

T.R.
GEBZE TECHNICAL UNIVERSITY
GRADUATE SCHOOL

PROBING THE ROLES OF SIK2 IN
HYPOTHALAMIC POMC NEURONS

MEHMET SONER TÜRKÜNER

A THESIS OF DOCTORATE
DEPARTMENT OF MOLECULAR BIOLOGY AND GENETICS

ADVISOR: PROF. DR. FERRUH ÖZCAN

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T.C.
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LİSANSÜSTÜ EĞİTİM ENSTİTÜSÜ

SIK2'NİN HİPOTALAMİK POMC NÖRONLARINDAKİ
ROLLERİNİN ARAŞTIRILMASI

MEHMET SONER TÜRKÜNER

DOKTORA TEZİ
MOLEKÜLER BİYOLOJİ VE GENETİK
ANABİLİM DALI

DANIŞMAN: PROF.DR. FERRUH ÖZCAN

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JURY

MEMBER

(THESIS ADVISOR) : Prof. Dr. Ferruh ZCAN

MEMBER

: Prof. Dr. Petek BALLAR KIRMIZIBAYRAK

MEMBER

: Assoc. Prof. Dr. İbrahim YAMAN

MEMBER

: Assist Prof. Dr. Can M. ERİŐTİ

MEMBER

: Assist Prof. Dr. Pınar PİR

APPROVAL

Gebze Technical University Graduate Administration Board
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To my family and my scientific dreams...

ABSTRACT

The neuronal etiology of obesity is centered around diet-induced inflammatory state in the arcuate nucleus of the hypothalamus which impairs the functionality of pro-opiomelanocortine neurons (POMCs) responsible for whole-body energy homeostasis and feeding behavior. Intriguingly, systemic salt inducible kinase 2 (SIK2) knockout mice demonstrated reduced food intake and energy expenditure along with modestly dysregulated metabolic parameters suggesting a causal link between the absence of SIK2 activity in POMCs and the observed phenotype. To be able to probe the role of SIK2 in POMCs, we conducted a comparative secretomics study from cultured POMC neurons lacking or overexpressing SIK2 depending on pharmacologically induced endoplasmic reticulum (ER) stress induction, a hallmark of metabolic inflammation and POMC dysregulation in diet-induced obese (DIO) mice. The data corroborated our hypothesis of POMC specific SIK2 activity in controlling the energy metabolism and feeding in DIO mice by regulating the nature of related POMC secretome in response to early-phase of the ER stress induction. On the other hand, we showed that SIK2 depletion increases the production and co-localization of α -MSH with secretory vesicles in the absence of ER stress. Our data also suggested that SIK2 may also negatively regulate cellular secretion in a stress-independent manner presumably through COPII vesicles formation. Overall, our data is consistent with the reduced food intake reported for the systemic SIK-KO mouse. Based on these data we proposed a physiological switch-like model wherein SIK2 acts as an adjustable knob that responds to ER stress both at physiological and pathological conditions and to maintain survival and cellular homeostasis downstream of UPR. If ER stress persists and is not resolved SIK2's ability to support cellular homeostasis and survival pathways is overpowered by counteracting mechanisms like inflammatory and apoptotic pathways leading to cell death.

Keywords: SIK2; POMC; Obesity; ER stress; Feeding; Secretome.

ÖZET

Obezitenin nöronal etiyolojisinde, vücudun enerji dengesinden ve beslenme davranışından sorumlu pro-opiomelanokortin nöronlarının (POMC'ler) işlevselliğini bozduğu bilinen, hipotalamusun arkuat çekirdeğindeki diyet kaynaklı inflamasyon hedef gösterilmektedir. Ayrıca, sistemik tuzla indüklenebilir kinaz 2 (SIK2) taşımayan fareler, düzensiz metabolik parametrelerle birlikte azalmış gıda alımı ve enerji harcaması fenotipi sergilemektedir, bu da POMC'lerde SIK2 aktivitesinin yokluğu ile gözlemlenen fenotip arasında nedensel olası bir bağlantıyı desteklemektedir. SIK2'nin POMC'lerdeki rolünü araştırabilmek için, farmakolojik olarak tetiklenen endoplazmik retikulum (ER) stres indüksiyonuna bağlı olarak SIK2'den yoksun veya aşırı ifade eden POMC nöronları üzerinde karşılaştırmalı sekretomik bir çalışma yürüttük. Verilerimiz, ER stres indüksiyonunun erken fazına yanıt olarak, POMC'ye özgü SIK2 aktivitesinin POMC sekretomunu düzenleyerek DIO farelerinde enerji metabolizmasını ve beslenmeyi kontrol etmedeki rolüne dair hipotezimizi desteklemiştir. Öte yandan, çalışmamızda ER stres yokluğunda SIK2'nin yokluğunun α -MSH üretimini ve salgı vezikülleriyle birlikte lokalizasyonunu artırdığını gösterdik. Bulgularımız ayrıca SIK2'nin hücresel salgıyı muhtemelen COPII vezikülleri oluşumu yoluyla stresten bağımsız bir şekilde olumsuz yönde düzenleyebileceğini de göstermektedir. Nihayetinde, verilerimiz sistemik SIK2 taşımayan fare için bildirilen azalmış gıda alımı fenotipi ile tutarlılık içerisindedir. Bulgularımıza dayanarak, SIK2 için hem fizyolojik hem de patolojik koşullarda ER stresine yanıt veren ve UPR'nin aşağı akışında hayatta kalma ve hücresel homeostazı sürdürmek için ayarlanabilir bir düğme görevi gören anahtar benzeri bir model önermekteyiz. Modelimize göre, ER stresi devam eder ve çözülmezse, SIK2'nin hücresel homeostazı ve hayatta kalma yollarını destekleme kabiliyeti, hücre ölümüne yol açan inflamatuvar ve apoptotik yollar gibi karşı mekanizmalar tarafından etkisiz hale getirilmektedir.

Anahtar Kelimeler: SIK2; POMC; Obezite; ER stres; Beslenme; Sekretom

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LIST OF SYMBOLS AND ABBREVIATIONS

SIK2	: Salt Inducible Kinase
POMCs	: mHypoA-POMC/GFP-2 cells
DIO	: Diet-induced obesity
ARC	: Arcuate nucleus
CNS	: Central nervous system
PVH	: Paraventricular nuclei of the hypothalamus
DMH	: Dorsomedial nucleus of the hypothalamus
LHA	: Lateral hypothalamic area
TGN	: Trans Golgi network
ISG	: Immature secretory granules
MSG	: Mature secretory granules
CSF	: Cerebrospinal fluid
PN	: Proteostasis network
UPR	: Unfolded protein response
ERAD	: Endoplasmic reticulum (ER) associated degradation
RIDD	: IRE1 α -dependent decay
ALS	: Amyotrophic lateral sclerosis
LC-MS/MS	: Liquid chromatography–mass spectrometry
IP-MS	: Immunoprecipitation-based mass spectrometry
PSM	: Peptide sequence mass
LQF	: Label-free quantification
GO	: Gene Ontology
FDR	: False discovery rate
1DE	: one-dimensional gel electrophoresis
DDA	: Data-dependent acquisition
DGE	: Differential gene expression

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1. INTRODUCTION

1.1. Obesity

Obesity is a complex global epidemic that is particularly prevalent among children and adolescents and has been the subject of extensive research [1]. It is defined as an excessive accumulation of body fat. Body mass index (BMI) is a standardized metric used to quantify body fat, calculated by dividing an individual's weight (kg) by the square of their height (m^2). An individual is classified as obese if their BMI is equal to or above 30 kg/m^2 [2]. Obesity is characterized by a state of positive energy balance, whereby energy intake exceeds energy expenditure (Figure 1.1). This imbalance can result from a number of factors, including environmental, dietary, behavioral, social, and genetic predispositions [3], [4],[5] [6], [7]. The excess energy is stored within the various depots of adipose tissue by increasing the storage capacity of existing adipocytes (hypertrophy) or the number of newly generated adipocytes (hyperplasia) [8]. Hypertrophied adipocytes especially in visceral tissues are considered the source of the local inflammatory response, which is thought to be a key link between obesity and the associated dysregulations including metabolic, cardiovascular, neurodegenerative, and cancerous conditions [9], [10], [11], [12].

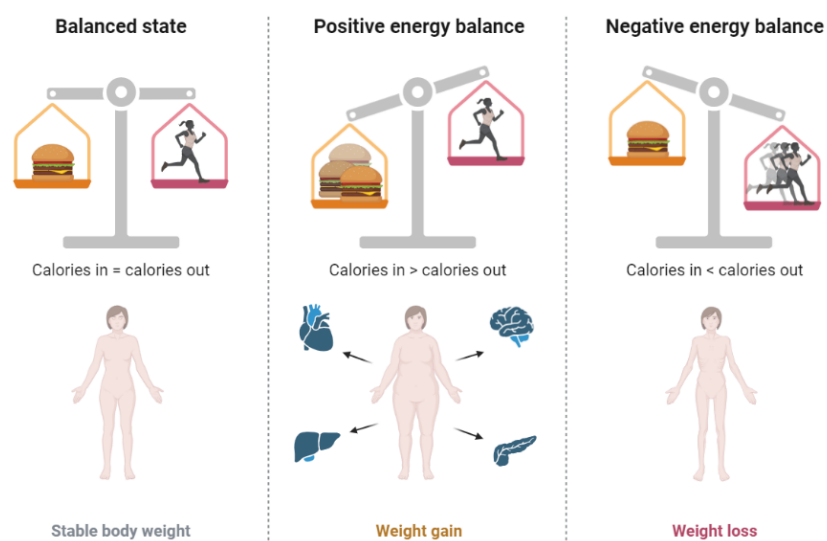


Figure 1.1: Schematic illustration of energy balance and its effects on body weight. Created in <https://BioRender.com>.

1.1.1. Hypothalamic Obesity

Hypothalamic obesity is caused by damage or dysfunction of the hypothalamus, a region of the brain that plays a crucial role in regulating energy balance, appetite, and body weight. Although the hypothalamic obesity paradigm can be traced back to earlier clinical studies based on hypothalamic injuries [13], [14], the concept has recently been revisited, especially in studies in which low-grade metabolic inflammation was detected in the hypothalamus prior to the onset of obesity. High-fat diet (HFD) exposure and the resulting metabolic dysregulation can trigger neuroinflammation in the hypothalamus, particularly in regions like the arcuate nucleus (ARC) and preoptic area (POA) [15]. This neuroinflammation involves peripheral macrophage infiltration from the visceral adipose tissue and the activation of microglia and astrocytes, leading to the release of pro-inflammatory cytokines such as TNF- α , IL-1 β , and IL-6 [16] [17]. The mechanisms underlying this hypothalamic inflammation appear to be multifactorial. Increased levels of saturated fatty acids, such as palmitic acid, can directly induce inflammatory responses in hypothalamic cells, they can also activate the TLR4 signaling pathway in hypothalamic neurons, leading to the production of pro-inflammatory cytokines and the induction of insulin and leptin resistance [18].

Interestingly, some studies have suggested that interventions targeting the reduction of hypothalamic inflammation, such as dietary modifications, exercise, or pharmacological approaches, may be effective in reversing or mitigating the effects of obesity on the brain [19], [20], [21], [22]. Therefore, understanding the mechanisms underlying this hypothalamic dysregulation and identifying interventions to prevent or reverse it are important areas of research for the treatment of obesity and its comorbidities.

1.2. Hypothalamic Regulation of Energy Hemostasis

The central nervous system (CNS) is crucial for monitoring and regulating the metabolic homeostasis of the organism. Genome-wide association studies (GWAS) have identified that a majority of genes associated with BMI are expressed in the CNS, with many of them located in the hypothalamus [23]. The hypothalamus facilitates communication between the peripheral organs and CNS via the hub of communication. Particularly the hypothalamic ARC is an integrator of nutrient-state communicating signals and plays a pivotal role in maintaining the overall energy balance and controlling feeding behavior [24], [25]. It contains two distinct antagonistic types of neurons; the first type is characterized by the expression of orexigenic (appetite-promoting) Agouti-related peptide/neuropeptide Y (AgRP/NPY), while the second type is marked by the expression of anorexigenic (appetite-suppressing) pro-opiomelanocortin/cocaine- and amphetamine-regulated transcript (POMC/CART). These neurons receive signals from both the peripheral organs and the CNS and communicate with postsynaptic melanocortin 4 receptor (MC4R)-expressing secondary neurons through the release of different neuroproteins [25], [26]. They exhibit distinct synaptic connections with the paraventricular nucleus of the hypothalamus (PVN/PVH) as well as the ventromedial hypothalamus (VMH), the dorsomedial nucleus of the hypothalamus (DMH), the lateral hypothalamic area (LH), and the medial amygdala [27], [28] (Figure 1.2).

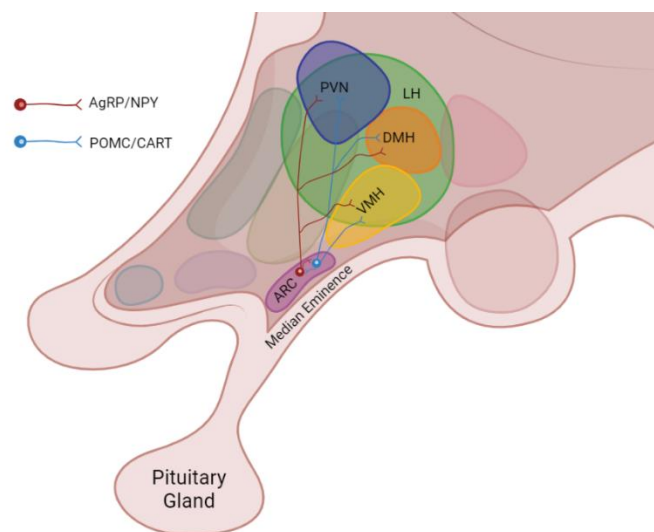


Figure 1.2: ARC and associated hypothalamic nuclei in energy regulation. Created in <https://BioRender.com>.

During hunger, a state of negative energy balance, specific hormones secreted by the gastrointestinal tract, such as ghrelin, stimulate AgRP/NPY neurons. These neurons promote food intake and reduce the metabolic rate by inhibiting both PVH and POMC neurons [26]. On the other hand, when satiety, a state of positive energy balance, hormones like leptin and insulin activate POMC and inactivate AgRP neurons. This stimulation increases energy expenditure while reducing food consumption by activating PVH neurons with α -melanocyte-stimulating hormone (α -MSH) released from the POMC neurons (Figure 1.3) [29]. Disruption of this finely tuned regulatory process leads to an imbalance between energy intake and expenditure as well as deregulation of peripheral metabolism [30], [31], [32].

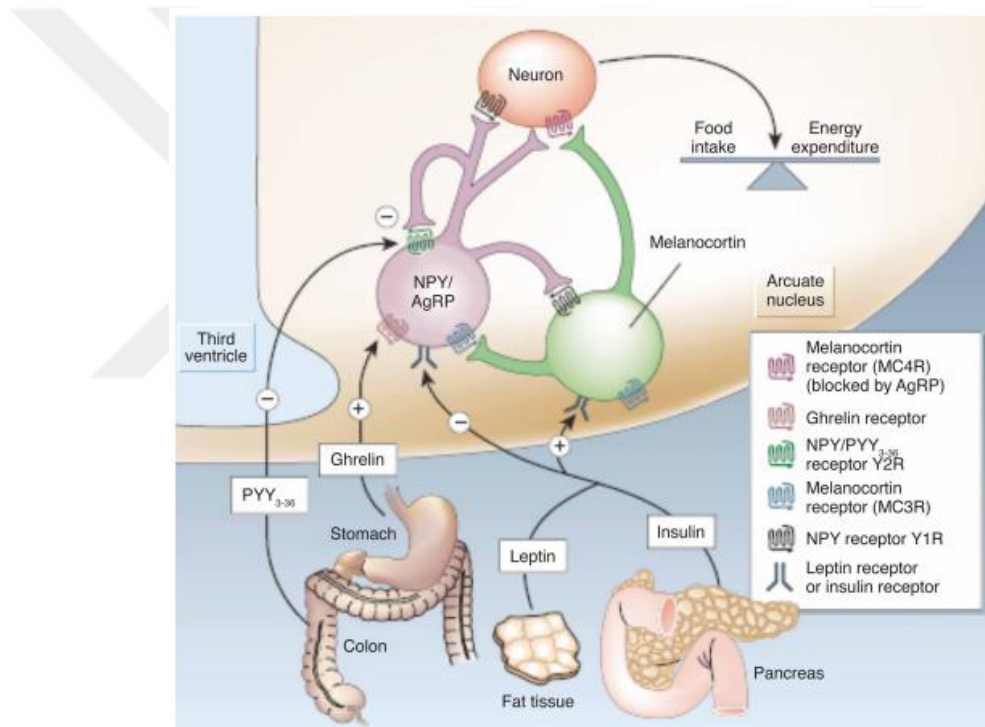


Figure 1.3: Hypothalamic control of global energy balance.

However, the evidence suggests that AgRP neuronal activity is not a prerequisite for the development of obesity and prolonged HFD has been shown to attenuate the response of AgRP to feeding-related stimuli [33], [34]. These findings suggest a potential for non-AgRP GABAergic neurons in chronic control of appetite. Indeed, it was discovered that neurons expressing prepronociceptin (PNOC), a recently identified GABAergic cell type in the ARC, were activated by acute HFD. PNOC^{ARC} inhibits anorexigenic POMC neurons and promotes feeding [35].

1.2.1. Hypothalamic Neuronal Heterogeneity of ARC

The heterogeneity of POMC and AgRP neurons in the hypothalamus is a critical research area that highlights the complexity of energy homeostasis regulation. Recent RNAseq and chemi/optogenetic studies have elucidated the distinct subpopulations within these neuronal groups, revealing their unique roles and interactions in metabolic processes. Early studies indicated that POMC neurons are classified according to their expression of the neurotransmitter glutamate or γ -aminobutyric acid (GABA) [36]. Single-cell RNA sequencing showed that approximately 75% of the POMC population was classified as ‘canonical’ POMC(P⁺), containing high levels of *Pomc* and little/no *Agrp*, while 25% contained low levels of *Pomc* and high levels of *Agrp* (A⁺P⁺). It was further observed that these neurons expressed leptin and insulin receptors at different rates [37]. Interestingly, POMC neurons expressing the cannabinoid receptor (CB₁R) increase food intake by releasing β -endorphins instead of α -MSH in response to cannabinoids. This is another level of plasticity [38]. Collectively, different studies comprising both biased and unbiased methodologies, have demonstrated a high degree of intracellular and intercellular POMC plasticity. This can be classified according to spatial heterogeneity, cell surface receptors, and expressed neuropeptides/neurotransmitters [24], [39], (Figure 1.4).

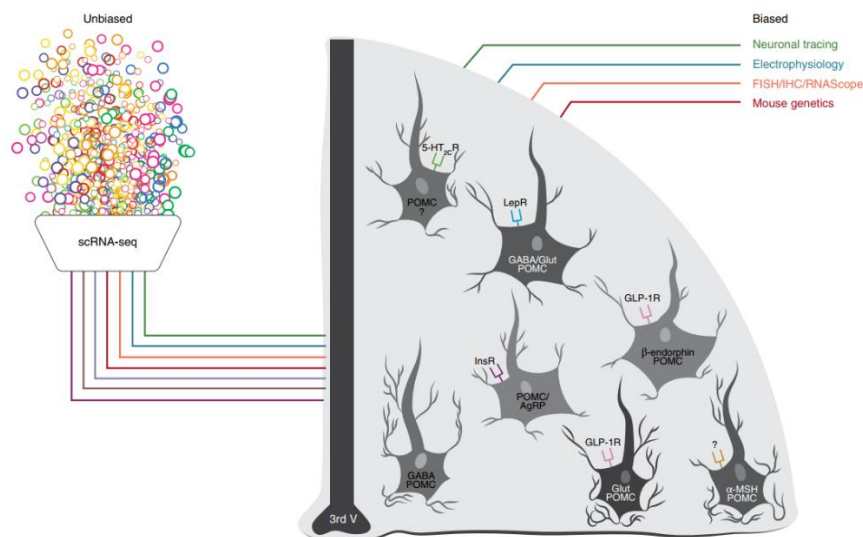


Figure 1.4: POMC Neuron subtypes identified with classification methods.

1.2.2. POMC Processing and Secretion

POMC, a member of the opioid/orphanin gene family, is a common precursor of melanocortin-related peptides (ACTH/ α -MSH, β -MSH and γ -MSH) and the opioid β -endorphin [40], [41]. A comprehensive review of the extant literature reveals the presence of POMC-derived peptides in certain species of invertebrates (*Theromyzon tessulatum*), as well as in vertebrates [42], [43], [44]. There is evidence of the expression of two or three different *pomc* transcripts and its receptor, MC4R, in various species of teleosts (e.g., zebrafish [45], carp [46], and barfin flounder [47]). Furthermore, the studies in fish injected with MRC agonists have demonstrated a dose-dependent decrease in food intake, indicating a conserved hypothalamic control of energy homeostasis analogous to that observed in mammals [48]. This finding underscores the notion that the melanocortin system has its origins in the early stages of evolution. Furthermore, studies have suggested an extremely ancient origin and a remarkably high level of conservation of POMC and derived peptides. Indeed, it has been demonstrated that the invertebrate sequences of POMC-derived peptides are highly like those of vertebrates, and that leech and human α -MSH can bind to the same receptor [42], [43].

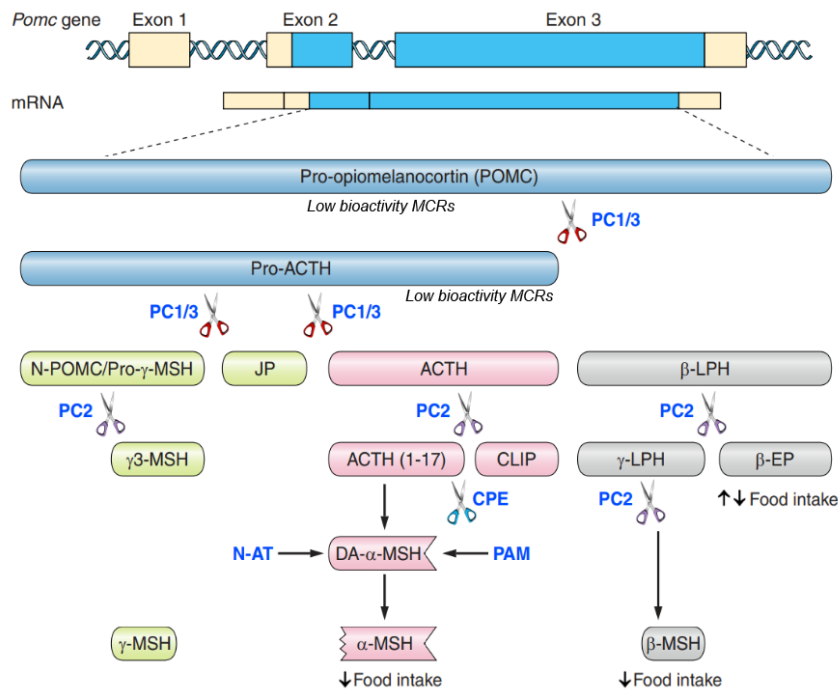


Figure 1.5: Pro-opiomelanocortin (POMC) processing generates numerous functional peptides.

POMC is initially expressed as a precursor protein and then undergoes a series of posttranslational enzymatic steps to form biologically active peptides, including melanotropins such as α -MSH, corticotropins, such as adrenocorticotropin hormone (ACTH), and endorphins such as β -endorphin [49]. Post-translational processing of POMC is primarily mediated by prohormone convertases (PCs), particularly PC1/3 and PC2, which cleave POMC at lysine and arginine-based dibasic amino acid sequences in organism and tissue-specific manner, with distinct cleavage patterns observed in the pituitary, hypothalamus, and skin. Despite the hypothalamic heterogeneity, most POMC neurons exhibit co-expression of both PC1/3 and PC2 for complete POMC maturation [37]. However, the Lys-Lys cleavage site is not present in all rodents, such as mice and rats, and hence, β -MSH is not produced [49]. Certain peptides undergo further modifications to become active peptides. To generate α -MSH from ACTH (1-17), C-terminal amino acids are removed by carboxypeptidase E (CPE) and then amidated by peptidyl-glycine α -amidating monooxygenase (PAM) to form less active des-acetyl (DA)- α -MSH. It is then acetylated at the N-terminal by N-acetyltransferase (N-AT) to give biologically active α -MSH [50] (Figure 1.5).

The processing of POMC into mature peptides occurs within the axis of ER, Golgi and secretory vesicles, respectively. After translation, the POMC pro-hormone proceeds into the ER via an N-terminal signaling peptide. A series of chemical reactions occur, including cleavage of the signal peptide, N-linked glycosylation, and disulfide bond formation. The pro-hormone is then transferred to the Golgi via the coat protein complex II (COPII) vesicles. It undergoes further modifications such as phosphorylation, while cleavage processes with peptidase activity in the Trans Golgi Network (TGN) where it is sorted into immature secretory granules (ISG). Further peptidase activity, acetylation, and amidation are performed within the mature secretory granules (MSG) before fusion with the plasma membrane [50] (Figure 1.6).

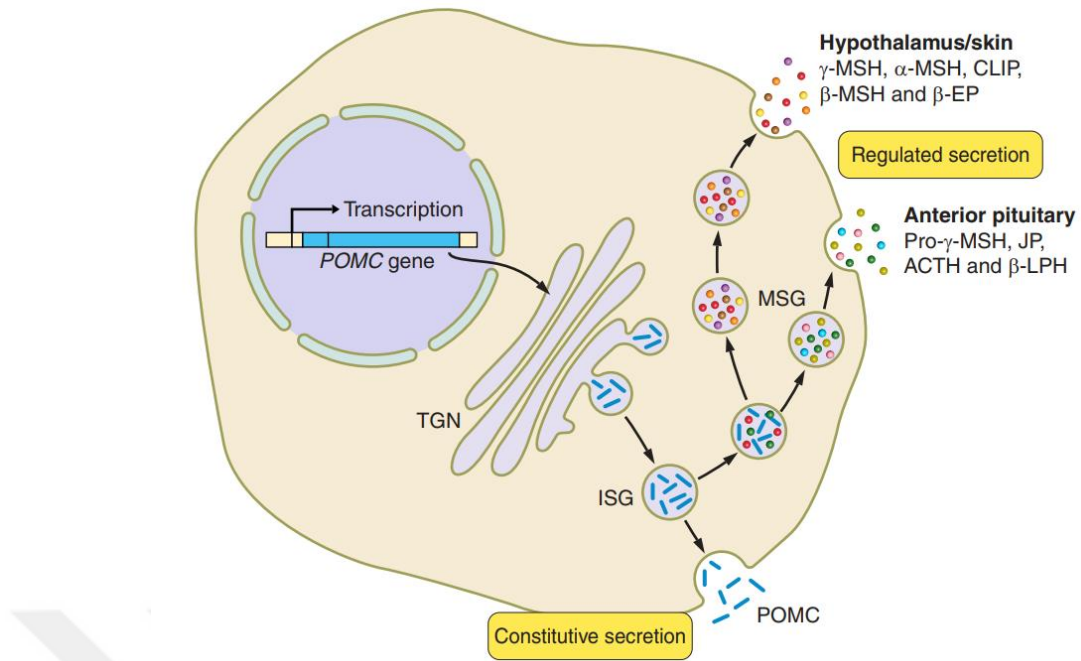


Figure 1.6: Pomc secretion pathways.

There are two distinct secretion pathways for POMC peptides. In the regulated secretion pathway, most of the neuropeptides and hormones are stored in the MSG until cells receive a signal for their release. The α -MSH can be released by this pathway, and it is most abundant in the hypothalamus. In the constitutive pathway from ISG, the release of peptides is not regulated by external factors. Incompletely processed POMC can be released by this pathway (Figure 1.6). Precursor POMC itself is highly detectable in the cerebrospinal fluid (CSF). It has been suggested that low levels of CSF POMC in obese animals can be used as a biomarker for POMC activity. [51], [52].

1.3. Proteostasis and Endoplasmic Reticulum Stress

Proteostasis, the maintenance of protein homeostasis, ensures proteins are correctly synthesized, folded, and degraded. It is coordinated by a multi-compartmental system termed the proteostasis network (PN). The three core functional axes constitute the PN: (1) protein synthesis at the ribosomes with translational regulation machinery, (2) protein folding through the molecular chaperones and co-chaperones, and (3) protein clearance by ubiquitin-proteasome pathway (UPS), endoplasmic reticulum (ER) associated degradation (ERAD) or autophagy-lysosome pathway [53] (Figure 1.7).

The PN is not static; It can adapt to different conditions through stress response pathways including the heat shock response (HSP), oxidative stress response (OxR) and the unfolded protein response (UPR). The ER plays a pivotal role in the proteostasis, acting as a central coordinator for quality control processes that ensure the optimal functionality of the secreted and membrane proteins in the cell. The process of protein folding within the ER is characterized by a relatively high degree of failure, with only a small proportion of proteins successfully overcoming the ER's protein quality control (ERQC) mechanisms. During the maturation process, misfolded and aggregated proteins are degraded via the ERAD pathway [54].

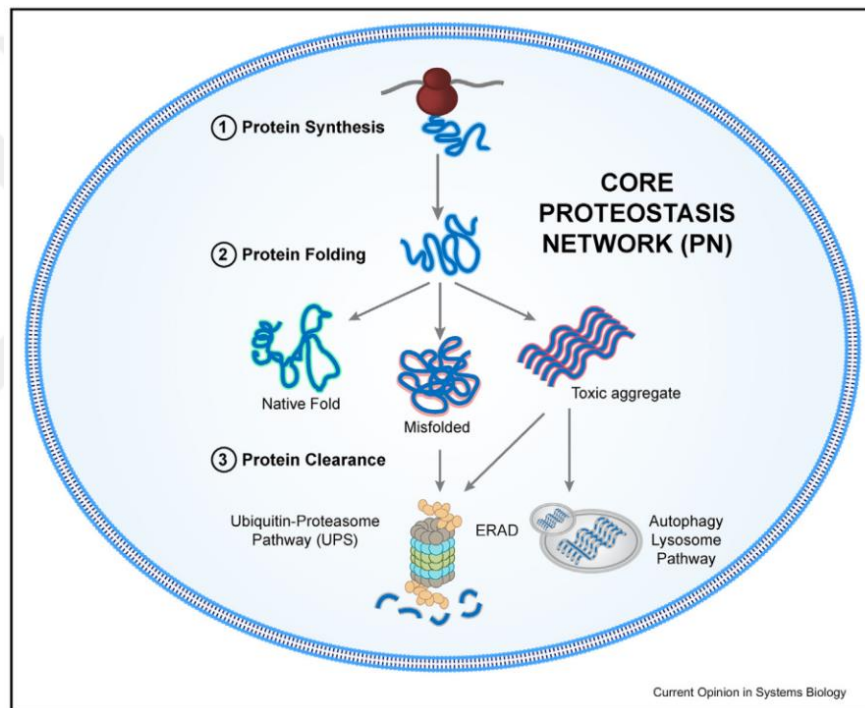


Figure 1.7: The core proteostasis network (PN).

ER stress is the proteostatic imbalance within the ER caused by overloading misfolded proteins which exceeds ER capacity. Consequently, this leads to the activation of the UPR, which is an integrated signaling route that travels from the ER lumen to the cytosol and nucleus in order to communicate information regarding the status of protein folding [55]. The UPR entails the activation of three ER transmembrane sensor proteins known as inositol-requiring enzyme 1 (IRE1), PKR-like ER kinase (PERK), and activating transcription factor 6 (ATF6) (Figure 1.8). The ER chaperone binding

immunoglobulin protein (BiP/Grp78) modulates the initiation of UPR in the presence of misfolded proteins. Under basal conditions, BiP interacts with the luminal domains of these transmembrane proteins and keeps them inactive, whereas in the ER stress, BiP is recruited by misfolded proteins, resulting in the activation of sensor proteins of the UPR branches. ATF6 is vesicular transported to the Golgi where it is cleaved by proteases into its soluble cytosolic fragment ATF6 (N). It then enters the nucleus and activates UPR genes (Figure 1.8A). PERK homodimerizes and phosphorylates itself and translation initiation factor eIF2 which in turn shuts down global translation and increases the expression of the transcription factor ATF4. The ATF4 then enters the nucleus and activates UPR genes for survival or apoptosis (Figure 1.8B). IRE1 is a bifunctional kinase/endoribonuclease (RNase) that oligomerizes and trans-autophosphorylates to activate its downstream pathways. RNase activity selectively reduces translation by degrading newly synthesized mRNAs through a pathway called regulated IRE1 α -dependent decay (RIDD). It also truncates the UPR-specific transcription factor X-box binding protein 1 (XBP1) mRNA. Spliced sXBP1 then enters the nucleus and activates UPR genes, especially for ERAD and ER biogenesis (Figure 1.8C) [56].

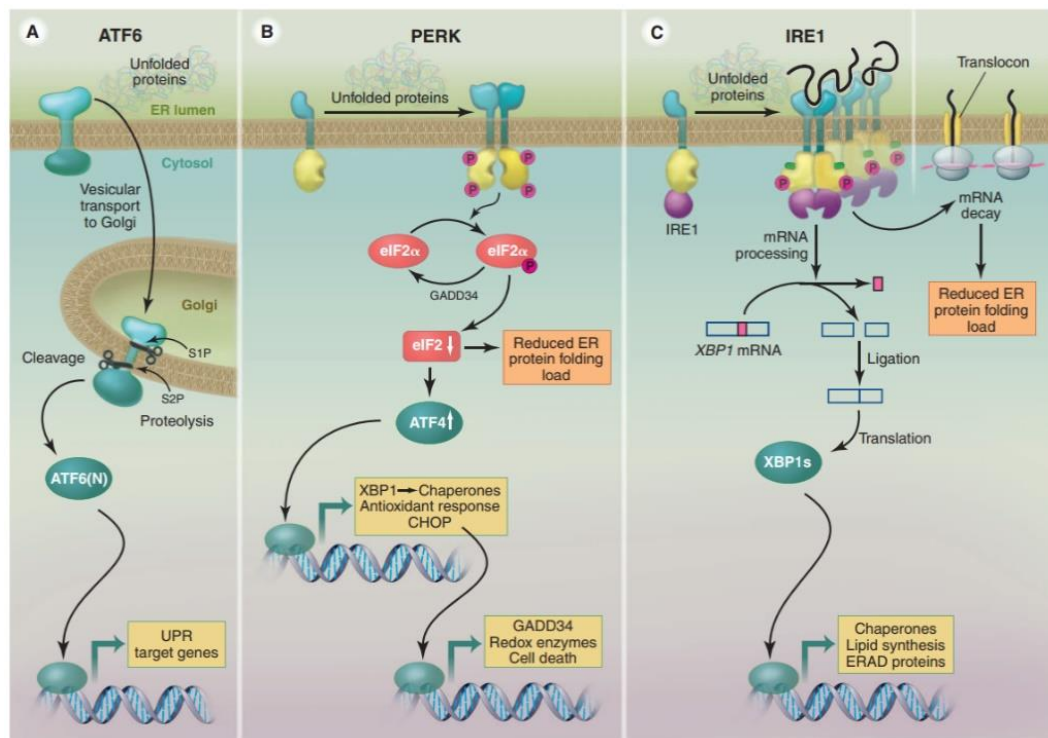


Figure 1.8: The three branches of UPR

Collectively, UPR signaling induces a series of events that inhibit global protein synthesis, increase the degradation of misfolded proteins, and improve their folding efficiency. Consequently, this reduces the overall load of misfolded proteins and allows the restoration of cellular proteostasis. If the cell is unable to cope with prolonged ER stress, the terminal UPR could trigger apoptotic signaling to destroy the damaged cell, thereby determining cell fate [57] [58].

1.4. Salt Inducible Kinases

Salt inducible kinases (SIKs) are serine/threonine kinases of the adenosine monophosphate activated protein kinase (AMPK) family [59], [60]. There are three SIK isoforms SIK1, SIK2 and SIK3 which are broadly expressed in vertebrate tissues. Based on human and mouse transcriptomic studies, they are mostly enriched within the adrenal gland, adipose tissue and brain, respectively [61], [62]. SIKs consist of an N-terminal kinase domain (KD), ubiquitin-associated (UBA) domain and a C-terminal domain (CTD). SIK1 also has a nuclear localization signal (NLS) sequence that enables it to exhibit functionality within both the nucleus and the cytoplasm (Figure 1.9). Activities of the SIKs are regulated by post-translational modifications.

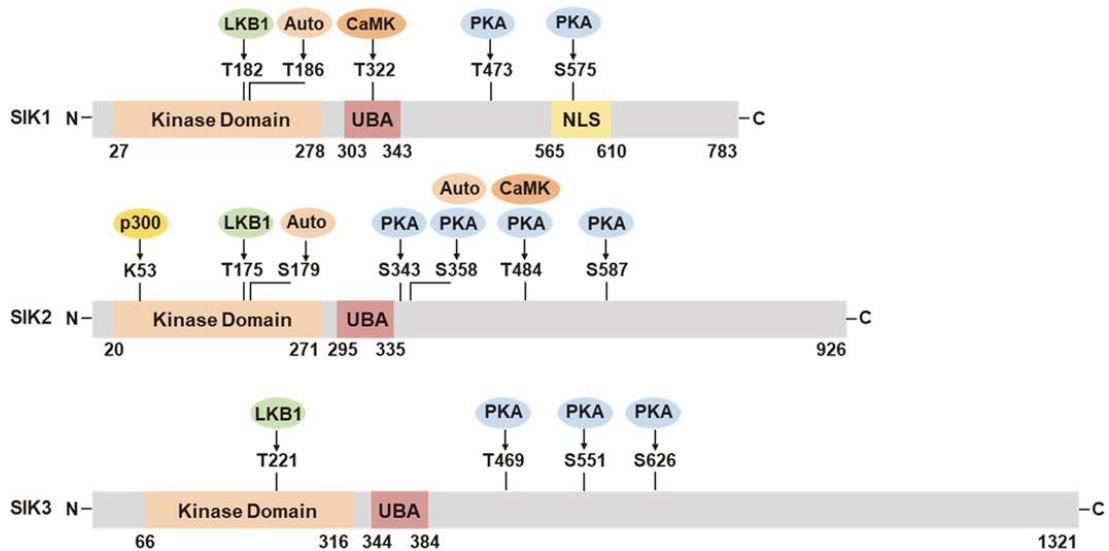


Figure 1.9: SIKs functional domains and post-translational modifications.

