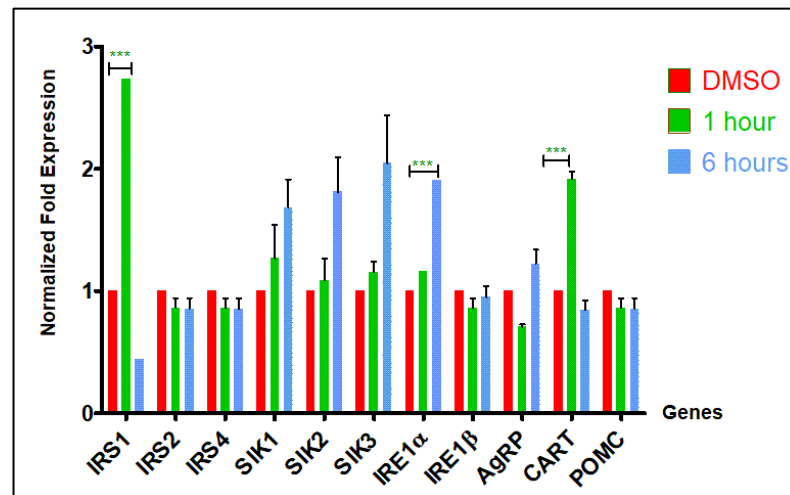


Figure 4.1. Characterization of mHypoA cells. Total RNA isolation, cDNA synthesis and real-time qPCR were performed from mHypoA cells following treatment of 100nM thapsigargin for 1 to 6 hours, respectively. The expression of the genes of interest at the mRNA level was normalized to GAPDH expression. P-values; ***<0,001.

We detected moderate increases in the transcript levels of IRS1, IRE1 α , all SIKs, and the orexigenic neurotransmitters AgRP and CART after one or six-hour treatment of cell with ER stress inducer thapsigargin. However, the mRNA levels of IRS2, IRS4,

POMC and IRE1 β remained relatively unchanged without respect to duration of ER stress induction.

Next, we checked the activation levels of SIK2 in response to treatment of mHypoA cells with the centrally acting metabolic hormones insulin and leptin as well as chemical ER stressor thapsigargin to represent physiological and pathological conditions in healthy and DIO mice respectively. The anti-pSIK2 antibody used in this study recognizes the phosphorylated activation loop Thr175 residue (in mouse) of SIK2. Although SIK2 exhibited a similar activation profile marked with a major peak observed 15 min after treatment with leptin, insulin, and thapsigargin, slightly different profiles in response of SIK2 to two anorexigenic hormones suggesting a common upstream pathway presumably responsible for the SIK2 activity but divergent at downstream regulations (Figure 4.2).

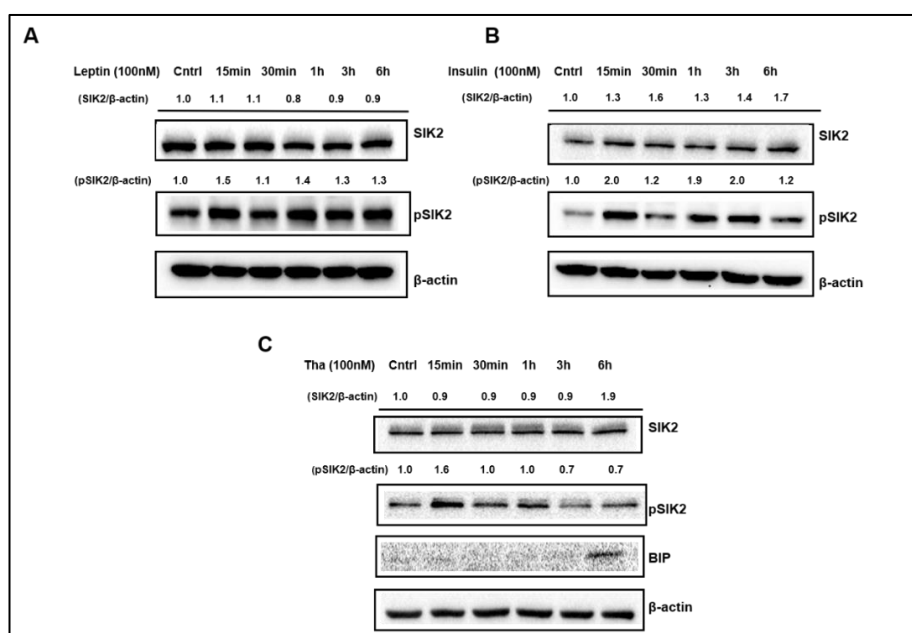


Figure 4.2. SIK2 activation profiles of HypoA cells in response to ER stress and anorexigenic hormones. mHypoA cells were treated with 100 nM leptin (A), 100 nM insulin (B), and 100 nM thapsigargin (C) for the indicated times (15 min-6 hrs). (Tha: thapsigargin)

It is interesting to note that, leptin treatment produced a more stable SIK2 activation profile compared to oscillatory cyclic response generated by insulin treatment. On the other hand, SIK2 activation in response to treatment of cells with thapsigargin produced a gradual and steady decrease in the level of pSIK2 returning

slightly below the basal level in 6 hours. Expectedly, we detected a moderate increase in the level of luminal hsp70 chaperone Grp78/BiP at six hours of thapsigargin treatment indicating the presence or appearance of persisting ER stress.

