THE ROLE OF PROTEIN KINASE R IN LIPOTOXICITY

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BY

BÜŞRA YAĞABASAN

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I certify that I have read this thesis and that in my opinion it is fully adequate,
in scope and in quality, as a thesis for the degree of Master of Science.

Assist.Prof.Dr.Ebru ERBAY (Advisor)

I certify that I have read this thesis and that in my opinion it is fully adequate,
in scope and in quality, as a thesis for the degree of Master of Science.

Assist. Prof. Dr. Ali GÜRE

I certify that I have read this thesis and that in my opinion it is fully adequate,
in scope and in quality, as a thesis for the degree of Master of Science.

Assoc. Prof. Dr. Sreeparna Banerjee

Approved for the Graduate School of Engineering and Science

Prof. Dr. Levent Onural

Director of the Graduate School

ABSTRACT

The Role of Protein Kinase R in Lipotoxicity

Büşra Yağabasan

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Supervisor Assist. Prof. Dr. Ebru Erbay

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Endoplasmic reticulum (ER) is a central organelle for cellular homeostasis through its myriad of functions including protein and lipid biosynthesis, protein folding and secretion and calcium homeostasis. When protein folding or secretion is disrupted, ER elicits a unique signaling response initiated at its membranes called the unfolded protein response (UPR). UPR attempts to restore cellular homeostasis and survival via reducing unfolded protein levels, however, if this cannot be achieved or the stress is prolonged the UPR could lead to apoptosis. Three specific ER membrane proteins, inositol-requiring enzyme-1 (IRE1), Protein Kinase R-resemble like ER kinase (PERK) and activating transcription factor 6 (ATF6), act as ER stress sensors and initiate distinct but interlaced signaling pathways to restore ER homeostasis. Recently, studies demonstrated that over nutrition, especially high amount of saturated fatty acids or cholesterol in the circulation, leads to the induction of ER stress in metabolic tissues, resulting in the activation of UPR signaling pathways. Furthermore, ER stress was shown to play a causal role in the pathogenesis of metabolic diseases such as obesity, insulin resistance, type 2 diabetes and atherosclerosis.

Interferon inducible double strand RNA activated protein kinase R (PKR) is also known to be activated during ER stress. Recent studies showed it can be activated by lipids during ER stress in cells and in metabolic tissues of obese mice. Genetic ablation or inhbition of PKR

enhances systemic glucose homeostasis and insulin sensitivity in obesity in rodent models. However, it is not known how PKR becomes activated by overnutrition or by ER stress. In fact, many of the specific cellular components and molecular mechanisms in lipid induced cellular stress or death, namely lipotoxicity, is not completely understood. PKR is one of the serine/threonine kinase that is known to be activated during lipid induced ER stress, but only a few specific downstream substrates are known and these fall short of explaining PKR's role in lipotoxicity in chronic metabolic disease pathogenesis. PKR also plays a crucial role in activation of inflammasomes through interacting with the inflammasome components. There is a gap in our knowledge regarding PKR's specific molecular actions in nutrient-induced inflammation and metabolism in chronic metabolic diseases.

In this thesis study, my major goal was to develop specific tools to modulate PKR's activity and search for its specific substrates in lipotoxicity and its role in mediating lipid-induced ER stress response. For this purpose, I developed a novel chemical-genetic approach to specifically modify PKR's kinase activity during ER stress. In this approach, the bulky side-chain of a gatekeeper amino acid (such as methionine) in the ATP binding cavity of PKR has been altered to a smaller side-chain amino acid (such as glycine) in order to slightly enlarge the cavity to accommodate bulky ATP analogs (activating or inhibiting). This mutant of the PKR has been named the analog sensitive kinase allele (ASKA) of PKR and was shown to utilize normal ATP as well as the bulky ATP analogs in kinase reactions. Furthermore, I demonstrated the specific inhibition of PKR kinase with the inhibitory, bulky ATP analogs such as4-Amino-1-tert-butyl-3-(1'-naphthyl)pyrazolo[3,4-d]pyrimidine (NAPP1) or 4-Amino-1-tert-butyl-3-(1'-naphthylmethyl)pyrazolo[3,4-d]pyrimidine (1-NMPP1). In order to move one step closer to identification of potential PKR substrates, I also optimized kinase reactions for immunoprecipitated PKR ASKA mutant and visualized several potential downstream substrates in my initial experiments.

Finally, I studied a unique relationship between two ER stress related kinases IRE1 and PKR in lipid induced ER stress conditions. I observed specific inhibition of IRE1's endoribonuclease activity with an inhibitor, but not its kinase activity, completely blocks PKR activation by lipids. These findings strongly support hat IRE1's RNAse activity is necessary for PKR kinase activation by lipids. This function of IRE1 RNAse domain is novel and unsuspected. The future goals of this research should be directed to discovering the RNA mediators of IRE1-PKR coupling and understanding their role in mediating the inflammatory and metabolic pathologies associated with chronic metabolic diseases.

In conclusion, in my thesis study, I developed novel chemical-genetic approach to specifically modify PKR kinase activity that could be useful in discovering novel PKR substrates. Based on the preliminary findings in this thesis, PKR appears to have many unidentified substrates regulated during lipid induced ER stress. Furthermore, using the chemical-genetic PKR as a tool as well as several other approaches I demonstrated the existence of a unique, functional relationship between IRE1 and PKR in lipotoxicity. In addition, the results in my thesis shows that IRE1's endoribonuclease activity is required and sufficient for PKR kinase activation by lipids. These findings and tools developed during my studies can be further utilized for analyzing the specific role of PKR in lipotoxicity, which is important for the health consequences of metabolic diseases.

Key words: UPR, ER stress, PKR, IRE1, Lipids.

ÖZET

Protein Kinaz R'nin Lipotoksisite'deki Rolü

Büşra Yağabasan

Moleküler Biyoloji ve Genetik Yüksek Lisansı

Tez Yöneticisi: Yrd. Doç. Dr. Ebru Erbay

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Protein ve yağ biosentezi, protein katlanması, kalsiyum salgılanması ve dengelenmesini de içeren sayısız fonksiyonuyla Endoplazmik Retikulum (ER) hücresel homeostaz için merkezi bir organeldir. Protein katlanması ya da salgılanmasıyla ilgili bir bozukluk olduğunda, ER zarlarından katlanmamış protein yanıtı (KPY) adı verilen özgün bir sinyal cevabı başlatır. KPY düzgün katlanmamış protein seviyesini azaltarak ER homeostazını eski haline getirmek için girişimde bulunur, fakat eğer ER homeostazı tekrar elde edilemezse ya da ER stresi uzun süreliyse KPY apoptoza yol açabilir. Üç özel ER zar proteini, inositol-gerektiren enzim-1 (IRE1), Protein Kinaz R'yi (PKR) anımsatan kinaz (PERK) ve aktifleştiren transkripsiyon faktör 6 bu yolakta stres sensörü olarak rol alır ve ER homeostazını tekrar sağlamak için ayrı fakat birbirinin içine geçmiş sinyal yolaklarını başlatır. Yakın zamanda, çalışmalar gösterdi ki aşırı beslenme, özellikle dolaşımda olan yüksek miktarda doymuş yağ asitleri ya da kolesterol, metabolik dokularda ER stresini indükler ve bu indüksiyon KPY sinyal yolaklarının aktifleşmesine yol açar. Ayrıca ER stresinin obezite, insülin direnci, tip 2 diyabet, ve aterosklerozis gibi metabolik hastalıklarının patogenezinde sebepsel bir rol oynadığı gösterilmiştir.

Interferon ile indüklenebilen ve çift sarmallı RNA ile aktifleşen protein kinaz R'nin (PKR) de ER stresi sırasında aktifleştiği bilinmektedir.Son çalışmalar ayrıca PKR'nin ER stresi boyunca

lipidler ile ve obez farenin metabolik dokularında aktifleşebileceğini göstermiştir. PKR'nin genetik ablasyon ya da inhibe edilmesi kemirgen modellerdeki obezitede insülin duyarlılığını ve sistemik glukoz homeostazını arttırır. Fakat PKR'nin aşırı beslenme ile ya da ER stresiyle nasıl aktifleştiği bilinmemektedir. Aslında yağ ile indüklenmiş hücresel streste ya da ölümdeki, birçok özel hücresel bileşen ve moleküler mekanizma, yani lipotoksisite tam olarak anlaşılamamıştır. PKR yağ ile indüklenen ER stresinde aktifleştiği bilinen serin/treonin kinazlarından biridir, fakat sadece birkaç özel substratı bilinmektedir ve bunlar PKR'nin kronik metabolik hastalık patogenezindeki lipotoksisitede PKR'nin rolünü açıklamada yetersiz kalmaktadır. PKR ayrıca inflamazom bileşenleriyle etkileşime girerek, inflamazom aktivasyonunda önemli bir rol alır. PKR'nin kronik metabolik hastalıklarda besin ile indüklenen iltihaplanma ve metabolizmadaki özel moleküler eylemlerine ilişkin bilgimizde bir boşluk bulunmaktadır.

Bu tez çalışmasında, benim ana amacım PKR'nin aktivitesini düzenlemek için özel araçlar geliştirmek ve onun lipotoksisitedeki özel substratlarını ve yağ ile indüklenen ER stresi cevabında oynadığı rolü araştırmaktı. Bu amaçla, PKR'nin ER stresi sırasındaki kinaz aktivitesini özel olarak mofidiye etmek için özgün bir kimyasal-genetik yaklaşımı geliştirdim. Bu yaklaşımda, ATP analoglarının (engelleyen ya da aktifleştiren) PKR'nin ATP bağlanma boşluğuna uyum sağlayabilmesi için PKR'nin ATP bağlanma boşluğundaki iri hacimli yan-zincir bekçi aminoasit (metiyonin gibi) daha küçük yan-zincir aminoasitine (glisin gibi) değiştirilir. PKR'nin bu mutantı ATP analoğuna duyarlı kinaz (AZKA) olarak adlandırılır ve bu mutantın kinaz reaksiyonlarında normal ATP'nin yanında iri hacimli ATP analoglarından da yararlandığı gösterilmiştir. Buna ek olarak, ben PKR kinazının inhibe eden iri hacimli-Amino-1-tert-butyl-3-(1'-naphthyl)pyrazolo[3,4-d]pyrimidine (NaPP1) or 4-Amino-1-tert-butyl-3-(1'-naphthylmethyl)pyrazolo[3,4-d]pyrimidine (1-NMPP1) gibi ATP analoglarıyla inhibe oluşunu gösterdim. Potansiyel PKR substratlarını tanımlamaya bir adım daha

yaklaşmak için, ayrıca immünoçökeltilmiş PKR AZKA mutantı için kinaz reaksiyonlarını optimize ettim ve başlangıçtaki deneylerimde potansiyel aşağı sinyal yolaklarındaki substratlarını görüntüledim.

Son olarak, yağ ile indüklenen ER stresi koşullarında, iki ER stresi ile ilgili kinasın arasındaki özgün ilişkiyi çalıştım. IRE1 kinazının kinaz değil ama endoribonükleaz aktivitesini bir inhibitör ile inhibe ettiğimde, yağlara bağlı PKR aktivasyonunun tamamen kaybolduğunu gözlemledim. Bu bulgular IRE1 kinazının RNAse aktivitesinin PKR kinazının yağ ile aktifleşmesi için gerekli olduğu bilgisini güçlü bir şekilde destekler. IRE1 kinazının RNAse bölgesinin bu fonksiyonu özgündür ve umulmadıktır. Bu araştırmanın gelecekteki hedefi IRE1-PKR bağlantısındaki RNA aracılarını keşfetmeye ve bu bunların kronik metabolik hastalıklarla ilşkili inflamatuar ve metabolik patolojilerdeki rolünü araştırmaya yönlendirilmelidir.

Sonuç olarak, tez çalışmamda, PKR'nin kinaz aktivitesini özel olarak modifiye etmek ve bu şekilde özgün PKR substratlarını keşfetmek için özgün bir kimyasal-genetik yaklaşım geliştirdim. Bu tezde bulunan ön bulgulara göre, yağ ile indüklenen ER stresi esnasında PKR'nin birçok tanımlanmamış substratı var gibi görünmektedir. Buna ek olarak, kimyasal-genetik PKR'yi kimyasal genetik aracı olarak kullanarak ve başka diğer yaklaşımlarla, lipotoksisite esnasında IRE1 ve PKR kinazları arasında benzersiz, fonksiyonel bir ilişkinin varlığını gösterdim. Ayrıca, tezimdeki sonuçlar göstermekte ki; IRE1 kinazının endoribonükleaz aktivitesi PKR kinazının yağlar tarafından aktifleştirilmesi için gerekli ve yeterlidir. Çalışmalarım esnasında geliştirilen bu bulgular ve araçlarında, metabolik hastalıkların sağlıksal sonuçları için önemli olan PKR'nin lipotoksisitedeki özel rolünün analizi için daha fazla yararlanılabilir.

Anahtar kelimeler: KPY, ER stresi, PKR, IRE1, Yağlar.

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Dedicated to my beloved grandma, rest in peace…

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

1. INTRODUCTION

1.1. Endoplasmic Reticulum, Its Structure and Function

The endoplasmic reticulum (ER) is a central organelle for cellular homeostasis. ER, a membrane-bound organelle, is comprised of stacks of tubules and vesicles that connect it to a network of other membranous organelles[2]. Two types of ER are classified depending on association with or without ribosomes and called rough ER and smooth ER, respectively. The rough ER is important for protein synthesis. Secretory and transmembrane proteins are folded in the ER lumen and reach maturation before they leave the ER[1]. The smooth ER is the site for lipid and sterol synthesis as well as the major intracellular storage site for calcium (Ca^{2+})[3]. Furthermore, ER is the merging site for many extracellular and internal signals and orchestrates adaptive cellular responses to alternating cellular conditions. For example, via storing or releasing Ca^{2+}, ER plays a role in muscle relaxation and contraction, learning and memory, metabolism and cell death[2].

As the major reservoir for intracellular Ca^{2+}, the ER can store up to three to four times more Ca^{2+} concentrations than in the cytosol. With the appropriate signal, this concentration gradient facilitates the exit of Ca^{+2} from the ER to the cytoplasm through one of the many ER membrane channels/receptors, such as Inositol triphosphate receptor (IP3R), leak channels and ryanodine receptor (RyR). In direct contrast to this, the sarcoplasmic-endoplasmic reticulum Ca^{2+} ATPase (SERCA) pumps take up calcium from cytosol into the ER lumen and as a result generate the high calcium gradient between ER and cytoplasm[5].

Lots of enzymes and molecular chaperons function in the ER to facilitate its central role in protein folding, maturation and secretion. Collectively these are called the reticuloplasmins.

Molecular chaperones facilitate proteins folding by increasing the yield of structural maturation and folding rather than increasing the rate. One of the ER chaperones known as Binding immunoglobulin protein (BiP) also known as 78 kDa glucose-regulated protein (GRP-78), recognizes and binds to unfolded proteins through these proteins' hydrophobic (specifically, aromatic) amino acids[6]. BiP also has an ATP hydrolysis function, acts as adenosinetriphopshatase (ATPase), it dissociates from its protein ligands when there is ATP. BiP/GRP78 protein and 94 kDa glucose-regulated protein (GRP94) bind to immunoglobulin heavy and light chains, and these ER stress proteins act as molecular chaperones and work together during folding and assembly of proteins[8]. Additionally, there are some ER-resident enzymes helping maturation and folding of proteins. For example, protein disulfide isomerase (PDI) acts as a catalyst in the formation of disulfide bridges. Another example is the calnexin/calreticulin/glycosyltransferase system, which helps the folding of proteins via interacting with nascent proteins and plays a role in ER-quality control system[7]. Newly synthesized unfolded proteins and proteins that reside in ER lumen are translocated into the lumen of the ER via one of the translocons called as Sec61 complex and are folded into mature forms with the assistance of molecular chaperones, helper folding enzymes and in the presence of high intralumenal Ca^{2+} concentrations. Then, these nascent polypeptides are N-glycosylated with N-glycosylating enzymes. Finally they mature into secondary and tertiary structures with the help of PDI. In addition, N-glycosylation enzymes and lectins also help the maturation of proteins[7]. After the mature forms of the proteins pass the ER quality control, they are then transported into the Golgi apparatus, whereas the unfolded or misfolded proteins are degraded by the highly conserved ER-associated degradation mechanism (ERAD).