(PTMs). Well conserved KD domain contains a liver kinase B1 (LKB1) threonine phosphorylation site in the activation loop (T-loop). The LKB1 activates SIKs by

phosphorylation of these sites with the aid of the UBA domain. It also stimulates autophosphorylation in the SIK1 (Ser186) and SIK2 (Ser179, Ser358), which maintain kinase activity and protein stability [63], [64]. The CTD domain contains multiple protein kinase A (PKA) phosphorylation sites. PKA-mediated phosphorylation deactivates SIKs by facilitating their binding to 14-3-3 proteins. Furthermore, histone acetyltransferase (HAT) p300-mediated inhibitory acetylation and HDAC6-mediated deacetylation of SIK2 at Lys53 (K53) regulate SIK2 activity [65] (Figure 1.9).

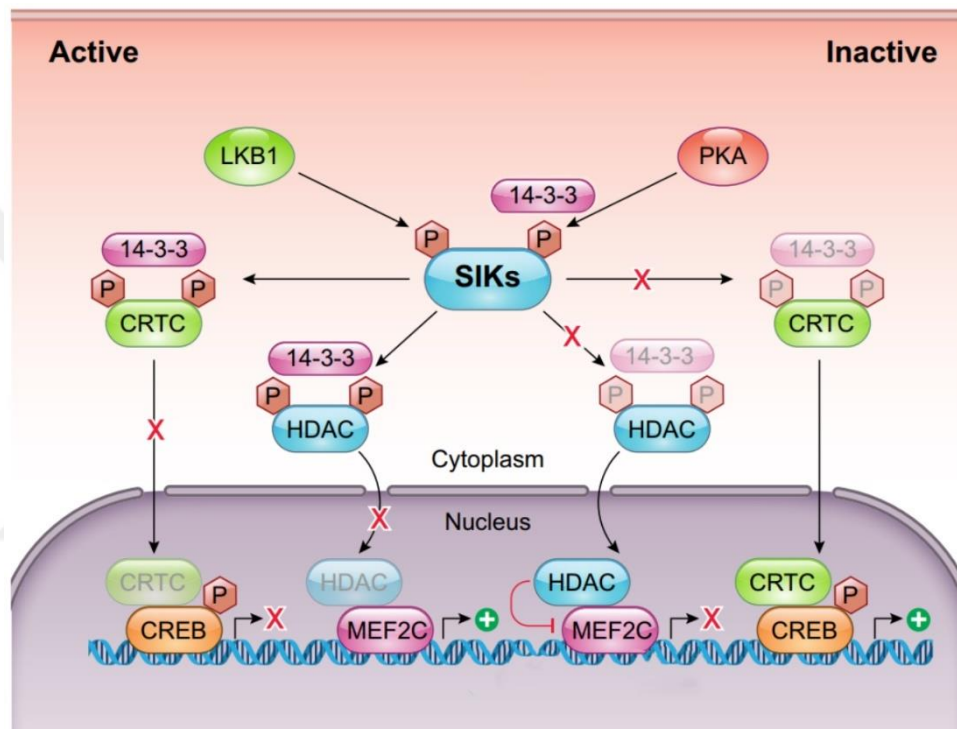


Figure 1.10: Targets of Salt-Inducible Kinases.

SIKs regulate physiological processes by phosphorylating their substrates. SIK phosphorylation causes the substrate to be retained in the cytoplasm by binding with 14-3-3. In response to various external physiological signals such as hormonal and neuronal factors, the second messenger cAMP signalling stimulates PKA-mediated phosphorylation of SIKs. Upon PKA-mediated SIK inhibition, substrates become unphosphorylated and translocated to the nucleus for target gene regulation. The well-characterized SIK substrates are the class II histone deacetylases (HDAC4,5,7,9) and the CREB-regulated transcriptional coactivators (CRTC1,2,3) [59], [60] (Figure 1.10).

1.4.1. SIK2

SIK2 is predominantly expressed in adipose tissue and plays a critical role in metabolic regulation. In adipose tissue, SIK2 expression is strongly induced during adipocyte differentiation. It contributes to insulin signaling by directly phosphorylating IRS-1 [66]. SIK2 positively regulates glucose uptake by inducing GLUT4 expression through HDAC4 and CRTC2,3 [67]. SIK2 has been demonstrated to function as a negative regulator of lipogenesis and gluconeogenesis in adipose and liver tissue, respectively [68]. SIK2 phosphorylates and inhibits p300 HAT activity which in turn leads to a reduction in acetylation of ChREBP that is the transcriptional activator of hepatic lipogenic genes [69]. SIK2 inhibits the expression of the lipogenic genes in adipocytes by potentially regulating SREBP1c [70]. The negative gluconeogenic regulation of SIK2 via CRCT2 is achieved through the insulin-mediated PI3K-AKT pathway, whereas glucagon inhibits this effect through PKA-CREB signaling [71], [72]. SIK2 participates in insulin secretion from pancreatic beta cells by promoting calcium influx through voltage-dependent calcium channels (VDCC) via inducing p35 degradation [73], [74]. SIK2 and SIK3 are implicated in the process of macrophage polarization as well as the release of inflammatory cytokines in monocytes and mast cells, respectively [75], [76]. Previous studies pointed out critical roles for SIK2 in various regions of the brain consistent with its high expression level in neuronal cells. SIK2 participates in neuronal survival after ischemia via CRTC1-CREB [77] and regulates corticotropin-releasing hormone (CRH) secretion in hypothalamic neurons via CRTC2 [78]. Hippocampal SIK2 plays a role in depression via brain-derived neurotrophic factor (BDNF) pathway [79].

Additionally, p53-mediated POMC transcription is triggered after DNA damage in UV-exposed keratinocytes in the skin. Secreted α -MSH and ACTH from keratinocytes activate MC1R in melanocytes, which in turn triggers PKA-mediated CREB phosphorylation and then MITF transcription. MITF increases melanogenesis through tyrosinase [80]. A recent study has shown that CRTCs can also stimulate this CREB-mediated MITF transcription. UV light also induces the nuclear localization of CRTC1 via SIK2 inhibition. Furthermore, systemic SIK2-KO changes the color from yellow to brown in Agouti mice, indicating that SIK2 may have a major role in pigmentation. This is important in terms of being the first study in which SIK2 and POMC were mechanistically demonstrated together [81].

The last but may be the most prominent role of SIK2 appears to be its switchlike behavior in regulating between proteostatic mechanisms such as ERAD and autophagy. The process of ERAD-mediated degradation is facilitated by SIK2's phosphorylation of valine-containing protein (VCP)/p97 [82]. SIK2 activity is also implicated in the initiation and fusion of the autophagosome with the lysosome, a process that was found to be coordinated by the acetylation-deacetylation cycle [65]. Our recent data suggest that SIK2 mediated switch is not restricted to ERAD-Autophagy pair but also operates in decision between autophagy and secretion via COPII vesicles. The molecular mechanisms that are responsible for the distinction between autophagosomes and vesicular secretion have not yet been fully characterized, even though autophagosomes have been involved in the secretion of leaderless proteins.

2. PURPOSE OF THE STUDY

Previous research has demonstrated that metabolic inflammation in the hypothalamic arcuate nucleus of diet-induced obese (DIO) animal models can influence the expression of anorectic POMC and associated neuropeptides, which in turn can result in alterations in feeding behavior and energy metabolism. POMC knockout mice exhibit adult-onset obesity, resulting from elevated feeding and reduced energy expenditure [83]. HFD-induced obese models show that defective regulation of hypothalamic POMC and associated α -MSH/ β -endorphin levels are the earliest markers distinguishing obesity-prone from obesity-resistant mice [84]. The results of experiments on mice and rats indicate that the intracerebroventricular (i.c.v.) administration of MC4R agonists, including α -MSH and ACTH, has a reducing effect on feeding behavior [50], [85]. These findings suggest that POMC peptides, particularly MSHs, and derivatives, play a pivotal role in the obesity. Another relationship has been shown between hypothalamic inflammation, proteostasis, and associated ER stress, and the lipid content of DIO animal models [86]. Dietary fats can induce serine-threonine kinases such as IKK and JNK by triggering ER stress in the hypothalamic neurons during the early stages of obesity [18], [87], [88]. HFD-induced mice exhibit defects in autophagy and the presence of activated I κ k β –Nf κ b signaling in their hypothalamic neurons [89]. Diet-induced obesity also impairs mitochondria-ER contacts in POMC neurons. This leads to ER stress, defective POMC processing, and reduced α -MSH production [90].

Systemic SIK2 knockout (KO) mice demonstrated reduced food intake and energy expenditure along with various dysregulated metabolic parameters such as adipose tissue dysfunction and impaired glucose/insulin tolerance [68]. This finding suggests a potential association between the absence of SIK2 in POMC functionalities in relation to disease etiopathogenesis. However, SIK2's pleiotropic effects on various cellular functions make it one of the most challenging kinases to study in the context of hypothalamic neurons. To tackle this complexity associated with SIK2 functionality in these apparently heterogeneous POMC populations, we used a relatively less complicated therefore most informative POMC cell line mHypoA-POMC/GFP-2. Our primary objective was to study the effect of ER stress and the downstream effector

kinase SIK2 on the secretome of these neurons paying specific attention to the secretion of the POMC-derived neuropeptide α -MSH. In the long term, the result of this study might prove to be useful targets for the treatment of obesity and related pathologies.



3. MATERIALS

3.1. Chemicals

Table 3.1: List of used chemicals in this study.

Name	Supplier
Acetic Acid	Merck, USA
Acetonitrile	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
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
Cello-IF	Cellorama, USA
DMSO	Sigma-Aldrich, USA
EDTA	AppliChem, Germany
Formic acid	Fisher Scientific, USA
Glycerol	AppliChem, Germany
Glycine	Appllichem, Germany
Halt protease and phosphatase inhibitor cocktail	Fisher Scientific, USA
HEPES	Sigma-Aldrich, USA
HG-9-91-01	ApexBio, USA
Hydrochloric acid	Merck, USA
Iodoacetamide	Sigma-Aldrich, USA

Table 3.1: List of used chemicals in this study (continue).

Isopropanol	Merck, USA
Kanamycin	AppliChem, Germany
Methanol	Emsure, Germany
Moviol 4-88	Sigma-Aldrich, USA
N, N, N', N'-tetramethylethylenediamine (TEMED)	AppliChem, Germany
N,N'-Methylenebisacrylamide	Sigma-Aldrich, USA
NP-40	AppliChem, Germany
Paraformaldehyde	Sigma-Aldrich, USA
PEI-MAX	PolyScience, USA
Phosphate Saline Buffer (PBS)	Lonza, Switzerland
Polybrene	Sigma-Aldrich, USA
ProLong Diamond Antifade Mountant	Invitrogen, USA
Puromycine	Santa Cruz, USA
Recombinant Human Leptin	R&D, USA
Sodium Deoxycholate	Sigma-Aldrich, USA
Sodium Dodecyl Sulfate (SDS)	AppliChem, Germany
Sodium Fluoride	Merck, USA
Sodium Hydroxide	Sigma-Aldrich, USA
SybroRuby Gel Stain	Fisher Scientific, USA
TCEP	Thermo Scientific, USA
Thapsigargin	Sigma-Aldrich, USA
Tris-Base	AppliChem, Germany
Triton X-100	AppliChem, Germany
Trypsin	Fisher Scientific, USA
Tween 20	Sigma-Aldrich, USA
Urea	Invitrogen, USA
Water Soluble Tetrazolium Salt (WST-1)	Roche, Germany

3.2. Buffers and Solutions

Table 3.2: Solutions and buffers used in this study.

Solutions/Buffers	Contents/Suppliers
Luria-Bertani medium (LB) (Applichem)	25 g in 1L distilled water Sterilized by autoclave
LB Agar (Applichem)	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)
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 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

Table 3.2: Solutions and buffers used in this study (continue).

4X SDS-PAGE Loading Dye	200mM Tris-HCl pH 6.8 8% SDS 40% Glycerol 4% β -mercaptoethanol 50mM EDTA 0,08% Bromophenol Blue
NP-40 lysis buffer	50 mM Tris-Cl pH 7.4 150 mM NaCl 2mM EDTA 1% NP-40 0.5% sodium deoxycholate 1X Protease Inhibitor Cocktail 1X Phosphatase Inhibitor Cocktail
SOC medium	2% tryptone 0.5% yeast extract 10 mM NaCl 2.5 mM KCl 10 mM MgCl ₂ 10 mM MgSO ₄ 20 mM glucose
4% (w/v) Paraformaldehyde (PFA)	10 g PFA Dissolved within 250ml 1X PBS Adjusted pH 7.4 by adding 1M NaOH

3.3. Plasmids, Antibodies, and Primers

Table 3.3: List of used plasmids in this study.

Construct Name	Origin	Backbone
Empty pCMV-3tag-6	Agilent #240200	
SIK2-FLAG	This study	pCMV-3tag-6
mOrange2 ER-5	Addgene #57956	
mTFP1-Golgi-7	Addgene #55486	
mOrange N1	Addgene #54499	
ER-mOrange	This study	mOrange N1
Golgi-mOrange	This study	mOrange N1
mCherry2_N1	Addgene #54517	
pEGFP N2	Addgene #6081	

Table 3.3: List of used plasmids in this study (continue).

EBFP2 N1	Addgene #54595	
pLKO.1-TRC-cloning	Addgene #10878	
pLKO.1-TRC SIK2-KD	This study	pLKO.1-TRC-cloning
pLKO.1-TRC Scrambled	This study	pLKO.1-TRC-cloning
psPAX2	Addgene #12260	Viral packaging
pCMV-VSVG	Addgene #84549	Viral packaging

Table 3.4: 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
LC3	Rabbit	1:1000	CST # 3868S	14, 16
BiP	Rabbit	1:1000	CST #3177	78
XBP	Rabbit	1:1000	Abcam #ab37152	50, 28
Cleaved Caspase3	Rabbit	1:1000	CST #9661	17, 19
POMC	Rabbit	1:1000	CST #23499	10, 12, 28, 32
AKT	Rabbit	1:1000	CST #9272	60
pAKT (Ser473)	Rabbit	1:1000	CST #9271	60
STAT3	Rabbit	1:1000	CST #	86
pSTAT3 (Tyr705)	Rabbit	1:1000	CST #	86
NF κ B	Rabbit	1:1000	Affinity # AF5006	65
pNF κ B (Ser536)	Rabbit	1:1000	Affinity #AF2006	65
APP	Rabbit	1:1000	Affinity #AF6084	117

Table 3.4: List of used antibodies in this study (continue).

Hsp90	Rabbit	1:1000	Affinity #AF6126	90
Calreticulin	Rabbit	1:1000	Affinity #DF3139	62
Fractalkine	Rabbit	1:1000	Affinity #DF12376	44
Gdf15	Rabbit	1:1000	Affinity #DF4096	37
GAPDH	Rabbit	1:3000	CST #2118	37
β -Actin	Rabbit	1:3000	CST #4970	43
α -Tubulin	Mouse	1:3000	CST #3873S	55
Secondary IgG-HRP	Mouse	1:3000	Santa Cruz #sc-2005	
Secondary IgG-HRP	Rabbit	1:3000	Santa Cruz #sc-2357	
Secondary antibody Alexa Fluor 488	Rabbit	1:500	CST #4412	

Table 3.5: List of used primers in this study.

Primer Name	Sequence (5'-3')	Application	Tm
mSIK2_KpnI_F	AAAAAGGTACCATGGTCATGGCGGATG	Cloning	61.2
mSIK2_KpnI_R	ATATATGGTACCAGGTCTCCCGGGCTAAGCAG	Cloning	64
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

Table 3.5: List of used primers in this study (continue).

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
mAgRP_F	GGCCTCAAGAAGACAACCTGC	qPCR	56.3
mAgRP_R	GACTCGTGCAGCCTTACACA	qPCR	57.4
mBLOC1S1_F	GAGGAGAGAAGCTATCGCTGCA	qPCR	58
mBLOC1S1_R	GAGGAGAGAAGCTATCGCTGCA	qPCR	56
mSCARA3_F	CCACGGAGAAATCCTTCGCAATG	qPCR	58.4
mSCARA3_R	TAGGTCCTCTGCTACCAACAGG	qPCR	57
mGAPDH_F	CATCACTGCCACCCAGAAGACTG	qPCR	59.8
mGAPDH_R	ATGCCAGTGAGCTTCCCGTTCAG	qPCR	61.6
mXBP1_F	GAATGGACACGCTGGATCCT	qPCR	57
mXBP1_R	GCCACCAGCCTTACTCCACTC	qPCR	60
(s)mXBP1_F	GAGTCCGCAGCAGGTG	qPCR	56
(s)mXBP1_R	GTGTCAGAGTCCATGGGA	qPCR	53
c-Fos_F	GGGAATGGTGAAGACCGTGTCA	qPCR	59
c-Fos_R	GCAGCCATCTTATTCCGTTCCC	qPCR	58
mSIK2_Exon2_ShRNA_F	CCGGAATCTACCGAGAAGTACAGATCTC GAGATCTGTACTTCTCGGTAGATTTTTTT G	shRNA- based knock down	-
mSIK2_Exon2_ShRNA_R	AATTCAAAAAAATCTACCGAGAAGTACA GATCTCGAGATCTGTACTTCTCGGTAGAT T	shRNA- System	-
Scrambled_F	CCGGCCTAAGGTAAAGTCGCCCTCGCTC GAGCGAGGGCGACTTAACCTTAGGTTTT TG	shRNA- System	-
Scrambled_F	AATTCAAAAAACCTAAGGTAAAGTCGCCC TCGCTCGAGCGAGGGCGACTTAACCTTA GG	shRNA- System	-

4. METHODS

4.1. Cell Culture

Mouse hypothalamic mHypo-A2/30 (#CLU190) and proopiomelanocortin cell line mHypoA-POMC/GFP-2 (#CLU501) were obtained from Cellutions Biosystems Inc. (Canada). The human embryonic kidney (HEK)-293FT (#R70007) cell line was acquired from Invitrogen (USA). The cells were grown according to the manufacturer's recommendations. The entirety of the cell culture operations were conducted within a cell culture cabinet, utilizing standard and sterilized culture materials. The cells were maintained in completed growth media, Dulbecco's Modified Eagle Medium (DMEM) high glucose (#41966029 Gibco, Canada), which was complemented with 10% fetal bovine serum (FBS) (Gibco) and 1% penicillin/streptomycin (10,000 U/ml, Gibco). They were incubated in a CO₂ incubator at 37 °C and with 5% CO₂. The purchased cell vial was taken out from liquid nitrogen and rapidly thawed in a 37°C water bath. It was diluted in the growth media (1:10 ratio) and then centrifuged at 1000 rpm for 5 min to remove DMSO. The cell pellet was resuspended with fresh growth media and seeded in a 100 mm tissue culture plate. The cells were subcultured (1:5-1:10 ratio, depending on the requirements) when they reached a confluence of 70-80%. During this subculture, 1x phosphate-buffered saline (PBS) (Lonza, Switzerland) was used to wash cells and 1x trypsin-EDTA (0,05% for neuronal cells and 0,25% for 293FT at 37°C for 1-5 min) (Gibco) to detach cells. After the initial growth of the cells, they were frozen (3×10^5 cells/ml for neurons and 3×10^6 cells/ml for Hek293FT) as cryovial aliquots in the freezing media to prevent the loss of the cell line and to use them in the biological replicates. The freezing media was identical to the growth medium mentioned earlier, but it contained an additional 10% DMSO. The cryogenic vials were placed in a freezing container (Mr. Frosty, NALGENE) for a period of approximately 14-16 hours (overnight) in a -80°C freezer. On the following day, they were transferred to a liquid nitrogen tank for long-term storage.

4.2. Chemical Treatments

For characterizations of neuron cell lines, ER stress inducer thapsigargin (THA) (Sigma Aldrich, USA), leptin (LEP), and insulin (INS) (#I6634, Sigma Aldrich) which are two crucial hormones involved in the hypothalamic regulation of energy homeostasis were used. 400.000 cells and 1.000.000 cells were seeded into 60 mm or 100 mm cell-culture dishes, respectively. THA, insulin and leptin were dissolved within DMSO, BSA-HCl and 20mM Tris-HCl solvents, respectively. 100 - 500nm THA and 10nm - 100nm insulin/leptin or their solvent vehicles were added to the media of the cells. After treatment cells were harvested at indicated time points for further analysis.

4.3. Molecular Cloning and Cell Manipulations

4.3.1. Transient Overexpression System

pCMV-3Tag-6 expression vector system (#240200 Agilent, USA) was used to achieve transient overexpression in the cells. The artificially created pDream_mSIK2_wild-type (WT) plasmid, containing the mouse SIK2 gene (Genescript, USA) was utilized as the source for subcloning. The Benchling cloud-based research and development platform (R&D) (San Francisco) and the Oligo Analyzer tool from Integrated DNA Technology (IDT) (USA) were used to design a suitable primer pair. Subsequently, it was synthesized by Macrogen Europe (The Amsterdam, NL) (Table 3.5).

The gene insert was generated from pDream_mSIK2 WT donor plasmids using the high-fidelity PrimeStar HS DNA Polymerase (#R010A Takara, USA) based polymerase chain reaction (PCR). Because of its high priming and short annealing time capacity, insert production was carried out using the specified reaction mixture and conditions in Table 4.1 and 4.3 without requiring any PCR optimization. After the PCR product was detected by the 1% agarose gel running and verified by comparing GeneRuler 1kb plus DNA ladder (#SM1331, Thermo Scientific, Germany), it was cleaned with MN Gel Extraction or PCR Clean up Kit (Macherey-Nagel, Germany) and made ready for digestion.

Restriction enzyme digestion reactions of the produced insert and targeted pCMV-3Tag-6 plasmid were performed by using fast digest KpnI enzyme (#FD0524 Thermo

Scientific, USA) with the appropriate FastDigest Green Reaction Buffer (10x) and condition (37°C for 30 minutes) according to manufacturer's recommendations. The digested insert and plasmid were extracted from 1% agarose gel by using the same extraction kit as mentioned above.

Ligation of linearized plasmid DNA and digested insert was carried out using T4 DNA Ligase (#M0202S NEB, USA). NEBioCalculator tool was used to calculate the required mass of the insert for the ligation reaction. For 20 µl of reaction volume, a mixture of 50 ng of digested plasmid and inserts was prepared using a molar ratio of 1:3 or 1:5. The reaction was performed at room temperature (RT) 25 °C for 2 hours by using T4 DNA Ligase reaction buffer.

The conventional transformation method was employed to transform the ligation products into competent DH5α. 50 µl aliquots of competent cells were thawed on ice and 10 µl ligation mix (50%) was added. After 30 min incubation on ice, they were transferred to a 42 °C heat block for 1min and then on ice for 2 min. Following the addition of the 750 µl Luria-Bertani (LB) Broth (#A0954, AppliChem) medium onto cells, they were incubated for 1 hour at 37 °C with gentle shaking (180 rpm) to allow the production of antibiotic resistance genes. After this incubation, cells were centrifuged at 3000 rpm for 5 min and then pellets were resuspended with 100 µl LB-Broth or SOC medium (if a stronger production is requested). The cell suspensions were spread on appropriate antibiotic-containing (50 µg/ml for kanamycin or 100 µg/ml for ampicillin) LB-Agar plates (#A0927, AppliChem) and cells were grown overnight at 37°C to obtain colonies.

Table 4.1: PCR reaction mixture 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 4.2: PCR reaction mixture for Abm 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 4.3: PCR reaction conditions for PrimeStar HS and Abm Taq DNA polymerase.

	PrimeStar HS		Abm 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	∞	

Colony PCRs were performed to identify positive clones on the agar plates by using the designed primer pair and Taq DNA Polymerase (ABM, Canada) according to the manufacturer's instructions. A total of 5-10 candidate colonies were picked from the agar plates using a sterilized toothpick and then mixed with 25ul of the reaction mixture. pDream_mSIK2 WT plasmid, the source of the insert, was used as a positive control. The PCR reaction was conducted within a thermal cycler device given in **Table 4.2** and **4.3**. The positive clones were identified by running the PCR products on 1% agarose gel. To further confirm cloning, recombinant plasmid candidates were isolated from identified colonies using DNA-spin Plasmid DNA Purification Kit (iNtRON Bio.). They were re-cut by using the identical restriction enzymes that were employed throughout the cloning process. A minimum of three of the confirmed clone candidates were selected and sent for sequencing at Macrogen. Once

the sequence had been verified, the next step was to obtain transfection-grade plasmids. This was achieved by purifying the confirmed pCMV-3Tag6-mSIK2_WT recombinant plasmid from its colony using the MN NucleoBond Xtra Midi Kit (MN, Germany), according to the manufacturer's protocol. The quality and quantity of plasmids were checked by Nanodrop Lite (Thermo) measurements and agarose gel electrophoresis.

Polyethyleneimine hydrochloride (MW 40,000) (PEI-MAX) (#24765, Polysciences, UK) based transfection method was used to generate overexpressed cells. As outlined in the preceding study, a series of optimizations were conducted on variables that influence PEI: DNA particle for increasing the efficiency of the transfection. Seeding the cells the day before the transfection was carried out to achieve 70-80% confluency on the day of transfection. The amounts of plasmid DNA: PEI (1:3) mixture and volume of 1x Tris/EDTA buffer with 150 mM NaCl (pH: 8,0) depending on the size of the used culture plate were indicated in the **Table 4.4**. After 10 min incubation at RT, PEI: DNA particles were dispersed equally over the cells. Six hours following the incubation of the transfection mixture, the culture media was replaced with fresh growth media to avoid potential toxicity. The transiently manipulated cells were suitable for utilization in subsequent experiments conducted between 48 and 72 hours post-transfection. The transfection of the empty backbone was used as a control group.

Table 4.4: PEI-based transfection mixture conditions.

Culture Plate	DNA amount	1x Tris/EDTA buffer
10 cm plate	15ug	750ul
6 cm plate	5ug	250ul
6-well plate	2,5ug	125ul

4.3.2. Lentiviral Based Stable shRNA System

One of the RNA interference, small hairpin RNA (shRNA) was used for gene knockdown. The three distinct shRNA oligonucleotides (Table 3.5) were generated by the use of the Whitehead Institute (WI) siRNA Selection Program (MIT, USA), which employed the designing rules established by the Broad Institute [91] to target particular

exon locations within the endogenous SIK2 mRNA, as illustrated in **Figure 4.1**. Scramble shRNA was designed as a negative control for RNAi. The synthesized oligonucleotides (Macrogen) were cloned into the pLKO.1 TRC Cloning vector (#10878 Addgene, USA) in accordance with the protocols provided by the manufacturer (Addgene). At least three of the verified colonies of clones were chosen and delivered to Macrogen for the purpose of sequencing. Transfection-grade plasmids were purified from its confirmed colony by using MN NucleoBond Xtra Midi Kit.



Figure 4.1: shRNA-based gene manipulation system for SIK2. Targets of designed CRISPR/shRNA oligos for different SIK2 gene sequence.

Hek293FT are fast-growing, highly transfectable cells modified with the SV40 large T antigen. They are optimal for producing high-titer lentivirus. The 293FT cells were seeded in 60 mm cell culture dishes at a density of 80–90% on the day of transfection. The 2nd Generation Packaging System, comprising 3 μ g of psPAX2 (#12260, Addgene), 2 μ g of pCMV-VSVG (#84549, Addgene), and 4 μ g of cloned pLKO.1-

TRC lentiviral expression vectors, was introduced into the cells by using PEI-based transfection, as previously described. Lentiviral particles were harvested 48 and 72 hours after transfection by centrifuging the conditioned media of the 293FT cells at 1500 rpm for 5 minutes, followed by filtration through a 0.45- μ m filter. The collected particles were divided into single-use 2ml vials and stored at -80 °C.

To generate stable SIK2-KD and Scrambled cell lines, POMC cells were seeded in a 6-well plate at a density of 50-70% on the day of transduction/infection. The single-use lentiviral aliquots were equilibrated to room temperature and mixed with 8 μ g/ml cationic polymer hexadimethrine bromide (Polybrene) (#H9268, Sigma Aldrich, USA). The growth media on the cells was removed and then the prepared viral mixtures were given to the cells for o/n incubation. Depending on cell density, either only the media were changed, or the cells were sub-cultured into 100 mm cell culture dishes (for dividing 1:5 ratio). Puromycin found in the pLKO.1-TRC backbone allowed us to select the cells stably expressing shRNA. A puromycin killing curve (1-10 μ g/ml) was therefore conducted on POMC cells in order to identify the optimum puromycin concentration for selection. 5 μ g/ml puromycin was used for 14 days to obtain stable manipulated cell lines. Their knockdown efficiency was then evaluated through immunoblotting. Confirmed ones were frozen as cryovial aliquots as previously mentioned in the cell culture section.

4.3.3. Reporter System

To investigate possible interaction and localization studies, we designed a reporter system based on a Red-Green-Blue (RGB) color model. We used mCherry2_N1 (#54517, Addgene), pEGFP_N2 (#6081, Addgene), and EBFP2_N1 (#54595, Addgene) plasmids and designed primers for the generation of fluorescent fused proteins (Table 3.5). Additionally, we subcloned ER (#36204, Addgene) and Golgi (#55486, Addgene) markers from commercial plasmids to the mOrange_N1 (#54499, Addgene) vector to obtain a compatible fluorescent scale (Figure 4.2A). Before the molecular cloning, we tested the system's operability via PEI-based transfection of empty fluorescent backbones into mHypo-A2/30 cells and then obtained a targeted RGB model under the Zeiss confocal microscope (Figure 4.2B). Recombinant reporter

plasmids that are specific to targeted proteins were obtained by the molecular cloning procedure described in detail earlier.

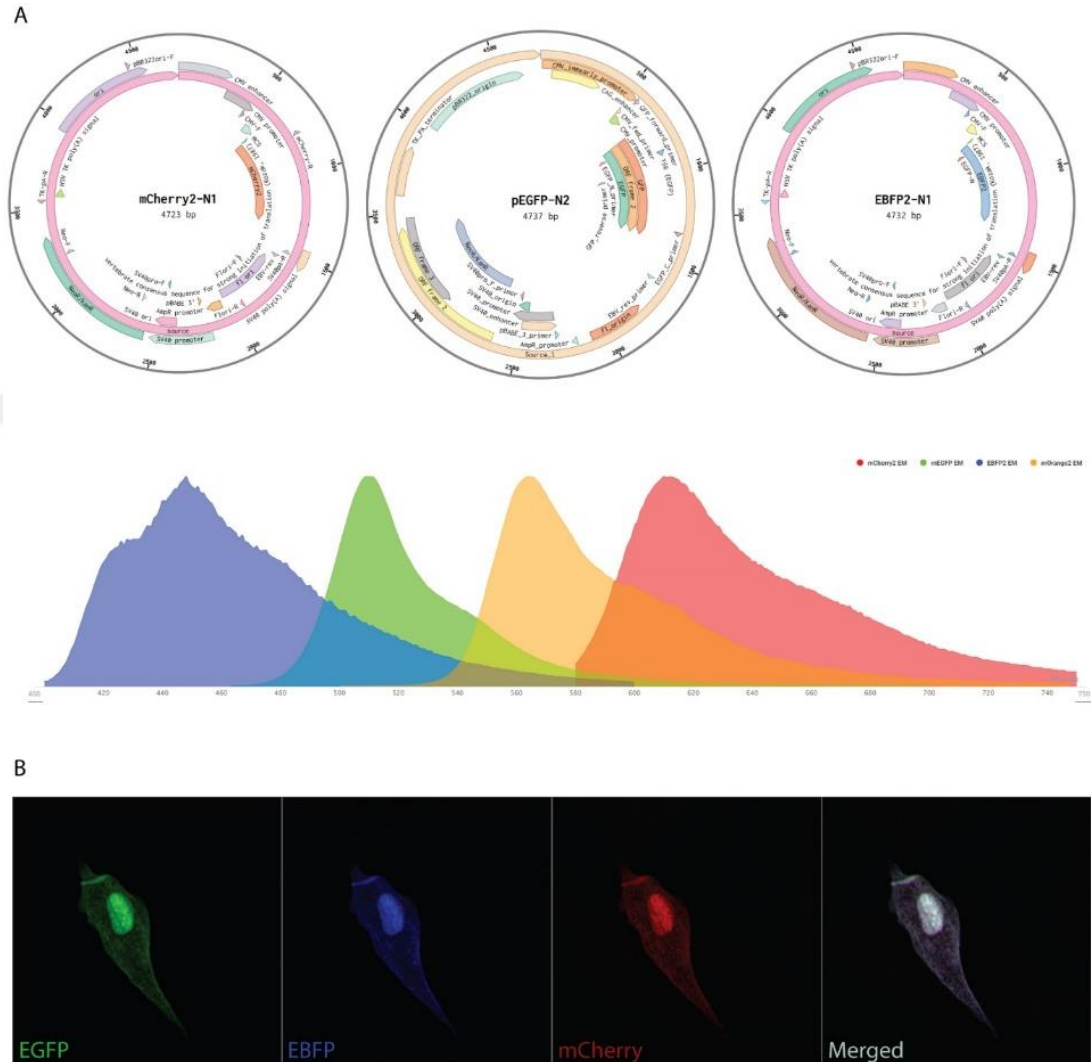


Figure 4.2: Reporter System for Co-localisation Studies. **A)** Fluorescent plasmids proper for Red-Green-Blue (RGB) color model and Fluorescent Emission chart of reporter proteins within these plasmid systems from FPbase Fluorescent Spectra Viewer. **B)** Imaging of co-transfected mHypo cell with empty fluorescent plasmid backbones by using Zeiss confocal microscope at 63X magnification.

4.4. Immunocytochemistry (ICC)

Because the especially co-transfection efficiency was low in mHypo-A2/30 and POMC cells, we decided to develop the ICC protocol for combining with the reporter

system. We used Cello-IF (Cellorama, USA) all-in-one immunolabeling reagent system. For a detailed examination of the neurons including axons and synaptic regions, cells were seeded in a 24-well plate with 15 mm cover glass (NEST, China) at 50-70% density on the day of ICC. Since all steps, except fixation, will be executed at 37 °C, all the solutions to be used were heated to 37 °C in the water bath. The cells were washed with 200µl of 1xPBS and fixed with 200µl of 4% paraformaldehyde (PFA) at RT for 20 min. After two 1xPBS washes for 5 min, the fixed cells can be stored in the PBS at 4 °C for up to one week. The fixed cells were washed with 100 µl of Cello-IF reagent for 5 min and then incubated with 100 µl of targeted protein-specific primary antibody solutions (1:100- 1:500) (CST) with gentle shaking (180 rpm) for 1 hour. Following three washes of 100 µl of Cello-IF for 5 min, the remaining steps were carried out in the dark because of the secondary antibodies are sensitive to light. The cells were incubated with 100 µl of Alexa fluor conjugated seconder antibody solutions (1:500) (CST) with gentle shaking (180 rpm) for 1 hour. They were finally washed twice with 200 µl of 1xPBS and then once with 200 µl of dH₂O. The samples on the cover glasses were closed onto slides by using ProLong™ Diamond Antifade Mountant with SYTOX™ Deep Red (#P36990, Invitrogen). Cells were examined under the Zeiss confocal microscope. Firstly, the ICC protocol was performed with GAPDH, POMC and SIK2 proteins to control specificity of ICC protocol, respectively (Figure 4.3).

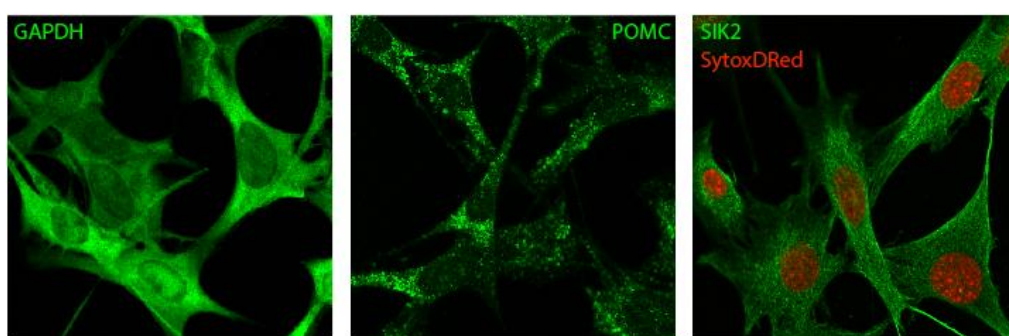


Figure 4.3: ICC imaging of POMC neurons. ICC was performed by using primary GAPDH, POMC, and SIK2 antibodies in the Cello-IF reagent. SytoxRed Dye was used to distinguish the nucleus. Imaging of fixed cells was obtained by using specific excitation and emission wavelength of their proteins/dyes under Zeiss confocal microscope at 63x magnification.

4.5. Quantitative real-time PCR (qPCR)

Total RNAs were isolated from the cells using RNeasy Mini Kit (#74104, Qiagen, Germany) according to the manufacturer's protocol. RNA isolation was performed in the fume hood, and RNase Wiper (#21131, iNtRON Biotechnology) was used for all sterilizations during the procedure. Nanodrop-Lite Spectrophotometer (Thermo Scientific) and agarose gel electrophoresis were used for the quality and the quantity of RNA. The OD_{260/280} ratio of each sample has been confirmed to be 2,0 - 2,2. In agarose gel electrophoresis, 0,5X Tris-Boric acid-EDTA (TBE) buffer and Red Safe nucleic acid staining solution (#21141, iNtRON Biotechnology) were used for 1% gel preparation. RNA samples were prepared with 5x RNA Loading Dye and then were loaded equally (~100-200 ng) into wells of the gel which was run at 80 Volt for 20 min within the 0,5X TBE buffer. The gels were visualized by Azure c600 imaging system. After RNA verifications, first-strand complementary DNA (cDNA) was synthesized from 1 µg of total RNA with the Maxima cDNA Synthesis Kit (#K1641, Thermo, USA) according to the instructions provided by the manufacturer. The qPCR amplification was performed using gene-specific primers (Table 3.5) and Luna Universal qPCR Master Mix (#M3003S, NEB, USA) under the specified reaction conditions (Table 4.5 and 4.6) within the LightCycler 480 (Roche Applied Science).