4.2. IRE1 a Putative Activating Kinase for SIK2

The acute and transient activation profile of SIK2 is a hallmark of its kinetic behavior invariably observed in response to hormonal induction and cellular stress conditions in neuronal cells and presumably many more. In order to test whether IRE1 could be the long-sought upstream activator kinase of SIK2, we exploited the availability of selective IRE1 inhibitors that target the kinase or RNase or both activities of the IRE1. Allosteric inhibitor KIRA6 suppress the kinase and the ensuing RNase activities of IRE1. On the other hand, APY29 by binding to ATP binding pocket in the N- terminal kinase domain suppresses the kinase activity while allosterically increasing the adjacent RNase activity. STF-083010 specifically binds to IRE1 RNase domain and blocks the RNase activity without interfering with the kinase activity. Firstly, we looked at the phosphorylation status of specific threonine residues found in the activation loop of IRE1 and SIK2 in the absence or presence of increasing concentrations of IRE1 inhibitors under ER-stress condition (Figure 4.3A). As expected, the allosteric IRE1 kinase inhibitors KIRA6 and APY29 significantly blocked the activation of SIK2 in a dose dependent manner while excluding the possible involvement of IRE1 α 's RNase domain and downstream effector kinases in the activation of SIK2. The latter is also confirmed when IRE1 α 's RNase specific inhibitor STF-083010 was included in a parallel reaction. The activator and inhibitory effects of APY29 and STF-083010 respectively on IRE1 RNase activity were further verified by measuring the level of XBP1s transcript via RT-PCR (Figure 4.3B).

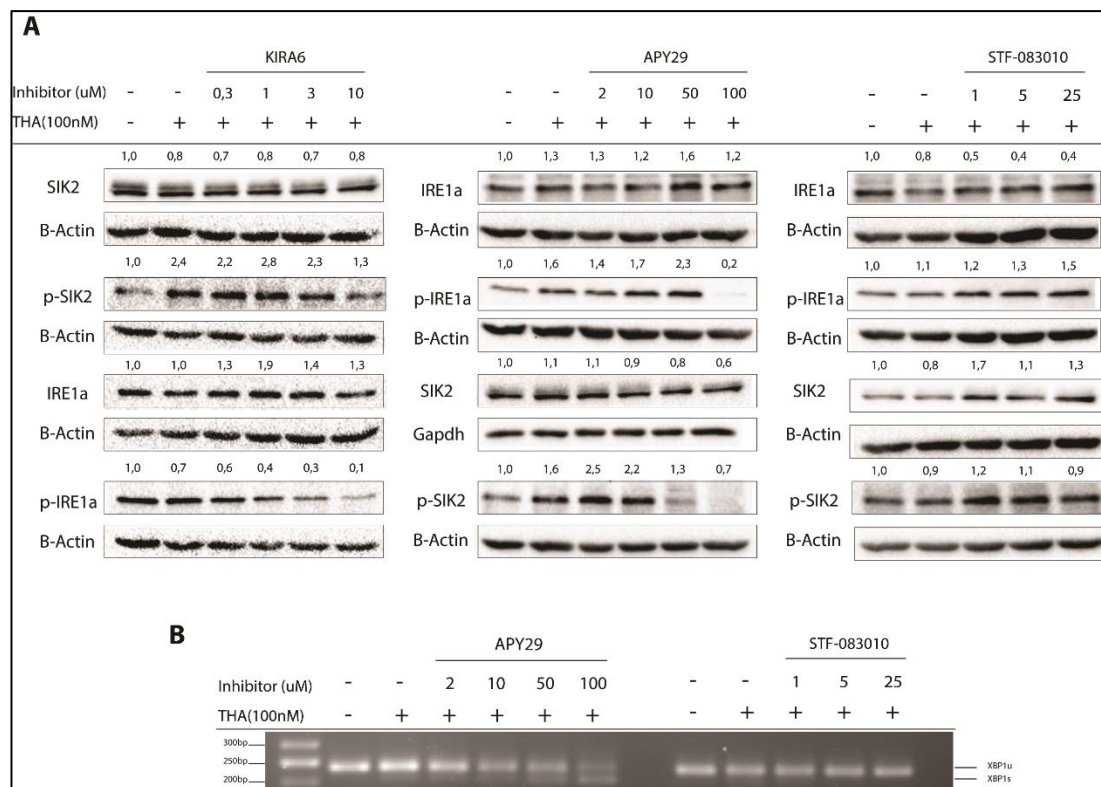


Figure 4.3. SIK2 activation levels depending on the presence of different IRE1 inhibitors in varying concentrations under ER stress conditions. A) KIRA6, APY29 and STF-083010 were incubated for 2 hours before treatment with 100nM thapsigargin for 15 minutes at the indicated concentrations in mHypoA cells. B) XBP1 amplicons amplified by RT-PCR from cDNAs omHypoA cells applied for 15 minutes after STF-083010 or APY29 treatment at the specified concentrations for 2 hours. (XBP1_u: unspliced XBP1, XBP1_s: spliced XBP1).

4.3. Identification of IRE1-IRS4-SIK2 Signalingosome

In order to investigate the mass spectrometry-based interaction between IRS4 and SIK2, we performed co-immunoprecipitations (co-IPs) studies with anti-SIK2 antibody from mHypoA cells transiently over-expressing IRS4. Following western blot analysis of the co-IPs reassured the interaction between endogenous SIK2 with IRS4 protein as also indicated in HEK293T [111] (Figure 4.4A). On the membrane the 160 kDa band is known to be specific for IRS4 and the two other smaller bands (95 and 72 kDa) represent the degradation products.