In the ERAD pathway the unfolded proteins translocate into cytosol through the translocon channels for proteasomal degradation[11]. It was also showed that, Sec61 is needed for ERAD-L pathway which is a specific ERAD pathway that degrades integral unfolded ER luminal

proteins[12,13]. There are also other ERAD pathways such as ERAD-C, which is responsible for degrading membrane proteins with cytosolic lesions, and ERAD-M, which degrades membrane proteins with misfolded transmembrane domains[143]. To conclude, ER plays a multitude of important roles to maintain cellular homeostasis including but not limited to promoting protein folding, maturation and secretion.

Another fundamental but less well appreciated function of the ER is in lipid biosynthesis. It is the central site for synthesis of cholesterol, ceramides and phospholipids. When intracellular cholesterol levels are lowered this leads to activation of the ER resident sterol regulatory element-binding protein-1 and 2 (SREBP-1 and 2), which can transcriptionally upregulate the expression of key cholesterol synthesis enzymes[15]. When intracellular cholesterol is low, SREBP is released from Sterol regulatory element-binding protein cleavage-activating proteins (SCAP), which normally tethers SREBPs to the ER membranes. The SREBP can then translocate into Golgi to get cleaved and activated as a functional transcription factor. In particular SREBP1a and -2 upregulates synthesis of cholesterol, whereas SREBP1c upregulates the transcription of key enzymes in fatty acid synthesis pathways[15]. Insulin induced gene proteins (Insig-1 and 2), another ER membrane-bound proteins, negatively regulate fatty acid synthesis through controlling the activity of SREBP and 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCoAR) degradation[14, 144,145]. In the presence of high amount of sterols, ER membrane proteins Insig-1 and Insig-2 interacts with HMGCoAR and recruits the tumor autocrine motility factor receptor protein gp78, a membrane-associated ubiquitin ligase, leading to ubiquitination of reductase. In result, the ubiquitinated reductase is degraded by the ERAD pathway[14]. Furthermore, when sterol levels are high, Insig-1 protein binds and changes the conformation of SCAP. Because of this conformational change, SCAP-SREBP-Insig-1 complex cannot be translocated to Golgi where SREBP is activated[145]. Another key lipid enzyme found the ER membrane is the Stearoyl-CoA desaturase (SCD), a

short lived enzyme which is needed for synthesizing monounsaturated fatty acids. SCD acts as a key component in hepatic synthesis of very-low-density proteins and triglycerides[16]. Also on the ER membrane, Diaclylycerol transferases (DGATs), act as catalysts in the final step of triacylglycerol (TAG) synthesis'[17]. The ER-bound Acyl-coenzyme A: cholesterol acyltransferases (ACAT) catalyze the formation of cholesterol esters from cholesterol[18]. Some ER enzymes such as DGATs and acyl-CoA: sterol acyltransferases (ASATs) for steryl esters, act as catalysts in elongating fatty acid synthase synthesized palmitate product, which is beyond 16-C length. They also lengthen dietary polyunsaturated fatty acids synthesized by fatty acid synthase. Desaturases such as Delta6-desaturase, and some ER-resident proteins, NADH-cyt b_5 Reductase and Cytochrome b_5 take role in formation of double bond in fatty acids[20]. To sum up, ER functions in lipid biosynthesis through these specific ER enzymes that regulate many cellular signaling pathways important in lipid biosynthesis.

Another essential role ER plays in lipid biology is contribution of the membrane components of the lipid droplets. Lipid droplets are major intracellular reservoir of mainly triacylglycerols (TAGs) and cholesterol esters (CEs). They consist of a hydrophobic neutral core containing these TAGs and CEs, surrounded by a phospholipid monolayer and cholesterol[21]. In addition, ER luminal proteins calnexin and BiP were detected in lipid droplet fractions[22],[147]. Still there is limited information about lipid droplet assembly and expansion mechanisms and most models remain theoretical. Researchers strongly came up with two specific models for lipid droplet formation. These are the classical model and the hatching model. In the classical model, the budding lipid droplet is surrounded by the cytoplasmic leaflet of the ER membrane[25]. As ER luminal proteins calnexin and BiP association with lipid droplet was shown, Plough proposed another model for lipid droplet formation called Hatching model. In this model, lipid droplet is covered by phospholipid monolayer, with both cytosolic and luminal leaflets of ER membrane, this structure is bicellar structure[22]. This model also

supports the role of lipid droplet in misfolded protein degradation, as lipid droplet associates with luminal ER protein, BiP, bind to misfolded proteins and target them for degradation[147]. Recently, it was shown that several ER-resident lipid enzymes including the neutral lipid synthetic enzymes diacylglycerol acyltransferase 1 (DGAT) or 1-acylglycerol-3-phosphate acyltransferase (AGAT) also have role in lipid droplet formation[24]. Furthermore, it was shown that ER Glycerol-3-phosphate acyltransferase 4 (GPAT4) relocalizes from ER to lipid droplets and mediates their expansion[2]. Therefore, it can be concluded that ER functions in lipid droplet formation with specific ER enzymes and structural proteins which functions in formation of lipid droplets[22,147].

Besides ER's general functions that have been discussed, in the pancreatic beta cells ER assumes an especially important metabolic role in pro-insulin biosynthesis. The freshly translated insulin mRNA prepro-insulin gets translocated into the lumen of the ER, where ER proteases cleave off the signal peptide of turning prepro-insulin into pro-insulin. Then pro-insulin is further folded by ER-enzymes via forming intramolecular double disulfide bonds. The properly folded pro-insulin is then translocated to Golgi and transferred into secretory granules. In secretory granules, pro-insulin is converted to mature insulin and released by exocytosis[26]. When the ER is stressed, however, insulin transcription, translation and secretion get blocked through several mechanisms involving the ER based stress response pathway[26].

1.2. Endoplasmic Reticulum Stress

1.2.1. The Causes and the Consequences of Endoplasmic Reticulum Stress

ER is a central homeostatic organelle that functions in membrane protein and lipid biosynthesis, calcium homeostasis, secretion, folding and maturation of proteins[41]. If the ER cannot adapt to the ever-changing intracellular conditions, such as a dramatic increase in

unfolded or misfolded proteins, it undergoes an elaborative stress response called Unfolded Protein Response (UPR)[41]. ER stress can be induced by many other physiological and pathological stimuli such as environmental toxins, glucose deprivation, elevated saturated lipids or free cholesterol, viral infections, defects in protein trafficking, mutations in chaperone genes, aberrant calcium regulation, hypoxia and complex conditions associated with aging and tumorogenesis[27,3,28]. When ER stress is induced, unfolded proteins are identified through the Endoplasmic Reticulum Quality Control (ERQC) mechanism[30]. Specifically, calreticulin, calnexin and BiP chaperone proteins identify the unfolded proteins by the surface exposure of their hydrophobic sites, reactive thiols and immature glycans and bind these proteins to promote their proper folding[30]. Once these proteins are refolded they are released from the ER. Defects in the N-linked glycosylation pattern are also carefully monitored and such proteins become targets for ER α-mannosidases, following degradation-enhancing α-mannosidase-like protein (EDEM) binding to them[30]. These are then translocated to cytoplasm and degraded via the ERAD pathway[12,29,30].

ER stress can be viewed in three phases: acute, periodic and chronic[32]. The most widely studied form is the acute form, which can be experimentally induced via chemical treatments such as tunicamycin (protein glycosylation inhibitor), thapsigargin (SERCA pump inhibitor) and Dithiothreitol (DTT) (protein disulfide bond inhibitor)[32]. Exposure to toxic levels of saturated fatty acids or free cholesterol can also induce acute ER stress, whereas simultaneous treatments with unsaturated free fatty acids like oleate and palmitoleic acid can block this response[32]. Rhythmic or transient physiological activities such as feeding-fasting cycle, or transient glucose/lipid infusions induce the “periodic” type of ER stress, and after each cycle, ER homeostasis is fully restored with help of UPR[32]. And the final form of ER stress is the “chronic” form and in which ER homeostasis is never fully achieved in the continuous presence of ER-stress inducer leading to uninterrupted UPR signaling[32]. Chronic ER stress is

widely observed in metabolic diseases such as insulin resistance, obesity, dyslipidemias and atherosclerosis, in which the ER stressor being chronic nutrient excess[32]. Extended exposure to saturated fatty acids and cholesterol, lead to chronic, lipotoxic ER stress, which cannot be easily resolved[32]. A recent study in obese mice documenting reduction in ER protein synthesis and increase in lipid biosynthesis underscores the pivotal changes that occur around ER metabolism upon chronic, nutrient excess. Furthermore, the same study shows the alterations in ER lipid metabolism is coupled to the inhibition of SERCA activity and causally associated with chronic ER stress[31].

1.2.2. The Unfolded Protein Response

The UPR is an elaborative, adaptive, intracellular signal transduction pathway initiated as a response to ER stress and first described in mammalian cells by Kozutsumi and colleagues in 1988[27, 33]. In this study, investigators noted that expression of two ER-stress resident, glucose modulated proteins; GRP78/BiP and GRP94 are induced upon the accumulation of misfolded proteins at high temperatures[33]. In the same study, expression of wild type and mutant forms of influenza virus haemagglutinin (HA) protein in simian cells also lead to upregulation of the GRP78/BiP and GRP94 expression[33]. Unfolded protein response pathway was first described in yeast; a 22 base pair, cis-acting element (UPR element) was found sufficient for the induction of KAR2 (BiP) gene transcription by the accumulation of unfolded proteins[34]. Later it was shown that this UPR element is found in the promoter regions of five UPR target genes in yeast (karyogamy gene (KAR2) (homolog of BiP), protein disulfide isomerase gene (PDI1), protein disulfide isomerase EUG1 gene (EUG1), peptidylprolyl isomerase family gene (FKB2), and LHS1 Hsp70 family chaperone gene (LHS1)) and is required for their induction. Finally, the molecular UPR pathway was described upon the discovery of the yeast Hac1p, a UPR- activated transcription factor which binds to these UPR elements and activates the

transcription of ER chaperone proteins[39,40]. Hac1p is a basic-leucine transcription factor, which binds to UPR elements on genes that encode ER molecular chaperones[38].

Peter Walter and his colleagues also identified IRE1, the key ER membrane protein that processes and activates Hac1p, in *S.Cerevisae*[35]. The human homolog of IRE1 gene, ERN1, encodes 1115 aminoacid length transmembrane kinase, Ire1p. Its glycosylated N-terminal side lies inside microsomes and its C-terminal cytosolic side has kinase activity[36]. Besides being kinase, Ire1p also has an endoribonuclease activity, which is responsible for the cleavage and activation of HAC1 mRNA[37,38].

Historically, yeast IRE1 is the first discovered and conserved arm of the UPR that leads to the activation of XBP1 and production of molecular chaperoes[50,28]. In mammalian cells, in addition to the IRE-1, two additional UPR branches maintain ER homeostasis and are regulated by two ER membrane-bound proteins: PKR-like eukaryotic initiation factor 2 α kinase (PERK) and activating transcription factor 6 (ATF6)[27]. (Figure 1.1) A well-excepted model suggests that in homeostasis conditions, the molecular chaperone BiP/GRP78 interacts with PERK and IRE1 to keep them in an inactive state[27]. When ER homeostasis is disrupted, BiP/GRP78 dissociates from both PERK and IRE1 in order to bind to unfolded proteins. These kinases are thus activated and initiate the UPR[41].

When ER homeostasis is disrupted, depending on the degree of disruption, adaptive or destructive downstream signaling pathways are activated upon the oligomerization and activating auto phosphorylation of IRE1 and PERK. In addition, ATF6 transcription factor translocates to Golgi, where it was cleaved by a serine protease site -1 protease (S1P), the metalloprotase site-2 protease (S2P) and it becomes fully activated. (Fig 1.1)

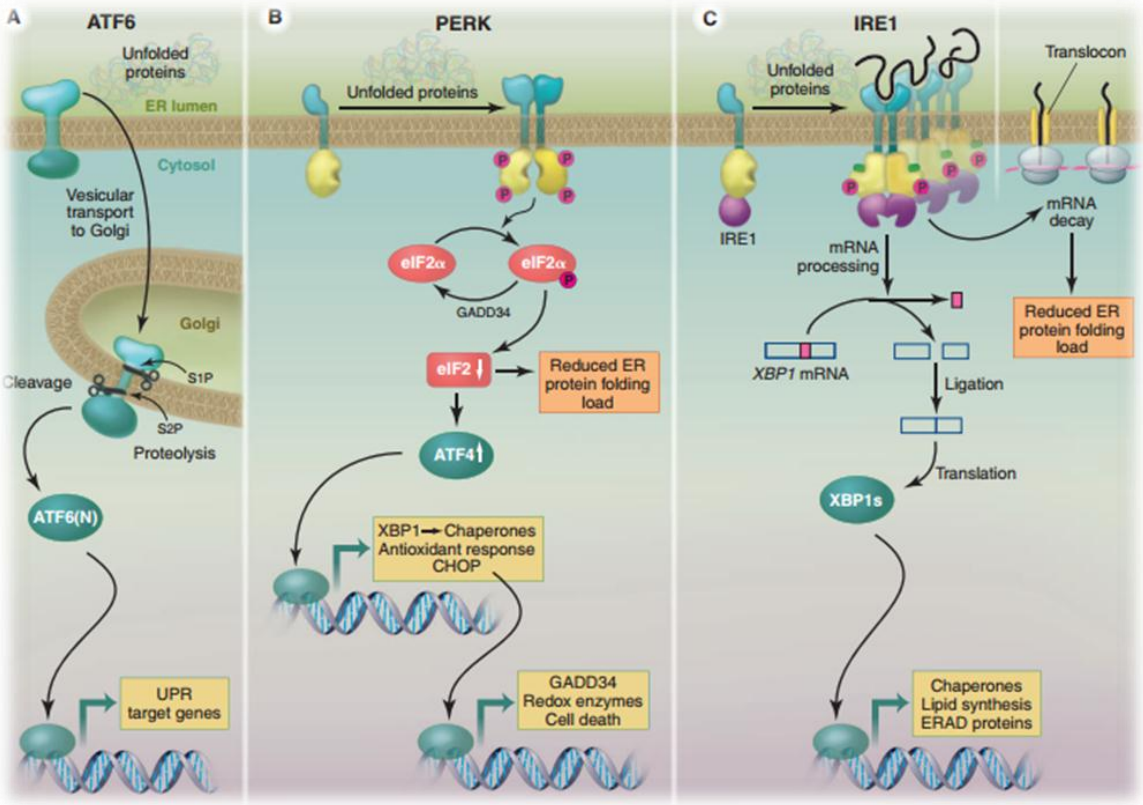


Figure 1.1. ER Stress and UPR pathway.
(reprinted with permission from Peter Walter and David Ron (2011) The Unfolded Protein Response: From Stress Pathway to Homeostatic Regulation. *Science Reviews*. Science 25 November 2011: Vol. 334 no. 6059 pp. 1081-1086 DOI: 10.1126/science.1209038.[41]) When there is ER stress, cell elicits a unique response called Unfolded Protein Response. During unfolded protein response three ER stress sensors PERK (PKR resemble ER kinase), activating transcription factor 6 (ATF 6) and Inositol requiring enzyme 1 (IRE1) are activated which represent three distinct arms of UPR. PERK and IRE1 are activated through auto phosphorylation and ATF6 is activated through cleavage by specific Golgi proteases S1P and S2P and when activated acts as transcription factor via upregulating UPR target genes' expression[49,76,81].When PERK is activated, it phosphorylates eIF2α, leading to translational inhibition and specific ATF4 mRNA translation. ATF4 acts as transcription factor and up regulates chaperones' genes expression such as XBP1 and CHOP. IRE1 splices XBP1 mRNA through its RNAse activity and leads to its translation and translated XBP1 protein acts as transcription factor via upregulating chaperones' lipid synthesis' ERAD proteins' gene expression[41].