Table 4.5: qPCR reaction mixture for Luna Universal qPCR Master Mix.

Component	Volume for 20ul Reaction	Final Concentration
Nuclease-free water	up to 20ul	
Luna Universal qPCR Master Mix	10ul	1X
Forward primer (10uM)	0,5ul	0,25uM
Reverse primer (10uM)	0,5ul	0,25uM
Template	Variable	<100ng

Table 4.6: qPCR reaction conditions for Luna Universal qPCR Master Mix.

Cycle Step	Temperature	Time	Cycle
Initial Denaturation	95°C	60 sec	1
Denaturation	95°C	15 sec	40-45
Extension	60°C	30 sec (+plate read)	
Melting Curve	60-95°C	various	1

4.6. Cell Viability Assays

Colorimetric-based cell proliferation reagent Water Soluble Tetrazolium Salt (WST-1) (Roche, Germany) was used for the non-radioactive quantification of cell viability and cytotoxicity of THA on the cells. POMC cells were seeded into a 96-well cell culture plate (TPP, Germany) at densities of ~5,000 cells per well. After 24 hours, they were treated with 100nM THA and its vehicle DMSO, respectively for the determined time points (6, 12, and 24 hours). A volume of 10 µl of WST-1 was added to each well during the final four hours of the treatment period. Following the incubation at 37 °C and 5% CO₂, the spectrophotometric measurements were conducted at two distinct wavelengths 450nm and 690nm (as a reference wavelength) utilizing the Varioskan Flash Reader (Thermo Scientific, USA).

To investigate the viability of POMCs in the presence of THA under serum-free conditions, cells also were seeded into a 24-well cell culture plate (NEST, China) at a density of ~25,000 cells per well. After 24 hours, cells in the serum-depleted group were washed three times with 1xPBS and then treated with 100nM THA and its vehicle DMSO, respectively in serum-free media for 6 and 12 hours. For serum-containing groups, treatment was performed directly in the completed media. The ratio of live to dead cells was determined by quantifying the viable cell count via the trypan blue stain under the DMi1 light microscope (Leica, Germany).

4.7. Conditioned Media (CM) Isolation

To perform a pure CM isolation, we optimized our protocol according to previous reports [92]. POMC cells were seeded into three 100 mm cell culture dishes (NEST,

China) at a density of approximately 800,000 cells per condition. After 24 hours, cells were washed three times with 1xPBS and then incubated with 100nM THA and its vehicle DMSO, respectively in 5ml of growth media containing 0% serum for 6 and 12 hours. The CMs collected from three 10cm cell culture dishes were combined into 15 mL of total medium for each condition. They were centrifuged at 500 x g for 5 minutes, followed by centrifugation at 2,000 x g for 10 minutes at 4°C. The supernatant was then filtered through a 0.22µm PES filter to exclude cellular debris and apoptotic bodies. At this point, the cleaned CMs can be stored in the short term at -20 °C and in the long term at -80 °C. The removal of extracellular vesicle contamination from the soluble secretome was conducted through a series of centrifugations at 10,000 x g for 30 minutes at 4°C (for the microvesicle fraction) and then at 100,000 x g for one hour at 4°C (for the exosome fraction). The resulting pure soluble secretome samples were concentrated to a volume of approximately 200-500 µl and buffer exchanged into 50 mM Tris-HCl (pH 8.0) by using the Amicon® Ultra Centrifugal Filter with a 3 kDa molecular weight cut-off (Merck, Germany). The Amicon centrifugation procedures were conducted by utilizing a fixed-angle rotor at 5,000 x g at 4°C. The Qubit 3.0 device with the Qubit Protein Assay Kit (#Q33211, Thermo Scientific, USA) was used to determine the total protein concentration, following the manufacturer's instructions.

4.8. One-Dimensional Gel Electrophoresis (1DE) and Immunoblotting

The 1DE and immunoblotting methods were used for qualitative and quantitative analysis of proteins, respectively. For cell line characterization assays and proteomics experiments, the total protein of cells was isolated by using a lysis procedure. Following two washes in 1xPBS, cell lysates were collected on ice by scraping within NP-40 lysis buffer (50 mM Tris-Cl pH 7.4, 150 mM NaCl, 2mM EDTA, 1% NP-40, 0.5% sodium deoxycholate), which was supplemented with Halt protease and phosphatase inhibitor cocktail (#78440, Thermo Scientific). The cell lysates were centrifugated at 18,000 x g for 15 minutes at 4°C. Harvested protein supernatants can be stored in the short term at -20 °C and in the long term at -80 °C. The concentrations of protein were determined through the utilization of the Bicinchoninic Acid (BCA) Assay Kit (#23225, Thermo Scientific) based on bovine serum albumin standards (BSA) (0,125-2 mg/ml) at 562 nm absorbance by using Varioskan Flash Reader (Thermo Scientific, USA) according to manufacturer's instructions.

SDS-PAGE gels were cast, run, and transferred utilizing the Mini Protean Tetra Cell and Criterion Blotter System (Bio-rad, USA). Although 4% stacking and 10% resolving gel were routinely prepared, 5% and 12% resolving gels were also used for further investigations of large and small molecular-weight proteins, respectively. The gel contents are shown in **Table 4.7**. After gel polymerizations, the protein samples were prepared (20-25 µg routinely) with 4x Laemmli Loading Dye and then denatured at 95 °C for 5min. 2-3 µl of the pre-stained protein markers Prime-Step™ Broad Range Protein Ladder (#773301, BioLegend, Canada) and Gangnam Stain Protein Ladder (#24052, iNtRON Biotechnology) were used as the molecular weight standards. Denatured proteins and markers were loaded equally into wells of stacking gel and run at 80-120 Volt within the 1x SDS running buffer.

Table 4.7: Component of the prepared SDS-PAGE gels.

	Resolving Gel (10%)	Resolving Gel (12%)	Resolving Gel (5%)	Stacking Gel
dH ₂ O	4,7ml	4,2ml	5,95ml	4,55ml
1,5M Tris-HCl (pH 8.8)	2,6ml	2,6ml	2,6ml	-
1M Tris-HCl (pH 6.8)	-	-	-	750ul
SDS 10% (w/v)	100ul	100ul	100ul	60ul
Acrylamide:Bis-acrylamide (37.5:1, 40%)	2,5ml	3ml	1,25ml	600ul
APS 10% (w/v)	100ul	100ul	100ul	30ul
TEMED	10ul	10ul	10ul	6ul
Total volume	10ml	10ml	10ml	6ml

Following the SDS-PAGE, the gels for total protein quality control were stained with SYPRO Ruby Protein Gel Stain (#S12000, Invitrogen, USA) according to the manufacturer's recommendation. They were visualized by ChemiDoc XRS+ (Biorad) with predefined settings. In the other gels, proteins were transferred to the PVDF membrane (Thermo Scientific) for 2 hours at 100V on the magnetic stirrer at 4 °C within the cold 1x transfer buffer. The membranes were blocked in 5% non-fat dried milk in 1x TBS-T for 1 hour at RT with shaking using an orbital shaker. After three times washing with 1x TBS-T for 5 min, they were incubated with an antibody solution (5% BSA in 1x TBS-T) containing targeted primary antibodies (Table 3.4) at 4°C on

an orbital shaker (or rotator for falcons). After overnight incubation, they were incubated with an antibody solution containing HRP-conjugated secondary antibodies (Table 3.4) at RT for 2 hours and then washed again three times with 1x TBS-T for 5 min. The protein bands were visualized using a chemiluminescence-based WesternBright ECL HRP substrate (Advansta, USA) according to the manufacturer's instructions. The images were then captured using the ChemiDoc XRS+ system (Biorad).

4.9. Proteomics Analysis

4.9.1. Sample Preparation for Bottom-up Proteomics

For trypsin-based bottom-up protein digestion, 5-10 µg of protein samples were dissolved in 50 mM Tris-HCL, pH:8.0 (Invitrogen, USA) containing 6M Urea (Invitrogen) which is required for full digestion. The reduction was conducted using 500 mM tris(2-carboxyethyl) phosphine (TCEP) (#PG82080, Thermo Scientific, USA) at a final concentration of 20 mM for 1 hour at 60 °C (or 10 min at 95 °C) in a thermoshaker with 400-500rpm. Subsequently, alkylation of them was performed using 1M iodoacetamide (IAA) (Thermo Scientific) at a final concentration of 40 mM for 30 min at RT. Because of the high concentration of urea affects trypsin activity, the samples were diluted (Urea <1M) with 50mM Tris-HCL (pH:8,0). Afterward, they were digested with Pierce trypsin protease (#90057, Thermo Scientific) at a ratio of 1:20 (w:w) for overnight at 37 °C in the thermoshaker with 400-500rpm. Formic acid (#A117, Thermo Scientific) was added to each sample at a final concentration of 1-2% in order to stop the tryptic digest. Pierce C18 tips (#87784, Thermo Scientific) and a speed-vac evaporator were used to desalt and concentrate the digested peptides.

4.9.2. Mass Spectrometry

The prepared 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, USA) coupled on-line to an LTQ Orbitrap ELITE mass spectrometer equipped with a nano-electrospray ion (ESI) source (Thermo Fisher Scientific). The peptides were diluted to 200ng/µl using a sampler buffer (5% acetonitrile and 0.1% formic acid in LC-MS Grade Water) (Thermo

Scientific) resulting in a total volume of 3 fractions. They were then injected into the liquid chromatograph with a 2 μ l injection volume. Mobile phase A was composed of 0.1% (v/v) formic acid in LC-MS grade water, whereas mobile phase B consisted of 0.1% (v/v) formic acid in 80% acetonitrile in LC-MS grade water. Gradient separation of peptides was carried out using a C18 column (Acclaim PepMap, RSLC 75 μ m x 250mm) (Thermo Scientific), starting with 4% of phase B over 5 min, 4–55% over 85 min, 55% over 10 min, 55–95% over 10 min, with final elution of 95% B for 5 min at a flow rate of 300nL/min. The LC-MS/MS analysis was performed on each sample in three fractions, using a data-dependent acquisition (DDA) methodology with the positive ion mode. MS1/MS2 scans were conducted in Orbitrap and ion trap analyzers, respectively, with a scan range of 350-1800 m/z at a resolution of 60,000. All MSn spectra were acquired using collision-induced dissociation (CID) with 35% normalized collision energy, 0.25 activation Q, a 10 ms activation time, and a 1 m/z isolation width. The data were acquired within the platform of Xcalibur 4.0 software (Thermo Scientific) and subsequently analyzed using the Proteome Discoverer (PD) 2.3 software (Thermo Scientific).

4.10. Bioinformatic and Statistical Analysis

The LS-MS/MS results for all samples are derived from three independent biological replicates and three technical fractions for each experimental condition and hence represent a robust and reliable dataset. The *mouse total protein* database, the *secretome* database (specifically the "Secreted" category from the cellular component of Gene Ontology), and the *shedome* database (specifically the "Transmembrane" and "GPI anchored" categories) from the UniProt protein resource were employed for the analysis of MS/MS spectra. This analysis was performed using the Sequest HT and msAmanda 2.0 search engines. The precursor ions were allowed to deviate from their expected mass by up to 10 parts per million (ppm), while the fragment ions were allowed to deviate by up to 0.5 Daltons (Da). The dynamic modification of *oxidation* (*M*) and the static modifications of *N-terminal acetylation* and *carbamidomethylation* were selected for analysis. The peptide sequence mass (PSM) was validated by utilizing the Percolator tool with a false discovery rate (FDR) and q value (< 0.05). For the study of proteins with small abundances, the spectral cluster node [93] was identified using the MS Amanda 2.0 engine [94], as recommended by the developers. The spectral clustering has the potential to enhance the ability to identify proteins with

low abundance while improving the accuracy of the resulting quantitative data, without simultaneously increasing the noise level of the datasets. Furthermore, the msAmanda search engine node consistently generates more precise spectra than the Sequest engine when analyzing datasets, while maintaining the same FDR. The utilization of these nodes allowed the examination of low abundant proteins (FDR > 0.05 and classified as LOW) which can also exhibit a significant fold change by label-free quantification (LFQ). For differential expression of protein analysis, the LFQ method was utilized with the Minora detection and precursor ion quantifier nodes of PD. The statistical significance of peptide abundances between experimental groups was evaluated using a one-way analysis of variance (ANOVA), with a p-value threshold of ≤ 0.05 . Proteins exhibiting a fold change of 2 or greater were identified as statistically significant. Because the preliminary results showed no significant differences between the secretomes of POMCs in the Scramble and pCMV-3tag6 groups, the secretome of the scrambled POMC was used as the control group (Appendix E). We investigated proteins that showed statistically significant variations in ER stress between the THA and DMSO experimental groups, which had different genetic profiles for SIK2 (Control, KD, and WT). In addition, we also calculated the differences that depend on the modification of SIK2 (e.g., KD versus Control) in both ER stress and non-stress circumstances.

For visually representing the hierarchical clusters between experimental groups, heat maps were created by using PD with the "*Euclidean*" distance function, which calculates the geometric distance between two data points in multidimensional space. The linking method was specified as "Complete," which computes the dissimilarity between two clusters as the greatest distance between any two data points in separate clusters. The ShinyGO software was used for gene ontology (GO) and biological pathway enrichment analyses [95]. For the source of terms and pathways, GO terms (cellular component, biological process, and molecular function), Reactome, KEGG, and WikiPathways were selected. The size of the pathway was specified between 2 - 5,000 genes. A Cytoscape plug-in ClueGO (v.2.5.10) software [96], was employed to identify and functionally visualize the nonredundant biological terms for the resulting hierarchical clusters within the grouped networks. The selection threshold was specified as "medium", in which at least 3 genes from the uploaded list and 4% of genes selected for gene ontology terms were determined to be related to a term by

referencing the databases. The CluePedia extension was also used for comprehensive pathway analysis, which displays common genes as functional groups of terms and pathways based on a kappa score of 0.4. Hypergeometric distribution and Bonferroni step-down statistical options were employed to calculate the minimum p-values via the FDR method. To visualize protein-protein interaction networks in functionally related groups, based on experimental or database-predicted information, the STRING platform was employed [97]. The threshold for statistical significance was defined as 0.05 for the p-value in all comparative analyses.

Immunoblotting and cell viability experiments were also conducted using samples from independent biological triplicates. Densitometric measurements for Western blot quantitation were performed using ImageJ software (version 1.48, USA). One-way ANOVA was used to perform statistical analyses for a Bonferroni-based multiple comparison of the control group with the treated/manipulated groups utilizing GraphPad Prism 9.3 software (USA). The obtained data were displayed as the mean values with the standard deviations. Additionally, the statistical parameters were included in the figures and accompanying figure legends.

As a result of qPCR, C_q values were found using regression analysis of the amplification traces. The average C_q values for triplicates were adjusted (ΔC_q) based on a reference gene. Ultimately, the expression levels were determined using the 2- ΔC_q equation. The one-way ANOVA analysis was conducted using the GraphPad software, as previously described.

5. RESULTS

5.1. Cell Lines

Commercially available hypothalamic murine cell line, mHypo-A2/30 was produced from two-month-old male C57Bl/6 mice by retroviral transfer of SV40 T-Ag. These cells were reported to express an ever-expanding variety of neuropeptides, enzymatic markers, and biologically active receptors in response to a wide range of hormones and stressors (CEDARLANE). As expected cells are sensitive to anorectic hormones insulin and leptin for neuronal activation and responded well to pharmacological stressor thapsigargin (THA) for ER stress induction [98]. We performed a series of dose response assays for THA (100nm and 500nm), insulin (10nm and 100nm) and leptin (10nm and 100nm) at the time period of 12 hours at given intervals.

Western blot studies with specific antibody indicated that 100nm thapsigargin was sufficient for ER stress induction as specified by the level of glucose-regulated protein 78 (GRP78/BiP) a well-known ER stress marker. SIK2 responded acutely to ER stress induction by reaching its peak value at 15 min of THA treatment followed by a gradual decline as indicated by the phosphorylation of its activation loop T175. POMC expression decreased in the early periods of treatment but it has reached the control level in the late period. This situation may be due to the stress response of the cell in the early period. Leptin and insulin were also sufficient for POMC production with different expression levels. They were also triggered the SIK2 activity, especially at the 15min similar to ER stress response. This intriguing intersection can contribute to revealing the role of SIK2 activity within these neurons between stressed and hormone induced conditions for our further investigations (Figure 5.1).

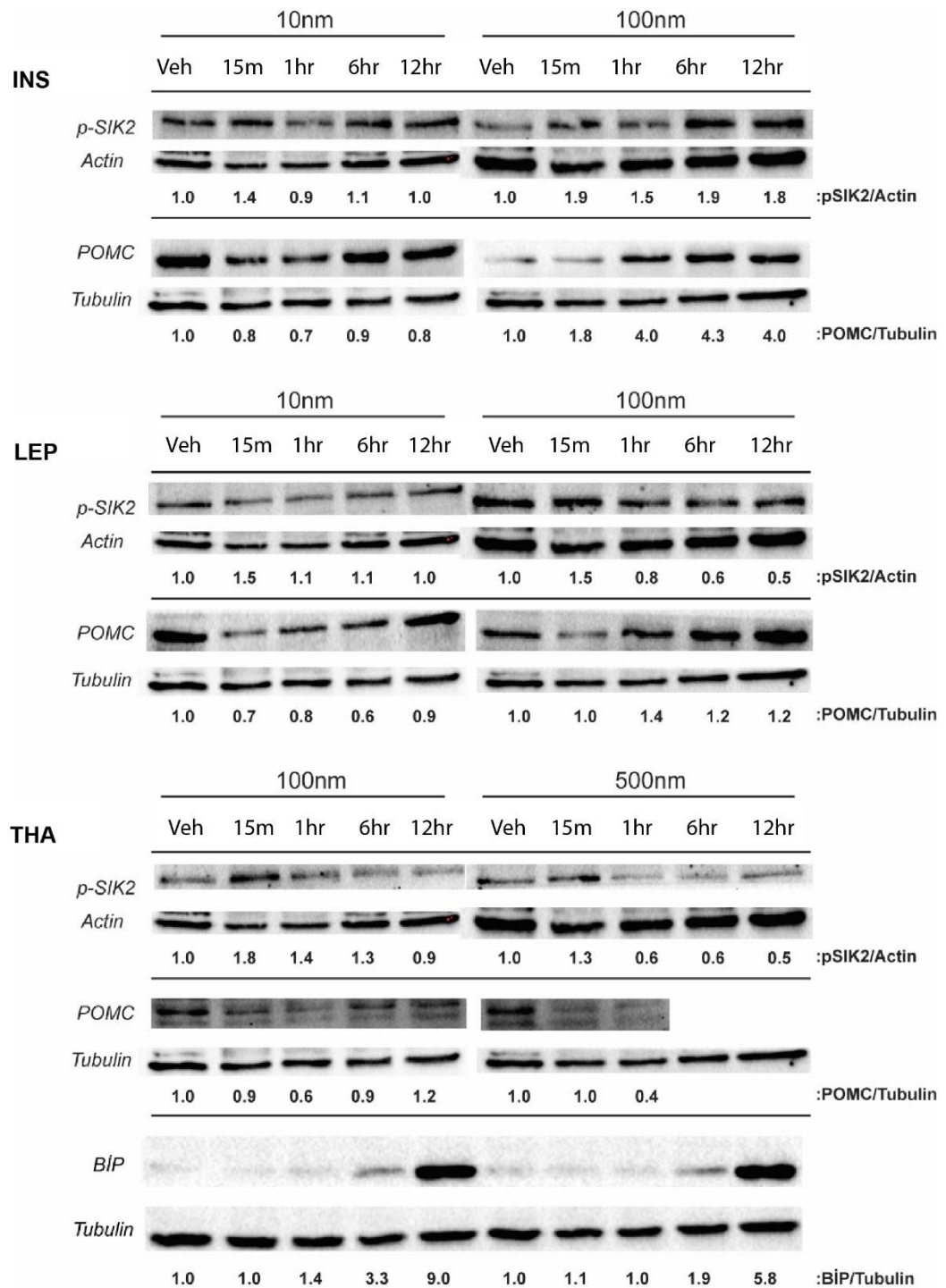


Figure 5.1: POMC expression and SIK2 phosphorylation profile of mHypo A2/30 cells. POMC expression and activation patterns of SIK2 were detected under stressed (THA) or hormonal-induced conditions (INS and LEP) via western blotting using specific antibodies against phospho-T175/T163 of SIK2 and POMC at the protein level. Additionally, tubulin and BIP are observed as housekeeping and ER stress marker genes, respectively. The fold change levels of proteins (n=6) were calculated according to vehicle control groups.

To further characterize the SIK family, POMC/AgRP expression, and UPR response, we performed SYBR-Green-based qPCR against the isoforms of SIK2, ER-resident sensor protein IRE1, and splicing of XBP1 under ER stress and insulin/leptin-induced conditions. This assay was performed by using 100nm THA, insulin, or leptin which was determined in the previous experiments at different time scales.

According to our qPCR data, *pomc* expression could not be detected at any time points of induction. This may be due to its low-level of expression in the cells that do not change with ER stress, insulin or leptin induction. On the other hand, *agrp* was significantly decreased at the 15 min of inductions and normalized thereafter. This may indicate an acute response. Because of the heterogenic properties of mHypoA2/30, which includes both POMC and AgRP-derived populations, this change in the *agrp* levels has not been surprising for us (Figure 5.2). All three isoforms of SIK showed a gradual increase approximately 2-fold within 6-hour of THA treatment and returned to preinduction level thereafter in 12 hours. This showed that we should consider the possibility of compensation among isoforms in POMC neurons. As we expected, Xbp1 splicing and *ire1* level increased proportional to duration of ER stress. (Figure 5.2A).

As result of these assays, because of the heterogenic properties of mHypoA2/30 cell line harboring both POMC (weak response) and AgRP-derived population and associated complications, we decided to use the company's new addition to their hypothalamic POMC lines so called *mHypoA-POMC/GFP-2*. This cell line represents a relatively more homogenous POMC cell line due to their isolation criteria based on the POMC expression. The POMCs were obtained and immortalized from the hypothalamus of four male POMC-GFP mice that were aged eight weeks old. The expression of the EGFP transgene was driven by the POMC promoter (strain C57BL/6J-Tg(Pomc-EGFP)1Low/J) (CEDARLANE). Previous studies with these cells further confirmed their identity by showing the secretion of α -MSH and their responses to anorectic hormones including insulin and leptin. Beside they responded to endoplasmic reticulum stressor palmitate as expected. Overall, these cells are reliable in vitro model for the diagnosis, development, and validation of novel treatments that target diseases related to neurodegeneration, obesity and metabolism [99], [100].

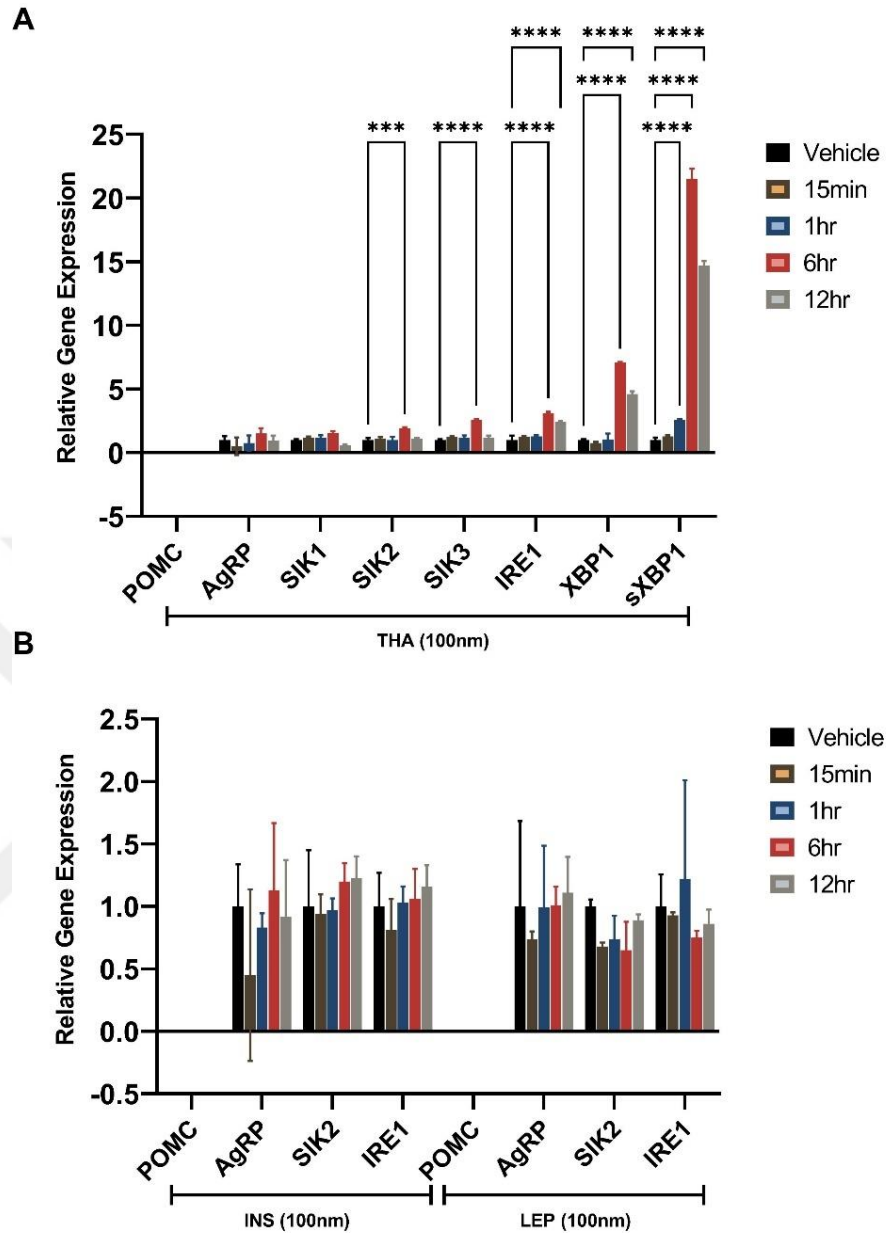


Figure 5.2: mRNA-based characterization of mHypo-A2/30. Expression patterns of interested genes at the mRNA levels under stressed (A) or hormone-induced conditions (B) via SYBR-Green-based qPCR using specific primers. GAPDH was used as the control for normalization. Vehicles were used as induction minus control of assays. Delta Ct ($n=6$) analysis was performed to determine relative mRNA levels. The error bars on the densitometric graphs represent the means $\pm$ standard deviation (SD). For the statistical significance is indicated as **** $P < 0.0001$, and *** $P < 0.001$.

For the characterization of POMCs, the expression level of genes specific to neuronal activation, energy metabolism-related neuropeptides, and SIK isoforms were analyzed at transcriptional level by qPCR. Despite the significant upregulation of *c-fos*, a marker of neuronal activation, was observed at 15 minutes and persisted for up to one hour, no statistically significant changes were observed in *agrp* and *pomc* levels in response to ER stress. All isoforms of SIKs showed a gradual increase up to the 3rd hour of THA treatment and returned back to uninduced level under long-term ER stress (Figure 5.3A). This indicates the possibility of compensation between isoforms against ER stress. Like in the stress response, it was observed that hormonal induction could activate neurons within 15 min. In response to insulin, there was an increase in *pomc* expression and a decrease in *agrp* expression. Among the SIK isoforms, only SIK2 was found to be increased by a minimum of 1.5-fold in response to insulin, with a significant level at 1 hour (Figure 5.3B). Although the neuronal activation was induced by leptin, only a significant decrease in *agrp* level was observed only at 15 min. No statistically significant change was detected in SIK isoforms or in the *pomc* level (Figure 5.3C). Considering the known POMC heterogeneity, the presence of *Agrp* expression within our cells is not unexpected. However, as anticipated, they exhibited an increase in *pomc* expression and a contrary decrease in *agrp* expression in response to insulin and leptin stimulation. The reason why leptin stimulation was not as effective as insulin may once again be attributed or linked to the known receptor heterogeneity. Our cell population may contain a significantly higher number of POMC cells that carry insulin receptors. Additionally, insulin may have a more acute effect on the cells, whereas leptin is more chronic. Therefore, the duration of leptin administration may not be sufficient for stimulation. Despite possible compensatory effects in the ER stress, no significant changes in isoforms other than SIK2 were observed following insulin and leptin induction. This suggests the potential involvement of SIK2 in hormonal induction processes.

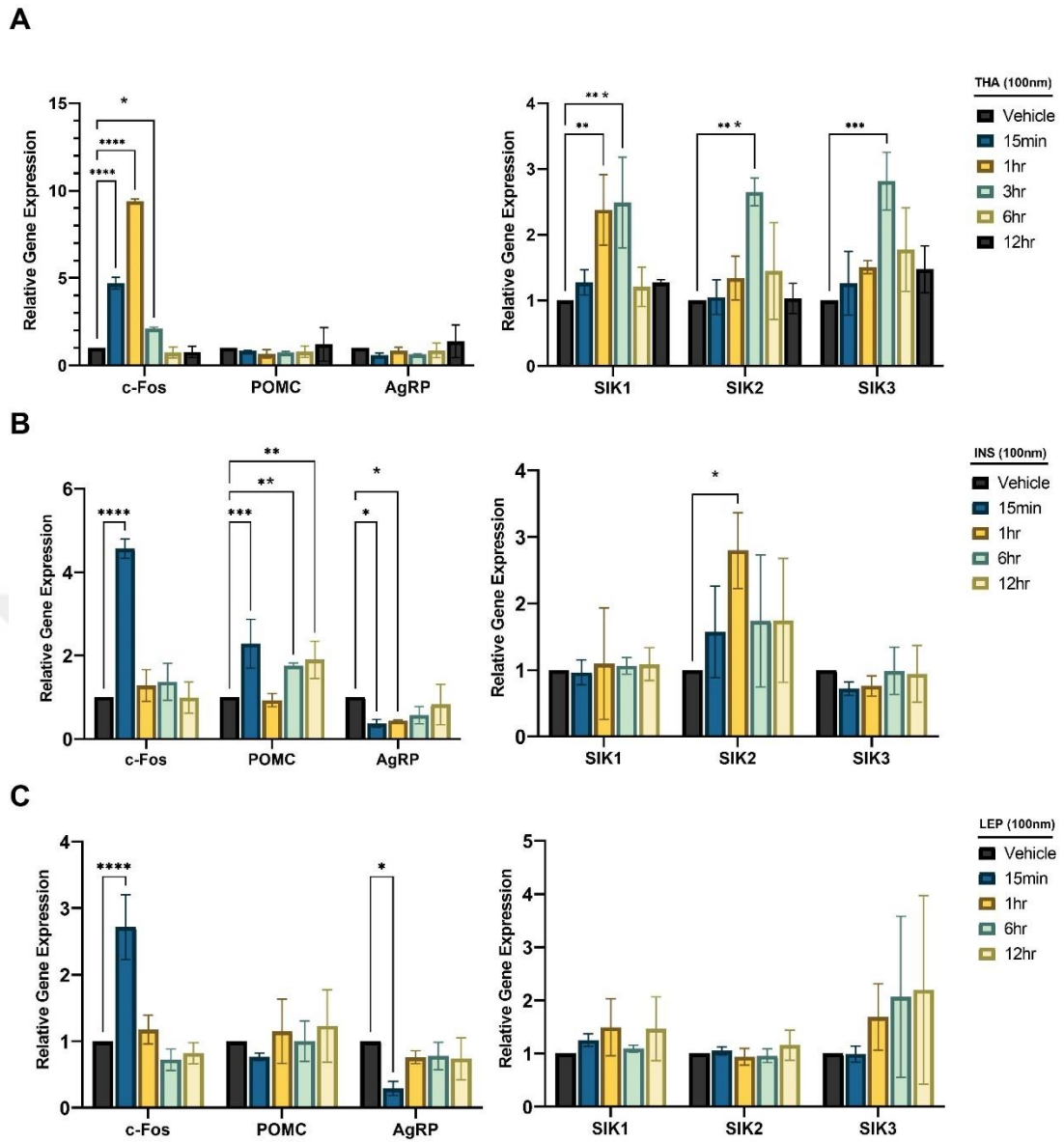


Figure 5.3: Characterization of POMCs. Expression patterns of interested genes at the mRNA levels under stressed (A) or hormone-induced conditions (B and C) via SYBR-Green-based qPCR using specific primers. GAPDH was used as the control for normalization. Vehicles were used as induction minus control of assays. Delta Ct ($n=6$) analysis was performed to determine relative mRNA levels. The error bars on the densitometric graphs represent the means $\pm$ standard deviation (SD). For the statistical significance is indicated as **** $P < 0.0001$, *** $P < 0.001$, ** $P < 0.01$ and, * $P < 0.05$.

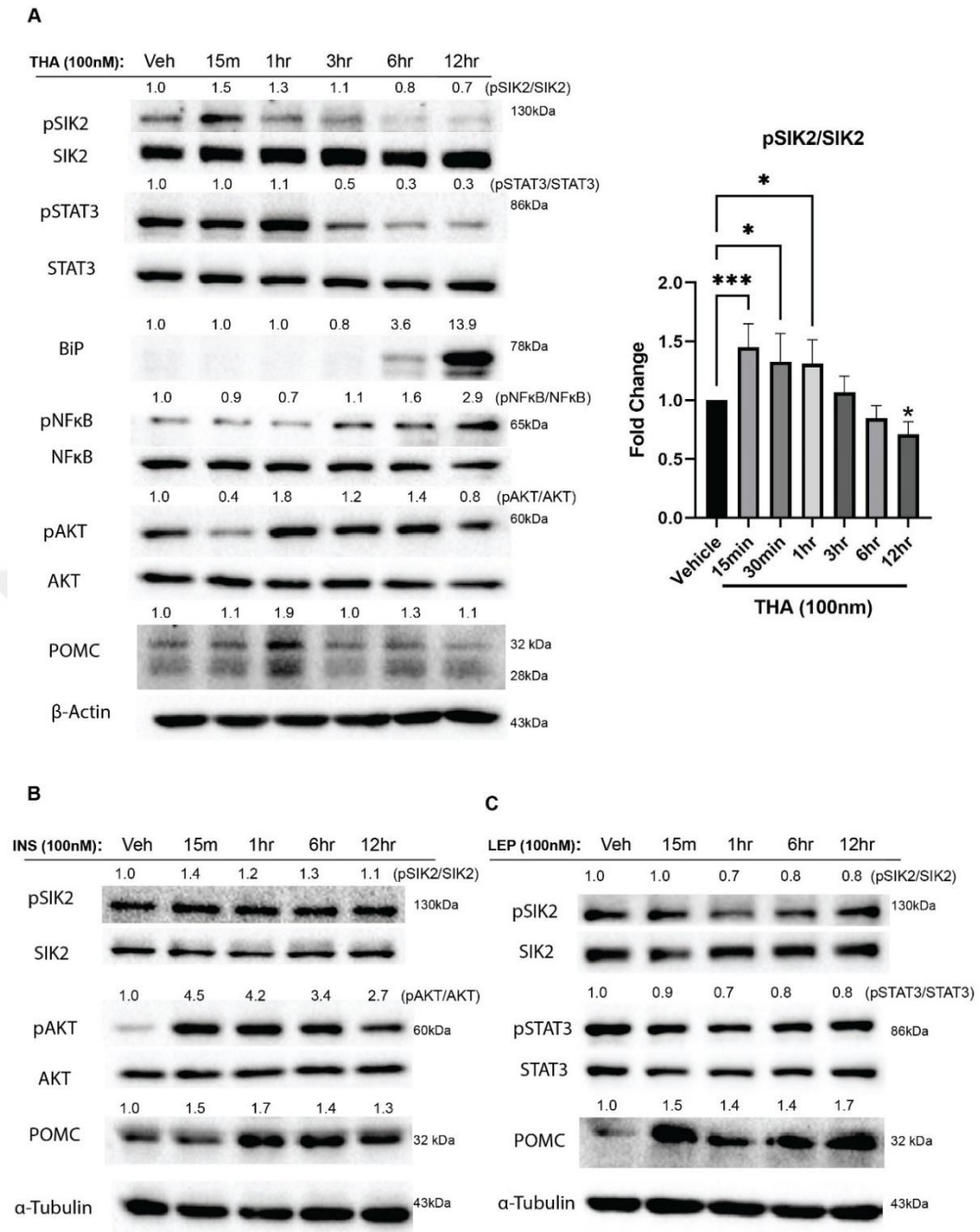


Figure 5.4: POMC expression and SIK2 phosphorylation profile of POMCs. POMC expression and activation patterns of SIK2 were detected under ER stress (A) or hormonal-induction conditions (B and C) by western blotting using specific antibodies against phospho-T175/T163 of SIK2 and POMC for the indicated time points. Additionally, the expression of BiP, phosphorylation ratio of NFkB, AKT, and STAT3 showed ER-stress, inflammation, insulin, and leptin signaling induction markers, respectively. The indicated fold change levels of proteins were calculated according to vehicle control groups ($n=6$). The error bars on the densitometric graphs represent the means $\pm$ standard deviation (SD). For the statistical significance is indicated as *** $P < 0.001$ and, * $P < 0.05$.

At the protein level, it was observed that ER stress triggered SIK2 activity for up to 1 h with a maximum level detected at 15 min. The precursor POMC level showed a critical increase in 1 h in parallel with SIK2 activity and then returned to the basal level, indicating protein accumulation in response to acute ER stress. Phosphorylation of STAT3 did not change up to 1 hour and showed a critical decrease in the following hours of stress. This is consistent with the finding that STAT3 inhibition is required for apoptosis induced by chronic ER stress [101]. AKT phosphorylation demonstrated a significant decrease at 15 minutes, coinciding with the peak in SIK2 activity. This was followed by a rapid increase, returning to the basal level after an extended period of stress. NF κ B phosphorylation, associated with inflammation and apoptosis pathways, exhibited a gradual increase in response to prolonged ER stress (Figure 5.4A). Insulin induction was achieved within 15 min by observing the phosphorylation of AKT. This phosphorylation demonstrated a gradual decrease in a time-dependent manner. SIK2 activity reached its maximum level at 15 min and then gradually decreased, as observed in cases of ER stress. The POMC expression significantly increased in response to insulin, as would be expected and consistent with qPCR (Figure 5.4B). Despite the absence of any alterations in SIK2 and STAT3 activity in response to leptin induction, a statistically insignificant increase in the POMC levels was observed (Figure 5.4C). This may be due to the previously mentioned receptor heterogeneity.