The investigation of the other interactors of IRS4 carried out using mHypoA cells transiently over-expressing FLAG-tagged IRS4 through co-IP assay. The interaction between IRE1 and IRS4 was authenticated by western blot analysis (Figure

4.4B). In the same immunoprecipitates, the presence of known IRE1 interaction partner TRAF2 was also detected defining a new IRE1 signaling complex incorporating IRS4 and the stress kinase SIK2 to achieve new biological functions. Interestingly, ER stress favors the physical interaction between IRS4 and TRAF2, while promoting dissociation of IRS4 and SIK2 from IRE1. Currently we are seeking answers to question of how these distinct IRE1 complexes are biologically significant especially in the content of POMC neurons. (Figure 4.4B).

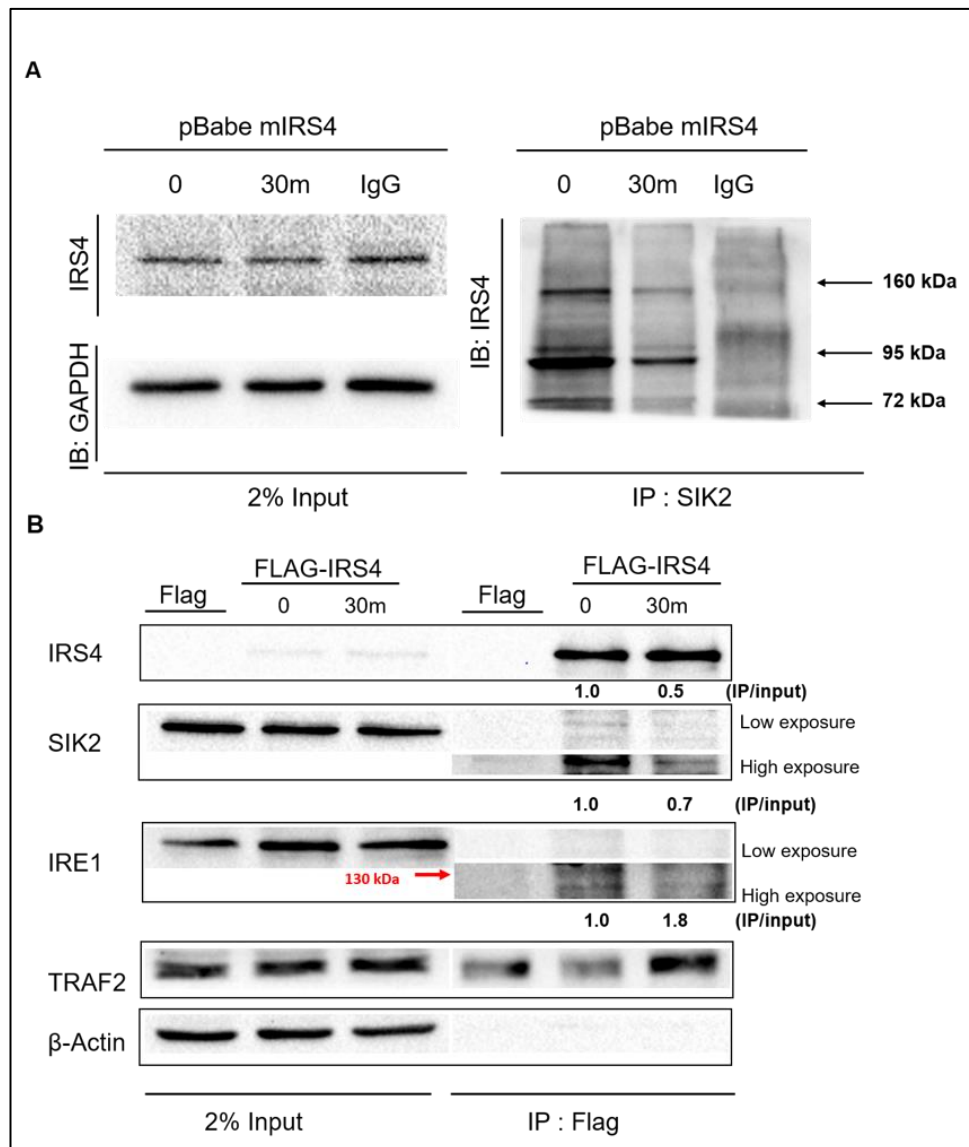


Figure 4.4. Co-immunoprecipitation assay indicates IRS4 interacts with endogenous IRE1 and SIK2 in mHypoA cells. 48 hours after transfection cells were either untreated or treated with 100 nM Tha for 30 minutes. IP's were carried out with anti-SIK2 (A) and anti-FLAG (B) antibody and analyzed by Western blotting using indicated antibodies. IP, immunoprecipitation; IB, immunoblot.

The authentication of IRE1-IRS4 sub-cellular localization is also provided with the fluorescent based co-localization study. The ER localization of newly identified IRE1-IRS4 complex was demonstrated by transiently co expressing mCherry-IRS4 and sYFP-IRE1 along with the fluorescent ER tracker a specific peptide signal fused to mTurquoise in mHypoA cells. The subcellular co-localization of the IRE1 and IRS4 were detected with confocal microscopy at the appropriate excitation wavelengths set for the respective fluorescence tags in the presence and absence of ER stress.