The first discovered arm of the UPR is regulated by IRE1 and conserved from yeast to mammals. There are two types of mammalian IRE1, which has differential tissue expression pattern. IRE1α is expressed in many tissues such as liver, skeletal muscle, lung, placenta and particularly in pancreas, whereas IRE1β is only expressed in intestinal epithelia[51,52]. For activation, IRE1 first homo-oligomerizes and then auto-phosphorylates itself at serine 724 in its C-terminal kinase domain[53]. The only known substrate of IRE1 kinase is itself. Besides its kinase activity, IRE1 also has a unique endoribonuclease activity, which cleaves HAC1

mRNA in yeast or the homologous XBP1 (X-box binding protein) mRNA in metazoans[53,54]. The cleavage of the 26 nucleotide from the intron of XBP1 produces 41 kDA size, active XBP1 transcription factor. IRE1 also nonspecifically cleaves mRNAs on ER membrane in order to reduce protein synthesis and loading of protein synthesis to ER (IRE1 dependent mRNA decay pathway (RIDD). Recently, it was shown that IRE1α endoribonuclease (RNAse) domain also leads to fast decay of pre-miRNAs 17, 34, 96 and 125. These miRNAs all target caspase-2 mRNA translation. Therefore it can be concluded that, under ER stress conditions, IRE1α positively regulates expression of apoptotic Caspase-2 protein via inhibiting maturation of select miRNAs that have role in mediating the apoptotic outcome of UPR[58].

Active XBP1 transcription factor up regulates the expression of UPR target genes including ER chaperones, ERAD proteins and ER membrane expansion related phospholipid synthesizing enzymes[54,55]. Furthermore XBP1 can work in coordination with ATF6 to induce the heat shock protein 40 member DnaJ (Hsp40) homolog, subfamily C, member 3 protein $P58_{IPK}$. This protein assists in protein folding by behaving like a co-chaperone and furthermore, can bind and inhibit PERK and PKR activities[49, 142].

Additionally, IRE1 is known to interact with adaptor proteins like such as TNF receptor-associated factor 2 (TRAF2) in order to stimulate apoptosis signal regulating kinase (ASK1). ASK1 activates the pro-apoptotic cJUN NH2-terminal Kinase (JNK), p38 mitogen activated kinase (p38MAPK) and caspase 12[57]. Under normal conditions caspase 12 is bound and inactivated by TRAF2, but when there is ER stress caspase 12 dissociates from TRAF2 complex and this activated form leads to cell death [57] . IRE1 also through interaction with TRAF2 regulates JNK-AP1 and nuclear factor κB (NF-κB) pathways (Figure 1.2). Finally, by activating JNK, IRE1 can indirectly regulate insulin synthesis proteins Insulin receptor

substrate 1 (IRS1) and Insulin receptor substrate 2 (IRS2) via its JNK-induced inhibitory phosphorylation[28].

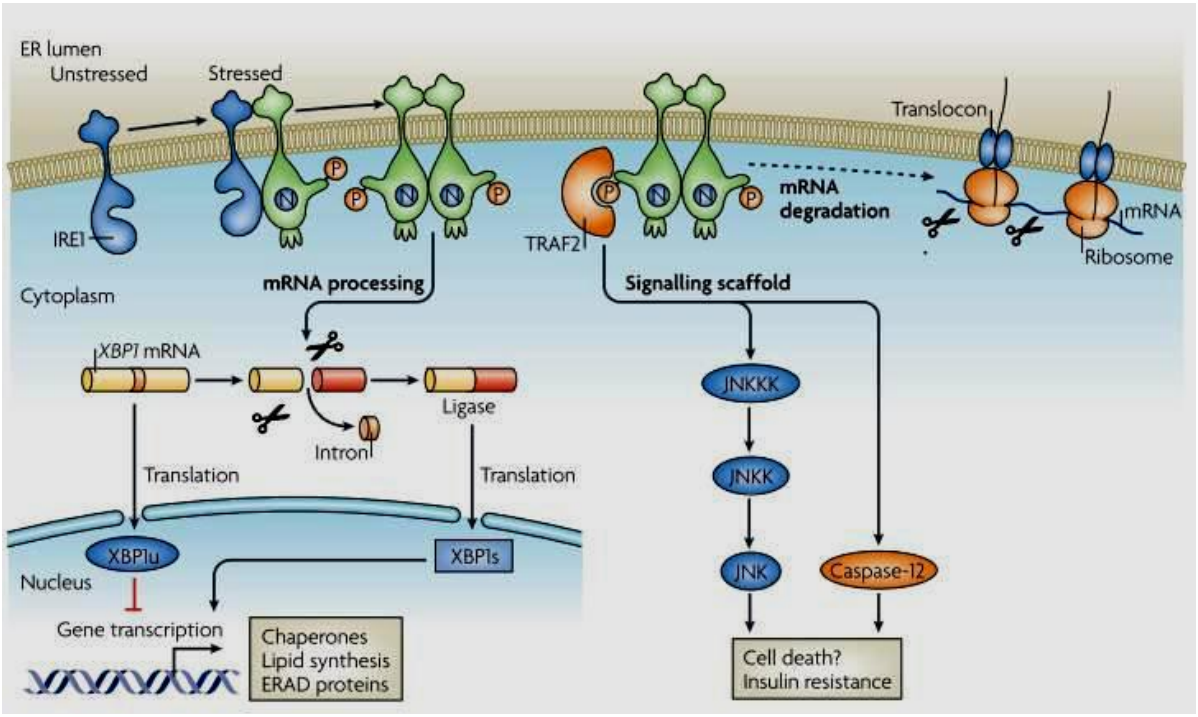


Figure 1.2. IRE1 arm of the Unfolded Protein Response.
(reprinted with permission from Ron, D., and Walter, P. (2007). Signal integration in the endoplasmic reticulum unfolded protein response. *Nat. Rev. Mol. Cell Biol*. 8, 519–529. © Nature publishing Group, 2007[50].). In the presence of ER stress, IRE1 oligomerizes and autophosphorylates itself, leading to its activation[50]. After it is activated, through its RNAse domain, it splices XBP1 mRNA, which is then translated and acts as a transcription factor for expression of chaperones', lipid synthesis' and ERAD proteins' genes[50]. It also interacts with TRAF2 through its kinase domain resulting in its activation and activated TRAF2 starts a signaling cascade, interacts and activates mitogen activated protein kinases leading to insulin resistance[154]. It also interacts with Caspase-12 leading to cell death[50].

The second UPR arm is maintained by PERK. PERK is a type I transmembrane serine/threonine kinase which has N-terminal luminal ER stress sensor and a cytosolic kinase domain. Similar to IRE1 activation mechanism, PERK can oligomerize and auto phosphorylate (at threonine 981) upon dissociation of BiP[42]. PERK shares similarities with the three other eIF2α phosphorylating kinases at its C-terminal kinase domain[43]. The other eIF2α kinases are the interferon inducible viral infection derived double strand RNA activated Protein Kinase R (PKR), the amino acid deprivation activated general control non-

depressible kinase 2 (GCN2) and the heme regulated eif2α kinase (HRI)[43] . Eif2α is a key protein that controls protein translation mechanism by transporting the initiator methionyl-transfer RNA (met-tRNA) to the ribosome in order to initiate protein translation[44]. When PERK is activated, it inactivates eukaryotic initiation factor 2 α (eIF2α) through phosphorylating it at Serine 51 site and thus, resulting in general protein translation inhibition. In this way, PERK reduces protein overload in the ER to alleviate ER stress. (Figure 1.3). PERK activity also induces activation of the nuclear factor kappa β (NF-κB) genes[45]. NF- κB has many functions especially as a key regulator of inflammation[50]. eIF2α phosphorylation leads to phosphorylation and translational suppression of inhibitory kappa B (IκB) resulting in release of inflammatory cytokines such as IL-6 and TNF-α[46,47]. Another protein PERK associates and phosphorylates is the Nuclear erytroid factor 2[28]. Phosphorylation of the Nrf2/Keap1 (Kelch-like ECH-associated Protein 1) complex leads to Nrf2 dissociation and transport to nucleus[28,]. Nrf2 is a transcription factor that has role in eliciting antioxidant response[146]. In contrary with its role in general protein translation inhibition, PERK activates Activating Transcription Factor-4 (ATF-4) translation leading to the transcription of UPR target genes and chaperones (Figure 1.3)[27,41]. ATF4 stimulates transcriptional activation of apoptosis, inflammation, glucose metabolism, ER redox control, negative feedback eIF2α phosphorylation inhibitory genes[28]. Transcription factor C/EBP homologous protein also called CCAAT/enhancer binding proiculutein (CHOP) [41] and growth arrest- and DNA damage inducible- 34 (GADD34) [41] , ER redox control (ERO1) and activating transcription factor 3 (ATF3) are four gene targets of ATF4[28,44]. GADD34 interacts with catalytic subunit of protein phosphatase (PP) and dephosphorylates eIF2α relieving its inhibition, leading to translational recovery[44]. Ero1 functions in balancing the redox potential in the ER[191]. CHOP is a transcription factor which regulates activation of apoptosis genes. ATF6 stimulated $P58_{IPK}$ interacts with cytosolic domain of PERK and leads to repression of its activity[48,49]. $P58_{IPK}$ is

expressed several hours after PERK activation, this shows that in the end of UPR $P58_{IPK}$ expression is induced and cells go apoptosis[48,49]. Therefore, it can be observed that besides its protective role and maintaining ER homeostasis through up regulation of UPR chaperones' expression to relieve ER stress, PERK can also contribute to cell death pathways when ER stress is prolonged[48,49,50].

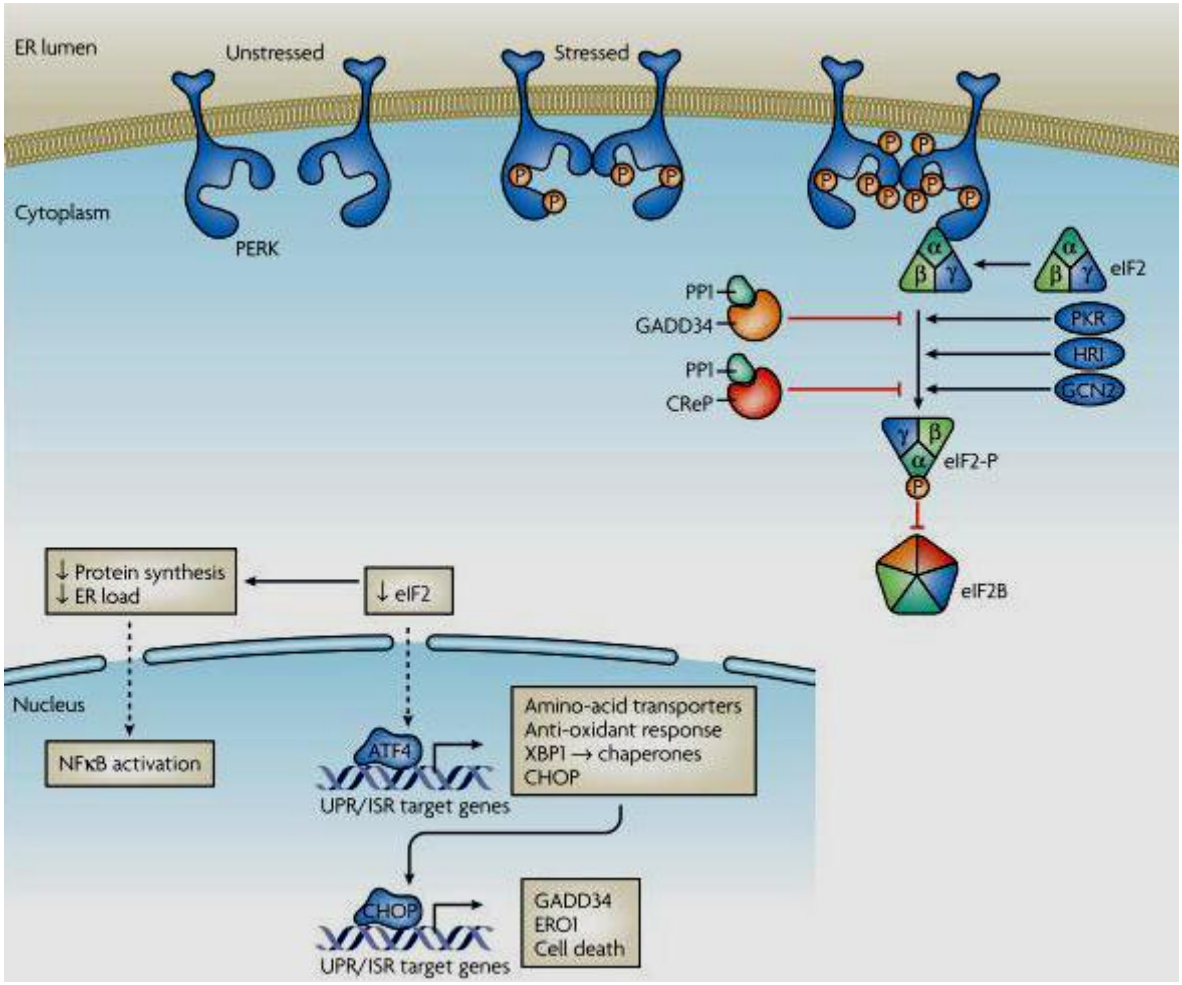


Figure 1.3. PERK arm of the Unfolded Protein Response.
(reprinted with permission from Ron, D., and Walter, P. (2007). Signal integration in the endoplasmic reticulum unfolded protein response. *Nat. Rev. Mol. Cell Biol*. 8, 519–529. © Nature publishing Group, 2007[50].**).** In the presence of ER stress, PERK is activated through auto phosphorylation, leading to phosphorylation and inactivation of eIF2α. Besides leading to translational suppression, eIF2α results in ATF4 mRNA translation, therefore leading to UPR/ISR target genes expression such as XB1 and CHOP [62,45,149]. CHOP then acts as transcription factor resulting in activation of GADD34, ERO1 genes and cell death genes expression [61,65,66,67].