5.2. SIK2 Activity and Localization Under ER Stress

To confirm SIK2 activity and examine its subcellular location in POMCs in response to ER stress, immunocytochemistry targeting pSIK2 was performed. We obtained an image consistent with the pSIK2 pattern of POMCs that we have seen at the protein level in the characterization assay (Figure 5.4A). SIK2 activity increased at 30 min around the nucleus and decreased after 6 hour of ER stress induction (Figure 5.5). Upon 1 hour pre-incubation of 1 μ M HG-9-91-01 SIKs inhibitor, this activity also decreased against the 30 min ER-stress conditions. This finding confirms the presence of SIK2-specific activity during the early ER stress period. The pSIK2 signal concentrated around the nucleus and may underly its ER localization consistent with the earlier reports. However, this has to be further delineated by its colocalization with ER specific markers.

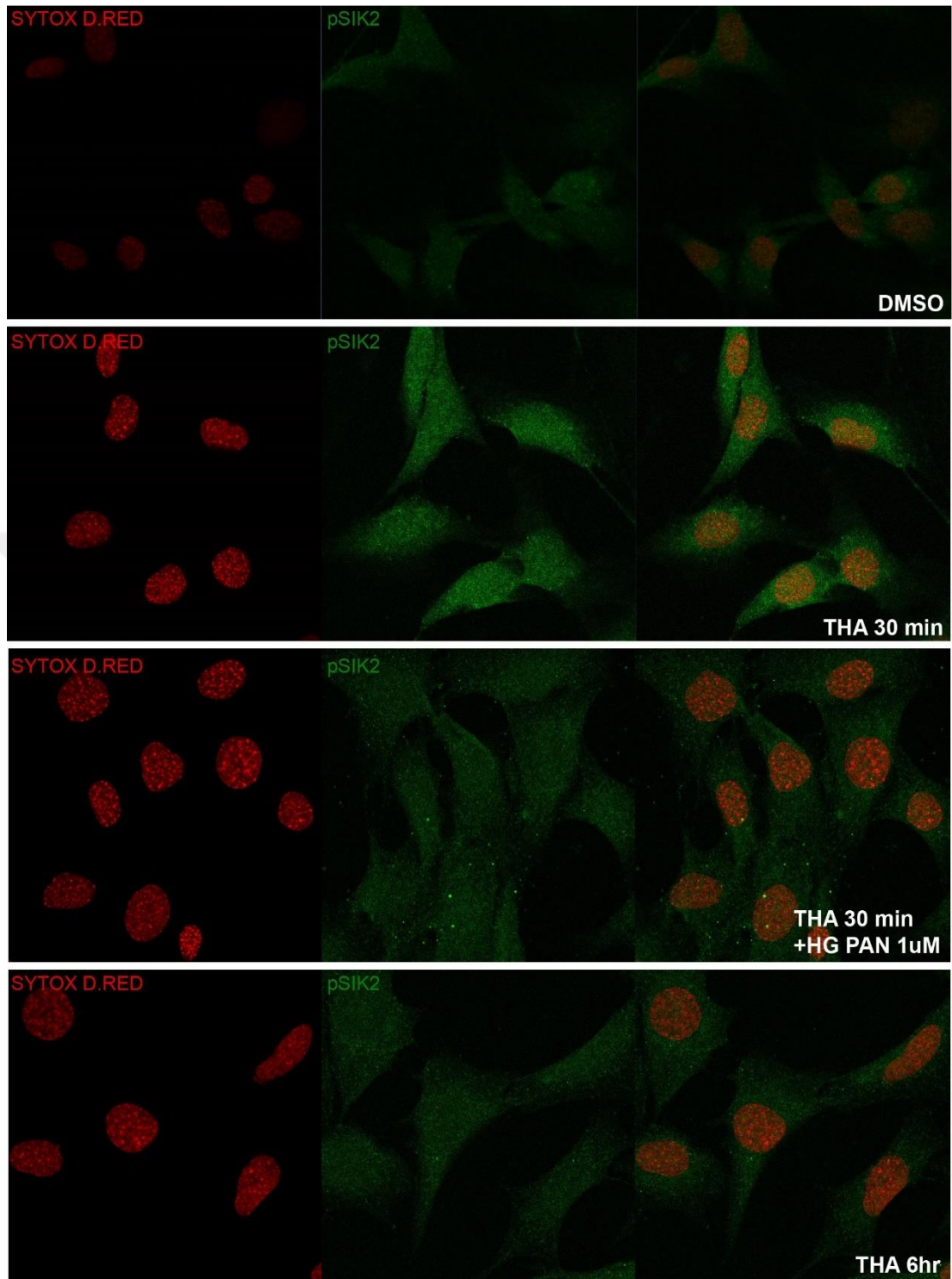


Figure 5.5: pSIK2 localizations in the POMCs. ICC was performed by using primary pSIK2 (T175) and secondary Alexa488 antibodies with Cello-IF reagent. Sytox Deep Red dye was used to distinguish the nucleus. Imaging of fixed cells was obtained by using specific excitation and emission wavelength of their proteins/dyes under Zeiss confocal microscope at 63x magnification. 100nm THA was used for ER stress induction. 1 μ M HG PAN (HG-9-91-01) was used as an SIK2 inhibitor.

5.3. The Preliminary Experimental Outcomes of the Secretome Studies

The secretomes containing soluble proteins secreted by POMCs in response to ER stress and the presence/absence of SIK2 were collected and subsequently subjected to LC-MS/MS analysis to determine their identities and relative amounts of protein content. For this, a series of optimization studies were conducted. In the present study, although the initial stages of ER stress (0-12 hours) were examined, the effects of stress conditions on the cells were observed over a 24–48-hour interval to ensure sufficient secretome collection. Initially, the impact of varying serum conditions (0-10%) on cellular outcomes was examined over 24 hours (Figure 5.6). This investigation aimed to assess the influence of serum conditions on secretome quality and cell survival, with a potential correlation to diverse stressors, including ER stress, autophagy, and apoptosis. Despite an increase in serum deficiency, tolerance of ER stress and autophagy was observed, as indicated by the expression of BiP, XBP1 splicing and LC3II/I ratio. However, there was a proportional increase in apoptosis as evidenced by caspase 3 cleavage (Figure 5.6A). Subsequently, the impact of 100 nM THA on cell viability was assessed over a 24-hour period under identical conditions. Microscopic observations revealed that only 10-20% of cells exhibited mortality under serum-deficient conditions. Upon THA induction, it was observed that the cells exhibited tolerance up to 12 hours, but at later time points, approximately 40-50% mortality was evident across all conditions (Figure 5.6B). Based on these findings, the decision was taken to collect secretomes under 12 hours of ER stress with serum-free conditions.

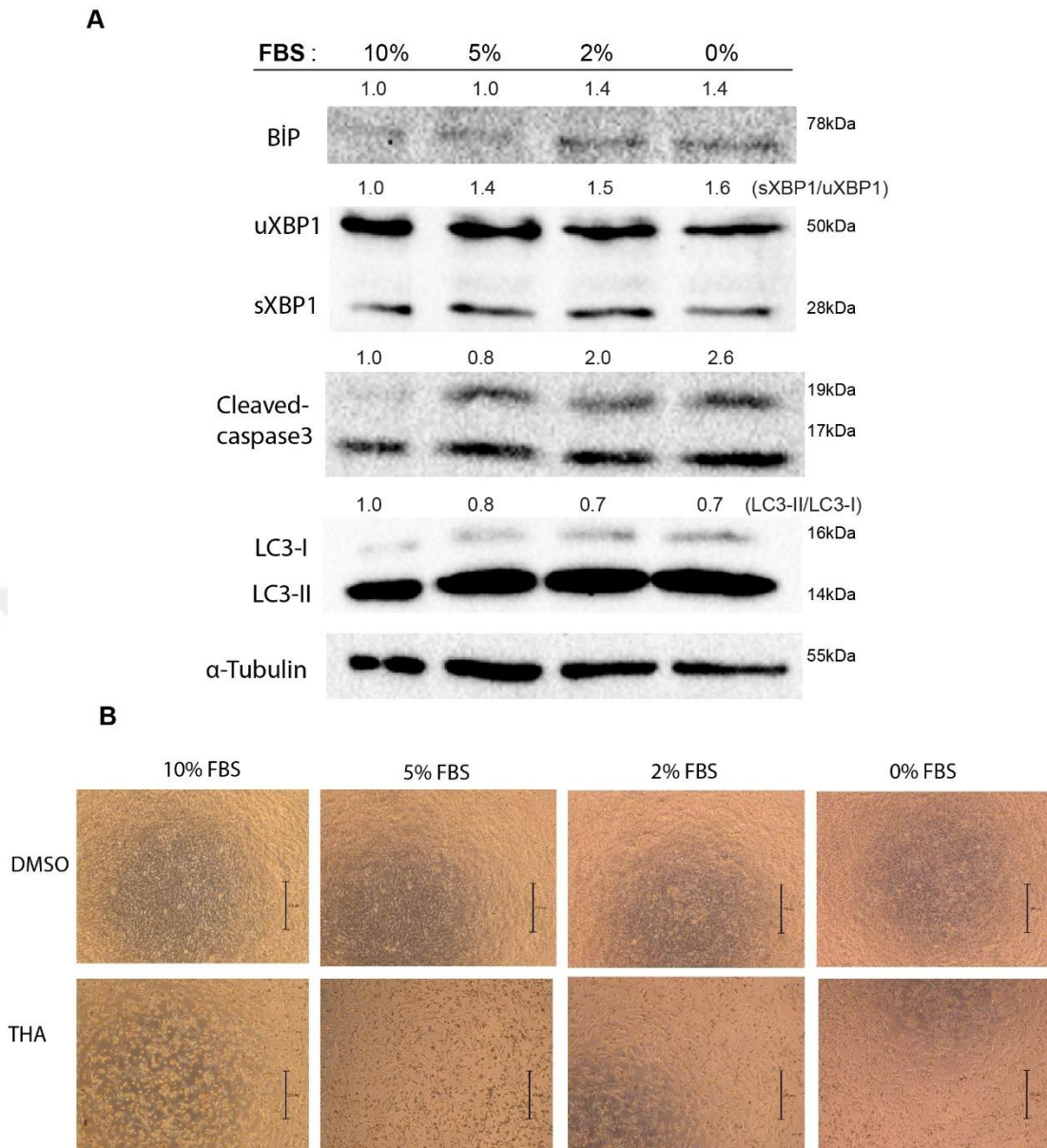


Figure 5.6: Stress tolerance of POMC neurons at varying serum levels. POMC cells were cultivated in varying serum conditions (0%-10%) for 24 hours to examine the potential stressors that may arise due to serum deprivation. The control of stress conditions was conducted via immunoblotting at the protein level, utilizing specific antibodies to stress markers (A), while morphological examinations under the ER stress (B) were observed under a light microscope (Leica, Dmi1) at 4x magnification. Immunoblot analysis was performed by calculating the band areas using Image J software, normalizing the reference protein α -tubulin, and determining the fold change relative to the 10% serum control group.

A preliminary secretome experiment was conducted under conditions of 12-hour ER stress. Western blot analysis was conducted on lysates obtained from the treated cells to ascertain the stress status. It was observed that ER stress was triggered only by thapsigargin, and that apoptosis gradually increased depending on the duration of stress. The total amount of LC3I and II increased with serum deficiency and decreased with thapsigargin. However, the conversion of LC3I to LC3II showed an opposite pattern. This result was unexpected and cannot be explained by the results of this experiment alone (Figure 5.7A). The SDS-PAGE confirmation of the secretome revealed the presence of a broad protein band with an approximate molecular weight of 60 kDa (Figure 5.7B). Given the possibility that this band may represent serum albumin residue with a molecular weight of 66 kDa, it was determined that performing at least two PBS washes during the transition to serum-free medium in subsequent experiments would be appropriate, considering the available literature.

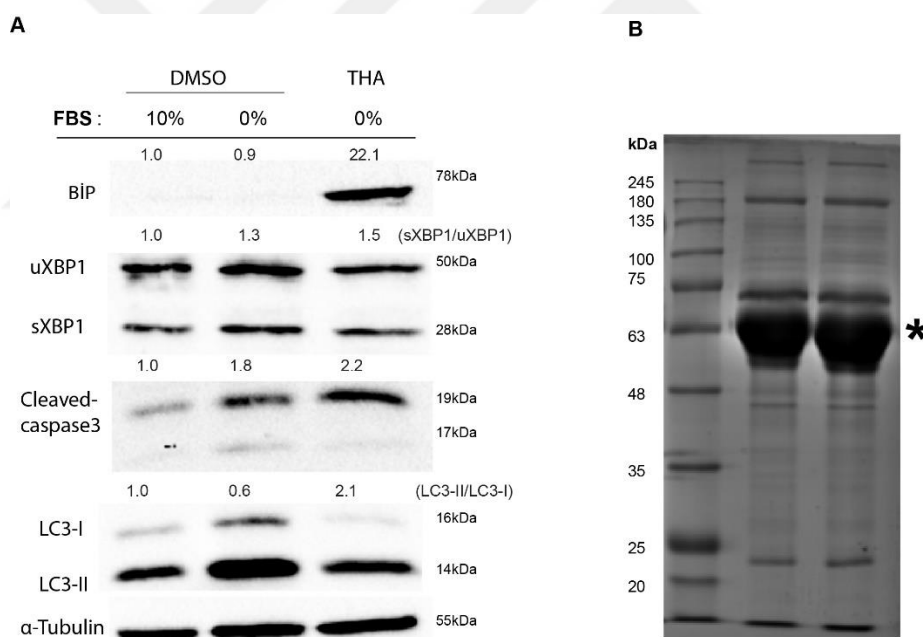


Figure 5.7: Analysis of the stress tolerance and secretome quality of 12-hour ER stress-induced POMC neurons. A) Cells whose secretome is taken were lysed and immunoblotted with specific antibodies to ascertain the presence of various stress markers. B) To ensure the quality of the secretome, gel-based control step was conducted. This entailed loading the same amount of protein sample (10 μ g) as that used in the LC-MS/MS procedure onto an SDS-PAGE gel and staining the total protein with Sypro Ruby. The immunoblot analysis was conducted by calculating the band areas using Image J software, normalizing to the reference protein, α -tubulin, and determining the fold change relative to the 10% serum control group.

The collected and verified secretome samples were analyzed by LC-MS/MS. The analysis conducted with Proteome Discovery software revealed that 177 of the 1,510 total proteins were extracellularly secreted proteins. Of these, 104 were secreted independently of ER stress, 20 were secreted only in the presence of ER stress, and 53 were secreted in the absence of ER stress (Figure 5.8A). The heatmap analysis of the 177 secreted proteins revealed the formation of two clusters, one representing ER stress-dependent decreased protein expression (cluster 1) and the other representing ER stress-dependent increased protein expression (cluster 2) (Figure 5.8B). Gene ontology terms, pathway analysis, and functional assignments were conducted on the protein lists of the two clusters separately using g: Profiler, a bioinformatics analysis server (Appendix-A). For each cluster, the g: GOST graph was employed to perform functional profiling of the gene lists, utilizing a range of biological evidence. A statistical enrichment analysis was conducted to determine the overrepresentation of information obtained from Gene Ontology (GO) terms, biological pathways, regulatory DNA elements, human disease gene annotations, and protein-protein interaction networks. The graph depicts the negative logarithm of the p-value, which represents the randomness of the intersection between the given gene lists and the ontology term, on the y-axis. The x-axis represents the color-coded functional terms grouped by data sources. Upon examination of the graph, it becomes evident that the cellular compartment assignments of both subsets indicate a significant level of association with the "extracellular matrix" and "synaptic cleft" (see *p*-values between 10^{-23} and 10^{-11}). Similarly, the molecular function assignments align with expectations, pointing to "signal receptor binding activity," "cytokine/chemokine activity," and "hormone activity" as being associated with extracellularly released proteins. This corroborates the findings of our studies targeting the secretome.

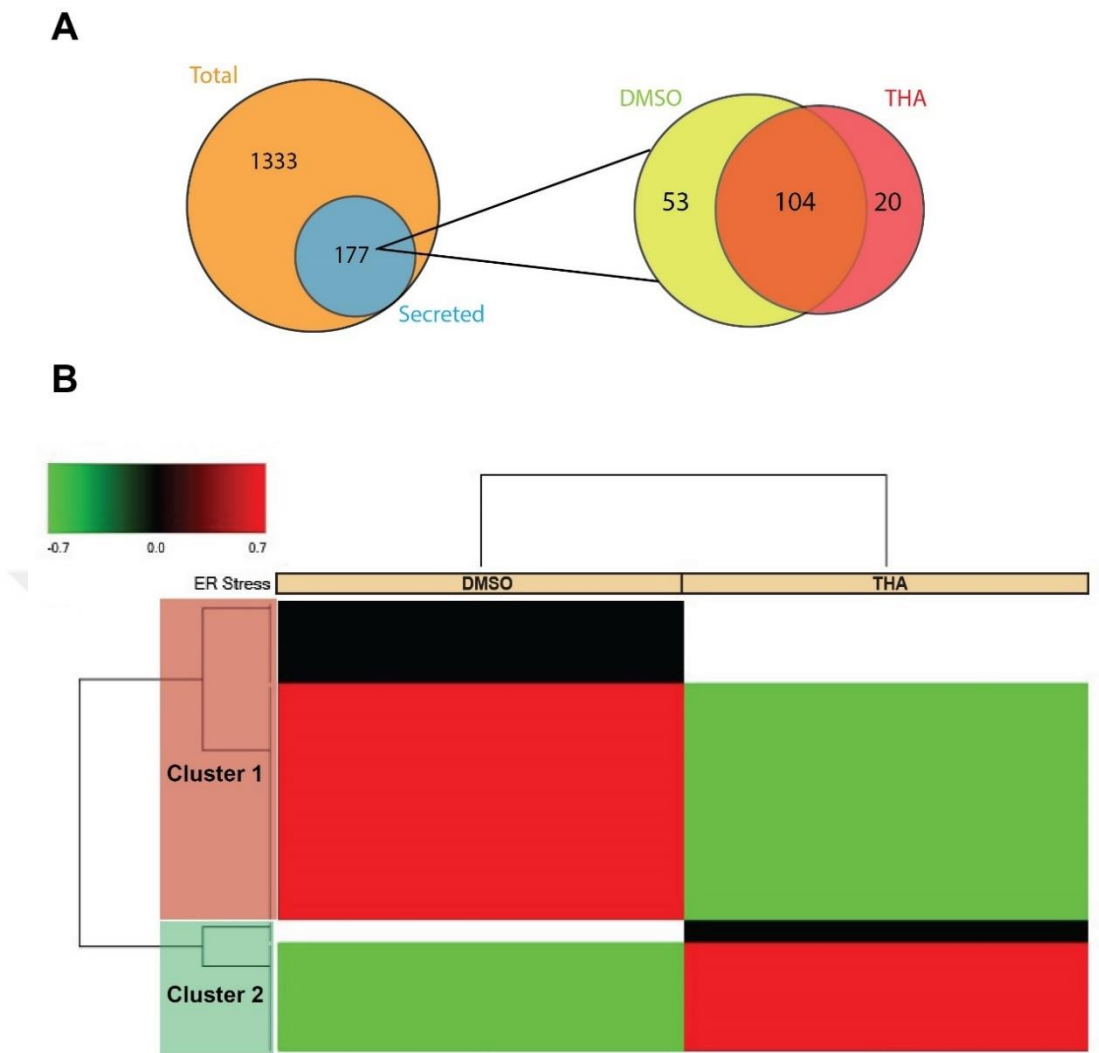


Figure 5.8: LC-MS/MS Analysis of the ER stress-dependent POMC secretome. A) Purified POMC secretome samples were subjected to LC-MS/MS (Orbitrap ELITE, Thermo) analysis, yielding 1,510 total protein (Uniprot "mus musculus") data. Of these, 177 were identified as being included in the secretome database (Uniprot "signal "+"secreted", GO Cellular Compartment "extracellular"). B) To illustrate the clustering of proteins under conditions of ER stress and DMSO control, a heat map of the POMC secretome was generated. The protein abundances were quantified using z-scores, which were visually represented by a color code.

An examination of the proteins in Cluster 1 revealed those proteins whose expression were decreased in response to ER stress induction belong to biological terms; "negative regulation of apoptosis/programmed cell death (heat shock protein 84b, secreted phosphoprotein 1, macrophage migration inhibitory factor, follistatin-related protein 1)," "TGF- β production," "acute inflammation response (complement C3,

metallopeptidase inhibitor 1)," and "positive regulation of proliferation." Furthermore, these proteins have been linked to molecular functions such as "cytokine binding" (Biglycan, Cartilage oligomeric matrix protein), "interleukin-1 binding" (Heat shock protein 84b), and "nerve growth factor binding," as well as biological pathways such as "PI3K-Akt signaling pathway." Additionally, it has been linked to cellular compartments such as secretory vesicles and phagosomes (protocathepsin1).

In cluster 2 proteins whose expression were increased in ER stress-dependent manner associated with several biological processes, including microglial cell proliferation, positive regulation of I-kappaB phosphorylation (Fractalkine), ER stress response, negative regulation of interleukin-1 production, and protein maturation (Protein disulfide-isomerase, Fibulin-1, Beta-2-glycoprotein 1, Gelsolin). Moreover, these proteins were linked to cellular compartmental functions, including the ER-dependent protein quality control apparatus and the ER chaperone complex (Protein disulfide-isomerase), as well as molecular functions such as PI3 kinase catalytic subunit binding (Gelsolin).

Then we performed series of LC-MS/MS-based secretome studies conducted in the presence of ER stress to better understand the effects of SIK2 on the secretome profile of POMCs. For this purpose, stable SIK2-KD POMC cell lines were first established by shRNA. Subsequently, the cells were cultivated in four 10-cm culture dishes, and conditioned media (CM) and simultaneous lysates of the cells were collected for further analysis after 24 and 48 hours with THA treatment. It was not feasible to collect 48-hour cell lysates from the experimental samples, as the mortality rate reached approximately 40-50% with ER stress at 24 hours and the viability decreased to approximately 10-20% at 48 hours. Thus, the CM were used from the cells transfected with scrambled shRNA or shSIK2 RNA and treated only with DMSO for 24 and 48 in the analysis of the secretome. The results demonstrated that SIK2 suppression was maintained at 70-80% in the cell lysates, as evidenced by immunoblotting. As anticipated, the expression of BiP and the splicing of XBP1 were diminished in SIK2-KD cells. The LC3II/I ratio was then analyzed to determine the flux of autophagy. LC3II accumulation and autophagy flux in SIK2 KD cells was reduced by ER stress compared to the control (Figure 5.9A). This result in autophagy flux observed in our experiments may be attributed to prolonged serum starvation (24 hours) or SIK2 deficiency. Additionally, SDS-PAGE gels were utilized to examine the isolated

secretome profiles, which demonstrated that PBS washing effectively reduced the albumin band, thereby facilitating the acquisition of protein bands of the secretome with enhanced quality (Figure 5.9B).

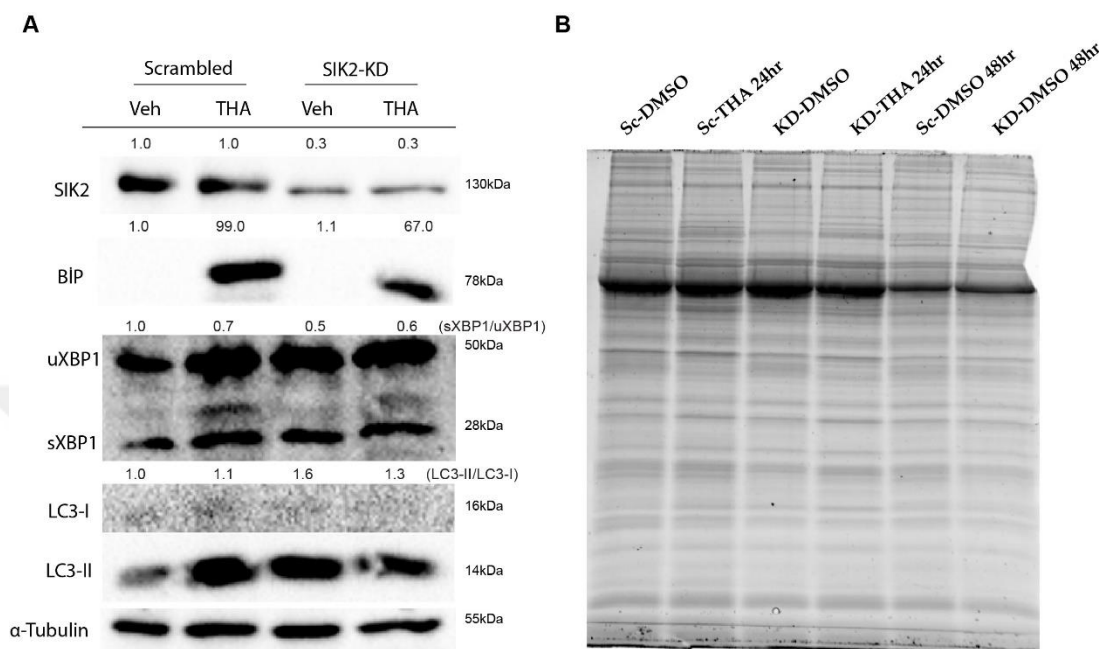


Figure 5.9: Effect of SIK2 on stress response and secretome of POMCs. A) Immunoblotting was conducted using specific antibodies to ascertain the SIK2 protein level and various stress markers. B) The 5 µg protein sample to be analyzed by LC-MS/MS was loaded onto an SDS-PAGE gel, and quality control was performed by staining total protein with Sypro Ruby. Immunoblot analysis was conducted by calculating the band areas using Image J software, normalizing to the reference protein α-tubulin, and determining the fold changes relative to the Scramble-DMSO control group.

The collected and verified secretome samples were analyzed by LC-MS/MS. Our analysis of the data using Proteome Discovery software revealed that 389 proteins out of a total of 1993 proteins were identified as extracellularly released proteins (Figure 5.10A). It is postulated that the elevated number of proteins associated with the secretome in comparison to the results displayed in **Figure 5.8** (see 177 proteins). This can be attributed to the fact that the sample collection periods were conducted later in the day (24 and 48 hours), serum could be effectively removed, and four 10 cm petri dishes were utilized for each condition instead of two. The data set did not include ER stress due to the high rate of cell death observed in the subset where ER stress was

triggered for 24 and 48 hours. A principal component analysis (PCA) of the data reveals that the proteins exhibiting the most significant changes in expression due to the gene effect between the control (scrambled) and SIK2 KD cells are predominantly present in the 24-hour data set. The orientations of the scrambled and SIK2 KD groups with respect to the point of origin exhibited a statistically significant difference at 24 hours. In contrast, at 48 hours, the protein expressions of the SIK2 KD and control groups were found to be similar (Figure 5.10B). This may be attributed to the fact that the cellular response to serum starvation over the subsequent 48 hours hinders the impact of genetic manipulation. As illustrated in the volcano graph, the expression of 30 proteins secreted from SIK2 KD cells exhibited a notable decline at 24 hours in comparison to the control. Conversely, the expression of 5 proteins increased by a minimum of twofold. At 48 hours, the expression of 12 proteins declined significantly, while the expression of 21 proteins increased by a minimum of twofold (Figure 5.10C). The observed decrease in protein expression at 24 hours, followed by an increase at 48 hours, may provide a potential explanation for the gene effect in the PCA analysis that was lost at 48 hours. As observed in the heat map, the proteins that underwent significant alterations were grouped into six clusters. In addition to the data represented in the PCA graph, it is notable that the rates of significant change in the 24-hour data are higher than in the 48-hour data (see Figure 5.10D, 1st and 2nd columns), with a discernible pattern difference. Gene ontology terms, pathway analysis, and functional assignments were conducted on the protein lists of the six clusters separately using g: Profiler (Appendix-B).

An examination of the proteins in Cluster 1 reveals that those exhibiting a significant reduction (≥ 3 -fold) in expression due to SIK2 depletion are associated with a number of biological processes, including "protein trimerization," "long-term neuronal plasticity," "vesicle-associated transport regulation," and "positive regulation of interferon- β and interleukin-1 β production." Moreover, these proteins have been linked to molecular functions such as "DNA-binding transcription factor binding" and "insulin receptor binding," as well as biological pathways such as "post-translational protein phosphorylation." Additionally, it has been linked to cellular compartments such as COPII-coated ER-Golgi transport vesicles and secretory vesicles. In examining the proteins within Cluster 2, it becomes evident that the proteins exhibiting

a significant reduction (≥ 2 -fold) in expression due to SIK2 depletion are similar to the protein assignments observed in Cluster 1.

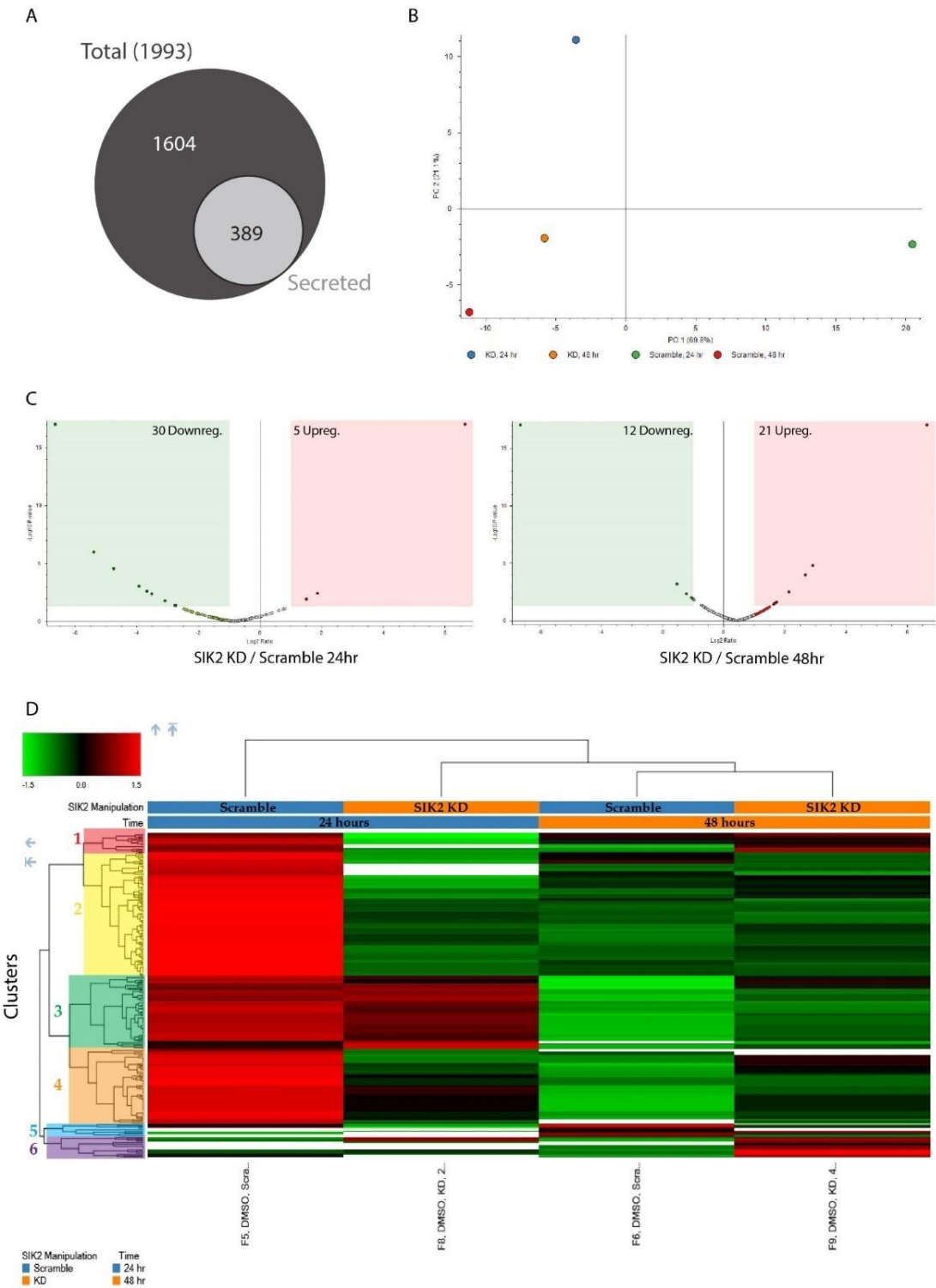


Figure 5.10: LC-MS/MS Analysis of SIK2-dependent POMC secretome. A) LC-MS/MS analysis of the SIK2 manipulation-dependent POMC secretome

using PD revealed 1993 proteins, of which only 389 were identified as belonging to the secretome database. B) The PCA plot was employed to examine the genetic manipulation and time-dependent parameters that resulted in notable alterations among all samples. C) A volcano plot was employed to illustrate the proteins whose secretion exhibited an increase or decrease over the 24- and 48-hour periods, depending on the influence of SIK2. D) A heat map of the POMC secretome was constructed to illustrate the clustering of proteins under 24 and 48 hours of SIK2 depletion. The protein abundances were quantified using z-scores, which were visually represented by a color code.

In contrast, Cluster 3 exhibited no alterations in its protein expression patterns. Cluster 4 comprises proteins whose expression decreased significantly (≥ 1.5 -fold) compared to the control up to 24 hours and increased significantly (≥ 2 -fold) compared to the control at 48 hours due to SIK2 reduction. These proteins are associated with a number of biological processes, including "regulation of lipid metabolism", "protein heterodimerization", "reduction of food intake in response to dietary excess", "positive regulation of microglia and macrophage proliferation" and "positive regulation of the MAPK pathway". Additionally, it has been linked to molecular functions such as cytokine activity and SMAD binding, as well as cellular compartments including the Golgi-mediated vesicle, the CSF1-CSF1R complex, and the ER-to-Golgi compartment.

Cluster 5 comprises proteins whose expression is significantly increased (≥ 2 -fold) in the control groups at 48 hours, as well as proteins whose expression is absent or significantly decreased (≥ 3 -fold) in both hours compared to the control, with the decrease in SIK2. These proteins are associated with a number of biological processes, including "negative regulation of RNA polymerase activity", "negative regulation of apoptotic cell clearance", "positive regulation of DNA repair", "positive regulation of Toll-like receptors 4 and 9" and "UV protection". Additionally, molecular functions such as "interleukin-22 receptor activity," "type 1 interferon receptor activity," and "DNA binding" were identified, as well as cellular compartments including "alpha v-beta3 integrin-HMGB1 complex."

In examining the proteins within Cluster 6, we observe that those expressed at 48 hours in the control groups and exhibiting a significant increase (≥ 2 -fold) in expression compared to the control with SIK2 reduction at both time points are associated with a range of biological processes, including "growth factor activity", "protein sequestering

activity”, “insulin-like growth factor I binding”, “complement C1q complex binding”, “neurexin family protein binding”, and “1-3 beta glucan binding”. Additionally, it has been linked to cellular compartments, including the “interleukin-5 receptor complex”. As demonstrated in **Table 5.1**, the selected proteins were identified as markers of clusters affected by SIK2 modification. Our preliminary results have indicated a correlation between the interested proteins and both SIK2 and ER stress.

Table 5.1: Differentially secreted protein list depends on SIK2 manipulation.

<i>Protein Name</i>	<i>Variation</i>
Nidogen-2	DOWN
Alpha-2-HS-glycoprotein	DOWN
Amyloid-beta precursor protein	DOWN
Heat shock protein 84b	DOWN
Spp1 protein	DOWN
Calreticulin	DOWN
Periostin	DOWN
Syntenin-1	DOWN
Slit homolog 1 protein	DOWN
C-C motif chemokine	UP
Growth/differentiation factor 15	UP
Angiopoietin-related protein	UP
Matrix metalloproteinase 10	UP
Stromelysin-1	UP

5.4. Establishment of the POMC Model for the Comparative Secretome Analyses

In order to understand how SIK2 affects the overall energy balance and eating habits of an organism, we aimed to discover the released proteins of ER-stressed POMCs, which are the main characteristic of hypothalamic obesity. In order to accomplish this objective, we conducted a differential secretome investigation using LC-MS/MS on the conditioned medium of POMCs that were either missing or overexpressing SIK2 under ER stress conditions. Before commencing comparative secretomic experiments, the effectiveness of SIK2 knockdown and overexpression in POMCs, as well as their

sensitivity to THA, were validated using immunoblotting employing particular antibodies (Figures 5.11A and C). Each of shRNAs suppressed SIK2 by approximately 70-80% in the SIK2-KD POMC neurons containing Exon2 design were selected for further experiments. SIK2 is a very sensitive sensor for ER stress. It quickly responded to THA treatment, reaching its active form within 15 minutes. However, it sharply decreased below the control level within 3 hours, then it gradually decreased despite ongoing stress induction (Figure 5.11B).