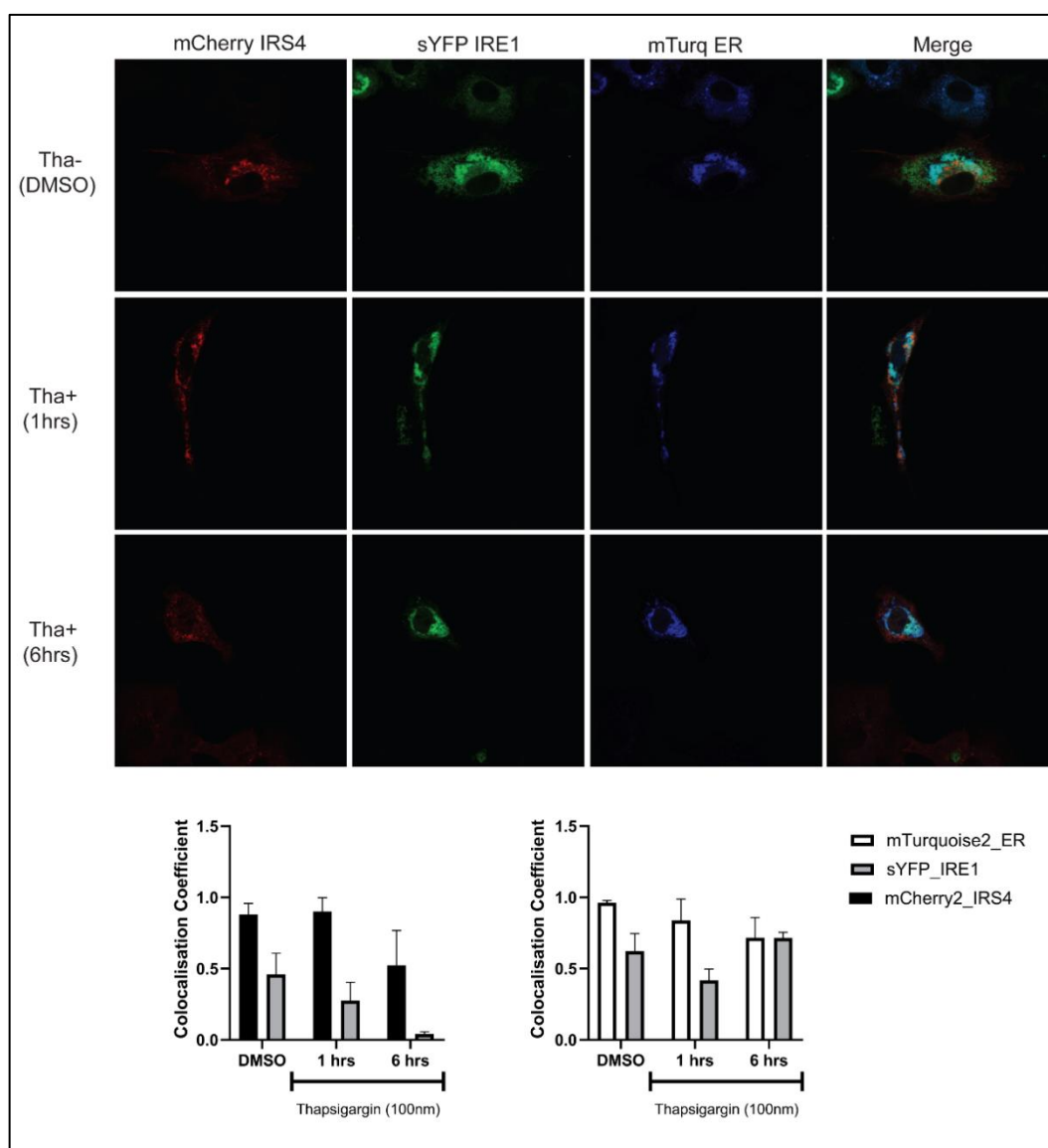


Figure 4.5. IRS4 and IRE1 localizations in ER stress conditions. Thapsigargin was applied to mCherry-IRS4 mHypoA cells co-transfected with ER specific signal carrying mTurq-ER and sYFP-IRE1 at a dose of 100nM for 1 hour and 6 hours. Control groups were treated with DMSO as a vehicle. The colocalization of IRE1 and IRS4 has $p < 0.0001$ (****) which was statistically significant.

Analysis of the images obtained one and six-hour after ER stress induction unveiled the constitutive existence of IRE1- IRS4 complex on the ER membrane. However, IRS4 started to disassociate from the IRE1 and thereof from the ER in a proportion to the duration of ER stress (Figure 4.5). Due to the introduction of a mutation into sYFP, we were able to get strong fluorescent from the GFP channel and to use the red-green-blue (RGB) color panel by showing mTurquoise in blue pseudo-color. Co-localization analysis was executed by evaluating at least 5-10 cells separately with ZEN software (ZEISS).

4.4. Identification of IRS4 and IRE1 Interactomes

In order to identify specific interaction partners of IRE1 and IRS4 related to the intensity and the duration of ER stress, IPs were prepared from 293T cells transiently overexpressing Flag-tagged IRS4 or IRE1. Following, two consecutive IPs were performed using cells at 30 min and 6 hours after ER stress induction with thapsigargin. First IP was executed at the time point where maximum SIK2 activity is detected whereas the second IP was performed at empirically determined time point where IRE1-IRS4-SIK2 complex started to fall apart coincident with the replacement of TRAF2 with IRS4 to form functionally distinct IRE1 signaling complex. Next the IPs were analyzed by Orbitrap Elite mass spectrometer as outlined under methods section 3.6.