ATF6 is another proximal sensor in the ER stress response and responsible for the initiation of the third branch of the UPR[50]. ATF6 is a 90 kDA Basic Leucine Zipper (bZIP) protein, which

is activated after cleavage by specific Golgi-resident proteases[50]. When ER stress is induced, ATF6 is reduced, and this monomeric, reduced ATF6 translocates to the Golgi, where it is cleaved by site-1 and site-2 protease (S1P and S2P)[50]. These proteases specifically cleave ATF6 from the luminal domain and its trans-membrane anchor. The active ATF6 (50 kDA after cleavage) translocates to the Golgi and binds to promoters that contain ER stress response element (ERSE; CCAAT(N)9CCACG), which are found in genes like ERAD pathway proteins, lipid biosynthesis, and ER chaperones[59]. There are two isoforms of ATF6, ATF6α and ATF6β which become activated with ER stress. Studies show that ATF6 also induces the expression of XBP1[59] and $P58_{IPK}$, thus intercepting both the IRE-1 and PERK branches[49]. On the other hand, the Wolfram Syndrome 1 (WSF1) protein can regulate ATF6 activity via targeting ATF6 for ubiquitylation and proteasomal degradation by HMG-CoA reductase degradation protein 1 (HRD1) and E3 ubiquitin ligase[60]. Recently, it was showed that ATF6 indirectly down regulates B-cell lymphoma 2 (BCL-2) family member myeloid cell leukemia sequence 1 protein (MCL-1) in myoblast cell line, pointing out the potential role of ATF6 in initiating apoptosis during ER stress[61]. It was also shown that, homologs of ATF6 like cAMP responsive element binding protein 3-like 3 (CREBH), old astrocyte specifically induced substance (OASIS), basic leucine zipper transcription factor (LUMAN also known as LZIP), cAMP responsive element binding protein 3-like 4 (CREB4) and box B-binding factor 2 human homolog on chromosome 7 (BBF2H7) are also similarly proteolytically cleaved in Golgi and give different responses under ER stress. (Figure 1.4)[50].

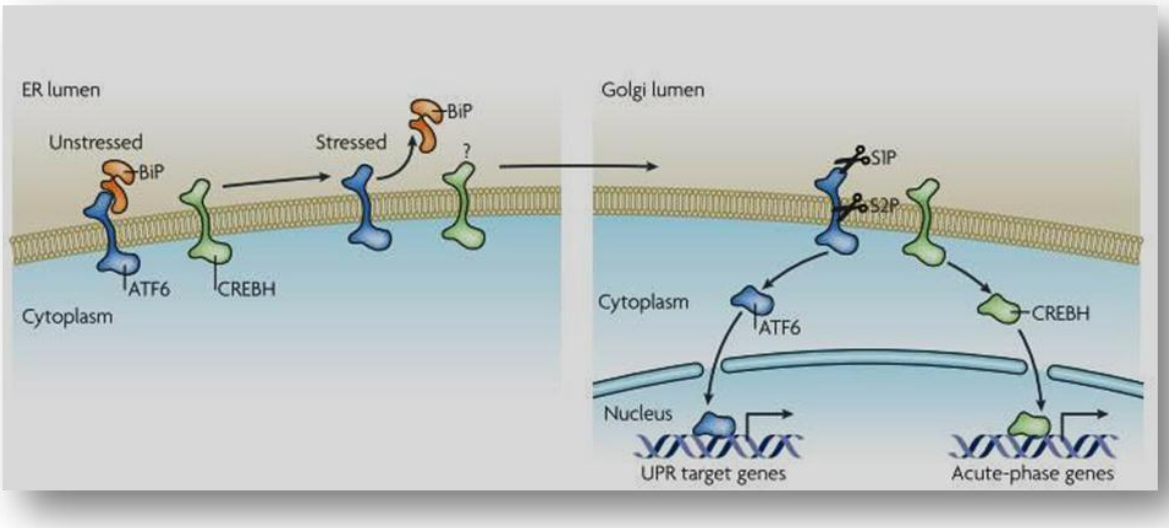


Figure 1.4. ATF6 branch of unfolded protein response.
(reprinted with permission from Ron, D., and Walter, P. (2007). Signal integration in the endoplasmic reticulum unfolded protein response. *Nat. Rev. Mol. Cell Biol*. 8, 519–529. © Nature publishing Group, 2007[50]). In the presence of ER stress, ATF6 dissociates from BiP and is transported to Golgi apparatus together with CREBH[49,76,81]. The lumenal site-1 protease and the intra-membrane site 2 protease cleave CREBH and ATF6 to activate these transcription factors[76,81]. These activated transcription factors then translocate to nucleus leading to ATF6 activation of UPR target genes and CREBH activation of acute phase genes' expression.

1.2.3. The Unfolded Protein Response Outputs: Adaptive or Pro-Survival, Destructive or Pro-Apoptotic

The accumulation of unfolded proteins in the ER activates the UPR[50]. UPR first tries to restore folding process by inducing transcription of ER chaperones and ERAD proteins and reducing protein overload by inhibiting general translation[61]. It also promotes cell cycle arrest in G1 phase[148]. However if ER stress is prolonged, this response also results in apoptosis[61]. There are many UPR mediated mechanisms for adaptation to ER stress. For example, PERK phosphorylates eIF2α, resulting in translational inhibition and reduction of the protein overload in the ER[50]. In addition, PERK activation leads to cell cycle arrest through the loss of short-lived cell cycle regulatory proteins, such as CyclinD1[62]. Even though general translation is inhibited as a result of PERK's activity, certain select mRNAs' translation continues in an increased manner due to their special regulation under the UPR[149]. One such

mRNA is ATF4 mRNA, whose translation increases in an eIF2α phosphorylation-dependent manner[149].

ATF4 translocates to nucleus and activates expression of many genes which then help restore ER homeostasis and provide amino acid sufficiency[149]. PERK also activates NRF2 by phosphorylation and leads to its dissociation from KEAP1[45,63]. Nrf2 then translocates into the nucleus and activates the expression of many antioxidant genes. This constitutes an important adaptive mechanism governed by PERK as NRF2 deficient cells showed increased rate of apoptosis under ER stress[63, 45].

Other adaptive responses to ER stress are managed by the ATF6 arm. After its activation, ATF6 translocates into the nucleus and induces the expression of many genes that encode ER chaperone and ERAD proteins[61]. ATF6 also induces XBP1 transcription, which is another important factor for cell survival[52].

The IRE1 arm also has a significant role in eliciting adaptive response. The main adaptive response of IRE1 is mediated by the spliced XBP1 mRNA, which generates the active XBP-1 transcription factor. XBP1 is a transcription factor that induces many genes that have role in protein folding and ERAD pathway[64]. XBP1 also regulates ER folding capacity itself[64].

Not all outcomes governed by UPR favor the cell's survival. UPR can also promote apoptosis when ER stress is prolonged[62,63,66,67]. For example, PERK activates ATF4, which transcribes the CHOP gene, an activating transcription factor in apoptosis. CHOP down regulates transcription of anti-apoptotic BCL-2 and it up regulates pro-apoptotic BH3-only protein Bim[65,66] and pro-apoptotic death receptor DR5 expression[65,66,67]. ERO1α, GADD34 and telomere repeat binding factor 3 (TRB3) are many other CHOP target genes have role in cell death mechanisms[61]. Recently, it was shown that the ATF6 arm also has an indirect role in triggering apoptosis via down regulation of anti-apoptotic BCL-2 family member MCL-1 in a myobast cell line[61]. Furthermore, studies also show that eventually IRE1 is turned off in

prolonged ER stress, whereas PERK arm remains on, suggesting the anti-survival role is dominated by PERK in continuous stress[50]. Nevertheless, IRE1 can also be pro-apoptotic by interacting with tumour necrosis factor receptor-associated factor 2 (TRAF2) and leading to Apoptosis signal-regulating kinase 1 (ASK1) and c-Jun N-terminal kinase (JNK) activation, which result in apoptotic death[57]. Under ER stress, JNK phosphorylates and suppresses BCL-2[150] and B-cell lymphoma extra-large protein (BCL-X_L)[150] anti-apoptotic activity and it also phosphorylates BH3 interacting-domain death agonist protein (BID) and proapoptotic BH3-only protein BIM, making these pro-apoptotic proteins active[151]. Recently Han et al., showed that IRE1 dependent RIDD pathway also have role in induction of cell death in the pancreatic beta cell line (INS-1)[68].

1.3. The Relationship between Endoplasmic Reticulum Stress and Inflammation

Unfolded protein response is associated with inflammation through multiple mechanisms including interaction of the UPR branches with JNK and NF- κB pathways, by promoting ER ROS production and Nitric Oxide formation during ER stress and inflammation[28].These pathways also play a major function in sterile inflammation observed metabolic diseases such as obesity, atherosclerosis, insulin resistance and diabetes[69]. Recent studies show that UPR signaling pathways result in production of many proinflammatory cytokines, through multiple mechanisms[69.] The historically first discovered and conserved branch of UPR, IRE1 pathway activates JNK leading to activation of the transcription factor activator protein 1 (AP1)[69,70]. AP1 translocates to the nucleus and activates transcription of inflammatory genes including tumor necrosis factor (TNF), keratinocyte growth factor (KGF), granulocyte macrophage colony (GM-CSF), interleukin-8 (IL-8) and some other cytokine receptors[70]. IRE1 also regulates inflammation related Mitogen activated protein kinase p38 and extracellular signal related kinase (ERK) via binding to non-catalytic region of tyrosine kinase adaptor protein

Nck[71]. Additionally, IRE1 signaling can interfere with NF-κB pathway by interacting with inhibitory IκB kinase (IKK) through TRAF2 complex[50]. Recently it was shown that IRE1 kinase activity and not endoribonuclease activity activates NF-κB pathway through interacting with TRAF2/IKK complex and maintaining IKK activity[72]. Also shown was that in JNK deficient cells, tumor necrosis factor alpha (TNF-alpha), interleukin (IL6) and monocyte chemotactic protein-1 (MCP-1) proinflammatory cytokines' expression are repressed[28,152,153,154]. As IRE1 can lead to the activation of JNK, it could induce the transcription of these pro-inflammatory cytokines[28]. Moreover, XBP1, downstream of IRE1, is necessary for B cells to secrete antibody, when they are induced with antigen[73,74]. XBP1 also has a role in production and release of antimicrobial peptides from Paneth cells[73,74]. NF-κB has also been shown to be regulated by the PERK arm through eif2α mediated inhibitor kappa B (IκB) translation suppression that results in NFκB activation[46,50,160]. Without IκB present, NFκB translocates to the nucleus and induces expression of inflammatory cytokines[69]. The third UPR arm, ATF6, has also been shown to activate NF-κB via Akt during subtilase cytotoxin induced ER stress[75] ATF6 appears to play an additional role in initiation of acute phase response (APR), a highly complex inflammatory process observed at the onset of serious diseases such as infection, trauma and inflammation[69]. One study shows that in the liver, cyclic-AMP responsive element binding protein H (CREBH) cooperates with ATF6 in APR activation[76].

Another link between ER stress and inflammation appears to be the ER-generated reactive oxygen species and oxidative stress[45]. Depending on UPR, Ero1p and endoplasmic reticulum flavin-linked sulfhydryl oxidase (Erv2p) enzymes up regulate chaperone proteins' expressions through forming disulfide bonds in proteins[77]. These enzymes can also reduce molecular oxygen. This reduced molecular oxygen leads to oxidative stress and ER stress[77]. The activation of PERK then initiates anti-oxidant response with help of Nrf2 activation by

PERK[45]. Moreover, ER stress induces the transcription of inducible nitric oxide synthase (iNOS) via NF-κB activation, but which of the UPR arms is responsible remains unknown[78].

1.4. The role of Endoplasmic Reticulum Stress and Endoplasmic Reticulum Stress related Metabolic Diseases

Over the past decade we have reached an understanding on the importance of ER homeostasis for metabolic well-being and an important target to prevent chronic, inflammatory and metabolic diseases such as obesity, diabetes, insulin resistance, fatty liver and atherosclerosis[32,155]. Early studies showed that the UPR arms are activated in the liver and adipose tissue of rodents during obesity and its reduction by chemical chaperons can prevent insulin resistance and metabolic disease[32,79,155]. These landmark studies by Hotamisligil' group were also extended to human obesity and has led to many groups to embark on research to understand the role of and the molecular mechanisms leading to ER Stress and UPR in chronic metabolic diseases[157,158,159].

Several mechanisms have been demonstrated regarding how ER stress is tied to the development of insulin resistance and diabetes in obesity. For example, upon induction of UPR, JNK-AP1 pathway is activated in an IRE1-dependent manner. JNK has been shown to phosphorylate the insulin receptor substrate 1 (IRS1) at Serine 307 position, leading to the disruption of insulin signaling and promoting insulin resistance[80]. PERK also increases JNK activity, through unclear mechanisms[28]. Additionally, ATF6[75] and PERK[46] activate NF-κB, which then transclocates into the nucleus and induces the expression of pro-inflammatory cytokines[50,190]. Other UPR-activated transcription factors can directly modulate expression of key lipogenic and gluconeogenic enzymes that contribute to the development of insulin resistance. For example, SREBP-1c[161,162,163], XBP1[160], and NRF2[164,165] are such transcription factors that can lead to abnormal expression of lipogenic genes. On the other hand, CREBH

upregulates gluconeogenic genes while ATF6 and XBP1 downregulate their expression to promote insulin resistance[81]. UPR may also indirectly promote hepatic insulin resistance and hepatic steatosis via leading fat accumulation in the ER and inhibition of VLDL export through disrupting VLDL production by increasing apolipoprotein B100 (apoB100) degradation[81,169,170]. PERK- activated ATF4 is another factor that disrupts insulin signaling via activating the pseudokinase tribbles homolog 3 (TRB3) expression that negatively regulating protein kinase B (PKB), downstream component of the insulin signaling pathway[82]. ER stress can be caused by hepatic steatosis in *ob/ob* mice and this stress results in proteolytic cleavage of SREBP1c and SREBP2 transcription factors which upregulates sterol synthesis[163]. When BiP is overexpressed adenovirally in hepatocytes of ob/ob mice, SREBP1c activation decreases resulting in reduced hepatic triglycerides, and improved insulin sensitivity[163].

Additionally, it was showed that XBP1 deficient mice developed insulin resistance upon high fat diet induced obesity[79].In hepatocytes, depending on insulin signaling, heterodimer structure formed by the regulatory subunits of phosphotidyl inositol 3-kinase (PI3K), p85α (encoded by *Pik3r1*) and p85β (encoded by *Pik3r2*) is altered leading to interaction of p85α and p85β with XBP1s. Then this p85-XBP1s complex translocates to nucleus, resulting in activation of expression of UPR target genes[167]. In insulin resistant, *ob/ob* mice, the XBP1s-p85 interaction is disrupted and when p85 is overexpressed in these mice, translocation of XBP1s to nucleus is retained, and glucose tolerance is improved[167].According to a recent study, XBP1 was also shown to regulate gluconeogenic transcription factor Forkhead box protein O1 (FoxO1) resulting in affection of glucose homeostasis in obese mice[166]. Independent from its role in UPR, XBP1 improves glucose homeostasis through ubiquitination and degradation of FoxO1 in obese mice[166]. To sum up, XBP1 can improve insulin sensitivity through inducing UPR target genes' expression such as *Dnajb9*[168]. It can

also improve glucose tolerance in insulin resistant *ob/ob* mice by blocking gluconeogenesis as it interacts and degrades FoxO1[166].

ER stress is also observed in adipose tissue of obese individuals. ATF6-regulated chaperones' expression[158], eif2α phosphorylation[158] and expression of IRE1[157] together with JNK[157] and XBP1s[157], increase in adipose tissue of obese individuals. In one research, it was shown that several ER-stress related proteins such as calreticulin, protein disulfide-isomerase A3, glutathione-S-transferase, calnexin, JNK-1 and XBP1s are upregulated in subcutaneous adipose tissue of obese individuals[172]. This result shows UPR activation in subcutaneous adipose tissue of humans[172].