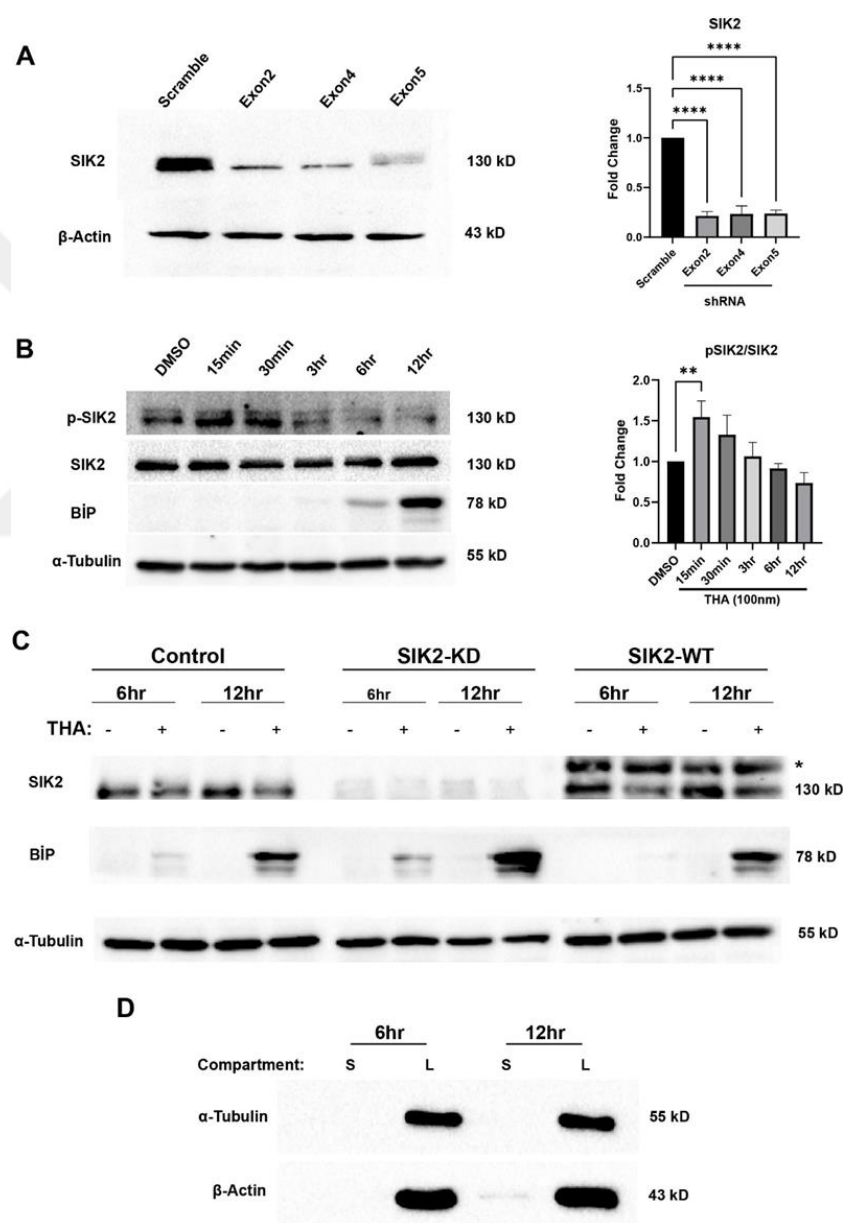


Figure 5.11: Validation of stable POMC cell lines and determination of secretome specificity. A) Three separate POMC lines were generated by utilizing shRNAs that specifically targeted different exons of the SIK2 mRNA. B)

The activation profile of SIK2 was identified at specific time points by inducing ER stress with 100 nM THA and utilizing an anti-pSIK2 (T175) antibody. SIK2 activation is measured by quantifying the relative intensities of pSIK2/SIK2 bands, which are represented as fold change in the bar graph (n=6). C) The modulation of SIK2 was verified by immunoblotting on SIK2-knockdown, SIK2-overexpressing, and control POMC cells that were treated for 6 and 12 hours with 100nm THA. ER stress was verified using the measurement of BiP expression (n=6). * The higher bands represent SIK2 that has been tagged with 3xflag. D) The purities of secretomes were assessed for the presence of cytoplasmic impurities using antibodies that target α -tubulin and β -actin. Cell lysates (L) were compared with secretomes (S), (n=6). The error bars on the densitometric graphs represent the means $\pm$ standard deviation (SD). For A, the statistical significance is indicated as ****P < 0.0001, and for B, it is indicated as **P < 0.01.

In the context of secretomics research, POMC cells containing either naturally occurring, excessively produced, or suppressed SIK2 were subjected to THA for durations of 6 and 12 hours in the absence of serum. The secretomes that underwent 24 hours of ER stress induction were not included in the data analysis since the neurons showed lower proliferation and viability (Figure 5.12A). There was no statistically significant difference in the percentage of viable cells between cultures containing serum and without serum. The cell viability remained consistently above 90% throughout the experiment, including ER stress conditions (Figure 5.12B). We did not find any statistically significant differences in cell viability and death when comparing SIK2 deletion and overexpressing cells in serum-free medium during the 12-hour THA treatment (data not shown). The secretomes from both control and modified POMCs were obtained by collecting the conditioned medium through a series of centrifugation steps. To check the integrity and purity of the secretome, immunoblotting was conducted using specific antibodies for cytoplasmic proteins actin and tubulin on both the POMC secretome and the entire cell lysate. β -actin was found in low quantities in the secretome samples in comparison to the entire lysate (Figure 5.11D). Although a 12-hour time frame is a commonly used interval that allows cells to synthesize their de novo secretomes, we also collected secretomes at 6-hour post-ER stress induction to more accurately represent changes attributable to SIK2 activity (peak level in the initial 30 min as shown in **Figure 5.11B**) in the resulting secretome. Moreover, supporting results have suggested that cultivated cells are capable of producing a

secretome efficiently and in adequate quantities within a time frame of 6 hours after being treated with pharmacological substances [102]

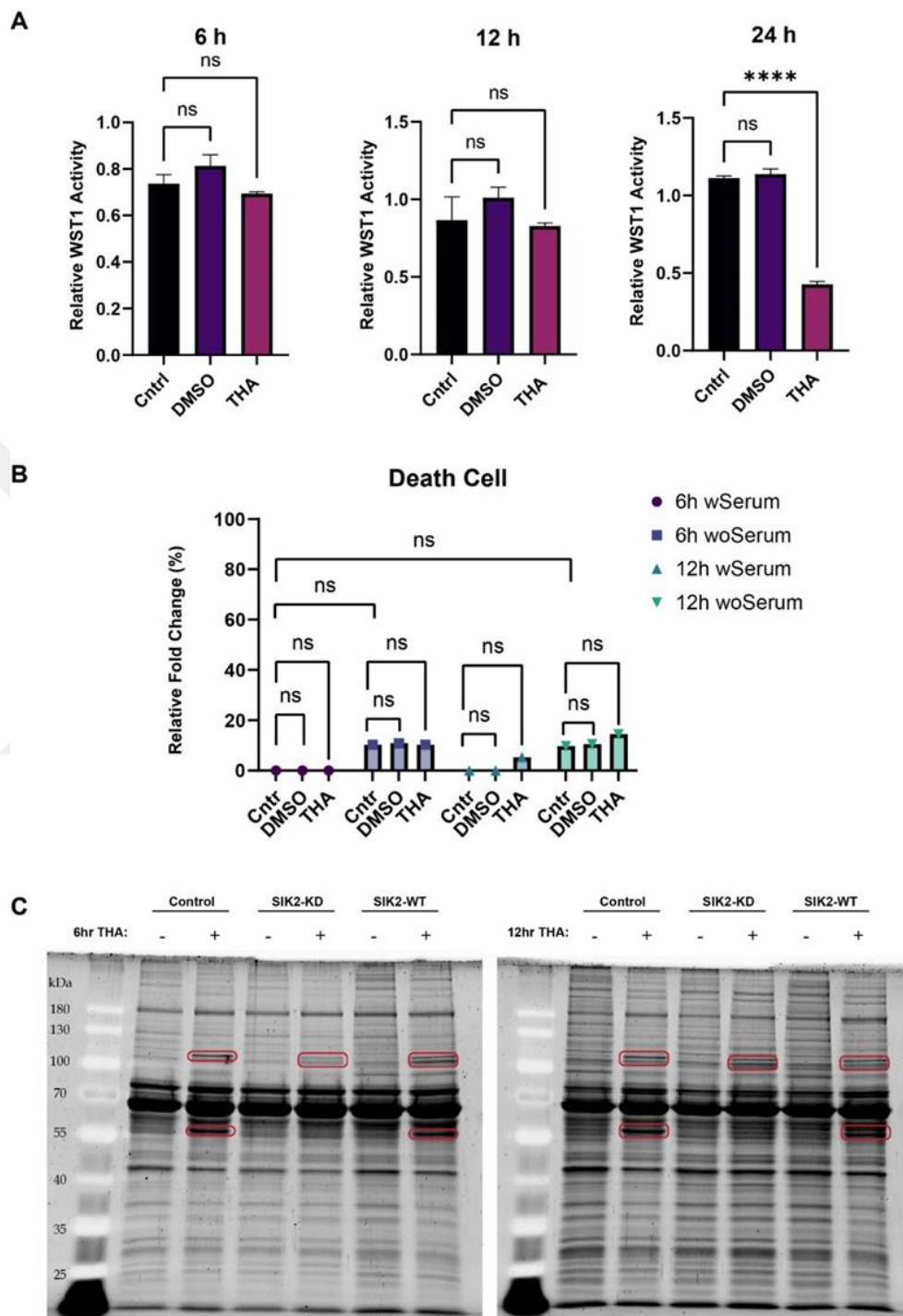


Figure 5.12: Assessing cell viability, stress tolerance and verifying POMC secretome quality. A) The WST-1 experiment was conducted to examine cytotoxicity. All conditions were compared to the control group (n=6). B) The effect of serum deprivation on the survival of cells was assessed using standard viable cell counts with trypan blue labeling for a duration of up

to 12 hours. The experimental settings were standardized to the control group of 6 hours with serum (n=6). **C)** Purified secretomes from control, SIK2 knockdown (SIK2 KD), and SIK2 overexpressing (SIK2 WT) POMCs, which were exposed to ER stress for 6 hours and 12 hours, were examined using 1D electrophoresis after staining the total protein with SyproRuby gel stain. The red rectangular signal represents protein bands that are secreted in a distinct manner. The error bars represent the means $\pm$ standard deviation (SD). The statistical significance is denoted by ****P < 0.0001, whereas "ns" indicates non-significance.

5.5. SIK2 modulates POMC Secretome in response to ER stress

Prior to mass spectrometry analysis, the integrity of isolated secretome samples was assessed by one-dimensional gel electrophoresis (1DE) and SYPRO-Ruby staining. As noted in the literature, after the treatment of the cells an adequate time of at least 12 hours allowed for transcription, translation and subsequent secretion to reach completion and fully represented in the ensuing secretome. Our prior analyses of CMs confirmed the differential expression of some secreted proteins that could even be spotted on the gel by the naked eye (Figure 5.12C). After the analysis of LC-MS/MS based data, a total of 1306 proteins that had FDR above 0.05 were identified, with a confidence interval ranging from medium to high. Among these proteins, 149 were soluble secreted proteins, 12 were shed proteins, and the rest were intracellular proteins. In order to identify proteins that are present in low abundance, we employed the analysis methods of msAmanda and spectrum clustering. **Figure 5.13A** illustrates that the utilization of this methodology led to the identification of 885 soluble proteins and 144 shed proteins. A heatmap was constructed based on the LFQ abundance intensities. Subsequently, we applied clustering methodology to these 1029 proteins whose secretion was either increased or decreased together concerning the SIK2 and ER stress variables. The heatmap displays hierarchical clusters of the comparative POMCs secretomes differentially regulated by ER stress and SIK2 separately and in combination as a function of stress duration (Figures 5.13B and C). In the absence of ER stress, SIK2 overexpression produced the most significant (≥ 2 -fold) increase in a group of related proteins (cluster 3) compared to SIK2 knockdown or control cells (Figure 5.13B). However, in response to (short term) 6-hour of ER stress induction, significant increases of at least two-fold in the secreted proteins listed in clusters 5 and 6 were detected in SIK2 knockdown cells compared to both control and SIK2

overexpression. That is, ER stress-mediated SIK2 activation in POMCs suppresses the level of these proteins, especially during the early phase of ER stress response (Figure 5.13C).

The comparative analyses of heatmap clusters revealed that SIK2 plays a pivotal role in regulating the secretion of proteins belonging to the extracellular matrix, immune response, axon guidance, neurite outgrowth, and feeding behavior-related terms and pathways. However, this connection between SIK2 and dictated secretome has been lost at prolonged (12 hours) ER stress induction consistent with the lowest SIK2 activity detected during this period (Figure 5.11B). The observed fluctuations between the 6-hour and 12-hour time points in the control group in the absence of stress may be attributed to the potential effects of serum starvation. However, there are no statistically significant differences calculated according to LFQ-based analysis for these fluctuations. On the other hand, the findings showed that the fraction of differentially secreted proteins that showed an increase at the 6-hour of ER stress was greater than those detected at 12-hour ER stress for both SIK2 knockdown and overexpressing cells, compared to the control (Figure 5.14). As an example, under no-stress conditions, the overexpression of SIK2 appeared to have a more extensive (about 70%) but distinct incremental influence on the secreted proteins in the clusters that were highlighted by two asterisk (Figure 5.14C). Collectively the data presented suggests that the effect of SIK2 on the POMC secretome is specific to the early phase of the ER stress response. The biological processes seem to be controlled by SIK2 through altering the related cellular secretome is in complete agreement with its known biological functions gathered from systemic SIK2 knockout animal models and various cell lines, including both neuronal and non-neuronal origin.

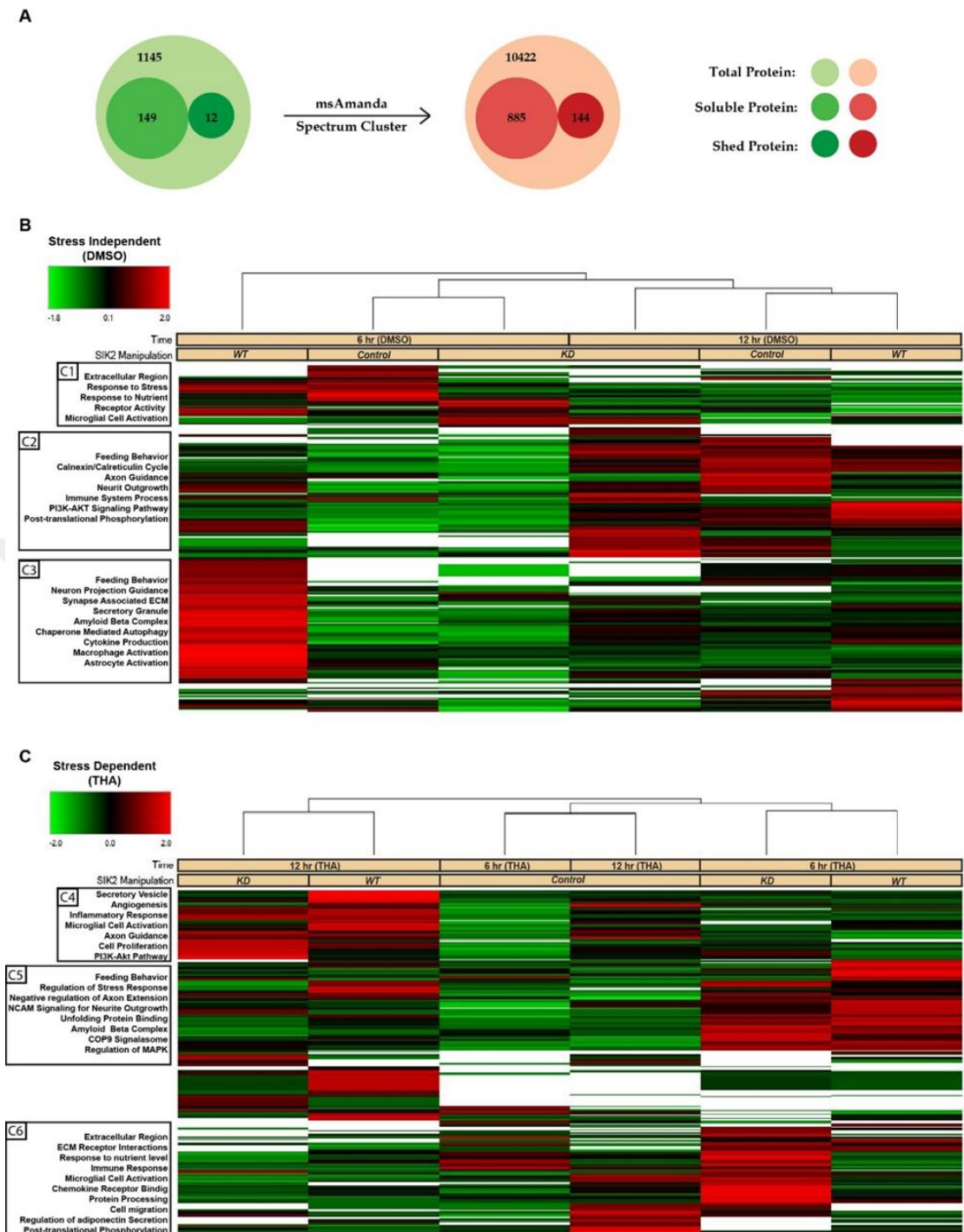


Figure 5.13: Visualization of the POMC proteins that are secreted differently depending on either ER stress or SIK2 or both in combination. A) The presence of soluble and shed secreted proteins is depicted. The size of the circles is directly proportional to the quantity of proteins. The heatmap displays the distribution of POMC secretome proteins with respect to SIK2 availability in the absence (B) and the presence of ER stress (C). The hierarchical clustering method used for this analysis by Proteome Discoverer 2.3. The proteins are represented by the rows, while the groups of replicates are shown by the columns. The protein abundances were

quantified using z-scores, which were visually represented by a color code (green shows the decrease; black shows no change; red shows the increase; white shows no detection). Functional annotation clustering with ShinyGO for the gene ontology terms/pathways is shown on the left, sorted by enrichment FDR score (<0.05).

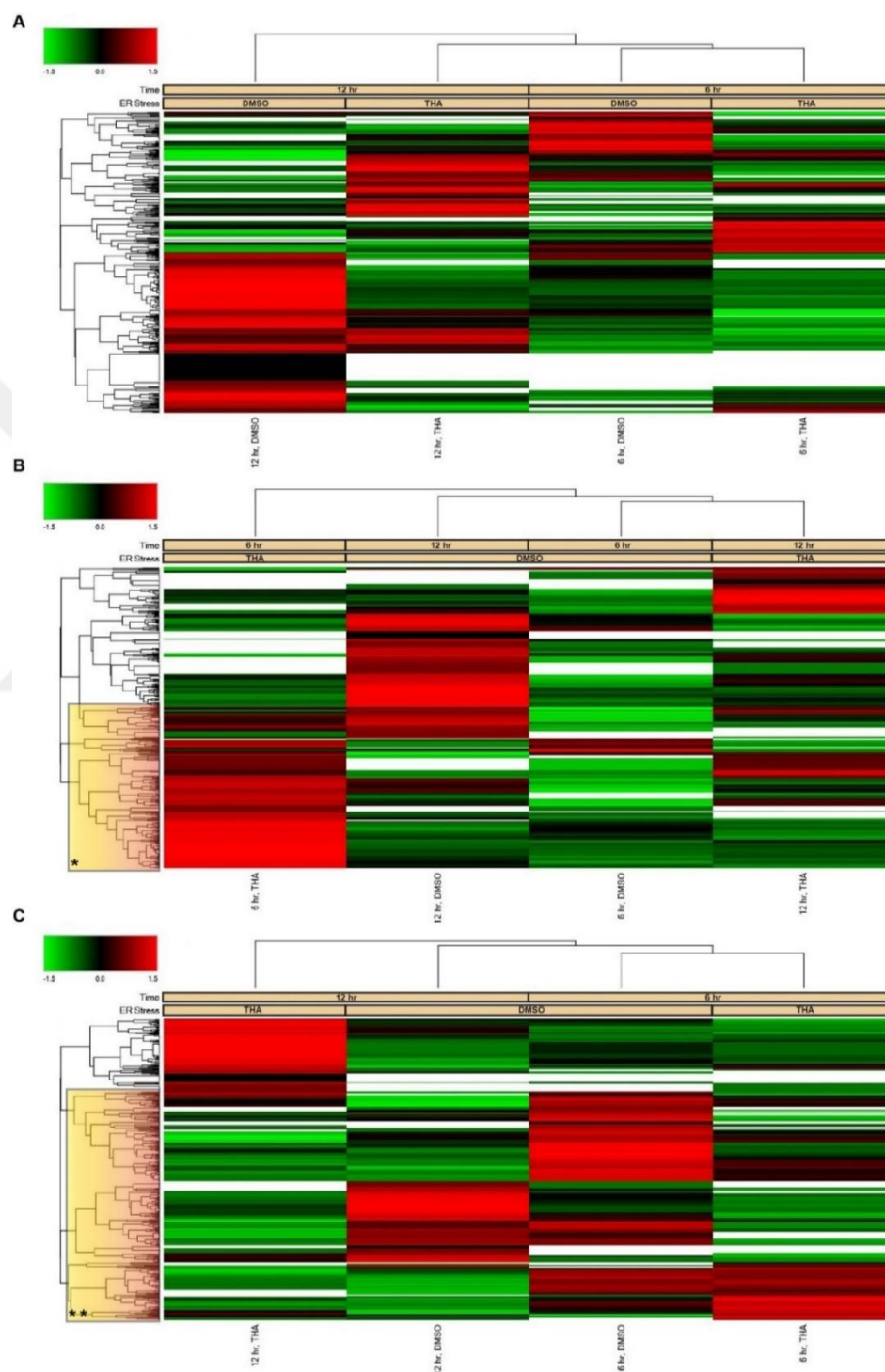


Figure 5.14: Heat maps depicting the ER stress responses that are specifically influenced by manipulations of SIK2. The heat map displays the

distribution of POMCs secretome proteins obtained at 6 and 12 hour of ER stress inductions for control (A), SIK2 KD (B), and SIK2 WT (C) samples. The hierarchical clustering was performed using Proteome Discoverer 2.3. Proteins are represented by rows, whereas groups of replicates are represented by columns. The colors represent z-scores, with green indicating a decrease, black indicating no change, and red indicating an increase, and white indicating no detection. *The proteins that showed the greatest increase in expression with 6 hours of treatment with THA in the SIK2 KD POMCs secretome. **The proteins that showed the greatest increase in expression with 6 hours of DMSO treatment in the secretome of SIK2 WT POMCs.

5.6. Functional Enrichment Analyses of Secretome

A study of functional enrichment was carried out on the gene lists derived from the proteins that were arranged in a hierarchy within the heatmap (which included Clusters 1-6). The analyses were performed using ShinyGO to determine functional pathways and biological processes with a high level of specificity defined as FDR of less than 0.05 (Figures 5.13B and C). Clusters 2-5, which were comprised of the secreted proteins that were influenced by SIK2 activity and displayed the lowest FDR values as assessed by ShinyGO, were subsequently moved to ClueGO, a Cytoscape plug-in, for further observations (Figures 5.15 and 5.16).

Most of the identified proteins that are released are involved in cellular functions ranging from receiving and processing signals from the external environment to the regulation of the extracellular matrix. In both physiological and ER stress conditions, SIK2 was found to be critical in determining both identity and quantity of majority of the secreted proteins that were categorized into functionally related groups (Figures 5.15A and 5.16A). In line with its systemic knockout phenotype, SIK2 activity has been consistently correlated with its ability to transiently switch on cascade of physiological processes including inflammatory macrophage polarization in support for maintaining cellular homeostasis and cell survival of various cell types including neurons and microglia. The effects of SIK2 on the identity and quantity of secreted proteins revealed the enrichment of functionally associated neuropeptides related to feeding behavior, cellular stress, and immune response, as well as neuron maturation and axon guidance in the absence of ER stress (Figure 5.15B). Furthermore, the secretion of chemotactic, inflammatory, appetite, and protein folding-associated proteins were found to be altered by SIK2 activity under ER stress conditions (Figure

5.16B). Using the LFQ abundance ratios, we have discovered 35 secreted proteins that are regulated by SIK2. These proteins are related to obesity and feeding behavior, neuroinflammation, axon guidance and neurite outgrowth pathways. These new findings have expanded the functional territories of SIK2 to include regulation of COPII mediated secretory vesicle formation and sorting physically in close proximity to its proposed role in phagophore formation. For a comprehensive list of secreted proteins affected by SIK2 is provided in **Appendix C** and **Appendix D**.



statistics. The various colors within a single node indicate the proportion of genes that exhibited significant enrichment within the term, categorized by different groups.

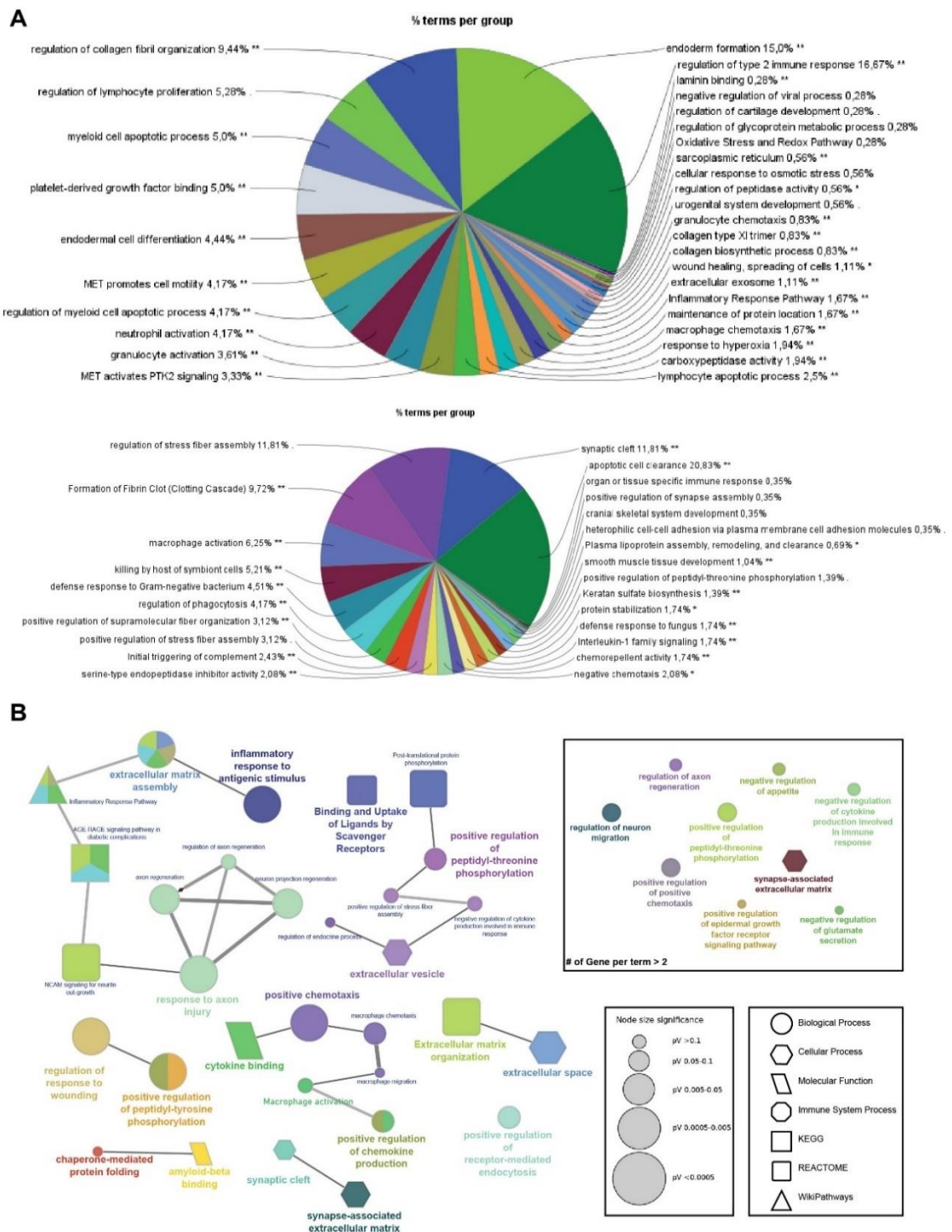


Figure 5.16: Assessment of SIK2-induced POMC secretome functional enrichment with ER stress. A) Pie charts developed by ClueGO and CluePedia display the Gene Ontology terms and pathway analysis for Cluster 4 (top) and Cluster 5 (bottom), indicating the percentage of terms/pathways per related

gene group. B) The network of specifically chosen functionally grouped terms/pathways. Every node in the graph represents a specific term or pathway. The size of each node corresponds to the p-value, and different databases are represented by distinct node shapes (please see the legend for information on node size and shape). The edge symbolizes the link between the processes that are functionally connected. The various colors within a single node indicate the proportion of genes that exhibited significant enrichment within the term, categorized by different groups. The network was established by defining the requirement as having a term annotation for a minimum of two genes, rather than three genes (as indicated in the square on the right).

During the early phase of the ER stress response, a set of proteins related to feeding and the maintenance of the energy balance such as growth/differentiation factor 15 (Gdf15), Serpinc1, amyloid precursor protein (App), TIMP metalloproteinase inhibitor 1 and 2 (Timp1 and Timp2), showed a significant increase in the secretome of SIK2-knockdown neurons. A comparable effect was also observed, albeit to a slightly larger extent, in the secretome of cells with SIK2 overexpression but no ER stress. This finding collectively provides evidence for SIK2 having a crucial role in determining the quantity and quality of these select neuropeptides secreted from POMCs. These secreted neuropeptides enable POMC neurons to communicate with its effector secondary neurons in the hypothalamus (PVN, LH etc.,) and immediate neighbor neurons including nonneuronal cells (such as microglia) to control energy homeostasis, expenditure and feeding behavior. However, calreticulin (Calr), which belongs to the same group, showed an opposite response to the SIK2 absence. It decreased by 2-3-fold compared to control cells when short-term ER stress was induced. However, when SIK2 was overexpressed, Calr returned to its original level. This may suggest that SIK2 act differentially on counteracting factors within the same group. (Refer to **Appendix C**, specifically the obesity and feeding behavior group highlighted in red.)

The release of proteins involved axon guidance and neurite growth, namely perlecan (Hspg2), dickkopf-like protein 1 (Dkk1), cathepsin B (Ctsb), matrilin-4 (Matn4), and nidogen-1 (Nid1), were found to be collectively elevated in the secretome of cells lacking SIK2 at the early phase of ER stress. This secretion pattern was quite similar to the one observed in cells with SIK2 overexpression but no ER stress. On the other another member of this group, Dkk1 displayed the opposite response. (Refer to

Appendix C in the section on axon guidance and outgrowth, which is highlighted in green).

Under physiological and early ER stress conditions, SIK2 strictly regulates the secretion of a specific group of proteins. These proteins belong to the proinflammatory cytokine and chemokine family. The family members, specifically macrophage migration inhibitory factor (Mif), annexin A1 (Anxa1), fraktalkine (Cx3cl1), and granulins (Grn), showed similar levels of secretome abundance in response to both SIK2 deficiency and short-term ER stress. During short-term ER stress, the secretome of cells with SIK2 knockdown exhibited sustained and significant elevation in the levels of Mif, Anxa1, Cx3cl1, and Grn. Under basal conditions in the absence of ER stress, SIK2 positively regulated the expression of three inflammatory secreted proteins: Mif, Grn, and Cx3cl1 (See **Appendix C** in the inflammation section, highlighted in blue).

In the listed three groups, the secretion levels of the proteins could be regulated by stress-activated SIK2 during the early phase of the ER stress response. However, during the late phase of the ER stress, coincident with the deactivation of SIK2 the secretion profile of the POMCs is irreversibly changed to represent abrogation of the cellular homeostasis to indicate the end of the cell survival period (Appendix D). Furthermore, functional enrichment analysis of differentially secreted proteins grouped into clusters 2 and 4 (Figures 5.13B and C) revealed a concerted effort to activate the major survival-related PI3K-AKT pathway during the early phase of the ER stress response [103] (Figure 5.17), whereas death-related C/EBP homologous protein (CHOP) signaling emerged as the activity of SIK2 decreased in response to prolonged ER stress induction[104]

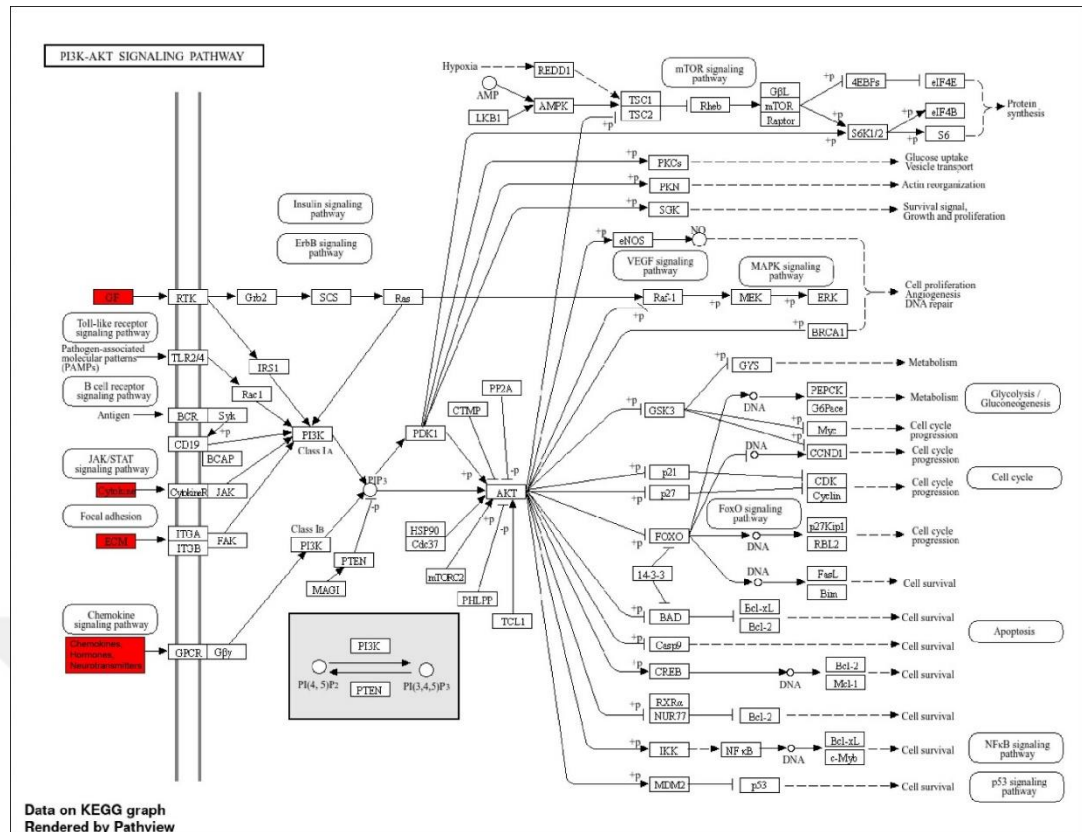
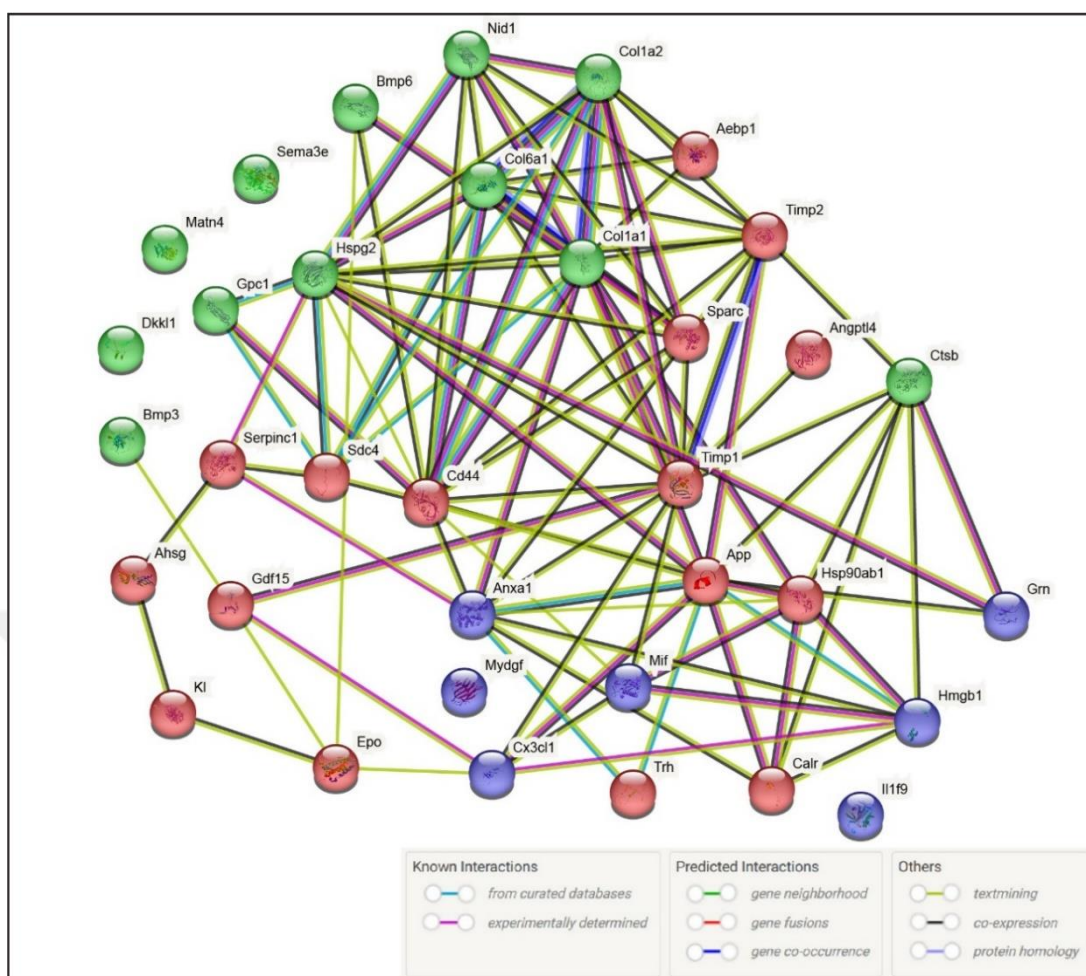


Figure 5.17: The PI3K-AKT pathway contains proteins that have been identified based on data from the KEGG database. The PI3K-AKT pathway is classified in both Cluster 2 and Cluster 4 based on the functional enrichment analysis of differentially secreted proteins. The graphic is produced by ShinyGo and depicts associated terms represented by red rectangles.