Gene enrichment and pathway analyses of IRS4 interactome from IP-MS pointed out mostly ER originated biological processes including initiation of protein synthesis, translocation, ERAD, secretion, the vesicles formation, apoptosis, and inflammation. However, due to the intractability of high number of proteins produced in the IP-MS of IRS4, we undertook an additional IP-MS study using flag-IRE1 as a bait to identify IRE1 specific interactome. Therefore, we assumed that IRE1 and IRS4 interactomes identified separately with IP-MS should produce an overlapping set of proteins common to IRE1-IRS4 signaling complex. This approach enabled us to generate a priority gene list for further studies. Indeed, analysis of the IRS4 interactome unveiled 480 and 195 proteins interacting with IRS4 in the presence and absence of ER stress, respectively, while 382 proteins were found to be constitutively bound to this adapter protein. Likewise, the analysis of IRE1 specific interactome

yielded 234 and 344 proteins selectively bound to IRE in the presence or absence of thapsigargin, respectively, while another 270 proteins were detected invariably in all conditions including the control as shown in the Venn diagram (Figure 4.6). Furthermore, we also included a venn representation of differential gene analysis of IRE1 and IRS4 interactomes generated at 30-minute and 6-hour after ER stress induction (Figure 4.7). A part of detailed comparative analysis of the IRE1 and IRS4 specific interactomes containing protein IDs and annotations were supplemented in the Appendix section.

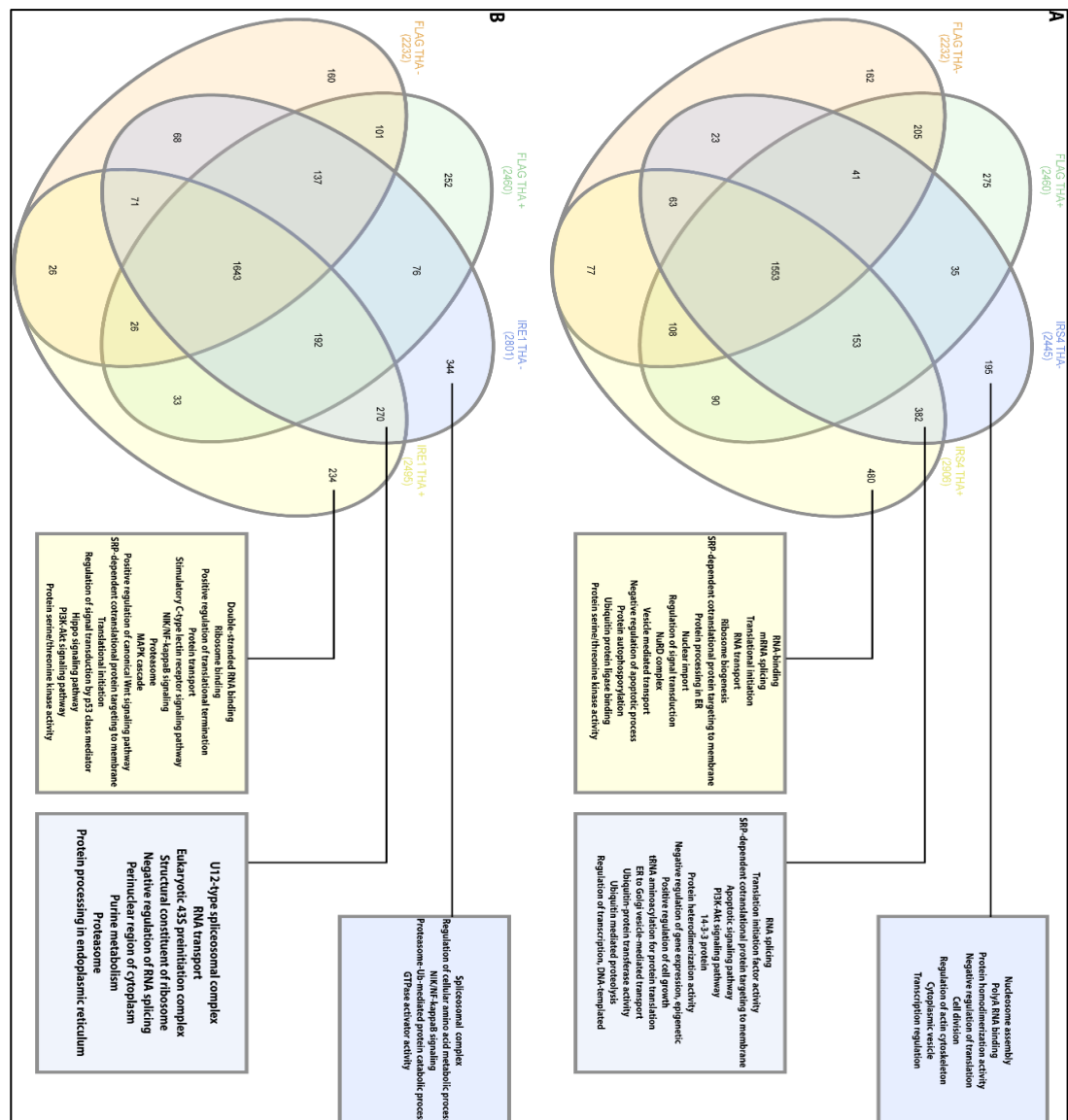


Figure 4.6. Functional gene annotation of proteins interacting with IRE1 and IRS4 in the ER stress-dependent manner. Interacted proteins with IRS4 (A) and IRE1(B) were FLAG-tagged, transiently overexpressed in 293T cells after 1uM thapsigargin treatment for 30 minutes were shown by IP-coupled LC-MS/MS.