Even researchers showed the presence of ER stress in mouse skeletal muscle after high fat diet for 4 weeks[173], the relationship between ER stress and insulin resistance is more complex compared to other tissues. For example, UPR elements' expression levels remain unchanged in muscle tissue after HFD, however insulin resistance and lipogenesis increases[174]. Interestingly, researchers showed that single physical exercise causes ER stress in muscle whereas exercise training result in adaptive UPR through peroxisome proliferators-activator receptor gamma coactivator-1 alpha (PGC-1α) dependent coactivation of ATF6[175]. Interestingly, it was demonstrated that, in human myotube cells, when palmitate inducible Stearoyl-CoA Desaturase 1 (SCD1) expression increases. Depending on the high inducibility of SCD1 expression depending on palmitate treatment, insulin sensitivity also increases[176].

The increase in IRE1 and PERK phosphorylation together with JNK and BiP activity seen in the liver and adipose tissue of obese mice, suggested similar signs of ER stress may be evident in the brain. Indeed, a study showed ER stress leads to the activation of the NFκB pathway and inflammation in the hypothalamus, by engaging the Toll like receptor signaling[83]. Saturated free fatty acids, such as palmitate lead to activation of mostly TLR4 and modestly TLR2[177]. It is not clear; however, what is the direct ligand that stimulates hypothalamic Toll

like receptors[177]. After TLR4 and TLR2 are activated, they activate JNK and IκB kinase resulting in inflammation and leptin/insulin resistance[177]. Furthermore, chemical chaperone application inhibited NFκB signaling and reduced leptin- resistance[84]. Another recent research demonstrated that unsaturated fatty acids as well as chemical chaperones can revert high fat diet induced hypothalamic inflammation and can reduce body mass and adipocity[178]. In addition, reduction in body fat mass by dieting leads to decrease in the hypothalamic PERK acitivity, whereas ATF6, BiP and Xbp1 mRNA expression remain induced[85] . Very recently, it was shown that during diet-induced obesity, post translational processing of proopiomelanocortin (POMC) mRNA is impaired by ER stress leading to reduced production of appetite-suppressing neuropeptide α-melanocyte-stimulating hormone (α-MSH) as this hormone's synthesis is catalyzed by POMC protein[179]. In this study, researchers demonstrated for the first time that in obesity, ER stress contributes in regulation of energy balance through altering neuropeptide processing[179]. From all these studies, it can be concluded that excess nutrients result in ER stress, which elicits an elaborative adaptive response UPR in hypothalamus, muscle, liver and adipose cells in obesity and diabetes. Even the mechanisms eliciting this response is not fully clarified, some results are common between these tissues. UPR causes inflammation and insulin/leptin resistance in the hypothalamus, liver and fat cells through some specific cellular pathways as explained above[83].

1.5. Restoring ER function

After discovering the causality of ER stress in obesity induced metabolic diseases, a search to define novel approaches to restoring ER function has begun and the ER is viewed as a promising therapeutic target. For example, one of the first trials in rodents consisted of applying 4-phenylbutyric acid (PBA), a chemical chaperone which inhibits ER-stress related apoptosis, to mouse models of obesity and insulin resistance. PBA has been known to have a

protective function in cerebral ischemia and liver ischemia reperfusion injury[86,87]. Studies show it is also effective means to reduce ER stress and improve systemic metabolic homeostasis and insulin sensitivity in mouse models of obesity[79]. Another chemical chaperone, Tauroursodeoxycholic acid (TUDCA) also inhibits ER –stress and improves metabolism in mice[79]. Furthermore, TUDCA blocks ER stress-induced apoptosis in human liver cells through yet unknown mechanism[88]. Furthermore, chemical chaperone PBA also has been studied in another chronic, metabolic disease model, atherosclerosis, in mice. When PBA was chronically administered to Apolipoprotein E null (ApoE−/−) mouse model of atherosclerosis, ER stress was significantly reduced in atherosclerotic plaques[88]. Treatment with PBA reduced PERK phosphorylation and ATF3 expression in macrophages infiltrating the atherosclerotic plaques, as well as in isolated macrophages, resulting in protective effect against hypercholestrolemic stress[88]. Furthermore, a genetic-deficiency in a macrophage lipid chaperone also protected against ER stress and atherosclerosis. In addition, the lipid product of aP2 activity, a monounsaturated fatty acid known as palmitoleate, was also found to be protective effects against lipid-induced ER stress in macrophages[82]. Diakogiannaki et al., in their research also showed that when palmitoleate is given to β cells for 18 hours, Tunicamycin and Palmitate induced ER stress can be reduced[89]. Collectively, these data show that when palmitate (saturated and toxic) and palmitoleate (monounsaturated and non-toxic) are given in a combination treatment, ER stress and cell damage are prevented and meanwhile, evidence suggests ER membrane expands less in the combination treatment when compared to only palmitate treatment. This result shows that palmitoleate, also quite bioactive when given systemically, has protective effect on ER structure and function and a promising dietary approach to restoring ER stress in chronic metabolic diseases like atherosclerosis. These and other possibilities to reduce ER stress need to be vigilantly tested.

Recently in 2010, researchers came up with an important finding that interferon inducible double strand RNA dependent protein kinase R (PKR) can respond to excess nutrients, pathogens as well as ER stress and elicits a unique inflammatory response and metabolic response through regulating insulin action and JNK activity which are important in metabolic diseases [128]. This critical role of PKR enabled researchers search more about small chaperones that can regulate its activity for treatment of metabolic diseases such as obesity, insulin resistance and diabetes[128].

1.6. Structure and Function of Protein Kinase R

During viral infections, interferon secretion is the early response. In order to maintain homeostasis, many specific proteins are activated, particularly by interferons, during this process. One such induced protein is the 68 kdA sized, interferon inducible double strand RNA activated Protein Kinase R (PKR). PKR is a serine/threonine kinase that is activated not only during viral infections but also during ER stress[90,91,92,]. After binding dsRNA, PKR dimerizes and auto phosphorylates itself at its threonine 446 and serine 451 sites, leading to its full activation. PKR can phosphorylate the eukaryotic initiation factor 2 α (eIF2α) at Serine 51 site, resulting in general protein translation inhibition, therefore inhibition of viral replication[115].

PKR has a distinct structure compared to well-known other kinases. It is known that PKR can be activated by binding of double stranded RNA of cellular, viral, or synthetic (such as polyrI: polyrC, pIC) origin (longer than 30 nucleotides), to its two N-terminal dsRNA binding motifs (DRBMs I and II) in an RNA sequence-independent manner[93]. After the binding of dsRNA, PKR dimerizes and auto phosphorylates itself at Threonine 446 and Threonine 451 sites in C-terminal kinase domain. The DRBMs I and II domains have specific α-β-β-β-α fold and they are connected through a 22 nucleotide length linker region (Figure 1.5)[93,94].

Besides dsRNA, PKR is also activated with some cellular activator proteins such as mouse Retina and anterior neural fold homeobox protein RAX; and its mammalian homolog p53 associated cellular protein (PACT)[97,98]. Melanoma differentiation-associated gene-7 protein (MDA7) also activates PKR[99]. Adenoviral expression of MDA7 in human lung cancer cells, led to activation of PKR and induction of apoptosis[99]. pRB-binding protein E2F-1 (E2F1) transcription factor, which is observed in many cancer cell types resulting in apoptosis by prolonged general protein translation inhibition, is another inducer of PKR activity,[100]. Recently it was shown that PKR can respond to excess nutrients as well as ER stress[128]. Nakamura and his colleagues showed that, ER stress inducers such as saturated fatty acid palmitate and chemical inducer thapsigargin induce activation of PKR[128].

PKR acts as an intermediate in Toll-like receptor signaling. When dsRNA binds to Toll like receptor 3 (TLR3), Tumor necrosis factor (TNF) receptor-associated factor 6 (TRAF6), Transforming growth factor beta (TGF-β) activated kinase 1 (TAK1) and TGF-beta activated kinase 1/MAP3K7 binding protein 2 (TAB2) proteins cumulate around this receptor and form a complex[130,131,132]. PKR is also attracted to this complex, which then translocates to cytosol. In the cytosol, TAK1 gets phosphorylated by TGF-beta activated kinase 1/MAP3K7 binding protein 1 (TAB1)[180] leading to NF-κB and MAPKs activation. When PKR kinase was silenced, poly I:C induced activation of TLR3 dependent activation of NFκB and MAP kinases was also inhibited, demonstrating PKR's significant role in this process[130]. In TLR4 dependent but myeloid differentiation primary response 88 (MyD88) adapter protein independent pathways, Toll–interleukin 1 receptor (TIR) domain–containing adapter protein (TIRAP) interacts with PKR and leads to NF-κB acitvation in TLR4 dependent signaling pathways[132]. However, it was also shown that PKR participates in MyD88 dependent TLR signaling pathways[133]. Furthermore, a study by Karin et al. demonstrated that PKR is a critical

component in TLR-4 signaling pathway dependent macrophage apoptosis due to bacterial infection[133].

As cellular expression of PKR is a threat for viruses, several viruses have evolved some mechanisms for specifically inhibiting PKR. Some viruses result in expression of dsRNA binding proteins leading to inhibition of dsRNA-PKR interaction and therefore activation of PKR[183]. For example vaccinia virus E3L gene expresses E3L dsRNA binding protein which inhibits dsRNA dependent PKR activation through interacting dsRNA and preventing its binding to PKR[181]. Some other viruses elicit a unique response for inhibiting PKR through forming RNA with secondary structure. This virus-formed RNA binds to PKR but does not activate the kinase domain[183]. For instance, the human immunodeficiency virus type 1 (HIV-1) has trans activation response element (TAR) stem loop structure which is found in all 5'end of all HIV-1 mRNA transcripts and these transcripts bind PKR more efficiently then dsRNA leading to inhibition of PKR[182].Some viruses synthesize proteins that interact with PKR and inhibits its dimerization and activation[183]. For instance Hepatitis C virus expresses Nonstructural protein 5A (NS5A) which interacts with PKR through its kinase domain leading to inhibition of its dimerization and activation[183]. Some viruses synthesize proteins similar to eIF2α, which is the substrate of PKR[128]. For example HIV-1 synthesized Trans-Activator of Transcription protein (Tat) was shown to bind to PKR from the site where cellular eIF2α bind[183]. In addition some cellular proteins also can be utilized by viruses to inhibit PKR function. In influenza virus, p58 dissociates from Hsp40 and is activated. Then it binds to PKR resulting in inhibition of its dimerization and activation[184]. Therefore, it can be concluded that, even PKR is very critical in viral defense, viruses evolved many mechanisms in order to inhibit PKR activity.

Besides its role in cellular response to viral infection, PKR also has a role in cell growth control and differentiation, cell cycle regulation and apoptosis it also acts as tumour

suppressor[106,117]. In addition to inhibiting general protein translation[50,106,117], PKR functions in many signaling pathways through interaction with specific proteins[94]. For example, PKR negatively regulates cell growth; studies have shown that when wild type PKR was overexpressed in mammalian or yeast cells, the proliferation rate of these cells decrease dramatically and moreover, PKR levels oscillate in different stages of cell cycle[95]. In one study, it was shown that PKR interacts with p53 tumour suppressor protein and phosphorylates it on serine 392 sites both *in vitro* and *in vivo* leading to activation of p53[106].

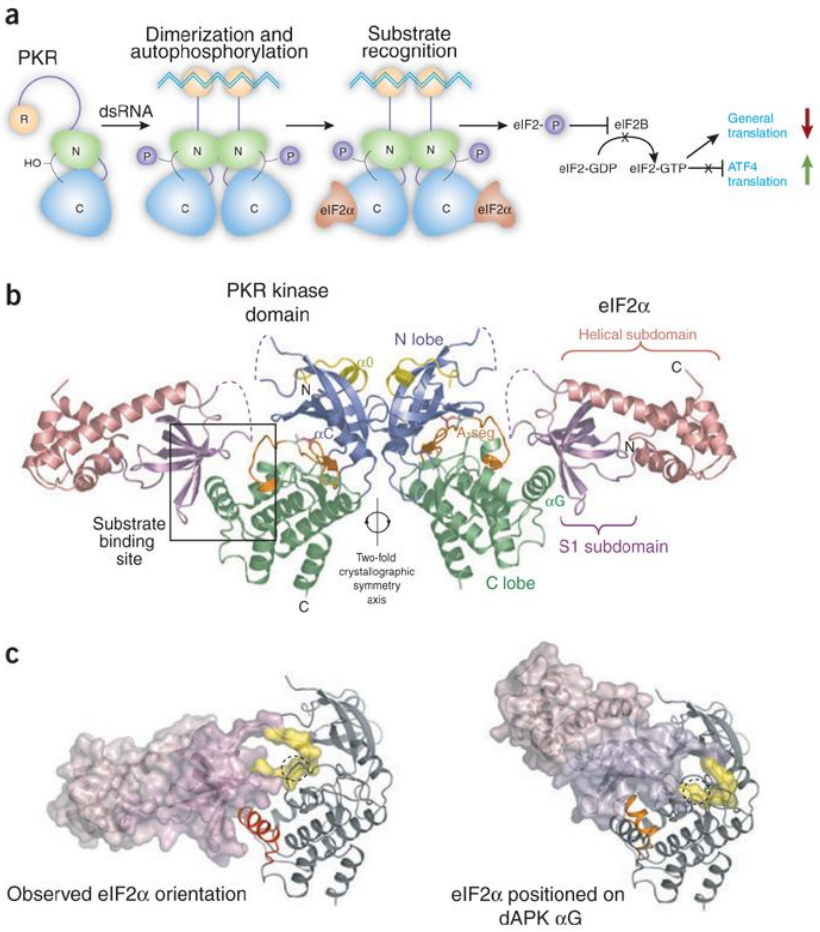


Figure 1.5. General structure of interferon inducible dsRNA activated Protein Kinase R.
(reprinted with permission from Hinnebusch et al., (2007). eIF2 kinases provide a new solution to the puzzle of substrate specificity. *Nature Structural and Molecular Biology*)[96]. When PKR is induced with interferons and activated with dsRNA, dsRNA binds N-terminal domain of PKR leading to its dimerization and auto phosphorylation from its C-terminus [93,94,96,115]. After auto phosphorylation, PKR binds and phosphorylated eIF2α through its C-terminal kinase domain whose phosphorylation leads to general translation inhibition and specific activation of ATF4 mRNA translation via eIF2α phosphorylation[96].

Additionally in another research it was showed that PKR knockdown by siRNA in human colon cancer cell lines resulted in resistance to therapeutic anti-cancer drugs and under DNA damaging conditions, PKR was found to be important for p53 activation[107, 109].

PKR is an important kinase in cellular defense and inflammation. It is necessary for mediating IRF1 interferon regulatory factor 1 (IRF1) induced cell growth inhibition[108]. Another important function of PKR is activation of NF-κB transcription factor via interacting and activating IKK[117] (Figure 1.6). PKR activates IKK complex via its kinase domain, as K296R (kinase inactive, dsRNA binding domain active) mutants cannot activate NF- κB pathway[109]. NF-κB transcription factor plays role in important cellular mechanisms including inflammation and apoptosis. It activates transcription of apoptosis inducing genes such as Fas TNF receptor superfamily member 6 ligand (FasL), Fas, Caspase1, IRF-1 and p53 and transcription of inflammatory cytokines[110]. Another factor in inflammation that PKR interacts, when inactive, is the signal transducers and activators of transcription protein (STAT-1)[111]. When PKR is induced with double stranded RNA (dsRNA) or interferons (IFNs), it dissociates from STAT-1, thereby leading to its inactivation[111]. Furthermore, PKR interaction with STAT-3 is also necessary for its activation by platelet derived growth factor. (PDGF)[112]. In their study Williams et al. showed that PKR is required for the activation of p38 mitogen-activated protein kinase (MAPK) and JNK by double-stranded RNA, lipopolysaccharide (LPS) and proinflammatory cytokine stimulation[113,115,116]. Recently it was also shown that, PKR kinase activity is necessary for activation JNK and p38 mitogen activated protein kinases[114].