5.7. Evaluation of Protein-Protein Interactions Network

The secreted proteins produced from POMCs, which are controlled by SIK2 during the initial stages of the ER stress response, were entered into the STRING software to analyze the protein-protein interactions (PPI) network (Appendix C). A network of PPIs that are functionally significant was presented, together with their functional annotations, derived from either experimental or database-predicted data. Analysis of the STRING revealed links among the members of the three distinct gene groups discovered in the functional enrichment analysis. The table below (Figure 5.18) lists the genes that are represented in the PPI network. Analysis of the PPI network showed a complex network of interactions among the members of three functionally related groups. This emphasizes the complex connection between the individuals in these

three separate but functionally interconnected groups, which are regulated by SIK2, presumably downstream of the unfolded protein response (UPR), in ER-stressed POMC neurons. For example, the stress alarmone and anorectic protein Gdf15 interact with members of two other categories, including the inflammatory Cx3cl1, axon guidance-related protein (Bmp3), and the obesity-related proteins Timp1 and erythropoietin (Epo). Timp1, a shed protein implicated in the inflammatory response plays a central regulatory role at the crossroads of protein-protein interactions between obesity-related proteins and feeding behavior-related proteins (Gdf15, Cd44, Sparc, and Angptl4) as well as axon guidance-related proteins (Ctsb, Hspg2, Col1a1, and 2) and neuroinflammation related proteins (Cx3cl1, Anxa1, and Mif). Furthermore, our investigation uncovered a correlation between cathepsin B (Ctsb), a type of cysteine protease located in lysosomes that is involved in protein degradation and the breakdown of amyloid precursor protein (App), and various proteins linked to obesity and feeding behavior, specifically Calr, App, Hsp90ab1, Timp1, and Timp2. Furthermore, cathepsin B also interacted with inflammation-related proteins Hmgb1 and Grn (Figure 5.18). Although APP is predominantly linked to the pathophysiology of Alzheimer's disease, a recent report pointed out its association with obesity phenotypes such as insulin resistance and inflammation, which are regulated by tumor necrosis factor α (TNF- α) [105] [106]. In conclusion section, we carried out an extensive and targeted examination of these PPIs.



Color Code	Cluster Name	Gene Count	Protein Names
	Obesity & Feeding Behavior	16	Kl, Serpinc1, Gdf15, Trh, Epo, Calr, App, Hsp90ab1, Cd44, Angptl4, Timp1, Timp2, Sdc4, Sparc, Ahsg, Aebp1
	Axon Guidance & Neurite Outgrowth	12	Matn4, Gpc1, Sema3e, Dkk1, Ctsb, Bmp3, Bmp6, Col1a1, Col1a2, Col6a1, Hspg2, Nid1
	Inflammation	7	Hmgb1, Mif, Cx3cl1, Il1f9, Mydgf, Anxa1, Grn

Figure 5.18: Visualization of the investigated protein-protein interactions using the STRING tool. The proteins in the PPI network are visualized as 3D spheres and identified by their corresponding gene names, with each protein representing a node in the network. The nodes are linked by edges that symbolize their interaction. The hue of the edges corresponds to their source of interaction knowledge, as illustrated. Proteins are organized into clusters based on their related biological terminology, with each group being depicted by a circle of distinct colors. The complete names of the proteins are provided in **Appendix C**.

5.8. The Confirmation of Secretome Protein Profiling

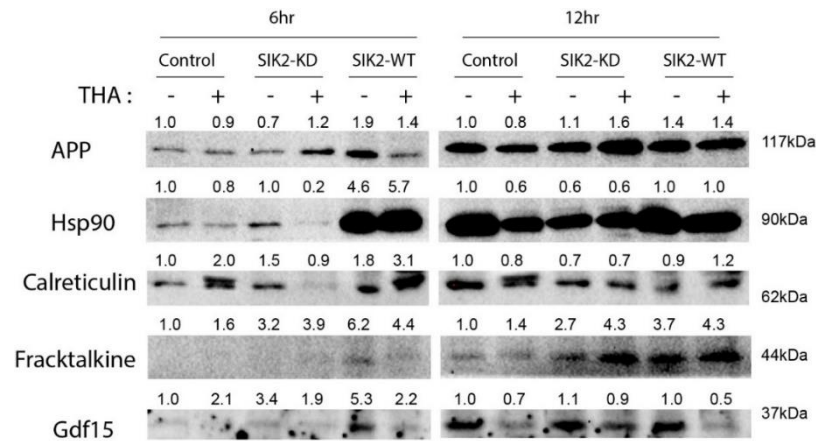


Figure 5.19: Confirmation of the detected secretome proteins by western blotting. The levels of secreted proteins were verified by immunoblotting on the secretome of SIK2-knockdown, SIK2-overexpressing, and control POMC cells that were treated for 6 and 12 hours with 100nmol/L THA. Immunoblot analysis was conducted by calculating the band areas using Image J software. The indicated fold change was calculated by normalizing each band intensity to the control of 6-hour or 12-hour THA induction, separately.

For further verification, immunoblotting was performed on secretome samples for some of the proteins found to be regulated by SIK2. Since the protein quantities in the secretome samples were lower than those in the standard cell lysates, three biological secretome samples were combined and analyzed. According to mass spectrometry analyses, proteins with high FDR classification were effectively detected in immunoblotting, while proteins with medium and low FDR values such as Fractalkine and Gdf15 could be poorly visualized, especially in the 6-hour point. Consistent with the findings of the LC-MS/MS, SIK2, and ER stress-dependent alterations in protein profiles were detected, predominantly in the 6-hour samples. However, these changes were largely disappeared within 12 hours. The levels of Calr and Hsp90 chaperones, secreted in response to ER stress, were diminished in the absence of SIK2, yet increased to critical levels upon SIK2 overexpression. As anticipated, the APP secretion demonstrated an opposite pattern in comparison to these chaperones. As far as they can be visualized, Fractalkine and Gdf15 can increase in the absence of SIK2 independently of ER stress, and their secretion is further increased

by SIK2 overexpression. While it is evident from the LC-MS data that these proteins can be restored to physiological levels in SIK2-overexpressed cells, their drastic increases in SIK2-KD could not be adequately demonstrated (Figure 5.19).

5.9. SIK2 and POMC Processing

To unravel the possible role of SIK2 on POMC processing, immunoblotting with few antibodies specific to overlapping but distinct regions of the POMC protein was performed on the cell lysates of secretomes samples treated or not with THA. Since POMC processing is a complex process involving the formation of different biologically active peptides and intermediates, antibodies that differentially recognize a subset of these peptides was used to decipher the POMC processing and resulting active secreted peptides in POMC neurons. POMC-N is an antibody that recognizes the synthetic peptide corresponding to the residues surrounding Pro69. It is therefore ideal for the detection of γ -MSH and its intermediates. POMC-C is an antibody that recognizes the synthetic peptide derived from human POMC corresponding to amino acid residues R236-E267. This peptide, with its conserved sequence, corresponds to mouse β -endorphin and its intermediates. α -MSH antibody recognizes synthetic α -MSH peptide (SYS MEHFRWGKPV) and is therefore ideal for the detection of α -MSH and its intermediates, including ACTH (Figure 5.20A). Because each peptide sequence recognized by these antibodies is also part of POMC and ACTH precursors, they can also detect these precursors (>20 kDa).

The obtained results indicate the presence of POMC precursors with a molecular weight greater than 20kDa and intermediates less than 20kDa (Figure 5.20B, C, D). In addition to the bands corresponding to the expected molecular weights, a variety of other bands of different sizes were observed. This phenomenon may be attributable to the presence of known or unknown variants that may be subject to posttranslational modifications. Detected intermediate and peptide regions by N-POMC (Figure 5.20B) and C-POMC (Figure 5.20D) antibodies are much weaker than α -MSH (Figure 5.20C). This is an expected result since it is known that γ -MSH and β -MSH are not secreted in rodents. Although the α -MSH bioactive peptide with a size of 1.5kDa could not be detected for methodological constraints, the prominent visualization of its intermediates indicated dominant α -MSH processing. In the absence of ER stress, an increase in POMC precursors is observed in the absence of SIK2, especially in the 6-h experimental conditions in which we previously saw that SIK2 exerts an effect. The

parallel increase in α -MSH intermediates suggests that the absence of SIK2 increases the expression of POMC rather than affecting its processing. This is consistent with the reduced appetite observed in the systemic SIK2 ko phenotype. Conversely, when SIK2 is overexpressed, there is no change in POMC precursors, but there is an ER stress-independent increase in intermediates. This may indicate that SIK2 increases POMC processing. In general, POMC intermediates were decreased by ER stress. This is consistent that ER stress cause defective POMC processing and reduced α -MSH production [90]. However, elucidation of the possible effects of SIK2 on the POMC process requires further studies.



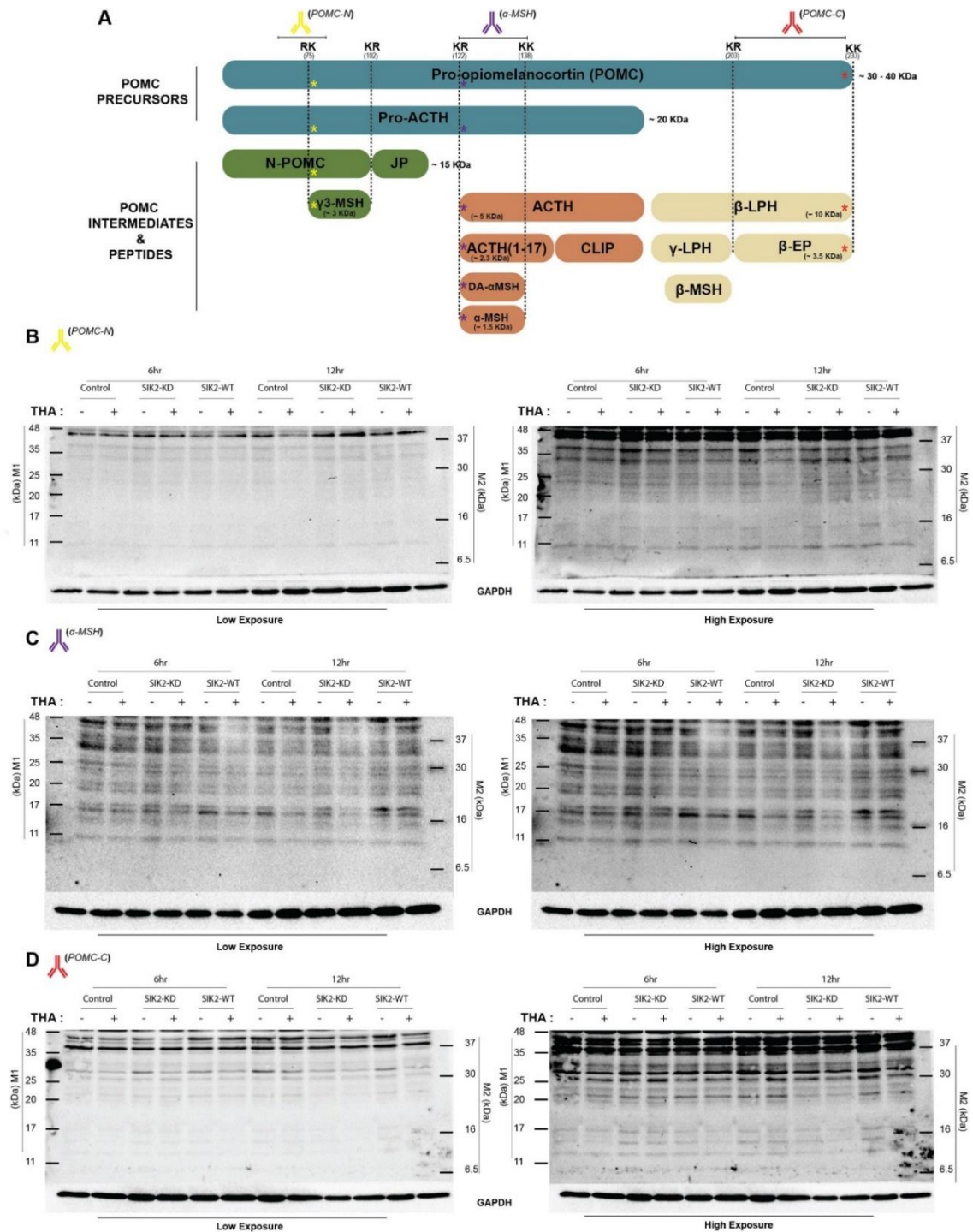


Figure 5.20: Assessing POMC processing under ER stress conditions in SIK2 Manipulated POMCs. A) Schematic representation of antibodies and recognition sites targeting different peptides in the POMC processing. B) The levels of POMC precursors and derived neuropeptides/intermediates were verified by immunoblotting on the SIK2-knockdown, SIK2-overexpressing, and control POMC cells that were treated for 6 and 12 hours with 100nm THA.

5.10. The effects of SIK2 and ER stress on POMC processing throughout the anterograde secretion pathway

Because of our protein extraction methodology which disregards rather small peptides, neuropeptides such as α -MSH could not be detected by mass spectrometry. To observe how α -MSH neuropeptide is affected by ER stress and SIK2, ICC was performed in SIK2-manipulated POMC neurons using an α -MSH specific antibody. The ER-Golgi axis was visualized by plasmid backbones that contained protein markers of these organelles fused to the fluorescent reporter protein. In the presence of ER stress, the co-localization of α -MSH with Golgi and secretory vesicles exhibited a time-dependent decrease compared to the DMSO control. In the absence of ER stress, SIK2 depletion increased this co-localization in the opposite direction. In the early period of stress, a decrease was observed, as seen in control cells. Conversely, in the case of prolonged stress, an increase was observed, and this remained relatively high compared to control (Figure 5.21 and 5.23A). Moreover, the total areas of Golgi and secretory vesicles also exhibited a pattern correlated with this co-localization (Figure 5.23B, left).

It is noteworthy that the α -MSH signal is concentrated around the nucleus when its colocalization with Golgi is reduced. This observation prompted us to investigate the potential ER accumulation of POMC precursors. As expected, co-localization of α -MSH with ER demonstrated a significant increase under conditions of ER stress, most notably during the initial phase, followed by a subsequent decrease as stress progressed. In the absence of ER stress, the SIK2 depletion caused this co-localization to be less pronounced. In the early phase of ER stress, a similar but weaker increase was observed, as in the control group. However, it was observed that their accumulation on the ER continued with prolonged stress and did not decrease contrary to control group (Figure 5.22 and 5.23A). Moreover, total ER areas show a correlated pattern with this colocalization (Figure 5.23B, right).

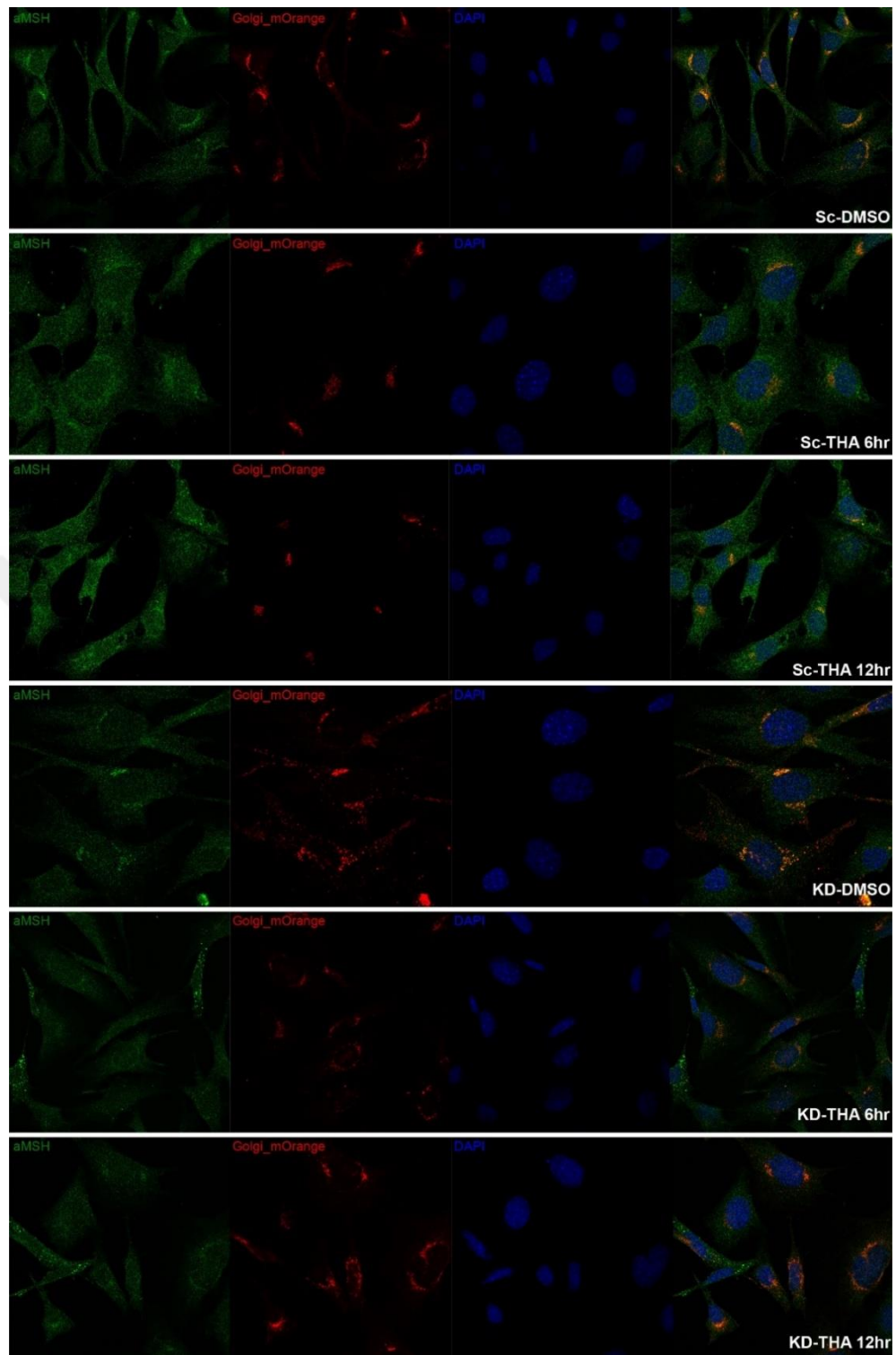


Figure 5.21: Colocalization of α -MSH with Golgi and secretory vesicles. The ICC procedure was conducted on transiently Golgi-mOrange overexpressing POMCs, which were SIK2-WT co-expressing, SIK2-knockdown, or control POMC. The ICC process was performed utilizing anti- α -MSH and secondary Alexa488 antibodies. DAPI was used to distinguish the nucleus. The imaging of fixed cells was obtained by using specific excitation and emission wavelength of their proteins/dyes under Zeiss confocal microscope at 63x magnification.

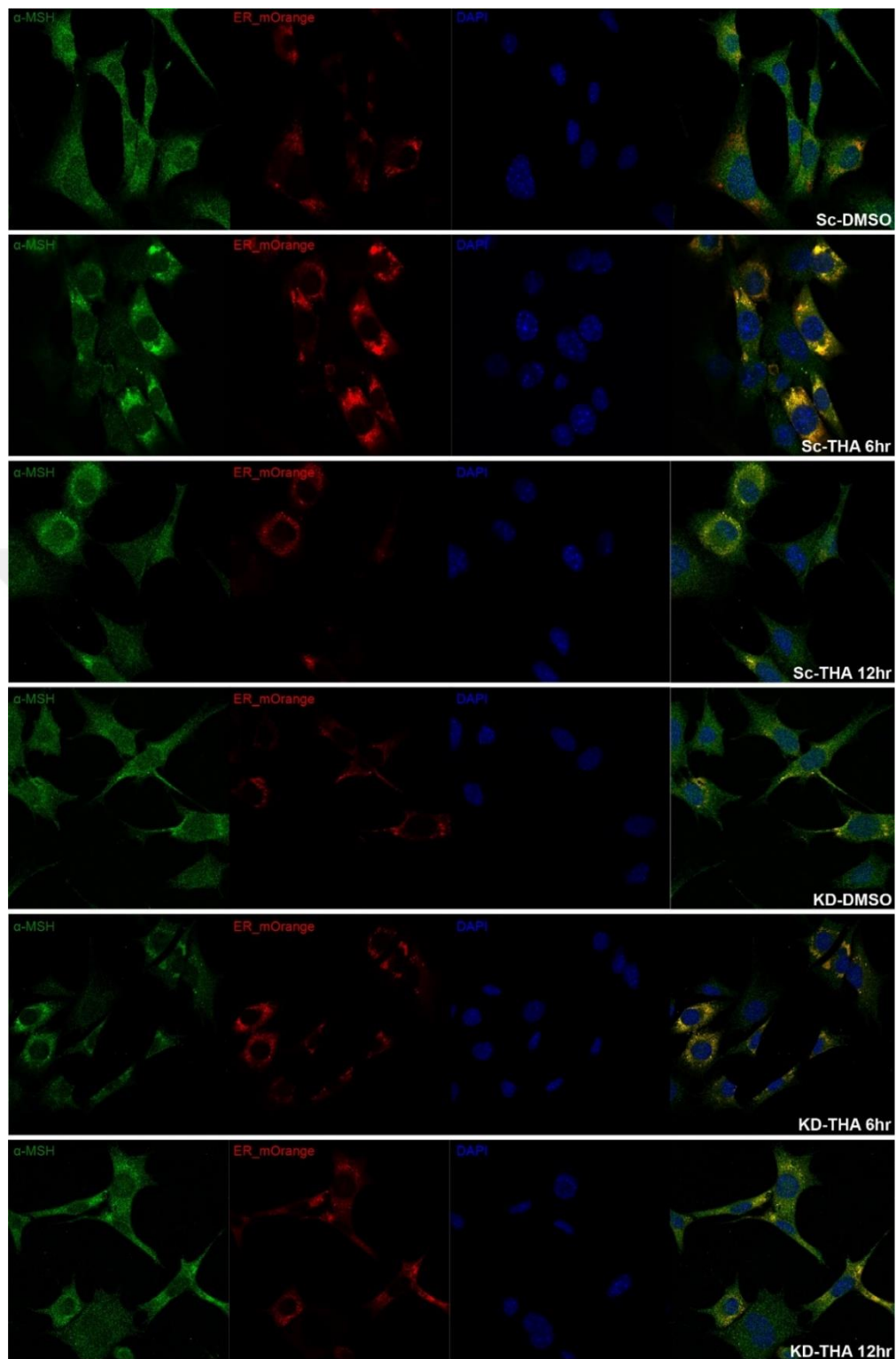


Figure 5.22: Colocalization of α -MSH with ER. The ICC procedure was conducted on transiently ER-mOrange overexpressing POMCs, which were SIK2-WT co-expressing, SIK2-knockdown, or control POMC. The ICC process was performed utilizing anti- α -MSH and secondary Alexa488 antibodies. DAPI was used to distinguish the nucleus. The imaging of fixed cells was obtained by using specific excitation and emission wavelength of their proteins/dyes under Zeiss confocal microscope at 63x magnification.

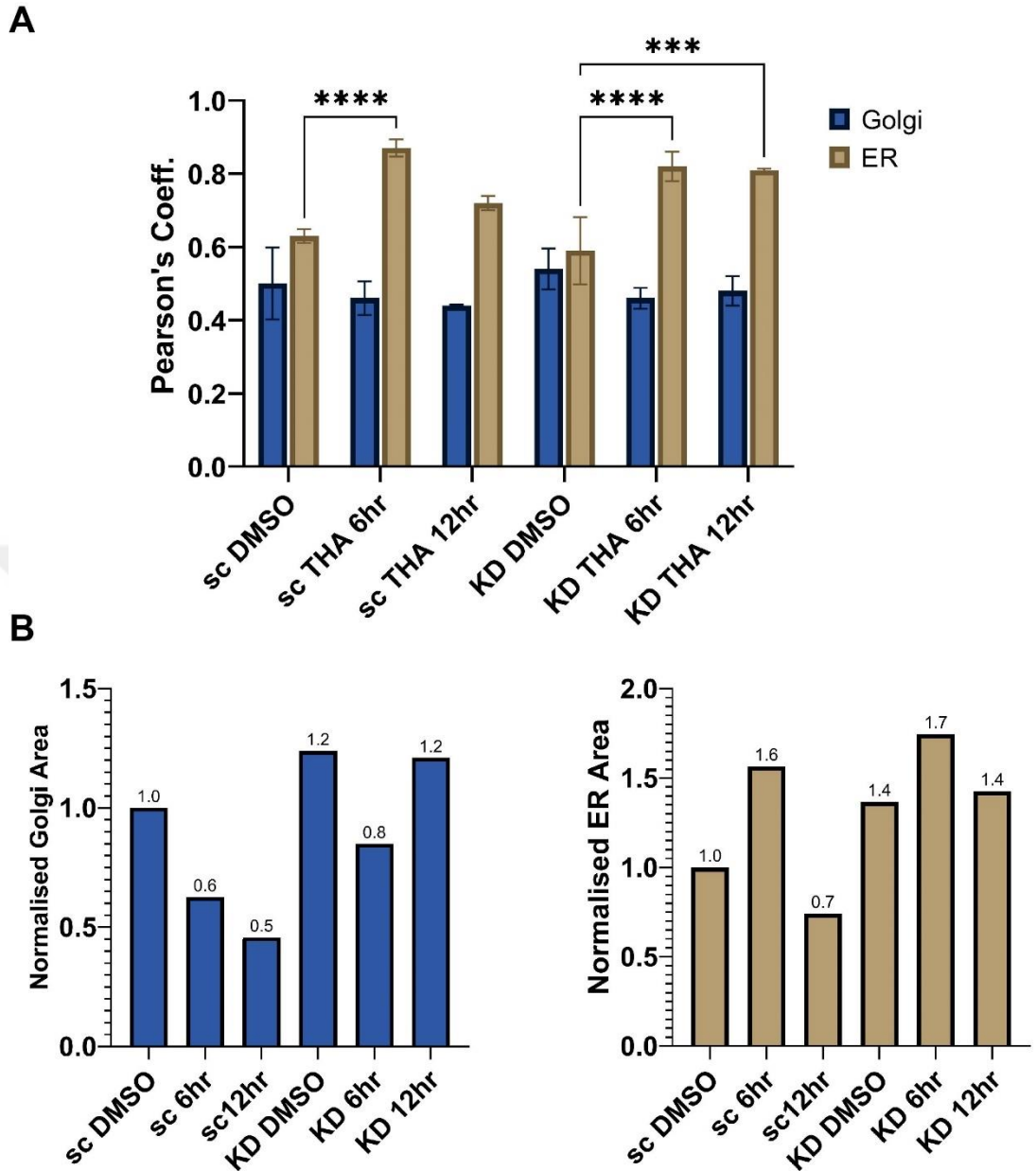


Figure 5.23: Statistical analysis of colocalization of α -MSH with ER and Golgi. The colocalization analysis (A) and calculations of the total Golgi and ER areas (B) was conducted on approximately 25–30 cells by BioJacop, a plugin of the Fiji. One-way ANOVA analyses were performed on the obtained Pearson coefficient values using GraphPad Prism software. Total organelle areas were normalized to scramble DMSO control group. A minimum of three distinct regions from each sample were imaged using a Zeiss confocal microscope at 63x magnification. The error bars on the densitometric graphs represent the means $\pm$ standard deviation (SD). For the statistical significance is indicated as **** $P < 0.0001$, and *** $P < 0.001$.

6. DISCUSSION

Hypothalamic obesity is frequently characterized by hypothalamic inflammation and specific neuroendocrine changes including hyperleptinemia and insulin resistance. However, a recent study has demonstrated that defective regulation of POMC neuropeptides occurs prior to hypothalamic inflammation in HFD [84]. Moreover, different studies have shown that AgRP neuronal activity is not a prerequisite for the development of obesity [33], [34]. In the light of these findings, we centered our hypothesis on POMC neurons in the ARC involved in energy homeostasis. In the preliminary characterization studies POMC cells exhibited good responsiveness to both insulin and leptin associated with expected SIK2 activation profiles not only to these anorectic hormones but also to ER stress induction validating their suitability for the further functional studies.

Interestingly, SIK2 is largely localized to the ER membrane and undergoes a quick and transient activation profile when treated with pharmacological ER stressor thapsigargin [107]. Our study (the data not shown) and other research suggest that the UPR plays a role in the activation of SIK2 in response to ER stress. Nevertheless, the precise location of its substrate in the ER, along with the specific upstream activator kinase, remains to be elucidated. Following the UPR, SIK2 has been consistently connected to the regulation of important ER stress response pathways, such as inflammation, ERAD, and autophagy. A recent study has demonstrated the presence of SIK2 in ER lipid rafts, which are essential for the proper packaging of secretory cargo proteins into export vesicles [108]. Collectively these findings suggest that SIK2 may also play critical roles in a molecular mechanism that enables it to regulate the quantity and type of secreted proteins in metabolically active cell types including neurons. We postulated that the impact of SIK2 on the POMC secretome could be easily probed by studying the alterations in secreted proteins by LC-MS/MS based quantitative secretome study. To accomplish this, we conducted a comparative secretomics investigation using SIK2 knockdown and overexpressing POMC neurons that were exposed to short- and long-term ER stress induction with thapsigargin. It was discovered through an analysis of the secretomes of POMCs, that SIK2 plays a key role in the regulation of protein secretion during the early phase of the ER stress induction coincident with the ER stress-induced activation pattern of SIK2 (Figure

5.11B). The secreted proteins regulated by SIK2 were grouped into six biologically relevant clusters. We specifically studied four clusters (Cluster 2 and 3, and Cluster 4 and 5) that are associated and grouped within biological terms obesity and feeding behavior, axon guidance and neurite outgrowth, and neuroinflammation. These clusters were selected based on their FDR values of functional enrichments and significant overlap with the biological functions of SIK2 reported for its systemic knockout mice as well as for its modified cell lines. While SIK2 is primarily responsible for determining the early phase responsive secretome of the POMC neurons, prolonged ER stress induction associated with below the threshold SIK2 activity creates an opportunity for the counter acting inflammatory and apoptotic pathways to gain dominance over homeostatic ones. That is, SIK2 pressure on these secreted proteins is simultaneously relieved by the deactivation of SIK2 during the prolonged ER stress response. This will make it possible for inflammatory and apoptotic mediators to settle in neuronal and non-neuronal cells that are part of the neuronal circuitries for feeding and energy homeostasis. In this new vein, SIK2 suppresses the inflammatory content of the POMC secretome primarily in the early phase of the ER stress response, parallel to its effect on two other groups, including obesity and feeding behavior and axon guidance and neurite outgrowth as shown in **Appendix C**.

Within the group of obesity and feeding behavior, GDF-15 is often regarded as one of the proteins that have the highest potential for therapeutic application. SIK2 and ER stress exert their influence on the basal level of GDF-15 in a manner that is both precise and independent. This protein is an anorexigenic substance that functions by binding to its specific receptor GFRAL on the PVN, the secondary effector neurons downstream of POMCs, to reduce feeding and increase energy expenditure [109]. According to our findings, the level of Gdf15 in the secretome of SIK2 KD POMCs increased significantly at the early phase of ER stress induction. However, when SIK2 is overexpressed, the level of Gdf15 returned to level of control cells. In the absence of ER stress, the increased expression of SIK2 led to an increment in the level of Gdf15 that was comparable to or even greater than the increase by the induction of short-term ER stress in the absence of SIK2. This pattern was largely confirmed by the immunoblot studies on secretome samples. Nevertheless, protein expressions other than this pattern were also detected in early phase of ER stress induction, especially in

the SIK2 KD samples. This may be attributable to the fact that immunoblotting is a less sensitive method in comparison to LC-MS, or alternatively, it may be due to biases that may result from combining three biological samples. It is commonly observed that the level of circulating Gdf15 is elevated in obese human subjects despite increased food intake [110]. This is not in agreement with the recent report that exogenous Gdf15 injection ameliorated the obesity phenotype in obese animal models [111]. This contradiction necessitates that the future therapeutic strategies targeting Gdf15 or its upstream regulator SIK2 in POMC neurons of the obese human brain will require the identification of differences in the Gdf15-GFRAL axis between the two species.

Calreticulin (Calr), which exhibits a response to short-term ER stress induction, is an example of a protein that deviates from the typical pattern observed in this group. It displayed a significant increase in the secretome of neurons overexpressing SIK2, compared to control cells. There was a significant decrease in the quantity of Calr that occurred when SIK2 was deficient independent of the ER stress. This pattern was also confirmed by immunoblot studies on secretome samples. Calreticulin is a multifunctional ER chaperone implicated in obesity and related metabolic disorders, and its secreted form acts as a molecular chaperone for App [112], [113]. App has been connected to a number of features of obesity and feeding behavior, in addition to its well-known involvement in the pathogenesis of Alzheimer's disease [105]. The decreased amount of Calr that was observed in the secretome of SIK2-deficient POMC neurons during the early stress response of the ER is likely to cause a disruption in the normal folding of App. The present findings are consistent with the reverse protein secretion pattern of App against calreticulin, as revealed by LC-MS analysis and confirmed by immunoblotting. Furthermore, Hsp90, another secreted chaperone, exhibits a similar pattern of secretion like Calreticulin. This observation suggests the potential for SIK2 to regulate the quality control of secreted proteins through the action of secreted chaperones. However, any pharmacological endeavor to enhance the secretion of Calr or Hsp90 by activating SIK2 should be approached cautiously due to the inverse regulation of other members within the same group.

Fractalkine/Cx3cl1, an inflammatory chemokine, plays a crucial role in regulating communication between neurons and microglia. It also has a dual role in neuroinflammation by both suppressing the generation of proinflammatory cytokines by microglia and attracting immune cells as a chemoattractant [114], [115]. Fractalkine

exhibits a common secretion pattern triggered by ER stress and SIK2 or both. This pattern was largely confirmed by immunoblot studies on secretome samples. However, protein expressions other than this pattern were also detected in early ER stress conditions, especially in the SIK2 KD samples. This may be due to the immunoblotting methodology previously mentioned in Gdf15 discussion. Fractalkine secretion pattern suggests that the capacity of SIK2 to reduce fractalkine levels in the POMC secretome at the beginning of the ER stress response relies on its activation through the UPR. However, in the absence of ER stress, SIK2 overexpression can potentially inhibit the normal activity of endogenous SIK2 due to the constant physiological level of ER stress and cause increment of Fractalkine.

Collectively, the data indicates that the natural activity of SIK2 is necessary for maintaining all these neuro proteins that are released. These proteins collectively promote cell survival and homeostasis under normal or physiological settings without any stress. We observed a clear association between the amounts of these released proteins and the levels of SIK2, as shown in **Figure 6.1** (left panel). However, when short-term ER stress is induced, the levels of the members in all three groups become inversely associated with SIK2. This means that their levels increase significantly in the secretome of SIK2 knockdown cells, while they decrease moderately (2-3-fold) in the secretome of SIK2 expressing cells, regardless of gene manipulation (Figure 6.1, right panel). The comparative analyses of the effects of SIK2 and ER stress on POMC secretomes clearly demonstrate that SIK2 is vital in controlling the fundamental levels of various biological processes necessary for maintaining cellular and organismal equilibrium under normal physiological conditions, independent of ER stress. Nevertheless, when short-term ER stress was induced, SIK2 show a significant ability to reduce the amount of secreted proteins in most of these groups, although there were a few instances where it had the opposite effect, as previously described. However, SIK2 seems to relinquish its regulation over these proteins when the ER stress is prolonged for 12 hours. This coincides with an increase in inflammatory and apoptotic proteins, which is consistent with decreased cell survival and significant cell death (Figures 5.12A and B). The observed augmentation in secreted proteins in cells with suppressed SIK2 during brief ER stress induction offers further substantiation of its function as a switch-like regulator in maintaining appropriate levels of protective biological activities in response to variations in cellular stress conditions.

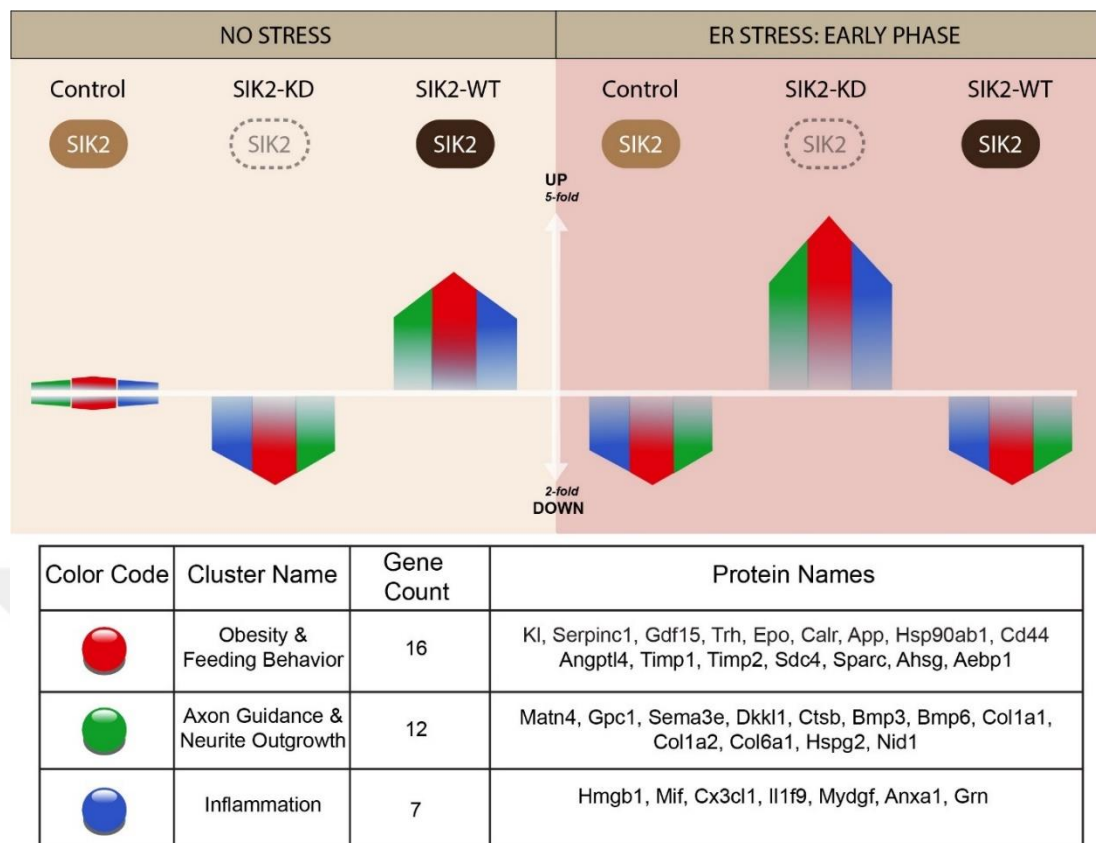


Figure 6.1: The illustration of the distinct and combined impacts of SIK2 and ER stress on specific proteins within three primary groups. The relative fold changes that are associated with the pattern of differential gene expression (DGE) are displayed using colored bars. These bars are used to show the changes. The proportional increase or decrease in the amount of proteins that were released is represented by the bars. In all of the experimental scenarios, the amount of protein that was present was shown to be comparable to that of the control group when there was no stress present. **Table 5.2** provides a full compilation of the fold changes for the genes that are not included in the graph.