5. CONCLUSION

Obesity is a global epidemic causally linked to sedentary lifestyle and consumption of energy dense food. Low-grade metabolic inflammation (meta-inflammation) invariably observed in the CNS neurons especially in the hypothalamus of obese animal models and presumably human subjects is thought to underlie etiopathogenesis of obesity. Interestingly, a recent work pointed out the significance of residential microgliosis in the arcuate nucleus as the primary site for the inflammatory response triggered by circulating saturated fatty acids. HFD-induced hypothalamic inflammation initiated by the resident microglia leads to propagation of inflammatory response by recruiting Bone Marrow Derived Macrophages (BMDMs) eventually leading to functional impairment and cellular loss of POMC and AgRP neurons responsible for the feeding and the energy homeostasis [106]. Although insulin and leptin resistances observed in these neurons are attributed to abrogation of protein homeostasis via ER stress, dysregulated autophagy and inflammation, the molecular mechanism underlying these processes have not been fully elucidated.

Using a LC-MS based substrate screening protocol, our group identified among others IRS4 as a novel substrate for the SIK2 in the ER membrane. SIK2 a member of AMPK like serine/threonine kinase family has been implicated in the regulation of anabolic pathways such as lipogenesis by regulating SREBP1c and CRTC1/2 activities, respectively. Recently SIK2 has been implicated as key player in the fine tunings of the protein homeostasis presumably by enhancing ERAD and autophagy flux through VCP/p97 and HDAC6 proteins, respectively. Earlier reports indicated that SIK2 level and activity in adipose tissue of obese animals increases contrary to robust decrease detected in obese human adipocytes indicating an divergent evolution in the metabolic engagements of this stress and hormone responsive kinase in different species [78]. A mild metabolic phenotype marked with the insulin resistance and associated impaired glucose, insulin tolerance, hypertriglyceridemia, reduced food intake and energy expenditure was reported for the systemic SIK2 knockout mice [72]. Despite the absence of accompanying body weight change the associated phenotype of reduced energy expenditure and food intake along with the enhanced thermogenesis points out significant neuronal roles for SIK2 especially in hypothalamic arcuate nucleus and in sympathetic neuronal activities, respectively. Taken together with the

critical role of IRS4 bearing PVN neurons in energy homeostasis and metabolism [7], the urgent need arise to understand biological functions of the newly identified IRE1-IRS4-SIK2 signalling complex in the neurons of the ARC, PVN, and sympathetic nervous system.

IRS4 is a signaling adaptor molecule and act as a signaling hub for various RTKs, and cytokine receptors and plays critical role in both insulin and leptin signaling pathways. Given support to our hypothesis IRS4 expression predominantly mapped to the hypothalamic region including the arcuate nucleus [107]. Beside, taken together with the abrogated energy metabolism and severe obesity phenotype of IRS4 null mice carrying CNS-specific IRS2 depletion and SIK2 KO mice is strongly suggestive for the genetic interaction between IRS4 and SIK2 in hypothalamic arcuate nucleus or its downstream neurons in the feeding circuitry [100].

An endoplasmic reticulum based phosphoproteomics study from 293T cells overexpressing SIK2 wild type (WT) or SIK2 kinase death (KD) mutant form unraveled an extensive change in the ER specific phosphoproteom depending on kinase activity and ER stress. Among others we decided to focus on ER stress enriched phosphopeptide representing the N terminal serine 64 residues of IRS4 in the 293T cells [111]. All together we proposed that SIK2 being a putative upstream kinase for the IRS4 which may act as an signaling adaptor for IRE1 to modulate its downstream signaling pathways and as a ER specific signaling hub to mediate crosstalk with insulin, leptin, and cytokine signaling pathways not excluding the possibility of regulating IRE1 mediated cell survival, apoptosis, ERAD and autophagy pathways primarily in POMC neurons. To test our this hypothesis, we investigated the effects of SIK2 on IRE1 signaling pathway through newly identified IRE1-IRS4 complex in POMC like neurons under chemically induced ER stress conditions. Firstly, commercially available mHypoA cells originally derived from adult hypothalamic arcuate nucleus were further characterized for the endogenous levels of the IRE1-IRS4-SIK2 signaling components and their isoforms at protein and mRNA levels. As expected the expression levels of both IRE1 and SIK2 were increased proportional to duration of chemically induced ER stress whereas IRS4 transcript level remained unchanged (Figure 4.1). A relative increase detected in the CART transcript may be related to its compensatory role in the attenuation of ER stress in the neurons [108]. Treatment of mHypoA cells with leptin and insulin individually produced different activation profiles for SIK2 as measured by its activation loop threonine 175

phosphorylation detected by specific antibody (Figure 4.2A, B). Although the maximum pSIK2 were attained invariably around 15 minutes of treatment with the two hormones and thapsigargin, pSIK2 response to treatment with insulin produced a perfect oscillation with 30 min intervals compatible with the inherent neuronal response to fed and fast cycle during animal feeding [20]. Although the peak SIK2 activity returned to a preinduction level within 30 minute of treatment with satiety hormone leptin producing almost identical profile with that of thapsigargin, the latter being more effective in restoring the basal SIK2 activity. Taken together SIK2 appears to respond acutely but transiently to the leptin and ER stress agent suggesting that cells create a very narrow time frame for SIK2 to complete its substrate phosphorylation presumably including IRS4 on the ER membrane. Although, we did not address the question of how pSIK2 oscillation is generated in response to treatment of mHypoA cells with insulin, we can speculate the possible involvement of SIK2 specific protein phosphatase 2A (PP2A) [109] (Figure 4.2C).