PKR also plays a key role in apoptosis. It interacts with Fas (TNFRSF6)-associated via death domain protein (FADD), leading to the activation of caspase-8. Active caspsase 8 sequentially converts procaspase 3 into active caspase 3, which results in apoptosis. In addition, caspase 8

promotes the interaction of Bid with Bax, leading to cytochrome c release from mitochondria and with this cytochrome release apoptosome is formed. (Figure 1.6)[117].

There are several other known substrates of PKR for example IRS-1 and B56α subunit of protein phosphatase 2A[118]. It was shown that PKR phosphorylates IRS-1 on its 307th Serine residue, which competes with the tyrosine phosphorylation of IRS-1. Consequently, this can lead to insulin resistance through impairment of the insulin signaling pathway [119]. PKR also phosphorylates the catalytic subunit of protein phosphatase 2 A protein's (PP2A) B56α subunit, resulting in PP2A activation and potentially in translational arrest due to PP2A mediated dephosphorylation of eukaryotic initiation factor 4 E[118].

Like PERK, PKR activates microtubule-associated protein LC3 (light chain 3 (LC3)) through eif2α phosphorylation, leading to autophagy[124,126]. In a recent research, it was also shown that Signal transducer and activator of transcription 3 (STAT3) binds to PKR inhibits its activity and therefore leads to autophagy. First they showed that STAT3 inhibitors lead to autophagy. They observed that inhibition of STAT3 led to overexpression of PKR. They finalize their experiments with immunoprecipitation analysis showing specific PKR-STAT3 specific interaction leading to autophagy. From their results, they concluded that STAT3 through its SH2 domain binds to catalytic domain of PKR, on the site where eIF2α binds, and instead of general protein translational inhibition, leads to autophagy.

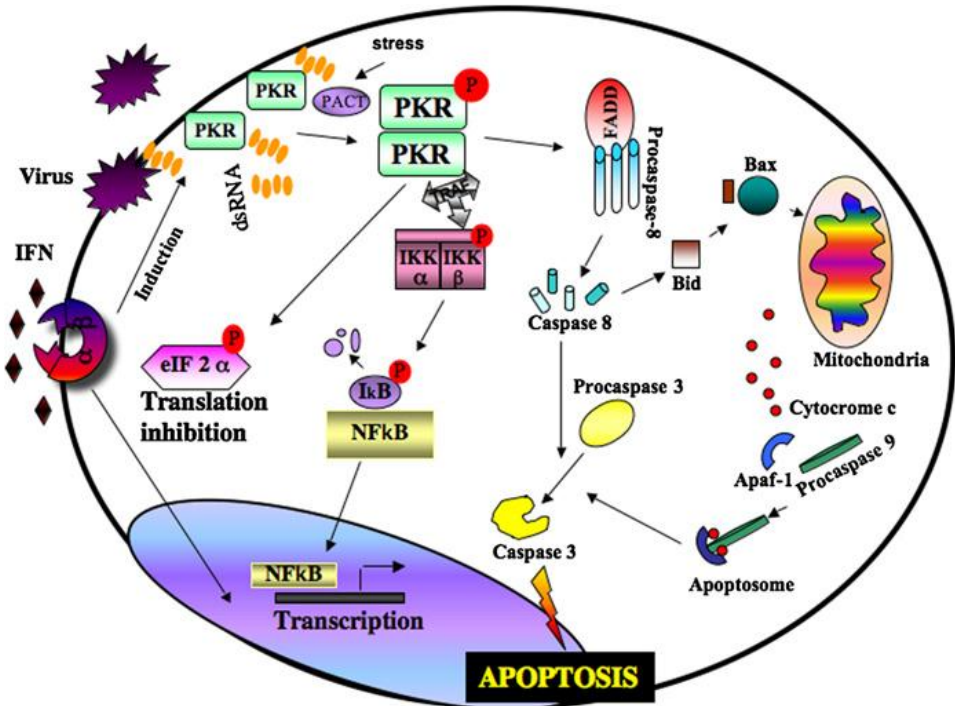


Figure 1.6. PKR induction, activation and its role in different cellular pathways.
(reprinted with permission from Esteban M., Meurs E.F., Garcia M.A. (2007) The dsRNA protein kinase PKR: Virus and cell control* *BIOCHIMIE*. 89 (2007) 799-811[117]). PKR is an interferon inducible, dsRNA[117] and PACT[98] activated kinase which activates many cellular signaling pathways. After it auto phosphorylates and activates itself, it phosphorylates eIF2α leading to general translational inhibition[50]. It interacts with TRAF complex and leads to phosphorylation of IKK complex, resulting in NFκB activation[50]. NFκB then translocates to nucleus and acts as transcription factor for expression of many inflammatory genes[83,130]. It also interacts with FADD leading to activation of caspase 8 and formation of apoptosome complex[50].

1.7. Role of Protein Kinase R in Inflammation and Metabolic Regulation

Nakamura et al., demonstrated recently that PKR can regulate the activity of inflammatory c-Jun terminal kinase[128]. JNK promotes the production of inflammatory cytokines like tumor necrosis α and interleukins (IL-1β, IL-6, and IL8) [121]. It has been shown that JNK regulates the transcription of these pro-inflamatory cytokines through interacting and modulating the activity of the transcription factor AP1. (Figure 1.7). Furthermore, through interacting and activating IKK complex, PKR activates another inflammatory transcription factor, NF-κB, which facilitates the synthesis of cytokines such as TNF-α, interleukins (IL-1β, IL-6, and IL8) as well as inducible enzyme cyclooxygenase 2 (Cox-2) that is highly abundant in sites of inflammation[121]. In addition PKR stimulates the activated transcription factor 2 (ATF-2),

which controls the transcription of pro-inflammatory gene expression. PKR also facilitates interferon regulatory factor protein 3 (IRF3) activation through mitochondrial antiviral signaling protein (MAVS, IPS1) adapter protein (Figure 1.7) [125]. Moreover, Lu et al. identified unique function of PKR in inflammasome activation. Inflammasome is a multiprotein, cytosolic caspase activating protein complex that leads to release of this caspase-1 activated cytokines including interleukin IL-1β, IL-18 and high-mobility group box protein (HMGB1)[122]. Through their experiments they showed that, stimulation of macrophages with inflammasome agonists leads to reduction in PKR auto phosphorylation. Furthermore, when PKR is inactivated by genetic deletion, silenced with shRNA or inhibited by specific PKR inhibitors, the secretion of IL-1β, IL-18 and HMGB1 were significantly reduced. Through immunoprecipitation experiments, they showed direct physical interaction of PKR with NOD-like receptor (NLR) family pyrin domain containing 3 (NLRP3), NLRP1, NLR family CARD domain containing protein 4 (NLRC4), absent in melanoma 2 (AIM2). They were able to reconstruct the inflammasome activity in a cell free system by combining recombinant NLRP3, PKR, pro-caspase-1 and apoptosis-associated speck-like protein containing a CARD (ASC) were present, NLRP3 inflammasome activity was reconstructed (Figure 1.7). Therefore it can be concluded that, besides its crucial role in metabolic homeostasis through regulating JNK activity and insulin signaling pathway, it also plays a critical role in innate immunity activating NLRP1, NLRP3, NLRC4 and AIM2 inflammasomes[122].

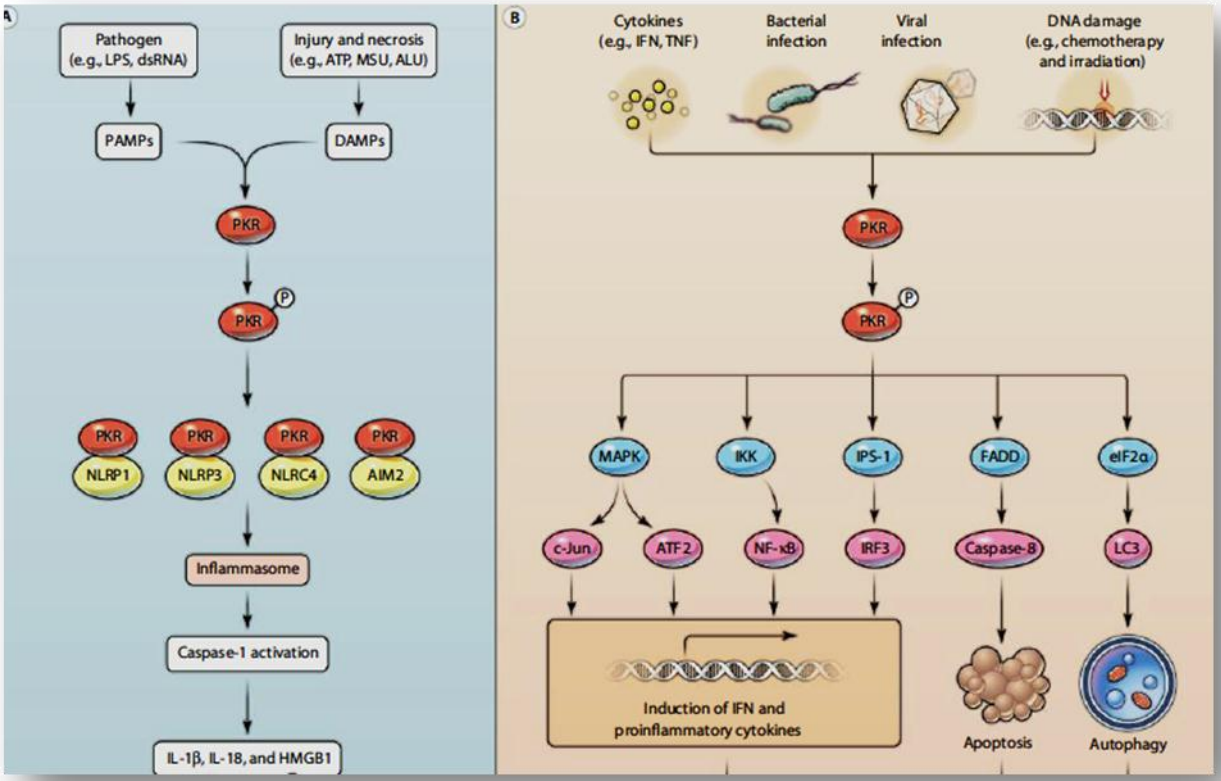


Figure 1.7. PKR related inflammation pathways.
(Reprinted with permission from Kang R.,Tang D. PKR-dependent inflammatory signals[124]. *Sci Signal*. 2012 Oct 23;5(247):pe47. doi: 10.1126/scisignal.2003511.) A) PKR senses and responds to PAMPs and DAMPs released through infection, injury and necrosis[124]. After activation, PKR interacts with NLRP1, NLRP3, NLRC4 and AIM2 inflammasomes resulting in caspase 1 activation and caspase-1 dependent secretion of inflammatory cytokines IL-1β, IL-18, and secretion of HMGB1[122]. B) Besides its role in inflammasome activation, PKR also has role in proinflammatory genes' expression induction, apoptosis and autophagy and it is also activated by bacterial and viral infections, cytokines and DNA damage[124].

Chronic inflammation is hallmark of metabolic diseases such as obesity, diabetes and insulin resistance. This low-grade inflammatory response in metabolic diseases has been referred to as metaflammation, due to its metabolic or nutrient origin[127]. Analogous to inflammasome a metaflammasome complex has been suggested to consist of some proteins which sense excess nutrients and metabolites and some proteins that mediate this inflammatory response to the metabolic stress. PKR may be viewed as one component of this hypothetical metaflammasome complex (Figure 1.8) [128].

PKR has been shown to respond to nutrients as well as ER stress that is induced by nutrient-excess and phosphorylates several proteins involved in metabolism such as JNK1[113,128] and IRS1[128,186.] Nakamura et al. showed that when PKR$^{-/-}$ mouse embryonic fibroblast cells (MEFs) were treated with saturated fatty acids (palmitate) ER stress was induced but not JNK. As a control, these cells were also treated with thapsigargin and the same results were observed leading to the conclusion that, PKR is a necessary, critical component for JNK activation in response to ER stress (whether induced by nutrients or chemicals)[128]. It is known that PKR and JNK can both phosphorylate IRS1 at 307th serine residue, blocking its tyrosine phosphorylation and lead to insulin resistance[128]. PKR not only regulates IRS1, but also IRS2, other critical component of insulin signaling pathway. Researchers demonstrated that PKR activates FoxO1 which leads to up-regulation of IRS2 gene expression[187]. The constitutively expressed FoxO1 is a hallmark in insulin resistance as this constitutive expression results in disruption of IRS–PI3K–Akt pathway finally leading to hyperglycemia and glucose intolerance[187].

Collectively these results demonstrate that both PKR and JNK are activated with excess nutrients and ER stress and control a combination of inflammatory and metabolic outcomes[128]. Whether PKR and JNK are part of a metabolic inflammasome like complex (metaflammasome) that senses the excess of nutrients and couples to inflammation needs to be rigorously tested in future experiments[128].

Finally, PKR activation increases in obese mice and PKR$^{-/-}$ mice exhibit increased insulin sensitivity, glucose tolerance and reduced fasting blood glucose levels, all signs of a better systemic metabolic homeostasis.[128,129]. It is thus now appreciated that PKR may play a critical role in obesity related insulin resistance and future efforts should be directed to finding optimal ways to block PKR-induced inflammation in chronic metabolic diseases.

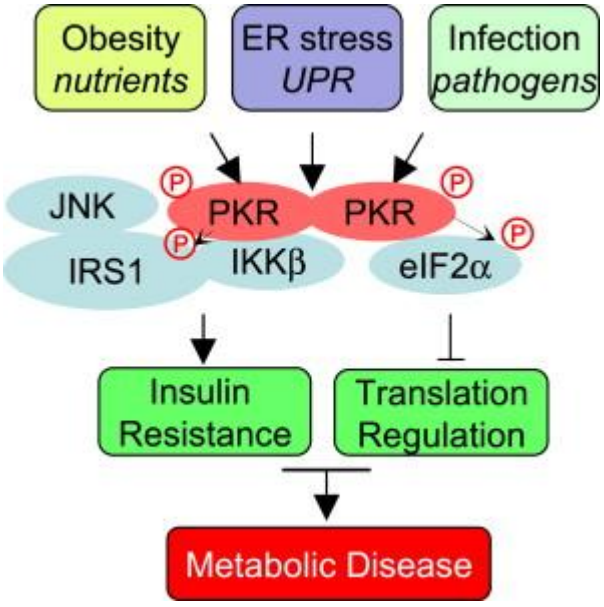


Figure 1.8. Hypothetical Metaflammasome complex and its components.
(Reprinted with permission from Nakamura T, Furuhashi M, Li P, Cao H, Tuncman G, Sonenberg N, Gorgun CZ, Hotamisligil GS. (2010). Double-stranded RNA-dependent protein kinase links pathogen sensing with stress and metabolic homeostasis. *Cell*. 2010 Feb 5;140(3):338-48. doi: 10.1016/j.cell.2010.01.001[128].)