In summary, we suggest using a physiological switch-like model for SIK2 due to its capacity to function as an adjustable control. This model can effectively detect changes in ER stress levels in both normal and pathological conditions, and subsequently initiate a corrective cellular response by precisely regulating various biological processes essential for cell survival and homeostasis. If the ER stress continues and is not reduced, the ability of SIK2 to maintain cellular balance and promote survival pathways is overpowered by opposing mechanisms. These mechanisms include the activation of inflammatory and apoptotic pathways, which occur because of UPR sensors and signal transducers IRE1, PERK, and ATF6. This aligns with the findings

of our data-driven physiological switch-like model shown in **Figure 6.1**. This study does not focus on the molecular mechanisms responsible for the changes in secreted protein levels induced by SIK2. However, we suggest that SIK2 may accomplish this function indirectly by regulating ERAD and autophagy processes, or directly by affecting the autophagy-related unconventional secretion pathway. This can be occurred by regulating the activity of downstream coactivators such as CRTC_s, p300/CREB-binding protein, and TFEB at the transcriptional level [65], [116]. However, it is equally or even more likely that SIK2 participates in the coat protein complex II (COPII) vesicle formation and/or cargo selection at the specified ER subdomains rich in lipid rafts [107], [108]. Our laboratory and others reported direct and indirect physical interactions between SIK2 and the outer coat protein of COPII vesicles in immunoprecipitation-based mass spectrometry (IP-MS)-driven protein–protein interaction data (These results have not been published yet). Additionally, ER-resident chaperones such as Calr and Hsp90 are known to contribute to the quality control of secreted proteins by participating in COPII vesicle-mediated transport through C-terminal conformational changes that hide their retention KDEL sequence. Our study has revealed that the secretions of these chaperones undergo significant changes in relation to SIK2. It is suspected that SIK2 in lipid rafts may have a role in directing the formation of secretion vesicles or the choice of cargo proteins for secretion.

The presence of biologically active neuropeptides including α -MSH could not be detected in the results of the LC-MS/MS analysis due to the neuropeptides being below the 3kDa limit, which was a consequence of the secretome collection methodology. Additionally, POMC precursor peptides, which are known to be released into the CSF, were not detected under any of the experimental conditions. Consequently, further studies were carried out to investigate the effects of SIK2 on POMC processing and α -MSH secretion. Previous studies have shown that diet-induced obesity leads to ER stress, defective POMC processing, and reduced α -MSH production [90] [117]. Consistent with these reports, our results demonstrated that in response to ER stress, Golgi and secretory vesicle co-localisations with α -MSH decreased and its precursors accumulated in the ER, accompanied by a decrease in POMC intermediates. Conversely, in the absence of ER stress, SIK2 depletion results in an increase in both the production and co-localization of α -MSH with Golgi and secretory vesicles. This

data aligns with the reported SIK2 knockout phenotype, which is characterized by decreased food intake and energy expenditure [68]. Furthermore, it was observed that this SIK2 effect in cells may be associated with the ER-Golgi vesicular transport. In the absence of SIK2, there was a significant increase in Golgi area and secretory vesicles in a stress-independent manner, consistent with our mechanistic proposal that COPII vesicle formation is regulated by SIK2. Additionally, the research showed that SIK2 moves to a particular subdomain of the ER in order to promote autophagy and protect cells from death under ER stress [96]. This suggests a close physical relationship between two separate but interconnected processes that are believed to be controlled by SIK2 during ER stress situations. Indeed, under prolonged ER stress conditions, the decreasing pattern in the ER-Golgi areas observed in the presence of SIK2 disappeared in the absence of SIK2. This phenomenon may be attributed to the disruption of the SIK2-mediated regulatory balance between autophagy and apoptosis. It is worthy of further studies to reveal the possible mechanisms of action of SIK2 to keep cells alive in the early stages of ER stress and apoptosis in the prolonged stages.

7. CONCLUSION

The proposed work is unique in the sense that it addresses the etiopathology of hypothalamic obesity by probing the quantitative and qualitative changes in the secretome of anorectic POMC neurons and the primary targets of the current therapeutic interferences. These neurons are responsible for controlling the energy balance and expenditure of the body. Both diet-induced obese animal models and humans show malfunction in POMC neurons, characterized by resistance to anorectic hormones such as leptin and insulin, or their loss due to apoptotic death. Hence, it is crucial to maintain healthy communication of POMCs with other components of the feeding circuitry in the hypothalamus and extra hypothalamic centers including secondary neurons located in PVN. We propose a new regulatory role for SIK2 in controlling the secretome of POMC neurons among which a few obesity-related secreted proteins GDF 15, calreticulin and APP need to be further elucidate for their contribution to disease pathogenesis especially in in vivo DIO models.

Finally, we need to get further insight into molecular mechanisms of the regulatory role of SIK2 in the generation and selection of secretory autophagosomes and COPII vesicles formation. Our comparative analysis of secreted proteins showed that POMC neurons secrete numerous valuable therapeutic target proteins. These proteins could influence both neuronal and non-neuronal cellular components of the hypothalamic and extra hypothalamic centers in the related neuronal circuits for feeding and energy homeostasis.

REFERENCES

- [1] C. L. Ogden, M. D. Carroll, B. K. Kit, and K. M. Flegal, "Prevalence of obesity and trends in body mass index among US children and adolescents, 1999-2010," *JAMA*, vol. 307, no. 5, pp. 483–490, Feb. 2012, doi: 10.1001/jama.2012.40.
- [2] "World Health Organization, Obesity and overweight, Fact Sheet." Accessed: Aug. 21, 2024. [Online]. Available: <https://www.who.int/news-room/fact-sheets/detail/obesity-and-overweight>.
- [3] D. Thivel, L. Isacco, C. Montaurier, Y. Boirie, P. Duché, and B. Morio, "The 24-h Energy Intake of Obese Adolescents Is Spontaneously Reduced after Intensive Exercise: A Randomized Controlled Trial in Calorimetric Chambers," *PLoS One*, vol. 7, no. 1, p. e29840, Jan. 2012, doi: 10.1371/journal.pone.0029840.
- [4] D. Thivel, L. Metz, J. Aucouturier, K. Brakoniecki, P. Duche, and B. Morio, "The Effects of Imposed Sedentary Behavior and Exercise on Energy Intake in Adolescents With Obesity," *Journal of Developmental & Behavioral Pediatrics*, vol. 34, no. 8, pp. 616–622, Oct. 2013, doi: 10.1097/DBP.0000000000000010.
- [5] J. Tamaroff, D. Williamson, J. C. Slaughter, M. Xu, G. Srivastava, and A. H. Shoemaker, "Prevalence of genetic causes of obesity in clinical practice," *Obes Sci Pract*, vol. 9, no. 5, pp. 508–515, Oct. 2023, doi: 10.1002/osp4.671.
- [6] J. O. Hill and J. C. Peters, "Environmental contributions to the obesity epidemic.," *Science*, vol. 280, no. 5368, pp. 1371–4, May 1998, doi: 10.1126/science.280.5368.1371.
- [7] N. L. Segal and D. B. Allison, "Twins and virtual twins: bases of relative body weight revisited.," *Int J Obes Relat Metab Disord*, vol. 26, no. 4, pp. 437–41, Apr. 2002, doi: 10.1038/sj.ijo.0801941.
- [8] J. Jo *et al.*, "Hypertrophy and/or Hyperplasia: Dynamics of Adipose Tissue Growth.," *PLoS Comput Biol*, vol. 5, no. 3, p. e1000324, Mar. 2009, doi: 10.1371/journal.pcbi.1000324.
- [9] L. Boutens and R. Stienstra, "Adipose tissue macrophages: going off track during obesity," *Diabetologia*, vol. 59, no. 5, pp. 879–894, May 2016, doi: 10.1007/s00125-016-3904-9.
- [10] F. A. Alnaser, "Risk Factors for Dementia," *Recent Advances in Clinical Trials*, vol. 1, no. 2, pp. 2–4, 2022, [Online]. Available: <https://www.alzheimers.org.uk/sites/default/files/pdf/>
- [11] J. Hirosumi *et al.*, "A central role for JNK in obesity and insulin resistance," *Nature* 2002 420:6913, vol. 420, no. 6913, pp. 333–336, 2002, doi: 10.1038/nature01137.

- [12] D. W. Haslam and W. P. T. James, "Obesity.," *Lancet*, vol. 366, no. 9492, pp. 1197–209, Oct. 2005, doi: 10.1016/S0140-6736(05)67483-1.
- [13] A. Bereket *et al.*, "Hypothalamic obesity in children," *Obesity Reviews*, vol. 13, no. 9, pp. 780–798, Sep. 2012, doi: 10.1111/j.1467-789X.2012.01004.x.
- [14] J. H. Kim and J.-H. Choi, "Pathophysiology and clinical characteristics of hypothalamic obesity in children and adolescents," *Ann Pediatr Endocrinol Metab*, vol. 18, no. 4, p. 161, Dec. 2013, doi: 10.6065/apem.2013.18.4.161.
- [15] S. Carmo-Silva and C. Cavadas, "Hypothalamic dysfunction in obesity and metabolic disorders," in *Advances in Neurobiology*, vol. 19, Springer New York LLC, 2017, pp. 73–116. doi: 10.1007/978-3-319-63260-5_4.
- [16] K.-H. E. Chen, N. M. Lainez, M. G. Nair, and D. Coss, "Visceral adipose tissue imparts peripheral macrophage influx into the hypothalamus," *J Neuroinflammation*, vol. 18, no. 1, p. 140, Dec. 2021, doi: 10.1186/s12974-021-02183-2.
- [17] N. F. Mendes and L. A. Velloso, "Perivascular macrophages in high-fat diet-induced hypothalamic inflammation," *J Neuroinflammation*, vol. 19, no. 1, p. 136, Dec. 2022, doi: 10.1186/s12974-022-02519-6.
- [18] M. Milanski *et al.*, "Saturated fatty acids produce an inflammatory response predominantly through the activation of TLR4 signaling in hypothalamus: Implications for the pathogenesis of obesity," *Journal of Neuroscience*, vol. 29, no. 2, pp. 359–370, 2009, doi: 10.1523/JNEUROSCI.2760-08.2009.
- [19] L. Della Guardia and R. Codella, "Exercise Restores Hypothalamic Health in Obesity by Reshaping the Inflammatory Network.," *Antioxidants (Basel)*, vol. 12, no. 2, p. 297, Jan. 2023, doi: 10.3390/antiox12020297.
- [20] E. Samodien *et al.*, "Diet-induced hypothalamic dysfunction and metabolic disease, and the therapeutic potential of polyphenols.," *Mol Metab*, vol. 27, pp. 1–10, Sep. 2019, doi: 10.1016/j.molmet.2019.06.022.
- [21] H. Jin *et al.*, "Effects of KGM and Degradation Products on Appetite Regulation and Energy Expenditure in High-Fat-Diet Mice via the Adipocyte-Hypothalamus Axis," *J Agric Food Chem*, vol. 72, no. 28, pp. 15765–15777, Jul. 2024, doi: 10.1021/acs.jafc.4c03819.
- [22] G. Srivastava and C. Apovian, "Future Pharmacotherapy for Obesity: New Anti-obesity Drugs on the Horizon," *Curr Obes Rep*, vol. 7, no. 2, pp. 147–161, Jun. 2018, doi: 10.1007/s13679-018-0300-4.
- [23] A. E. Locke *et al.*, "Genetic studies of body mass index yield new insights for obesity biology," *Nature*, vol. 518, no. 7538, pp. 197–206, Feb. 2015, doi: 10.1038/nature14177.

- [24] A. Jais and J. C. Brüning, “Arcuate Nucleus-Dependent Regulation of Metabolism-Pathways to Obesity and Diabetes Mellitus.,” *Endocr Rev*, vol. 43, no. 2, pp. 314–328, Mar. 2022, doi: 10.1210/endrev/bnab025.
- [25] G. J. Morton, D. E. Cummings, D. G. Baskin, G. S. Barsh, and M. W. Schwartz, “Central nervous system control of food intake and body weight.,” *Nature*, vol. 443, no. 7109, pp. 289–95, Sep. 2006, doi: 10.1038/nature05026.
- [26] M. J. Krashes *et al.*, “An excitatory paraventricular nucleus to AgRP neuron circuit that drives hunger,” *Nature* 2014 507:7491, vol. 507, no. 7491, pp. 238–242, Feb. 2014, doi: 10.1038/nature12956.
- [27] D. Wang *et al.*, “Whole-brain mapping of the direct inputs and axonal projections of POMC and AgRP neurons,” *Front Neuroanat*, vol. 9, p. 135563, Mar. 2015, doi: 10.3389/fnana.2015.00040.
- [28] E. Kwon and Y.-H. Jo, “Activation of the ARCPOMC→MeA Projection Reduces Food Intake.,” *Front Neural Circuits*, vol. 14, p. 595783, 2020, doi: 10.3389/fncir.2020.595783.
- [29] S. Gowda, T. Seibert, N. Uli, and R. Farrell, “Pediatric Obesity: Endocrinologic and Genetic Etiologies and Management,” *Curr Cardiovasc Risk Rep*, vol. 13, no. 12, p. 39, Dec. 2019, doi: 10.1007/s12170-019-0632-y.
- [30] C. Quarta, X. Fioramonti, and D. Cota, “POMC Neurons Dysfunction in Diet-induced Metabolic Disease: Hallmark or Mechanism of Disease?,” *Neuroscience*, vol. 447, pp. 3–14, Nov. 2020, doi: 10.1016/j.neuroscience.2019.09.031.
- [31] M. A. Cowley *et al.*, “Leptin activates anorexigenic POMC neurons through a neural network in the arcuate nucleus,” *Nature*, vol. 411, no. 6836, pp. 480–484, May 2001, doi: 10.1038/35078085.
- [32] B. Lizarbe *et al.*, “Hypothalamic metabolic compartmentation during appetite regulation as revealed by magnetic resonance imaging and spectroscopy methods,” *Front Neuroenergetics*, vol. 5, p. 49872, Jun. 2013, doi: 10.3389/FNENE.2013.00006.
- [33] R. G. P. Denis *et al.*, “Palatability Can Drive Feeding Independent of AgRP Neurons.,” *Cell Metab*, vol. 22, no. 4, pp. 646–57, Oct. 2015, doi: 10.1016/j.cmet.2015.07.011.
- [34] L. R. Beutler *et al.*, “Obesity causes selective and long-lasting desensitization of AgRP neurons to dietary fat,” *Elife*, vol. 9, Jul. 2020, doi: 10.7554/eLife.55909.
- [35] A. Jais *et al.*, “PNOCARC Neurons Promote Hyperphagia and Obesity upon High-Fat-Diet Feeding,” *Neuron*, vol. 106, no. 6, pp. 1009–1025.e10, Jun. 2020, doi: 10.1016/j.neuron.2020.03.022.
- [36] B. C. Jarvie and S. T. Hentges, “Expression of GABAergic and glutamatergic phenotypic markers in hypothalamic proopiomelanocortin neurons,” *Journal of*

Comparative Neurology, vol. 520, no. 17, pp. 3863–3876, Dec. 2012, doi: 10.1002/cne.23127.

[37] B. Y. H. Lam *et al.*, “Heterogeneity of hypothalamic pro-opiomelanocortin-expressing neurons revealed by single-cell RNA sequencing,” *Mol Metab*, vol. 6, no. 5, pp. 383–392, May 2017, doi: 10.1016/j.molmet.2017.02.007.

[38] M. Koch *et al.*, “Hypothalamic POMC neurons promote cannabinoid-induced feeding,” *Nature*, vol. 519, no. 7541, pp. 45–50, Mar. 2015, doi: 10.1038/nature14260.

[39] C. Quarta *et al.*, “POMC neuronal heterogeneity in energy balance and beyond: an integrated view,” *Nat Metab*, vol. 3, no. 3, pp. 299–308, Feb. 2021, doi: 10.1038/s42255-021-00345-3.

[40] B. A. Eipper and R. E. Mains, “Structure and Biosynthesis of Pro-Adrenocorticotropin/ Endorphin and Related Peptides,” 1980.

[41] R. M. Does, S. Lecaude, D. Bauer, and P. B. Danielson, “Analyzing the evolution of the opioid/orphanin gene family,” *Mass Spectrom Rev*, vol. 21, no. 4, pp. 220–43, 2002, doi: 10.1002/mas.10029.

[42] O. , Duvaux-Miret, C. , Dissous, J. P. , Gautron, E. , Pattou, C. , Kordon, and A. Capron, “The helminth *Schistosoma mansoni* expresses a peptide similar to human beta-endorphin and possesses a proopiomelanocortin-related gene,” *New Biol*, vol. 2, no. 1, pp. 93–99, 1990.

[43] M. Salzet *et al.*, “Leech immunocytes contain proopiomelanocortin: nitric oxide mediates hemolymph proopiomelanocortin processing,” *The Journal of Immunology*, vol. 159, no. 11, pp. 5400–5411, Dec. 1997, doi: 10.4049/jimmunol.159.11.5400.

[44] I. Rønnestad, A. S. Gomes, K. Murashita, R. Angotzi, E. Jönsson, and H. Volkoff, “Appetite-controlling endocrine systems in teleosts,” Apr. 18, 2017, *Frontiers Media S.A.* doi: 10.3389/fendo.2017.00073.

[45] V. Gonzalez-Nunez, R. Gonzalez-Sarmiento, and R. E. Rodríguez, “Identification of two proopiomelanocortin genes in zebrafish (*Danio rerio*).,” *Brain Res Mol Brain Res*, vol. 120, no. 1, pp. 1–8, Dec. 2003, doi: 10.1016/j.molbrainres.2003.09.012.

[46] R. J. Arends, H. Vermeer, G. J. M. Martens, J. A. M. Leunissen, S. E. Wendelaar Bonga, and G. Flik, “Cloning and expression of two proopiomelanocortin mRNAs in the common carp (*Cyprinus carpio* L.),” 1998.

[47] A. Takahashi *et al.*, “Nucleotide sequence and expression of three subtypes of proopiomelanocortin mRNA in barfin flounder,” *Gen Comp Endocrinol*, vol. 141, no. 3, pp. 291–303, May 2005, doi: 10.1016/j.ygcen.2005.01.010.

[48] J. Schjolden, H. B. Schiöth, D. Larhammar, S. Winberg, and E. T. Larson, “Melanocortin peptides affect the motivation to feed in rainbow trout (*Oncorhynchus mykiss*).,” *Gen Comp Endocrinol*, vol. 160, no. 2, pp. 134–8, Jan. 2009, doi: 10.1016/j.ygcen.2008.11.003.

- [49] I. Lindberg and L. D. Fricker, "Obesity, POMC, and POMC-processing Enzymes: Surprising Results from Animal Models," Dec. 01, 2021, *Endocrine Society*. doi: 10.1210/endocr/bqab155.
- [50] E. Harno, T. G. Ramamoorthy, A. P. Coll, and A. White, "Pomc: The Physiological Power of Hormone Processing," *Physiol Rev*, vol. 98, pp. 2381–2430, 2018, doi: 10.1152/physrev.00024.2017.-Pro-opiomelanocortin.
- [51] L. E. Pritchard *et al.*, "Proopiomelanocortin-derived peptides in rat cerebrospinal fluid and hypothalamic extracts: Evidence that secretion is regulated with respect to energy balance," *Endocrinology*, vol. 144, no. 3, pp. 760–766, Mar. 2003, doi: 10.1210/en.2002-220866.
- [52] G. Page-Wilson *et al.*, "Proopiomelanocortin, agouti-related protein, and leptin in human cerebrospinal fluid: correlations with body weight and adiposity," *American Journal of Physiology*, vol. 309, no. 5, pp. 458–465, 2015, doi: doi.org/10.1152/ajpendo.00206.2015.
- [53] M. Brehme, A. Sverchkova, and C. Voisine, "Proteostasis network deregulation signatures as biomarkers for pharmacological disease intervention," Jun. 01, 2019, *Elsevier Ltd*. doi: 10.1016/j.coisb.2019.03.008.
- [54] B. WE, M. RI, D. A, and K. JW, "Adapting proteostasis for disease intervention," *Science*, vol. 319, no. 5865, 2008, doi: 10.1126/SCIENCE.1141448.
- [55] C. Hetz, E. Chevet, and S. A. Oakes, "Proteostasis control by the unfolded protein response," *Nature Cell Biology* 2015 17:7, vol. 17, no. 7, pp. 829–838, Jun. 2015, doi: 10.1038/ncb3184.
- [56] W. P and R. D, "The unfolded protein response: from stress pathway to homeostatic regulation," *Science*, vol. 334, no. 6059, 2011, doi: 10.1126/SCIENCE.1209038.
- [57] M. Wang and R. J. Kaufman, "Protein misfolding in the endoplasmic reticulum as a conduit to human disease," *Nature* 2016 529:7586, vol. 529, no. 7586, pp. 326–335, Jan. 2016, doi: 10.1038/nature17041.
- [58] C. Hetz and F. R. Papa, "The Unfolded Protein Response and Cell Fate Control.," *Mol Cell*, vol. 69, no. 2, pp. 169–181, Jan. 2018, doi: 10.1016/j.molcel.2017.06.017.
- [59] A. Jagannath *et al.*, "The Multiple Roles of Salt-Inducible Kinases in Regulating Physiology," Jul. 01, 2023, *American Physiological Society*. doi: 10.1152/physrev.00023.2022.
- [60] F. Shi, "Understanding the roles of salt-inducible kinases in cardiometabolic disease," *Front Physiol*, vol. 15, p. 1426244, Jul. 2024, doi: 10.3389/fphys.2024.1426244.

- [61] L. Fagerberg *et al.*, “Analysis of the human tissue-specific expression by genome-wide integration of transcriptomics and antibody-based proteomics.,” *Mol Cell Proteomics*, vol. 13, no. 2, pp. 397–406, Feb. 2014, doi: 10.1074/mcp.M113.035600.
- [62] F. Yue *et al.*, “A comparative encyclopedia of DNA elements in the mouse genome,” *Nature*, vol. 515, no. 7527, pp. 355–364, Nov. 2014, doi: 10.1038/nature13992.
- [63] Y. K. Hashimoto, T. Satoh, M. Okamoto, and H. Takemori, “Importance of autophosphorylation at Ser186 in the α -loop of salt inducible kinase 1 for its sustained kinase activity,” *J Cell Biochem*, vol. 104, no. 5, pp. 1724–1739, Aug. 2008, doi: 10.1002/jcb.21737.
- [64] E. Henriksson *et al.*, “The AMPK-related kinase SIK2 is regulated by cAMP via phosphorylation at Ser 358 in adipocytes,” *Biochemical Journal*, vol. 444, no. 3, pp. 503–514, Jun. 2012, doi: 10.1042/BJ20111932.
- [65] F.-C. Yang *et al.*, “Reversible acetylation regulates salt-inducible kinase (SIK2) and its function in autophagy,” *J Biol Chem*, vol. 288, no. 9, pp. 6227–37, Mar. 2013, doi: 10.1074/jbc.M112.431239.
- [66] N. Horike *et al.*, “Adipose-specific expression, phosphorylation of Ser794 in insulin receptor substrate-1, and activation in diabetic animals of salt-inducible kinase-2,” *Journal of Biological Chemistry*, vol. 278, no. 20, pp. 18440–18447, May 2003, doi: 10.1074/jbc.M211770200.
- [67] H. E *et al.*, “SIK2 regulates CRTCs, HDAC4 and glucose uptake in adipocytes,” *J Cell Sci*, vol. 128, no. 3, 2015, doi: 10.1242/JCS.153932.
- [68] J. Park *et al.*, “SIK2 Is Critical in the Regulation of Lipid Homeostasis and Adipogenesis In Vivo,” *Diabetes*, vol. 63, no. 11, pp. 3659–3673, Nov. 2014, doi: 10.2337/db13-1423.
- [69] J. Bricambert, J. Miranda, F. Benhamed, J. Girard, C. Postic, and R. Dentin, “Salt-inducible kinase 2 links transcriptional coactivator p300 phosphorylation to the prevention of ChREBP-dependent hepatic steatosis in mice,” *Journal of Clinical Investigation*, vol. 120, no. 12, pp. 4316–4331, 2010, doi: 10.1172/JCI41624.
- [70] J. Du, Q. Chen, H. Takemori, and H. Xu, “SIK2 can be activated by deprivation of nutrition and it inhibits expression of lipogenic genes in adipocytes,” *Obesity (Silver Spring)*, vol. 16, no. 3, pp. 531–8, Mar. 2008, doi: 10.1038/oby.2007.98.
- [71] R. Dentin *et al.*, “Insulin modulates gluconeogenesis by inhibition of the coactivator TORC2,” *Nature*, vol. 449, no. 7160, pp. 366–369, 2007, doi: 10.1038/nature06128.
- [72] K. Patel *et al.*, “The LKB1-salt-inducible kinase pathway functions as a key gluconeogenic suppressor in the liver,” *Nat Commun*, vol. 5, no. 1, p. 4535, Aug. 2014, doi: 10.1038/ncomms5535.

- [73] J. I. Sakamaki *et al.*, “Role of the SIK2–p35–PJA2 complex in pancreatic β -cell functional compensation,” *Nature Cell Biology* 2014 16:3, vol. 16, no. 3, pp. 234–244, Feb. 2014, doi: 10.1038/ncb2919.
- [74] C. Iorio *et al.*, “Silencing the G-protein coupled receptor 3-salt inducible kinase 2 pathway promotes human β cell proliferation,” *Commun Biol*, vol. 4, no. 1, p. 907, Jul. 2021, doi: 10.1038/s42003-021-02433-2.
- [75] N. J. Darling, R. Toth, J. S. C. Arthur, and K. Clark, “Inhibition of SIK2 and SIK3 during differentiation enhances the anti-inflammatory phenotype of macrophages.,” *Biochem J*, vol. 474, no. 4, pp. 521–537, Feb. 2017, doi: 10.1042/BCJ20160646.
- [76] N. J. Darling, J. S. C. Arthur, and P. Cohen, “Salt-inducible kinases are required for the IL-33-dependent secretion of cytokines and chemokines in mast cells.,” *J Biol Chem*, vol. 296, p. 100428, 2021, doi: 10.1016/j.jbc.2021.100428.
- [77] T. Sasaki *et al.*, “SIK2 is a key regulator for neuronal survival after ischemia via TORC1-CREB.,” *Neuron*, vol. 69, no. 1, pp. 106–19, Jan. 2011, doi: 10.1016/j.neuron.2010.12.004.
- [78] Y. Liu, V. Poon, G. Sanchez-Watts, A. G. Watts, H. Takemori, and G. Aguilera, “Salt-Inducible Kinase Is Involved in the Regulation of Corticotropin-Releasing Hormone Transcription in Hypothalamic Neurons in Rats,” *Endocrinology*, vol. 153, no. 1, pp. 223–233, Jan. 2012, doi: 10.1210/en.2011-1404.
- [79] B. Jiang *et al.*, “Hippocampal Salt-Inducible Kinase 2 Plays a Role in Depression via the CREB-Regulated Transcription Coactivator 1-cAMP Response Element Binding-Brain-Derived Neurotrophic Factor Pathway.,” *Biol Psychiatry*, vol. 85, no. 8, pp. 650–666, Apr. 2019, doi: 10.1016/j.biopsych.2018.10.004.
- [80] A. Jagannath *et al.*, “THE MULTIPLE ROLES OF SALT-INDUCIBLE KINASES IN REGULATING PHYSIOLOGY,” Jul. 01, 2023, *American Physiological Society*. doi: 10.1152/physrev.00023.2022.
- [81] N. Horike *et al.*, “Downregulation of SIK2 expression promotes the melanogenic program in mice,” *Pigment Cell Melanoma Res*, vol. 23, no. 6, pp. 809–819, Dec. 2010, doi: 10.1111/j.1755-148X.2010.00760.x.
- [82] F. C. Yang *et al.*, “Interaction between salt-inducible kinase 2 (SIK2) and p97/valosin-containing protein (VCP) regulates endoplasmic reticulum (ER)-associated protein degradation in mammalian cells,” *J Biol Chem*, vol. 288, no. 47, pp. 33861–33872, Nov. 2013, doi: 10.1074/JBC.M113.492199.
- [83] L. Yaswen, N. Diehl, M. B. Brennan, and U. Hochgeschwender, “Obesity in the mouse model of pro-opiomelanocortin deficiency responds to peripheral melanocortin,” *Nat Med*, vol. 5, no. 9, pp. 1066–1070, Sep. 1999, doi: 10.1038/12506.
- [84] G. F. P. J. Souza *et al.*, “Defective regulation of POMC precedes hypothalamic inflammation in diet-induced obesity,” *Sci Rep*, vol. 6, no. 1, p. 29290, Jul. 2016, doi: 10.1038/srep29290.

- [85] A. V. Vergoni, R. Poggioli, D. Marrama, and A. Bertolini, “Inhibition of feeding by ACTH-(1-24): behavioral and pharmacological aspects,” 1990.
- [86] A. Ajoolabady *et al.*, “ER stress in obesity pathogenesis and management.,” *Trends Pharmacol Sci*, vol. 43, no. 2, pp. 97–109, Feb. 2022, doi: 10.1016/j.tips.2021.11.011.
- [87] X. Zhang, G. Zhang, H. Zhang, M. Karin, H. Bai, and D. Cai, “Hypothalamic IKK β /NF- κ B and ER Stress Link Overnutrition to Energy Imbalance and Obesity,” *Cell*, vol. 135, no. 1, pp. 61–73, Oct. 2008, doi: 10.1016/j.cell.2008.07.043.
- [88] L. Ozcan *et al.*, “Endoplasmic Reticulum Stress Plays a Central Role in Development of Leptin Resistance,” *Cell Metab*, vol. 9, no. 1, pp. 35–51, 2009, doi: 10.1016/j.cmet.2008.12.004.
- [89] Q. Meng and D. Cai, “Defective Hypothalamic Autophagy Directs the Central Pathogenesis of Obesity via the I κ B Kinase β (IKK β)/NF- κ B Pathway *,” *Journal of Biological Chemistry*, vol. 286, no. 37, pp. 32324–32332, Sep. 2011, doi: 10.1074/JBC.M111.254417.
- [90] M. Schneeberger *et al.*, “Mitofusin 2 in POMC neurons connects ER stress with leptin resistance and energy imbalance.,” *Cell*, vol. 155, no. 1, pp. 172–87, Sep. 2013, doi: 10.1016/j.cell.2013.09.003.
- [91] “WI siRNA Selection Program.” Accessed: Dec. 24, 2024. [Online]. Available: <http://sirna.wi.mit.edu/>
- [92] Anton Posch, *Proteomic Profiling*, vol. 2261. in *Methods in Molecular Biology*, vol. 2261. New York, NY: Springer US, 2021. doi: 10.1007/978-1-0716-1186-9.
- [93] J. Griss *et al.*, “Spectral Clustering Improves Label-Free Quantification of Low-Abundant Proteins,” *J Proteome Res*, vol. 18, no. 4, pp. 1477–1485, Apr. 2019, doi: 10.1021/acs.jproteome.8b00377.
- [94] V. Dorfer *et al.*, “MS Amanda, a universal identification algorithm optimized for high accuracy tandem mass spectra,” *J Proteome Res*, vol. 13, no. 8, pp. 3679–3684, Aug. 2014, doi: 10.1021/pr500202e.
- [95] S. X. Ge, D. Jung, and R. Yao, “ShinyGO: a graphical gene-set enrichment tool for animals and plants,” *Bioinformatics*, vol. 36, no. 8, pp. 2628–2629, Apr. 2020, doi: 10.1093/BIOINFORMATICS/BTZ931.
- [96] G. Bindea *et al.*, “ClueGO: a Cytoscape plug-in to decipher functionally grouped gene ontology and pathway annotation networks.,” *Bioinformatics*, vol. 25, no. 8, pp. 1091–3, Apr. 2009, doi: 10.1093/bioinformatics/btp101.
- [97] D. Szklarczyk *et al.*, “The STRING database in 2023: protein–protein association networks and functional enrichment analyses for any sequenced genome of interest,”

Nucleic Acids Res, vol. 51, no. D1, pp. D638–D646, Jan. 2023, doi: 10.1093/nar/gkac1000.

[98] M. H. Jäntti *et al.*, “Palmitate and thapsigargin have contrasting effects on ER membrane lipid composition and ER proteostasis in neuronal cells,” *Biochim Biophys Acta Mol Cell Biol Lipids*, vol. 1867, no. 11, Nov. 2022, doi: 10.1016/j.bbalip.2022.159219.

[99] A. Nazarians-Armavil, J. A. Chalmers, C. B. Lee, W. Ye, and D. D. Belsham, “Cellular insulin resistance disrupts hypothalamic mHypoA-POMC/GFP neuronal signaling pathways,” *Journal of Endocrinology*, vol. 220, no. 1, pp. 13–24, Jan. 2014, doi: 10.1530/JOE-13-0334.

[100] E. K. Tse and D. D. Belsham, “Palmitate induces neuroinflammation, ER stress, and Pomc mRNA expression in hypothalamic mHypoA-POMC/GFP neurons through novel mechanisms that are prevented by oleate,” *Mol Cell Endocrinol*, vol. 472, pp. 40–49, Sep. 2018, doi: 10.1016/J.MCE.2017.11.017.

[101] M. Song *et al.*, “P-STAT3 Inhibition Activates Endoplasmic Reticulum Stress-Induced Splenocyte Apoptosis in Chronic Stress,” *Front Physiol*, vol. 11, p. 525130, Jun. 2020, doi: 10.3389/fphys.2020.00680.

[102] J. Goodman, “Characterizing the secretome of adipose tissue in metabolic stress,” 2024, doi: 10.7916/5XGQ-2917.

[103] R. B. Hamanaka, E. Bobrovnikova-Marjon, X. Ji, S. A. Liebhaver, and J. A. Diehl, “PERK-dependent regulation of IAP translation during ER stress,” *Oncogene*, vol. 28, no. 6, pp. 910–920, Feb. 2009, doi: 10.1038/onc.2008.428.

[104] K. Hyoda, T. Hosoi, N. Horie, Y. Okuma, K. Ozawa, and Y. Nomura, “PI3K-Akt inactivation induced CHOP expression in endoplasmic reticulum-stressed cells,” *Biochem Biophys Res Commun*, vol. 340, no. 1, pp. 286–90, Feb. 2006, doi: 10.1016/j.bbrc.2005.12.007.

[105] J. Kang *et al.*, “The precursor of Alzheimer’s disease amyloid A4 protein resembles a cell-surface receptor,” *Nature*, vol. 325, no. 6106, pp. 733–736, Feb. 1987, doi: 10.1038/325733a0.

[106] G. Sommer *et al.*, “Amyloid precursor protein expression is induced by tumor necrosis factor α in 3T3-L1 adipocytes,” *J Cell Biochem*, vol. 108, no. 6, pp. 1418–1422, Dec. 2009, doi: 10.1002/jcb.22382.

[107] C. Zhang *et al.*, “Activation of salt Inducible Kinases, IRE1 and PERK leads to Sec bodies formation in Drosophila S2 cells,” 2021.

[108] H.-H. Wang *et al.*, “Activation of salt-inducible kinase 2 promotes the viability of peritoneal mesothelial cells exposed to stress of peritoneal dialysis,” *Cell Death Dis*, vol. 7, no. 7, pp. e2298–e2298, Jul. 2016, doi: 10.1038/cddis.2016.79.

- [109] L. Yang *et al.*, “GFRAL is the receptor for GDF15 and is required for the anti-obesity effects of the ligand,” *Nat Med*, vol. 23, no. 10, pp. 1158–1166, Oct. 2017, doi: 10.1038/nm.4394.
- [110] I. Cimino and A. P. Coll, “The role of GDF15 in food intake and appetitive behaviour,” Feb. 01, 2022, *Elsevier Ltd*. doi: 10.1016/j.coemr.2021.100299.
- [111] C. Hale and M. M. Véniant, “Growth differentiation factor 15 as a potential therapeutic for treating obesity,” Apr. 01, 2021, *Elsevier GmbH*. doi: 10.1016/j.molmet.2020.101117.
- [112] E. Jeffery, L. R. Peters, and M. Raghavan, “The polypeptide binding conformation of calreticulin facilitates its cell-surface expression under conditions of endoplasmic reticulum stress,” *J Biol Chem*, vol. 286, no. 4, pp. 2402–15, Jan. 2011, doi: 10.1074/jbc.M110.180877.
- [113] R. J. Johnson, G. Xiao, J. Shanmugaratnam, and R. E. Fine, “Calreticulin functions as a molecular chaperone for the-amyloid precursor protein,” 2001. [Online]. Available: www.elsevier.com/locate/neuaging
- [114] K. Biber, H. Neumann, K. Inoue, and H. W. G. M. Boddeke, “Neuronal ‘On’ and ‘Off’ signals control microglia,” Nov. 2007. doi: 10.1016/j.tins.2007.08.007.
- [115] J. F. Bazan *et al.*, “A new class of membrane-bound chemokine with a CX3C motif,” *Nature*, vol. 385, no. 6617, pp. 640–4, Feb. 1997, doi: 10.1038/385640a0.
- [116] F. Negoita, J. Säll, B. Morén, K. Stenkula, and O. Göransson, “Salt-inducible kinase 2 regulates TFEB and is required for autophagic flux in adipocytes,” *Biochem Biophys Res Commun*, vol. 508, no. 3, pp. 775–779, Jan. 2019, doi: 10.1016/j.bbrc.2018.11.177.
- [117] C. Cavadas, C. A. Aveleira, G. F. P. Souza, and L. A. Velloso, “The pathophysiology of defective proteostasis in the hypothalamus - From obesity to ageing,” Dec. 01, 2016, *Nature Publishing Group*. doi: 10.1038/nrendo.2016.107.