Although, our original hypothesis is based on the idea that SIK2 by interacting and presumably phosphorylating IRS4 could regulate the IRE1-mediated downstream signaling pathways, we were overly occupied with an extremely provoking idea that IRE1 may be the long sought upstream kinase for the SIK2. In order to test this hypothesis we employed three different inhibitors each producing different effects on the kinase and RNase activities of IRE1. The allosteric IRE1 inhibitor KIRA6 binds to kinase domain and suppress kinase and thereof RNase activities of IRE1. On the other hand, APY29 binding to kinase domain blocks kinase activity while allosterically activating RNase activity of IRE1. STF-083010 binds to RNase domain and suppress its enzymatic activity. Surprisingly, the preliminary results obtained from the parallel use of these three inhibitors unambiguously pointed out the IRE1 kinase activity as an elusive upstream kinase responsible for the phosphorylation dependent activation of SIK2 while excluding the possibility of putative effector kinases downstream of IRE1 α 's RNase activity (Figure 4.3A).

Next we demonstrated the physical interaction between endogenous IRS4 and SIK2 by performing co-IPs sequentially with antibodies specific to these two proteins. Similarly, the interaction between IRS4 and IRE1 and their co-localization on ER membrane were demonstrated both in the presence of endogenous and exogenous IRE1 using IP and confocal imaging, respectively. The authenticity of IRE1 complex was confirmed by co-immunoprecipitating the IRE1 α signaling components TRAF2-

JNK with anti IRS4 antibody. It is noticeable that IRE1 and IRS4 are constitutively co-exists on the ER membrane however, IRS4 dissociates from IRE1 thereof from the ER membrane proportional to duration of ER stress. Coincident with this dissociation SIK2 from IRS4 also unraveled with a similar kinetic suggesting that SIK2 could determine the dynamic behavior and constellation of the IRE1 signalosome as well. Similar regulatory switches mediated by SIK2 have been exemplified in the regulation of various metabolic processes [75], [79], [80], [81].

Finally, we carried out IP-MS based interactome studies to determine IRS4 and IRE1 interacting proteins in 293T cells treated or not with the thapsigargin. First, the presence of IRE1 and IRS4 in the IPs were confirmed based on respective peptide abundance scores. Then, we applied GO-term and pathway enrichment analyses to interacted (UP) and non-interacted (DOWN) genes with respect to 30-minutes and 6-hours ER stress induction with thapsigargin (THA). The most remarkable enriched terms in the IRS4/UP/THA (30 min) group were: "Protein serine/threonine kinase activity", "KEGG-Protein processing in ER", and "GO-BP-Protein autophosphorylation" (Figure 4.6A), "Protein serine/threonine kinase activity" and "KEGG- PI3K-Akt signaling pathway" terms were found to be overrepresented in the IRE1/UP/THA (30 min) group (Figure 4.6B). It is noteworthy that the term "protein serine / threonine kinase activity" is commonly assigned to both IRE1/DOWN/THA (6 hours) and IRS4/DOWN/THA (6 hours) groups. Comparative analysis of IP-MS identified IRE1 and IRS4 interactomes resulted in a list of overlapping proteins belonging to either 30 min or 6 hours of ER stress induction. For example, the "AP-3 adaptor protein" term was found to be overrepresented in both IRS4 and IRE1 interactomes indicating the possibility that AP3 may be utilized by IRE1-IRS4 signaling complex to mediate its unknown function. PP2A, which previously identified to interact with SIK2 [109], protein was come from IRS4 interactomes in the ER stress condition (30 min), also from IRE1 interactomes in absence of ER stress. (Figure 4.7). Moreover, RNF114, which is an E3 ubiquitin-protein ligase and involved in the pathway of protein ubiquitination, protein was observed in IRE1 interactomes in ER stress condition (6 hours) but in IRS4 intractomes in absence of ER stress.

Therefore, in the next step we are planning to further investigate the biological role of IRE1-IRS4 signaling complex in conjunction with its newly identified components such as AP3, PP2A, and RNF114 in the POMC neurons. We believe that

the preliminary data created in this work will pave the way for the development of new therapeutic approaches for the treatment of metabolic and neurodegenerative diseases.

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Ayşe Yazıcı was born in Istanbul, Turkey, in 1995. She graduated from Gökşen Mustafa Yücel high school with a first degree in the numerical department in 2012. She received a bachelor's degree in Molecular Biology and Genetics from Gebze Technical University with a first degree in 2017 with a GPA of 3.76/4.00. In the same year, she got admission to the Department of Cell Biology and Genetics, Gebze Technical University, and graduated in 2021 with a master of science degree. Since 2018, she has been working as a research assistant at GTU under the Faculty of Basic Sciences.

APPENDIX

Appendix A. List of proteins belonged to interactome of IRS4 and IRE1 in HEK293T cells.

Table A1.1. The list of common interacting proteins with both IRE1 (in the absence of the ER stress stimulus) and IRS4 (in the presence of only 30-minutes of the ER stress stimulus).