1.8. The Relationship between Protein Kinase R and Endoplasmic Reticulum Stress

ER stress plays a major role in the pathogenesis of metabolic diseases such as obesity, insulin resistance, atherosclerosis and diabetes, at least partially by inducing JNK activation and eif2α phosphorylation[3,15]. As JNK is also known to be regulated by PKR, Nakamura et al asked whether there is a specific role of PKR in ER-stress related metabolic diseases. Dependent on thapsigargin and palmitate treatment, ER-stress was induced and simultaneously PKR activity was found to increase in response to ER stress both *in vitro* and *in vivo*[128]. PKR activity increased in primary isolated WT MEFs when they were treated with 500 μM palmitate for two hours or with 100nM thapsigargin for one hour. ER stress activation in these experiments was confirmed with increased PERK phosphorylation in wild type MEFs[128]. They also reported $PKR^{-/-}$ mice have reduced body weight, leptin, adiponectin and blood glucose levels compared to $PKR^{+/+}$ mice, when fed with high fat diet[128]. p-eIF2α, JNK1 activity, inflammatory cytokines remained lower in High Fat Diet fed $PKR^{-/-}$ when compared to

$PKR^{+/+}$ on the same diet. In summary, these findings show that PKR is activated by nutrients, ER stress and infectious pathogens and coordinates inflammatory and metabolic responses that are critical for metabolic health and disease[128].

1.9. Overview of Chemical Genetics and Its Applications

The major approach that will be introduced in the studies that make this thesis is chemical-genetics. Chemical genetics is a growing and vibrant field, which aims to study biological systems through modulation with small molecules[136]. The major goal when using this technique is to elucidate the molecular mechanisms, cellular pathways and discover novel substrates of specific kinases[136]. By combining chemistry with the traditional genetic approach, it can be used to analyze the function of a single protein in an organism and in real-time. Knock-down and knock-out processes are alternative approaches with some major disadvantages to chemical-genetics. First, when a gene is knocked down or knocked out, its whole function is inhibited. This could lead to unprecedented problems for example if the target protein encoded by knocked down gene is a scaffold protein that controls the interaction of many other proteins. One can envision that if a scaffold protein is knocked out, these association and expression of all the other interacting proteins could change. Therefore this knock-out may have indirect and direct effects in cell which could not be differentiated. To solve this problem, inducible knockouts and RNA based approaches have been widely used, however, these approaches do not initiate a response as quickly as seen in signaling pathways. Furthermore, these approaches may also lead to side effects such as off-target issues. For example, Causton et al., in their research showed that a temporal shift in yeast (from 25 ^{o}C and 37 ^{o}C) results in a significant change in the genomic expression[136]. In sum, classical genetic methods are not very suitable to study a protein's function partially because of their lack of temporal control. On the contrary, the purely chemical approach could bring

about the necessary temporal control over the protein. But this time, a major problem is lack of specificity or off-target issues. But a powerful tool called chemical genetics combines the ability for temporal control by chemistry with target specificity achieved by genetics[137]. This method is subdivided in two groups as forward or reverse chemical genetics[136]. In forward chemical genetics method, target identification is performed through phenotype screening (from phenotype to protein) whereas in reverse chemical genetics method target identification starts with protein, it is isolated and tested against many small molecules.

Protein kinases act as critical components through activating and maintaining of specific signaling pathways that have role in many cellular processes and diseases. Therefore finding targets for specific kinases can be a novel therapeutic approach for many diseases. For this purpose, recently chemical genetics method has begun to be highly utilized by researchers. To identify targets of specific protein kinases, analog sensitive alleles (ASKAs) and novel small compounds that regulate kinase activity of these mutants are used[138]. In order to obtain ASKA mutants, the candidate protein kinases are engineered (via site-directed mutagenesis) to have a slightly enlarged ATP binding cavity that can accommodate bulky ATP analogs[139]. The procedure involves determining the "gatekeeper residue" (bulky amino acid residues at conserved sites that interact with ATP) by first aligning the sequences of the ATP binding cavity of the candidate kinase is with previously identified ASKA mutants of other kinases and also modeling the fit between the ASKA mutant and the bulky ATP analog with the help of PyMoL and SwissProt softwares. Then the ATP binding pocket is mutated at the identified gatekeeper residue via standard site-directed mutagenesis protocol. These large amino acids (such as methionine and isoleucine) are basically converted into small side chain hydrophobic amino acids (such as glycine and alanine). Importantly, this mutation most of the time does not lead to a compromise in the phosphotransfer capacity of the kinase. When combined with bulky ATP analogs, the mutant can be temporally regulated. When combined with a tractable

ATPgS (that leads to thiophosphate transfer on potential substrates) the ASKA technology can be used for disoverin novel, direct targets of the kinases. This involves the mass spectroscopic identification of the thiophoshorylated substrates from *in vitro* and *in vivo* kinase assays using the ASKA mutants. For this method, ASKA mutants are incubated with N6-alkylated ATPγS and which thiophosphorylates its substrates. Then p-nitro benzyl mesylate (PNBM) alkylation is performed to create thiophosphate esters on substrates. These specific thiophosphate esters can be recognized by a rabbit monoclonal thiophosphate ester-specific antibody and immunoprecipitated. Then mass spectrometry analyses are performed to identify the novel substrates (Figure 1.9).

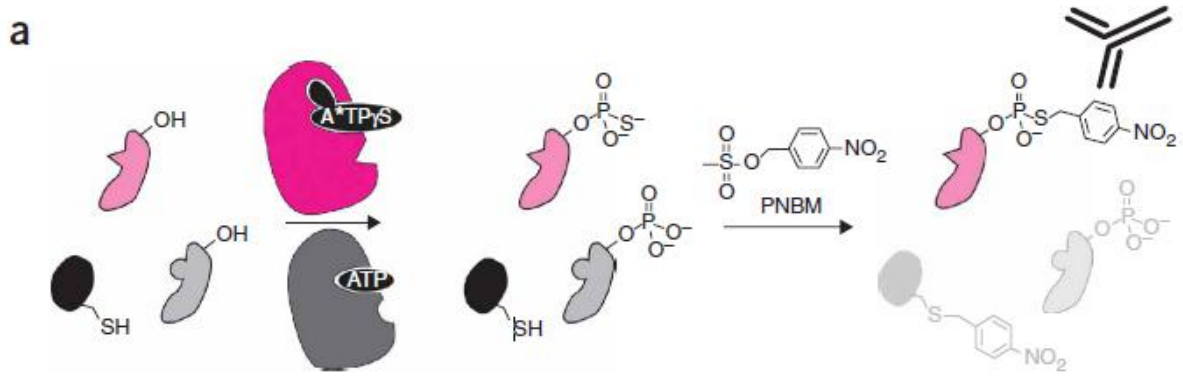


Figure 1.9. Strategy for labeling and recognizing individual kinase substrates through ASKA approach. (reprinted with permission from Allen JJ., Li M., Brinkworth CS., Paulson JL., Wang D., Hübner A., Chou WH., Davis RJ., Burlingame AL., Messing RO, Katayama CD., Hedrick SM, Shokat K.M. (2007). A semisynthetic epitope for kinase substrates. *Nat Methods*. Jun;4(6):511-6.© Nature Publishing Group, 2007 [140].)

The ASKA is depicted in pink, and the wild type kinase is depicted in grey. In the first step, analog sensitive kinase uses N6-alkylated ATPγS (A*TPγS) to thiophosphorylate its substrates. Then these thiophosphorylated substrates are alkylated with PNBM yielding formation thiophosphate esters on substrates that are recongnized with thiophosphate ester specific antibodies[140].

ASKA strategy is both suitable for *in vitro* and *in vivo* applications. It can be directly applied to cells via transient or stable transfections of ASKA mutants followed by treatment with ATP

analogs in cell culture. For *in vivo* applications, transgenic mice harboring ASKA mutation can be created and ATP analogs (only inhibitors like NAPP1 and NMPP1) can be given to them directly through intraperitoneal or subcutaneous injections or drinking water[140].

2. OBJECTIVES AND RATIONALES

Interferon inducible double strand RNA dependent protein kinase R (PKR) was shown to be activated by over nutrition, pathogens and endoplasmic reticulum (ER) stress[128]. It has been shown to play an important role in the pathogenesis of metabolic diseases likely through its functions in immune and metabolic responses[128]. When activated PKR phosphorylates eukaryotic initiation factor 2 alpha (eIF2α) leading to general translational inhibition and likely inhibition of insulin secretion, and phosphorylates IRS-1 at 307th Serine site leading to insulin resistance[128]. PKR also regulates activation of inflammatory kinases c-Jun N-terminal mitogen activated kinase (JNK) and nuclear factor kappa-light-chain-enhancer of activated B cells kinase NFκB, which results in the production of inflammatory cytokines. Very recently, another role of PKR in inflammation was demonstrated by researchers. PKR acts as a critical component in activation of inflammasomes through physically interacting inflammasome components[122]. Therefore it can be concluded that there is through its roles in inflammation, ER stress response, and by directly intercepting metabolic signaling PKR appears to be a kinase of interest in metabolic diseases research[128]. These properties of PKR show that its activity is important for integrating immune and metabolic response which is crucial in development and pathogenesis of metabolic diseases[128]. Genetic ablation of PKR in mice clearly demonstrated its central role in the development of insulin resistance and type 2 diabetes[128]. Therefore for metabolic diseases such as obesity, type 2 diabetes, insulin resistance and atherosclerosis, PKR or its substrates could become novel targets.

In my thesis study, I developed a novel chemical-genetic approach in order to temporally and specifically control the catalytic activity of PKR during lipid induced ER stress. I created a bulky ATP analog sensitive kinase allele (ASKA) mutant, which harbors a slightly enlarged ATP binding cavity that can accommodate the bulky analogs of inhibitory and activatory

nature. I planned to use this mutant to searches for novel PKR substrates and also to study PKR's role in mediating lipotoxic response. While it was not the scope of my master thesis study scope, this mutant will be useful in the future to generate a transgenic mouse model of PKR-ASKA mutant, allowing for specific modulation of PKR in chronic metabolic disease pathogenesis**.**

3. MATERIALS AND METHODS

3.1. MATERIALS

3.1.1. General Laboratory Agents

The reagents used during this thesis work were purchased from companies such as AppliChem (Dermstadt, Germany), SIGMA-ALDRICH (St.Louis, MO, USA), Merck (NJ, USA), Fisher Scientific (NJ, US), AMRESCO (Ohio, US). Ethanol, Methanol, Isopropanol were purchased from SIGMA-ALDRICH (St.Louis, MO, USA). DMSO was purchased from AppliChem (Dermstadt, Germany). HEPES and Tween were purchased from AMRESCO (Ohio, US). Agarose was purchased from PRONA (The European Economic Community). Ammonium persulfate was purchased from CARLO ERBA (Italy). Trizma Base and Sodium Chloride were purchased from SIGMA-ALDRICH (St.Louis, MO, USA). EDTA and DMSO were purchased from AppliChem (Dermstadt, Germany). Tryptone, Yeast extract, Bacto Peptone and Agar, were purchased from CONDA (Madrid, Spain). ECL Prime Western Blot Detection Kit was purchased from Amersham Pharmacia Biotech Company. (AMERSHAM, UK). 40% Acrylamide Reagent was purchased from Fisher BioReagents (Fisher Scientific, NJ, US). Ampiccilin, Kanamycin, Bradford and EGTA were purchased from SIGMA-ALDRICH (St.Louis, MO, USA). Plasmid mini- and midi-prep kits for plasmid isolation were purchased from Invitrogen by Life Technologies (Germany). Bovine Serum Albumin (BSA) was bought from Santa Cruz Biotechnology (Dallas, USA). Polyethylenimine was purchased from PolySciences (Warrington, PA, USA). PCR purification kit was purchased from MO-BIO Company (CarlsbadCA, USA) and Gel DNA Recovery Kit for DNA extraction from Agarose Gel was purchased from ZymoClean (Zymo Research Corporation, CA, USA). High Grade Pure Water was purchased from HyClone (Rockford, USA). Dulbecco's Phosphate Buffered Saline (DPBS) was purchased from GIBCO by Life Technologies (Invitrogen, Carlsbad, CA,

USA). PageRuler Plus Prestained Protein Ladder and Spectra Multicolor Broad Range Protein Ladder were purchased from Thermo Scientific. (Rockford, USA). TRIsure for RNA isolation was purchased from BIOLINE (A Meridian Life Science® Company). (London,UK).

3.1.2. Tissue Culture Materials and Reagents

Serological Pipettes were purchased from Sarstedt Inc (Newton, NC, USA) and Costar Corning Incorporated (NY, USA). Cell culture plates, dishes, scrapers and flasks were purchased from Greiner Bio One (Monroe, NC, USA) and Costar Corning Incorporated (NY, USA). Dulbecco's modified Eagle's Medium was purchased from Thermo Scientific HyClone (Rockford, USA) and Lonza (Basel, Switzerland). Rosswell Park Memorial Institute Medium (RPMI) was purchased from Lonza (Basel, Switzerland.) Trypsin, Fetal Bovine Serum (FBS) and L-Glutamine were purchased from GIBCO (Invitrogen, Carlsbad, CA, USA) and HyClone (Rockford, USA) respectively.

3.1.3. Bacterial Strains

DH5α *Escherichia Coli (E.Coli)* Strain was used.

3.1.4. Enzymes

KpnI-HF, XhoI, BamHI, HindIII-HF, EcoRI enzymes which were used in restriction enzyme digestion during this research, were purchased from New England Biolabs (Ipswich, MA, UK). NEB4 Buffer was brought with them and was used during these experiments. For cloning and colony PCR, Phire Hot Start enzyme purchased from (Thermo Scientific, Vantaa, Finland) and Taq Hot Start Enzyme purchased from Fermentas (Thermo Scientific, Vantaa, Finland) were used. Phusion Hot Start II High Fidelity enzyme was used for site-directed mutagenesis experiments and it was also purchased from FINNZYMES (Thermo Scientific,

Vantaa, Finland). For elimination of methylated bacterial plasmid, DpnI enzyme was used and it was bought from Thermo Scientific (Vantaa, Finland).

3.1.5. Nucleic Acids:

1 kb DNA ladder was purchased from Fermentas (Thermo Scientific Life Science Research, Rockford, USA). Mouse PKR commercial plasmid (PCMV-SPORT6) was obtained from Open Biosystems (Thermo Scientific, Rockford, USA). Human PKR commercial plasmid was a kind gift from Charles Samuel (UCSB, USA). pcDNA5F-FLAG and pcDNA3.1 (Hygromycin Resistant) plasmids were purchased from Invitrogen. (Carlsbad, CA, USA).

3.1.6. Oligo Nucleotides

Table 3.1. The primer list (sequences and Tm) used during this research.