BIOGRAPHY

Mehmet Soner TÜRKÜNER is a research assistant at the Department of Molecular Biology and Genetics at Gebze Technical University (GTU). He completed his Bachelor's degree in Molecular Biology and Genetics at Gebze Technical University, Department of Molecular Biology and Genetics, as a high honor student (valedictorian) in 2015. During this period, he carried out his 2209A-Tubitak Project on the investigation of the toxic effects of monosodium glutamate in liver cells and its possible relationship with insulin resistance in vitro cell culture. He received his Master's degree from the same department in 2017 with thesis regarding effect of monosodium glutamate on adipogenic potential of 3T3-L1. He published his article based on this thesis (DOI: 10.1002/cbin.11269). In addition, he participated as a researcher in the Gebze Technical University - Scientific Research Project (BAP-2017) on the possible effects of chemically induced endoplasmic reticulum stress on human adipocyte secretome profile. As an output of this project, he made an abstract/poster presentation on the possible SIK2-based effects of endoplasmic reticulum stress on human adipocyte secretome profile at GTU Graduate Research Symposium. He is currently a Ph.D. student in the same department. He continues her studies effect of SIK2 and proteostasis on hypothalamic neurons and adipogenesis in the context of obesity etiology as a research fellow in Tubitak 1001 projects (118Z154 (completed) and 122Z021(Continue)). He is also working as the High Resolution Mass Spectrophotometry instrument manager in Gebze Technical University - Central Research Application Laboratory (GTU-MAR) Cellular Proteomics Laboratory. He has extensive experience in LC-MS/MS based proteomic approaches (Secretome, interactome and PTM screen etc.), cell culture, confocal based cell Imaging, molecular cloning, lentiviral-based gene manipulation systems (CRISPR/Cas9 and RNAi), recombinant protein production and purification, protein-protein interactions, adipogenesis and bioinformatic approaches (pathway-network analysis, protein modelling, omics analysis etc.).

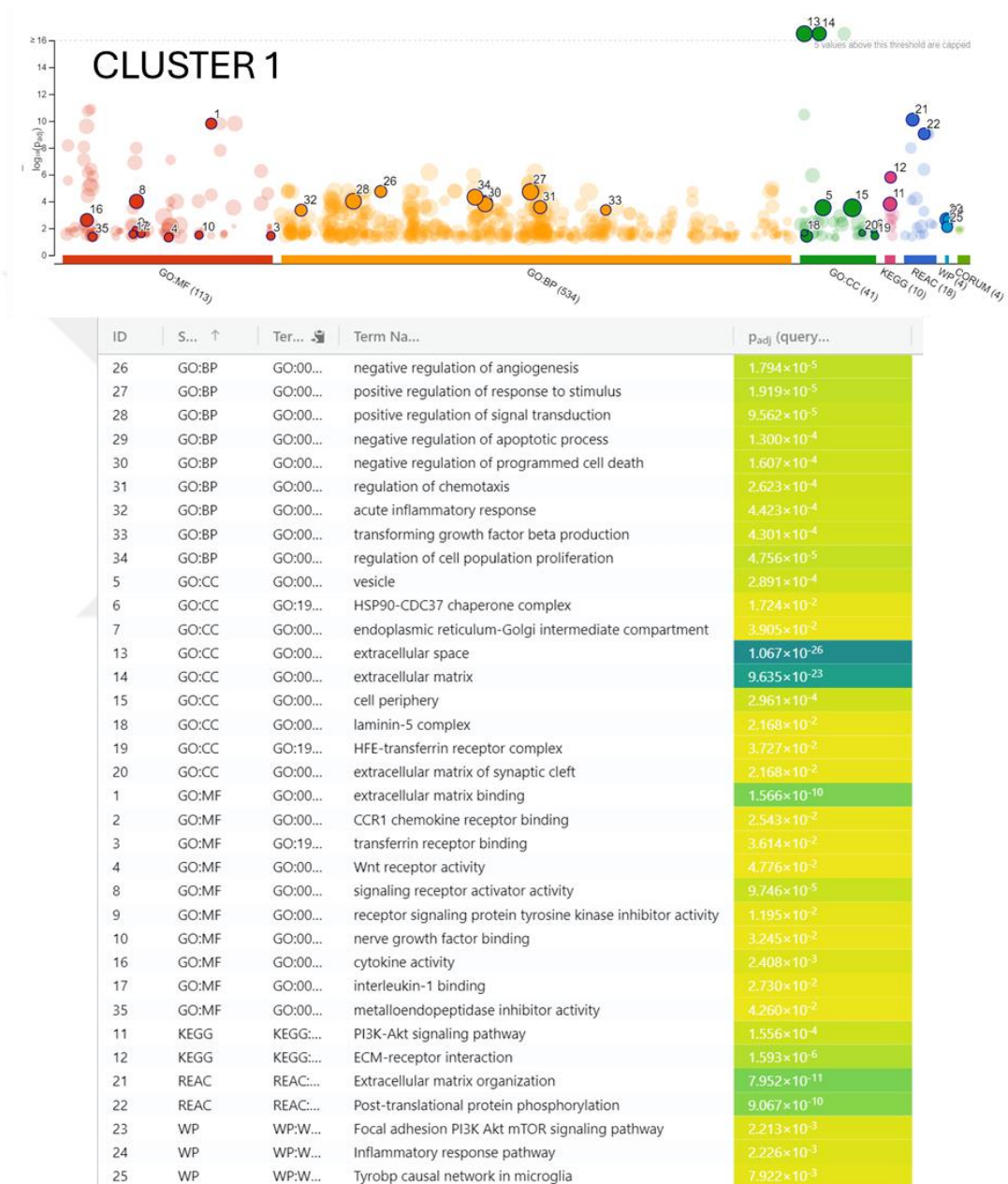
PUBLICATIONS AND PRESENTATIONS FROM THE THESIS

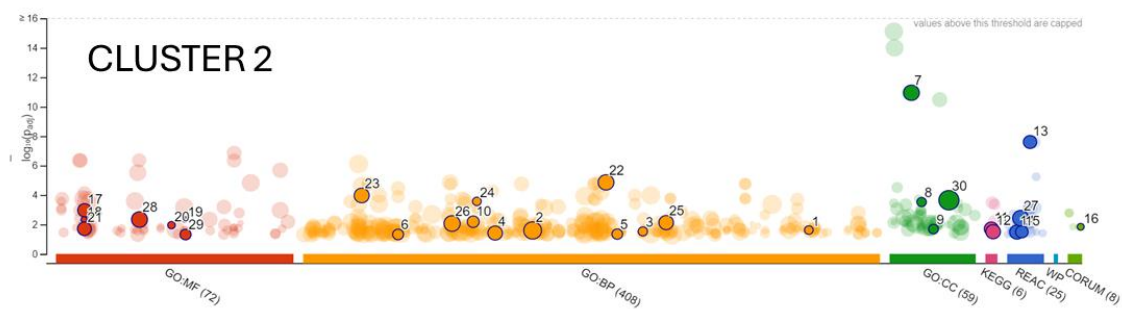
Türküner, M.S.; Yazıcı, A.; Özcan, F. “SIK2 Controls the Homeostatic Character of the POMC Secretome Acutely in Response to Pharmacological ER Stress Induction.” Cells 2024, 13, 1565. <https://doi.org/10.3390/cells13181565>



APPENDICES

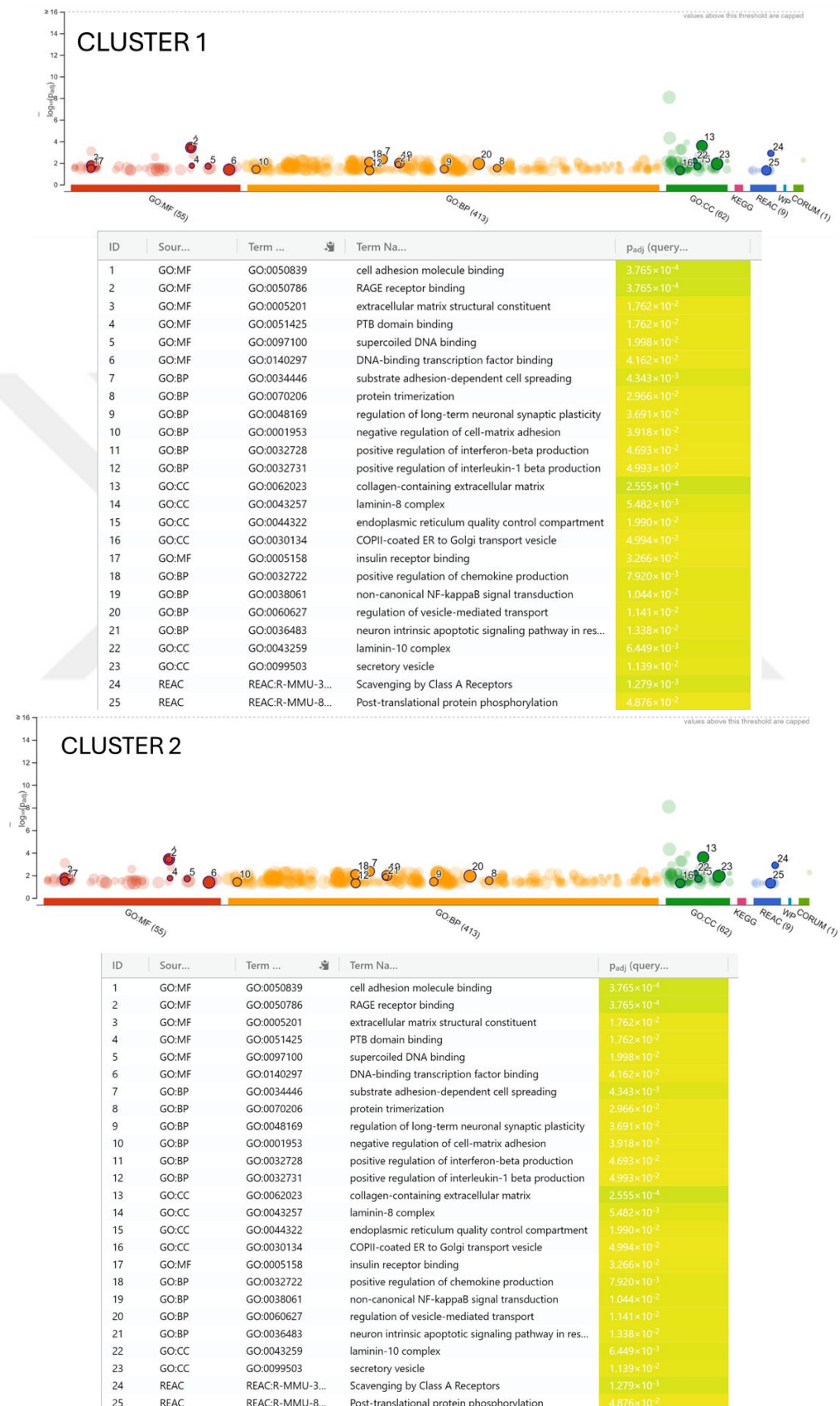
APPENDIX-A: The functional annotation analyses of the proteins belonging to the two clusters in the heat map shown in **Figure 5.8**.

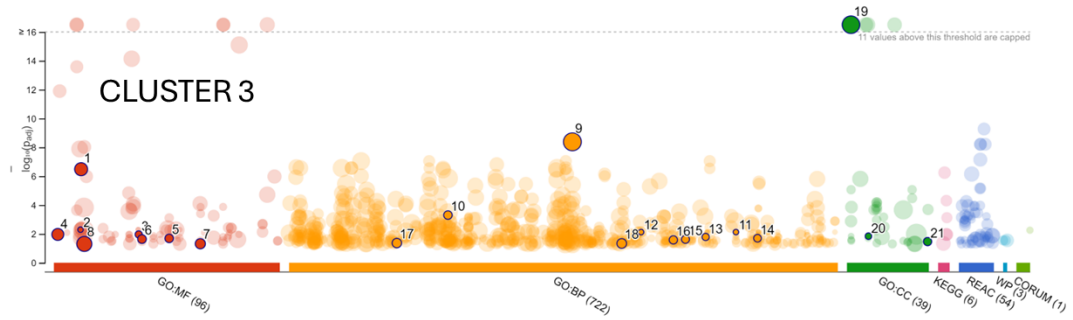




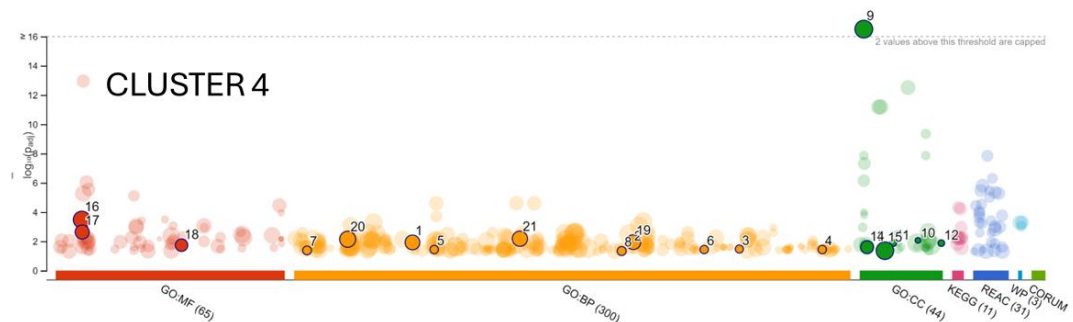
ID	Sour... ↑	Term ...	Term Na...	Padj (query...
16	CORUM	CORUM:6877	Hsp90b1-Cnpy3 complex	1.422×10^{-2}
1	GO:BP	GO:1903721	positive regulation of I-kappaB phosphorylation	2.390×10^{-2}
2	GO:BP	GO:0042981	regulation of apoptotic process	2.536×10^{-2}
3	GO:BP	GO:0061518	microglial cell proliferation	2.929×10^{-2}
4	GO:BP	GO:0034976	response to endoplasmic reticulum stress	3.823×10^{-2}
5	GO:BP	GO:0060080	inhibitory postsynaptic potential	4.508×10^{-2}
6	GO:BP	GO:0010804	negative regulation of tumor necrosis factor-medi...	4.759×10^{-2}
10	GO:BP	GO:0032692	negative regulation of interleukin-1 production	6.449×10^{-3}
22	GO:BP	GO:0051604	protein maturation	1.369×10^{-5}
23	GO:BP	GO:0007162	negative regulation of cell adhesion	1.071×10^{-4}
24	GO:BP	GO:0032914	positive regulation of transforming growth factor ...	2.669×10^{-4}
25	GO:BP	GO:0071356	cellular response to tumor necrosis factor	7.388×10^{-3}
26	GO:BP	GO:0030335	positive regulation of cell migration	8.545×10^{-3}
7	GO:CC	GO:0031012	extracellular matrix	1.120×10^{-11}
8	GO:CC	GO:0034663	endoplasmic reticulum chaperone complex	2.970×10^{-4}
9	GO:CC	GO:0044322	endoplasmic reticulum quality control compartment	2.055×10^{-2}
30	GO:CC	GO:0071944	cell periphery	2.234×10^{-4}
17	GO:MF	GO:0005201	extracellular matrix structural constituent	1.079×10^{-3}
18	GO:MF	GO:0005153	interleukin-8 receptor binding	4.549×10^{-3}
19	GO:MF	GO:0045237	CXCR1 chemokine receptor binding	4.549×10^{-3}
20	GO:MF	GO:0036313	phosphatidylinositol 3-kinase catalytic subunit bin...	1.098×10^{-2}
21	GO:MF	GO:0005179	hormone activity	1.960×10^{-2}
28	GO:MF	GO:0030546	signaling receptor activator activity	4.549×10^{-3}
29	GO:MF	GO:0045499	chemorepellent activity	4.787×10^{-2}
11	KEGG	KEGG:04141	Protein processing in endoplasmic reticulum	1.969×10^{-2}
12	KEGG	KEGG:04810	Regulation of actin cytoskeleton	3.111×10^{-2}
13	REAC	REAC:R-MMU-8...	Post-translational protein phosphorylation	2.475×10^{-6}
14	REAC	REAC:R-MMU-1...	Extracellular matrix organization	3.282×10^{-2}
15	REAC	REAC:R-MMU-2...	Integrin cell surface interactions	3.137×10^{-2}
27	REAC	REAC:R-MMU-1...	Hemostasis	3.702×10^{-3}

APPENDIX-B: The functional annotation analyses of the proteins belonging to the six clusters in the heat map shown in **Figure 5.9**.

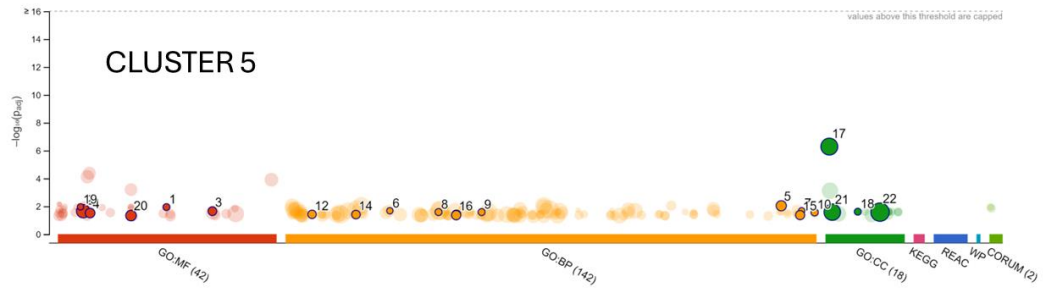




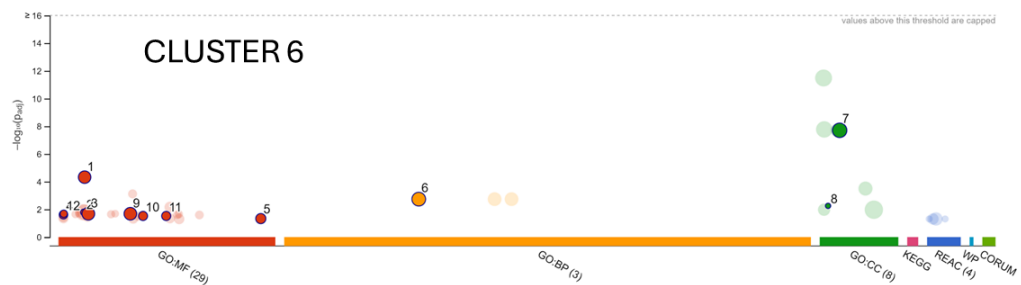
ID	Sour...	Term ...	Term Na...	Padj (query...
1	GO:MF	GO:0005201	extracellular matrix structural constituent	3.271×10^{-7}
2	GO:MF	GO:0005153	interleukin-8 receptor binding	5.066×10^{-3}
3	GO:MF	GO:0031737	CX3C chemokine receptor binding	1.088×10^{-2}
4	GO:MF	GO:0001540	amyloid-beta binding	1.088×10^{-2}
5	GO:MF	GO:0043120	tumor necrosis factor binding	2.052×10^{-2}
6	GO:MF	GO:0031995	insulin-like growth factor II binding	2.282×10^{-2}
7	GO:MF	GO:0048156	tau protein binding	4.801×10^{-2}
8	GO:MF	GO:0005543	phospholipid binding	4.949×10^{-2}
9	GO:BP	GO:0051239	regulation of multicellular organismal process	4.146×10^{-9}
10	GO:BP	GO:0032489	regulation of Cdc42 protein signal transduction	4.943×10^{-4}
11	GO:BP	GO:1901627	negative regulation of postsynaptic membrane organization	7.469×10^{-3}
12	GO:BP	GO:0071831	intermediate-density lipoprotein particle clearance	7.469×10^{-3}
13	GO:BP	GO:0150062	complement-mediated synapse pruning	1.623×10^{-2}
14	GO:BP	GO:1902951	negative regulation of dendritic spine maintenance	1.990×10^{-2}
15	GO:BP	GO:0099183	trans-synaptic signaling by BDNF, modulating synaptic transmission	2.370×10^{-2}
16	GO:BP	GO:0097114	NMDA glutamate receptor clustering	2.604×10^{-2}
17	GO:BP	GO:0017038	protein import	4.311×10^{-2}
18	GO:BP	GO:0070207	protein homotrimerization	4.596×10^{-2}
19	GO:CC	GO:0005576	extracellular region	1.576×10^{-35}
20	GO:CC	GO:0031232	extrinsic component of external side of plasma membrane	1.431×10^{-2}
21	GO:CC	GO:1990712	HFE-transferrin receptor complex	3.401×10^{-2}



ID	Sour...	Term ...	Term Na...	Padj (query...
1	GO:BP	GO:0019216	regulation of lipid metabolic process	1.187×10^{-2}
2	GO:BP	GO:0070208	protein heterotrimerization	1.405×10^{-2}
3	GO:BP	GO:1901143	insulin catabolic process	3.367×10^{-2}
4	GO:BP	GO:1990379	lipid transport across blood-brain barrier	3.658×10^{-2}
5	GO:BP	GO:0022417	protein maturation by protein folding	3.658×10^{-2}
6	GO:BP	GO:0120041	positive regulation of macrophage proliferation	3.658×10^{-2}
7	GO:BP	GO:0002023	reduction of food intake in response to dietary excess	4.215×10^{-2}
8	GO:BP	GO:0061518	microglial cell proliferation	4.621×10^{-2}
9	GO:CC	GO:0005576	extracellular region	6.153×10^{-29}
10	GO:CC	GO:0090498	extrinsic component of Golgi membrane	8.672×10^{-3}
11	GO:CC	GO:0035692	macrophage migration inhibitory factor receptor complex	1.333×10^{-2}
12	GO:CC	GO:1990682	CSF1-CSF1R complex	1.333×10^{-2}
13	GO:CC	GO:0005798	Golgi-associated vesicle	2.491×10^{-2}
14	GO:CC	GO:0005793	endoplasmic reticulum-Golgi intermediate compartment	2.528×10^{-2}
15	GO:CC	GO:0031982	vesicle	4.379×10^{-2}
16	GO:MF	GO:0005102	signaling receptor binding	3.339×10^{-4}
17	GO:MF	GO:0005125	cytokine activity	2.341×10^{-3}
18	GO:MF	GO:0046332	SMAD binding	1.839×10^{-2}
19	GO:BP	GO:0070371	ERK1 and ERK2 cascade	1.140×10^{-2}
20	GO:BP	GO:0006954	inflammatory response	7.225×10^{-3}
21	GO:BP	GO:0043410	positive regulation of MAPK cascade	6.639×10^{-3}



ID	Source	Term ID	Term Name	P _{adj} (query_1)
1	GO:MF	GO:0042018	interleukin-22 receptor activity	1.132×10^{-2}
2	GO:MF	GO:0005125	cytokine activity	2.227×10^{-2}
3	GO:MF	GO:0050786	RAGE receptor binding	2.227×10^{-2}
4	GO:MF	GO:0008301	DNA binding, bending	3.037×10^{-2}
5	GO:BP	GO:1905562	regulation of vascular endothelial cell proliferation	9.101×10^{-3}
6	GO:BP	GO:0017055	negative regulation of RNA polymerase II transcrip...	2.081×10^{-2}
7	GO:BP	GO:2000426	negative regulation of apoptotic cell clearance	2.081×10^{-2}
8	GO:BP	GO:0032425	positive regulation of mismatch repair	2.587×10^{-2}
9	GO:BP	GO:0038098	sequestering of BMP from receptor via BMP binding	2.587×10^{-2}
10	GO:BP	GO:2001200	positive regulation of dendritic cell differentiation	2.682×10^{-2}
11	GO:BP	GO:0034137	positive regulation of toll-like receptor 2 signaling...	3.719×10^{-2}
12	GO:BP	GO:0002840	regulation of T cell mediated immune response to...	3.755×10^{-2}
13	GO:BP	GO:0034165	positive regulation of toll-like receptor 9 signaling...	3.755×10^{-2}
14	GO:BP	GO:0009650	UV protection	3.900×10^{-2}
15	GO:BP	GO:2000343	positive regulation of chemokine (C-X-C motif) lig...	4.272×10^{-2}
16	GO:BP	GO:0034145	positive regulation of toll-like receptor 4 signaling...	4.325×10^{-2}
17	GO:CC	GO:0005576	extracellular region	5.158×10^{-7}
18	GO:CC	GO:0035868	alphav-beta3 integrin-HMGB1 complex	2.435×10^{-2}
19	GO:MF	GO:0004905	type I interferon receptor activity	1.090×10^{-2}
20	GO:MF	GO:0019956	chemokine binding	4.614×10^{-2}
21	GO:CC	GO:0005783	endoplasmic reticulum	2.604×10^{-2}
22	GO:CC	GO:0071944	cell periphery	2.604×10^{-2}



ID	Source ↑	Term ID	Term Name	P _{adj} (query_1)
6	GO:BP	GO:0030198	extracellular matrix organization	1.822×10^{-3}
7	GO:CC	GO:0031012	extracellular matrix	1.955×10^{-8}
8	GO:CC	GO:0005895	interleukin-5 receptor complex	5.538×10^{-3}
1	GO:MF	GO:0005201	extracellular matrix structural constituent	4.711×10^{-5}
2	GO:MF	GO:0005137	interleukin-5 receptor binding	1.608×10^{-2}
3	GO:MF	GO:0008083	growth factor activity	2.060×10^{-2}
4	GO:MF	GO:0001849	complement component C1q complex binding	2.407×10^{-2}
5	GO:MF	GO:0140311	protein sequestering activity	4.623×10^{-2}
9	GO:MF	GO:0019838	growth factor binding	2.060×10^{-2}
10	GO:MF	GO:0031994	insulin-like growth factor I binding	2.978×10^{-2}
11	GO:MF	GO:0042043	neurexin family protein binding	2.978×10^{-2}
12	GO:MF	GO:0001872	(1->3)-beta-D-glucan binding	2.060×10^{-2}

APPENDIX-C: Relative Fold Changes of three major groups with SIK2 modification and early phase (6 hour) ER stress-dependent manner.

	Accession ID	Protein Name (Abbreviation)	FDR Class [*]	HeatMap Cluster	Relative Abundance Ratio						
					6hr ER stress response (THA/DMSO) ^a		6hr Stress independent (DMSO)		6hr Stress dependent (THA)		
					Cntrl	KD	WT	KD/Cntrl ^b	WT/Cntrl ^c	KD/Cntrl ^b	WT/Cntrl ^c
Obesity and Feeding Behavior	Q35082	Klotho (Kl)	Low	C1-C5	1,402	100	1,046	1,174	100	100	17,604
	Q543.6	Antithrombin-III (SerpinC1)	Medium	C1-C5	1,026	1,652	0,648	0,97	1,84	2,225	1,238
	Q620.7	Growth/Differentiation Factor 15 (Gdf15)	Low	C2-C5	1,645	2,185	1,133	0,871	4,022	2,455	1,368
	Q62361	Pro-thyrotropin-releasing hormone (Trh)	Low	C2-C5	0,01	1,036	0,766	1	1	100	100
	B7ZMY9	Erythropoietin (Epo)	Low	C2-C4	18,99	0,01	12,678	9,936	1	0,066	0,969
	B2MMW9	Calreticulin (Calr)	High	C1-C5	4,119	0,468	11,924	0,45	0,917	0,178	3,571
	P12023	Amyloid-beta precursor protein (App)	Medium	C2-C6	0,965	2,27	0,706	0,498	3,189	3,072	1,518
	P15379	CD44 antigen (Cd44)	High	C1-C4	0,566	2,069	0,897	2,002	0,567	5,964	1,285
	Q71LX8	Heat shock protein HSP 90-beta (Hsp90ab1)	High	C3-C5	1,208	2,368	0,651	0,927	4,802	2,361	1,933
	Q621P8	Angiotensin-related protein 4 (Angptl4)	Low	C2-C5	1,126	1,136	1,238	7,973	12,277	7,305	4,612
	P12032	Metalloproteinase inhibitor 1 (Timp1)	High	C2-C4	0,127	0,36	0,073	0,373	4,778	1,626	2,862
	P25785	Metalloproteinase inhibitor 2 (Timp2)	High	C2-C5	0,611	1,144	0,276	0,733	4,885	1,374	1,454
	Q39988	Syndecan-4 (Sdc4)	Medium	C1-C5	0,376	0,313	0,56	0,318	1,542	0,212	1,096
	Q6NCU4	SPARC (Spac)	High	C2-C6	0,055	0,187	0,037	0,799	1,654	2,794	1,032
Axonal Guidance & Neurite Outgrowth	A0A338P7H5	Alpha-2-HS-glycoprotein (Ahsig)	High	C3-C5	0,975	1,519	0,916	0,467	2,74	2,549	2,523
	Q640N1	Adipocyte enhancer-binding protein 1 (Aebp1)	High	C2-C4	0,01	0,01	0,021	0,711	14,224	0,01	9,171
	Q69029	Matrilin-4 (Matn4)	Low	C1-C6	0,767	2,938	0,788	0,804	1,616	2,458	1,914
	Q69072	Glypican-1 (Gpc1)	Medium	C2-C6	0,01	0,01	0,345	0,01	1,181	0,01	12,078
	P70275	Semaphorin-3E (Sema3e)	Low	C2-C5	7,148	1,32	0,869	1,519	4,832	1,386	4,177
	Q602L9	Dickkopf-like protein 1 (Dkk1)	Low	C2-C4	0,452	4,363	0,01	6,27	1	17,097	0,01
	P10605	Cathpsin B (Ctsb)	High	C3-C6	0,128	0,283	0,079	0,754	1,737	1,884	1,005
	A0A0G2JE75	Bone morphogenetic protein 3 (Bmp3)	Low	C3-C5	0,984	6,54	1,566	0,348	0,731	3,47	3,068
	P20722	Bone morphogenetic protein 6 (Bmp6)	Low	C1-C6	1,142	3,508	0,708	1,929	0,406	0,872	0,359
	P11087	Collagen alpha-1(I) chain (Col1a1)	High	C2-C4	0,238	0,552	0,292	0,994	2,571	4,522	2,359
	Q01149	Collagen alpha-2(I) chain (Col1a2)	High	C2-C5	0,387	0,576	0,251	0,83	4,04	4,526	3,055
	Q04857	Collagen alpha-1(V) chain (Col1a1)	High	C2-C6	0,836	2,229	0,604	0,329	0,994	1	0,891
	B1B0C7	Perlecan (heparan sulfate proteoglycan 2)(Hspg2)	High	C1-C6	0,287	1,066	0,103	0,673	1,529	2,135	0,596
	P10483	Nidogen-1 (Nid1)	High	C2-C4	0,121	0,342	0,104	0,593	3,745	3,005	2,665
Inflammation	Q268V5	High mobility group protein B1 (Hmgbl1)	Medium	C2-C5	1,027	1,904	0,787	0,633	1,679	3,271	1,944
	P34884	Macrophage migration inhibitory factor (Mif)	High	C2-C4	0,721	2,153	0,817	0,864	2,139	5,431	3,303
	Q35188	Fractalkine (Cx3cl1)	Medium	C1-C6	0,802	2,532	0,506	0,549	1,786	2,386	1,016
	Q6R460	Interleukin-36 gamma (Il36g)	Medium	C1-C6	1,131	2,242	0,862	0,791	1,432	2,021	1,937
	Q6CPT4	Myeloid-derived growth factor (Mydgl)	High	C2-C4	29,652	24,199	26,057	1,423	1,431	1,28	1,348
	Q4FJ44	Annexin (Anxa1)	Medium	C2-C5	0,068	0,966	0,01	0,067	0,084	1,578	0,01
	Q544Y8	Granulin (Gm)	High	C2-C6	0,417	1,921	0,267	0,669	6,055	2,43	1,878

*FDR classifies Low as having a q-value greater than 0.05, while High and Medium are classified as having a q-value less than 0.05.

a: The ratio of secreted protein quantity in ER-stressed cells to unstressed cells.

b: The ratio of secreted protein by SIK2 knockdown cells relative to control cells.

c: The ratio of secreted protein by SIK2 overexpressing cells in relative to control cells.

APPENDIX-D: Relative Fold Changes of three major group with SIK2 modification and late phase (12 hour) ER stress-dependent manner.

	Accession ID	Protein Name (Abbreviation)	FDR Class [*]	HeatMap Cluster	Relative Abundance Ratio						
					12hr ER stress response (THA/DMSO) ^a			12hr Stress independent (DMSO)		12hr Stress dependent (THA)	
					Cntrl	KD	WT	KD/Cntrl ^b	WT/Cntrl ^c	KD/Cntrl ^b	WT/Cntrl ^c
Obesity and Feeding Behavior	C05082	Klotho (Kl)	Low	C1-C5	0,315	1,211	1,066	1,12	1,225	4,306	4,15
	Q543.6	Antithrombin-III (SerpinC1)	Medium	C1-C5	1,062	0,785	1,184	1,174	0,896	0,886	1,072
	Q620.7	Growth/Differentiation Factor 15 (Gdf15)	Low	C2-C5	0,348	0,963	0,957	1,179	0,702	1,303	1,274
	Q62361	Pro-thyrotropin-releasing hormone (Trh)	Low	C2-C5	100	12,618	10,397	0,886	1,005	1,239	0,961
	B7ZMY9	Erythropoietin (Epo)	Low	C2-C4	1	1	30,091	10,686	1	0,716	14,771
	B2MMW9	Calreticulin (Calr)	High	C1-C5	7,743	2,196	26,093	0,254	0,527	0,089	2,485
	P12023	Amyloid-beta precursor protein (App)	Medium	C2-C6	0,546	0,939	0,799	1,032	0,957	1,073	1,243
	P15379	CD44 antigen (Cd44)	High	C1-C4	1,459	1,752	1,419	1,945	1,003	2,169	0,607
	Q71LX8	Heat shock protein HSP 90-beta (Hsp90ab1)	High	C3-C5	0,378	1,076	1,265	0,545	0,87	0,72	1,319
	Q621P8	Angiotensin-related protein 4 (Angptl4)	Low	C2-C5	0,466	0,609	1,783	1,434	1,355	1,594	2,578
	P12032	Metalloproteinase inhibitor 1 (Timp1)	High	C2-C4	0,053	0,077	0,083	0,95	0,827	1,288	1,136
	P25785	Metalloproteinase inhibitor 2 (Timp2)	High	C2-C5	0,119	0,178	0,29	0,938	0,812	1,424	1,673
	Q39988	Syndecan-4 (Sdc4)	Medium	C1-C5	1,007	1,22	0,988	0,237	0,685	0,397	1,408
	Q6NCU4	SPARC (Spac)	High	C2-C6	0,052	0,034	0,034	1,053	0,734	0,882	0,484
Axonal Guidance & Neurite Outgrowth	A0A338P7H5	Alpha-2-HS-glycoprotein (Ahsig)	High	C3-C5	1,35	0,689	1,143	1,425	0,533	0,357	0,727
	Q640N1	Adipocyte enhancer-binding protein 1 (Aebp1)	High	C2-C4	0,02	0,062	0,037	0,554	1,213	1,122	1,404
	Q69029	Matrilin-4 (Matn4)	Low	C1-C6	1,135	0,685	0,841	1,593	1,048	0,508	0,828
	Q69072	Glypican-1 (Gpc1)	Medium	C2-C6	0,409	0,01	1,832	0,069	0,389	0,052	1,706
	P70275	Semaphorin-3E (Sema3e)	Low	C2-C5	0,567	0,28	1	2,388	0,091	1,604	0,01
	Q602L9	Dickkopf-like protein 1 (Dkk1)	Low	C2-C4	0,594	1,899	2,188	1,061	0,763	2,106	0,983
	P10605	Calhespin B (Ctsb)	High	C3-C6	0,115	0,156	0,095	0,95	0,892	1,339	0,571
	A0A0G2JE75	Bone morphogenetic protein 3 (Bmp3)	Low	C3-C5	0,702	10,963	1,037	0,11	0,823	1,046	1,133
	P20722	Bone morphogenetic protein 6 (Bmp6)	Low	C1-C6	12,367	0,59	29,513	1,556	0,488	0,077	1,858
	P11087	Collagen alpha-1(I) chain (Col1a1)	High	C2-C4	0,187	0,264	0,246	1,256	0,796	1,857	0,678
	Q01149	Collagen alpha-2(I) chain (Col1a2)	High	C2-C5	0,116	0,445	0,148	1,317	1,112	4,769	1,005
	Q04857	Collagen alpha-1(V) chain (Col1a1)	High	C2-C6	0,321	0,719	0,543	0,649	0,642	1,229	0,989
	B1B0C7	Perlecan (heparan sulfate proteoglycan 2) (Hspg2)	High	C1-C6	0,156	0,15	0,254	0,717	0,736	0,607	1,133
	P10483	Nidogen-1 (Nid1)	High	C2-C4	0,09	0,081	0,256	0,787	0,751	0,782	1,591
Inflammation	Q268V5	High mobility group protein B1 (Hmgbl1)	Medium	C2-C5	0,772	1,189	0,823	1,103	1,067	1,204	1,131
	P34884	Macrophage migration inhibitory factor (Mif)	High	C2-C4	1,428	1,822	1,709	1,382	0,896	2,248	1,073
	Q35188	Fractalkine (Cx3cl1)	Medium	C1-C6	0,931	0,969	1,353	1,257	1,13	0,642	0,92
	Q6R460	Interleukin-36 gamma (Il36g)	Medium	C1-C6	1,016	0,759	0,882	1,359	0,972	0,676	0,844
	Q6CPT4	Myeloid-derived growth factor (Mydgl)	High	C2-C4	15,375	6,446	23,569	1,148	0,581	0,486	0,457
		Annexin (Anxa1)	Medium	C2-C5	1,001	1,086	1,344	0,53	0,548	0,888	0,565
		Granulin (Gm)	High	C2-C6	0,176	0,265	0,216	0,859	1,283	1,431	0,737

*FDR classifies Low as having a q-value greater than 0.05, while High and Medium are classified as having a q-value less than 0.05.

a: The ratio of secreted protein quantity in ER-stressed cells to unstressed cells.

b: The ratio of secreted protein by SIK2 knockdown cells relative to control cells.

c: The ratio of secreted protein by SIK2 overexpressing cells in relative to control cells.

APPENDIX-E: Heat maps depicting the secretome profile of control vector expressing POMCs in response to ER stress.