Protein ID	Protein Name
Q53HR0	Eukaryotic translation initiation factor 3, subunit 3 gamma, 40kDa variant (Fragment)
G3V5Q1	DNA-(apurinic or apyrimidinic site) lyase (EC 4.2.99.18) (Fragment)
F8VZU9	Myosin light polypeptide 6
G3V1Y7	Myosin light polypeptide 6 (Myosin, light polypeptide 6, alkali, smooth muscle and non-muscle, isoform CRA_d)
E5RJT0	Eukaryotic translation initiation factor 3 subunit H (Fragment)
D6RBK5	Matrin-3 (Fragment)
Q68D11	Uncharacterized protein DKFZp686K23100
Q05BV5	OGFR protein
A0A0A0MRN5	Opioid growth factor receptor (Opioid growth factor receptor, isoform CRA_d)
Q9NZT2	Opioid growth factor receptor (OGFr) (Protein 7-60) (Zeta-type opioid receptor)
B3KMQ6	cDNA FLJ12172 fis, clone MAMMA1000684, highly similar to Opioid growth factor receptor
G3V3Y6	DNA-(apurinic or apyrimidinic site) lyase (Fragment)
G3V5D9	DNA-(apurinic or apyrimidinic site) lyase (Fragment)
Q96HI4	Glycylpeptide N-tetradecanoyltransferase (EC 2.3.1.97) (Fragment)
H7C5A0	Replication factor C subunit 2 (Fragment)
Q75MT5	Replication factor C (Activator 1) 2, 40kDa (Replication factor C (Activator 1) 2, 40kDa, isoform CRA_a)
P35250	Replication factor C subunit 2 (Activator 1 40 kDa subunit) (A1 40 kDa subunit) (Activator 1 subunit 2)
O00139	Kinesin-like protein KIF2A (Kinesin-2) (hK2)
P35249	Replication factor C subunit 4 (Activator 1 37 kDa subunit) (A1 37 kDa subunit) (Activator 1 subunit 4)
Q9ULR0	Pre-mRNA-splicing factor ISY1 homolog
Q96KR1	Zinc finger RNA-binding protein (hZFR) (M-phase phosphoprotein homolog)
Q06210	Glutamine--fructose-6-phosphate aminotransferase [isomerizing] 1 (EC 2.6.1.16)
H0YCC8	Polyadenylate-binding protein 4 (Fragment)
Q6PK82	AP3D1 protein (Fragment)

Table A1.1. Continue.

O14617	AP-3 complex subunit delta-1 (AP-3 complex subunit delta) (Adaptor-related protein complex 3 subunit delta-1) (Delta-adaptin)
Q6I9T8	Serine/threonine-protein phosphatase (EC 3.1.3.16)
P67775	Serine/threonine-protein phosphatase 2A catalytic subunit alpha isoform (PP2A-alpha) (EC 3.1.3.16) (Replication protein C) (RP-C)
Q8IXW5	Putative RNA polymerase II subunit B1 CTD phosphatase RPAP2 (EC 3.1.3.16) (RNA polymerase II-associated protein 2)
J3QSY4	Ribonucloprotein
Q9NX24	H/ACA ribonucleoprotein complex subunit 2 (Nucleolar protein family A member 2) (snoRNP protein NHP2)
P62875	DNA-directed RNA polymerases I, II, and III subunit RPABC5 (RNA polymerases I, II, and III subunit ABC5)
B1ANS9	WD repeat-containing protein 64
K7ERM6	Interleukin enhancer-binding factor 3 (Fragment)
Q9UGR2	Zinc finger CCCH domain-containing protein 7B (Rotavirus 'X'-associated non-structural protein) (RoXaN)
A0A024R1N2	Zinc finger CCCH-type containing 7B, isoform CRA_b

Appendix B. List of primers using in this study

Table B1.1: List of used primers in this study.

Primer Name	Sequence (5'-3')	Application	Tm
7F_EcoRV_mIRS4	ATGTGATATCATGGCGAGTTGCTCC TTC	Cloning	60.4
7R_HindIII_mIRS4	ATATAAGCTTCTTCCTGTAGTCTCGT CTGGCA	Cloning	61.3
mIRS1_F	AGCGCGCCTGGAGTATTATGAGAA	qPCR	60.1
mIRS1_R	GTCAGCCCGCTTGTTGATGTTGAA	qPCR	60.2
mIRS2_F	AGCCAGGAGACAAGAACTCC	qPCR	56.6
mIRS2_R	AGTGATGGGACAGGAAGTCG	qPCR	56.5
mIRS4_F	TCCTGTACCAATGCTTCTCCG	qPCR	56.8
mIRS4_R	CGCGAAGTATTCGTCCTGGG	qPCR	58.0
mSIK1_F	GCGACTACAACGAACAGGTGCT	qPCR	59.7
mSIK1_R	GTAGGAGGTAGTAAATGGCGGC	qPCR	57.3
mSIK2_F	GCTGCCTTTATGGAGGAAGAGTG	qPCR	57.7
mSIK2_R	GAGGTCTCCATCATACTGCTGG	qPCR	56.8
mSIK3_F	AACGGCAGCTAGGACAACAGTC	qPCR	59.4
mSIK3_R	GTGTTGGAGTCCTTGTAGGTGG	qPCR	57.4
mIRE1 α _F	GCCGAAGTTCAGATGGAATC	qPCR	53.6
mIRE1 α _R	ATCAGCAAAGGCCGATGA	qPCR	54.0
mIRE1 β _F	GGCATTGGACTTAGTGAGAGCC	qPCR	57.7
mIRE1 β _R	CCTTCTCCAACCAGTCACTGAC	qPCR	57.3
mAgRP_F	GGCCTCAAGAAGACAAGTGC	qPCR	56.3
mAgRP_R	GACTCGTGCAGCCTTACACA	qPCR	57.4
mCART_F	AAACGCATTCCGATCTACGA	qPCR	54.0
mCART_R	TCACAAGCACTTCAAGAGGA	qPCR	54.0
mGAPDH_F	CATCACTGCCACCCAGAAGACTG	qPCR	59.8
mGAPDH_R	ATGCCAGTGAGCTTCCCGTTCAG	qPCR	61.6
h/mXBP1_F	TGCTGAGTCCGCAGCAGGTG	RT-PCR	62.6
h/mXBP1_R	GCTGGCAGGCTCTGGGGAAG	RT-PCR	63.3