Primer	Sequence	Tm
mPKR M328A Fwd	5’ GTGCCTCTTTATTCAAGCGGAATTCTGTGATAAAGG 3’	61.7
mPKR M328A Rev	5’ CCTTTATCACAGAATTCCGCTTGAATAAAG AGGCAC 3'	61.7
mPKR M328G Fwd	5’ GTGCCTCTTTATCAAGGGGAATTCTGTGATAAAGG 3’	61.7
mPKR M328 G Rev	5' CCTTTATCACAGAATTCCCCTTGATAAAGAGGCAC 3'	61.7
hPKR M366A Fwd	5’ GTGCCTTTTCATCCAAGCGGAATTCTGTGATAAAGG 3’	63.2
hPKR M366A Rev	5’ CCTTTATCACAGAATTCCGCTTGGATGAAAAGGCAC 3’	63.2
hPKR M366G Fwd	5’ GTGCCTTTTCATCCAAGGGGAATTCTGTGATAAAGG 3’	63.2
hPKR M3666G Rev	5’ CCTTTATCACAGAATTCCCCTTGGATGAAAAGGCAC 3’	63.2 °C

Primer	Sequence	Tm
hPKR Seq 1 Fwd	5' CAGCAGGTTTCTTCATGGAGGAAC 3'	58 °C
hPKR Seq 2 Fwd	5' TTATGAACAGTGTGCATCGGG G 3'	57.5 °C
hPKR Seq 3 Fwd	5' AGATCTTTGGCACCCAGATTTGAC 3'	57.5 °C
hPKR Seq 4 Fwd	5' AGAAGAGGCGAGAAACTAGACAAA G 3’	56.4 °C
hPKR Seq Rev	5' GCGGCCAATTGTTTTGCTTCC 3'	58.3 ºC
mPKR Seq1 Fwd	5' GACCTCCACATGACAGA GG 3'	54.7 ºC
mPKR Seq2 Fwd	5' AATATACCTTGGACGCCAGG 3'	54.4 ºC
mPKR Seq3 Fwd	5' GCTTTGGGCCTTATTCTAGC 3'	53.7 ºC
mPKR Seq Rev	5' ATGTAGTTACCATAGGGCAAGC 3'	54.6 ºC
Spliced XBP1 For	5' AGTTAAGAACACGCTTGGGAAT 3'	54.6 ºC
Spliced XBP1 Rev	5' AAGATGTTCTGGGGAGGTGAC 3'	56.6 ºC

3.1.7. Electrophoresis, Photograpy, Spectrophotometry and NanoDrop

Agarose Basica and Agarose Reducta were purchased from PRONA. Gel Electrophoresis tank and power supply were bought from Hoefer Inc (Holliston, MA). To determine nucleic acid concentration, NanoDrop (ThermoScientific, Wilmington, USA) was used. To determine protein concentration, Bradford Reagent (SIGMA-ALDRICH, Germany) was used and measurement was performed with spectrophotometer Beckman Du640 (Beckman Instruments Inc, CA, USA).

3.1.8. Electroporation

Electroporation apparatus, Transfection Systems Pipette Station &Pipette and Transfection System 10ul and 100 ul Kit were purchased from NEON Transfection System Invitrogen (Carlsbad, CA, US).

3.1.9. Antibodies

Table 3.2. The list of antibodies that were used during this research; including catalog number, working dilution and incubation times.

Antibody	Catalog Number and Company	Dilution	Incubation Time
Total PKR	B-10, sc6282 Santa Cruz	1:1000	1 hour at Room Tempeature
Anti-PKR (phospho T446) antibody	[E120] (ab32036) Abcam	1:1000 (For MEFs) 1:2000 (For HEKs)	1 hour at room temperature for HEKs Overnight incubation for MEFs.
Anti-IRE1 (phospho S724) antibody	(ab48187) Abcam	1:1000	1 hour room temperature
Beta Tubulin	(H-235): sc-9104 Santa Cruz	1:1000	1 hour at Room Temperature
Goat Anti-mouse IgG-HRP	sc2005, Santa Cruz	1:5000	1 hour at Room Temperature
Goat Anti-rabbit IgG-HRP	sc2004, Santa Cruz	1:5000	1 hour at Room Temperature
Rabbit (polyclonal) Anti-PKR [pT451] Phosphospecific Antibody.	44668G, Invitrogen	1:1000	Overnight Incubation 4°C
Anti-phospho-PKR (Thr451) Antibody |	07-886	1:1500	Overnight Incubation at 4°C
Thiophosphate Ester (WB version) antibody RabMAb®	2686-1 (Abcam)	1:5000	Overnight incubation at 4°C
IRE1α(14C10) Rabbit mAb #3294	Cell Signaling	1:1000	Overnight incubation at 4°C

3.2. SOLUTIONS AND MEDIA

3.2.1. General Solutions

50X Tris-Acetate-EDTA (TAE)

242 g Trizma Base, 37.2 g EDTA (Tritiplex 3) and 57.1 ml Glacial Acetic Acid was dissolved in 1 liter ddH_20 for 1 day and autoclaved before use.

10X Phosphate Buffered Saline (PBS)

80 g NaCl, 2 g KCl and 15.2 g sodium phosphate dibasic dehydrate and dissolve it in 1 liter ddH_20 and adjust pH to 6.8.

Ethidium Bromide

From 10 mg/ ml stock solution, 1X was used for Agarose Gel Electrophoresis. For 75 ml Agarose gel, 5 ul Ethidium Bromide was used.

3.2.2. Competent Cell Solutions

$CaCl_2$ solution

Stock Solutions

PIPES (Molecular weight = 307.37 g/mol) → pH: 6.4 0.1 M stored at Room Temperature

$CaCl_2$ (Molecular weight = 147 g/mol) → 2 M stored at Room Temperature

Glycerol → 87 % stored at Room Temperature

The preparation of Mixture for 500 ml from Stock Solutions

Mix:

15 ml 2 M $CaCl_2$ (60 mM in the mixture)

50 ml 0.1 M PIPES (10 mM in the mixture)

86.2 ml 87% Glycerol

Mixed with 348.8 ml ddH2O to make volume 500 ml. Then the mixture was autoclaved or filtered before use, it was kept at 4ºC.

3.2.3. Bacteria Solutions

Luria-Bertani Medium (LB medium)

10 g NaCl, 10 g Bacto-Tryptone, 5 g Bacto-yeast extract were dissolved with 1 liter ddH2O, and then was autoclaved.

Agar medium (For Agar plates)

100 mg/ml stock solution in ddH_2O (1000X). 2 g Ampicciline was dissolved in 20 ml autoclaved ddH_20. Working solution was 100 μg/ml (1X). It was filtered, aliquots were made and kept at -20ºC.

Kanamycin (1000X, 50 mg/ml)

1 g Kanamycin was dissolved in 20 ml autoclaved ddH_2O, then was filtered, aliquots were made and kept at -20ºC.

Glycerol Stock Solution

50% Glycerol→ 10 ml Glycerol and 10 ml autoclaved ddH_2O were mixed and filtered.

500 μl 50 % Glycerol was mixed with 500 μl bacteria culture, it was vortexed and kept at -80 ºC.

3.2.4. Tissue Culture Solutions

DMEM/RPMI medium

The complete medium had 10 % Fetal Bovine Serum (FBS) , 1 % L-Glutamine and it was stored at 4 ºC.

1X Phosphate Buffered Solution

It was purchased from GIBCO (Invitrogen).

0.25% Trypsin-EDTA (1X), Phenol Red

It was purchased from GIBCO (Invitrogen).

L-Glutamine (200 mM)

It was purchased from GIBCO (Invitrogen).

Fetal Bovine Serum (FBS)

It was purchased from GIBCO (Invitrogen).

HEPES Buffer (1M)

It was purchased from SIGMA Life Science.

Albumin, from Bovine Serum (Fatty Acid Free)

It was purchased from SIGMA Life Science.

Thapsigargin

300 μM Thapsigargin solution was prepared in DMSO as stock solution. Working solution was 300 nm.

4-Amino-1-tert-butyl-3-(1'-naphthyl)pyrazolo[3,4-d]pyrimidine (NaPP1) , 4-Amino-1-tert-butyl-3-(1'-naphthylmethyl)pyrazolo[3,4-d]pyrimidine (1-NMPP1)

NaPP1 and 1-NM-PP1 were purchased from Taconic Artemis. 50 μM NaPP1 and 50 μM 1-NM-PP1 were dissolved in DMSO. Working solution was 40 μM.

Palmitic Acid (palmitate)

500 mM palmitate was dissolved in absolute ethanol. As working solution, 500 μM and 1000 μM palmitate were dissolved in filtered 1 % BSA (Fatty acid free) containing RPMI. It was heated up to 55ºC, for complete dissolution.

IRE1 RNAse Inhibitor

IRE1 RNAse inhibitor (N-[(2-Hydroxy-1-naphthalenyl)methylene]-2-thiophenesulfonamide) (STF-083010) was purchased from SIGMA-ALDRICH (St.Louis, MO, USA). It was dissolved in appropriate volume of DMSO to make final concentration 30 mM. The working concentration was 300 μM.

HBS Buffer for Electroporation (Instead of R Buffer)

Recipe is obtained from Batu Erman (Sabancı University).

21 mM commercial HEPES (pH; 7.05)

137 mM NaCl

5 mM KCl

0.7 mM Na_2HPO_4

6 mM Glucose

Solution was filtered before use and is kept at 4°C.

3.2.5. Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western Blotting Solutions

For this research, Mini PROTEAN Tetra Cell Western Blotting System (Bio Rad, CA, USA) was used for the Western Blot Experiments. The gels used were prepared as 10% and 12 % Resolving and 5 % Stacking Gels.

20% Ammonium persulfate

1 g Ammonium persulfate was dissolved in 5 ml ddH_2O.

1.5 M Tris-HCl (pH: 8.8)

54.45 g Tris Base (Trizma) was dissolved in 150 ml ddH_2O, pH was adjusted to 8.8 with 1 N HCl and volume was made up to 300 ml with ddH_2O. It was stored at 4 °C.

1.5 M Tris-HCl (pH: 6.8)

12 g Tris Base (Trizma) is dissolved with 60 ml ddH_2O, pH was adjusted to 6.8 with 1 N HCl and volume was made up to 100 ml with ddH_2O.

10% SDS Solution

10 g SDS was dissolved in 100 ml ddH_2O.

10% Resolving Gel

40% Acrylamide mix, 1.5 M Tris-HCl (pH: 8.8), 10% SDS and 20% Ammonium persulfate, 0.08% TEMED completed with suitable volume of ddH_20.

5 % Stacking Gel

40% Acrylamide mix, 1.5 mM Tris-HCl (pH: 6.8), 10% SDS and 20% Ammonium persulfate, 0.1% TEMED completed with suitable volume of ddH_2O.

5X Running Buffer

124 mM Tris Base (15 g/liter), 960 mM Glycine (72 g/liter) and 17.4 mM SDS (5 g/liter) were dissolved in suitable volume of ddH_2O. Working Solution was 1X.

5X Transfer Buffer

64.4 mM Glycine (14.5 g/liter), 80 mM Tris Base (29 g/liter), 2.14 mM (1.85 g/liter) SDS, were dissolved in suitable volume of ddH_2O. Working solution was 1X. (600 ml ddH_2O, 200 ml 5X Transfer Buffer, 200 ml Methanol)

Phospholysis Buffer

50 mM HEPES (pH: 7.9), 100 mM NaCl, 4 mM Tetra Sodium pyrophosphate ($Na_4P_2O_7$), 10 mM EDTA, 10 mM NaF, 1% Triton, 2 mM Sodium orthovanadate (Na_3VO_4), 1 mM phenylmethanesulfonylfluoride (PMSF), Phosphatase Inhibitor Cocktail 3 (Sigma, P0044) and 1X Protease Inhibitor Cocktail (10µM/ ml)

1M HEPES (pH: 7.9)

23.83 g HEPES was dissolved in 80ml ddH_20, Adjust pH 7.9 with KOH, Complete volume to 100ml.

10X Tris Buffered Saline (TBS)

1.5 M NaCl (87.76 g), 100 mM Trizma Base adjust pH 8.0 with 1N HCl complete volume to 1 liter with ddH_2O

1X-TBS-T

450 ml ddH_2O, 50 ml TBS, 500 µl Tween were mixed with stirrer.

Blocking Solution (5 % Bovine Serum Albumin in TBS-T)

2.5 g Bovine Serum Albumin (Santa Cruz) was dissolved in 50 ml 1X TBS-T.

Blocking Solution (5 % Milk in TBS-T or PBS-T)

2.5 g milk powder was dissolved in 50 ml TBS-T or PBS-T

10X Phosphate Buffered Saline

80 g NaCl, 2 g KCl, 15.2 g $Na_2HPO_4.2H_2O$ were dissolved in 1 liter ddH_2O and pH was adjusted to 6.8.

1X PBS-T

450 ml ddH_2O, 50 ml PBS, 500 µl Tween were mixed with stirrer

3.2.6. In Vitro Kinase Assay Solutions

PKR Kinase Buffer

15 mM commercial Hepes (300 µl) (pH7.4), 10 mM $MgCl_2$ (200 µl), 40 mM KCl (800 µl) were dissolved in 18.7 ml nuclease free water and the solution is filtered before use.

3.3. METHODS

3.3.1. The use of Software Programs

3.3.1.1. Pymol

3D hypothetical model of Protein Kinase R (PKR) was created with SwissModel Tools program on web and then it was aligned with other kinases (whose crystal structures were solved and whose ATP binding pockets were identified previously) in order to find the conserved ATP binding residues. Then ASKA mutants of these were created with PyMOL program. With help of PyMOL program, wild type and ASKA mutants of PKR were aligned with specific kinases in a complex with ATP or 1 NM-PP1.

For these alignments 2 proteins were used. The first one is GCN2. (Crystal Structure of eIF2alpha Protein Kinase GCN2: Wild-Type Complexed with ATP. RSCB ID:1ZYD (http://www.rcsb.org/pdb/explore/explore.do?structureId=1ZYD) and Crystal Structure of

eIF2alpha Protein Kinase GCN2: R794G Hyperactivating Mutant Complexed with AMPPNPRSCB ID: 1ZY5. (http://www.rcsb.org/pdb/explore/explore.do?structureId=1ZYD) Padyana et al., 2005) The second one is TgCDPK1. (Calcium-Dependent Protein Kinase 1 from Toxoplasma gondii (TgCDPK1) in complex with bumped kinase inhibitor NM-PP1. http://www.rcsb.org/pdb/explore/explore.do?structureId=3I7B) RSCB ID: 3I7B. Ojo et al., 2010). These GCN2-ATP and TgCDPK1 complexes were aligned with PKR WT and PKR ASKA mutants via using PyMOL Software program in order to show determine the exact ATP binding cavity and show that NMPP1 can perfectly fit in mutants' ATP pocket whereas it cannot fit in wild type form.

3.3.1.2. BioLign

BioLign 4.0.6 software program was used for sequence analysis of PKR wild type and mutants, according to manufacturer's recommendations. (http://en.bio-soft.net/dna/BioLign.html). The sequences for PKR wild type and mutant forms were in chromatogram format from the sequencing machine.

3.3.1.3. ClustalW2

Homo Sapiens (NP_001129123.1) and *Mus Musculus (*NP_035293.1) PKR sequences were aligned with *Mus Musculus* PERK (NP_034251.2), *Homo Sapiens* PERK (NP_004827.4), *Mus Musculus* IRE1 (NP_036146.2), *Homo Sapiens* IRE1 (NP_001424.3), *Mus Musculus* ERK1 (NP_001157143.1), *Homo Sapiens* ERK1 (NP_002736.3), *Mus Musculus* JNK2 (NP_001157143.1), *Homo Sapiens* JNK2 (NP_001128516.1), *Mus Musculus* JNK1 (NP_057909.1) and *Homo Sapiens* JNK1 (NP_002741.1) in order to compare and analyze the conserved ATP binding pocket sequences (Clustal W2, EMBL-EBI; http://www.ebi.ac.uk/Tools/msa/clustalw2/).

3.3.1.4. NCBI (National Center Biotechnology Information)

NCBI website was used in order to reach nucleotide and aminoacid (FASTA) sequence information of PKR.

(http://www.ncbi.nlm.nih.gov/gene?term=(eif2ak2[gene])%20AND%20(Homo%20sapiens[orgn])%20AND%20alive[prop]%20NOT%20newentry[gene]&sort=weight)

(http://www.ncbi.nlm.nih.gov/gene?term=(eif2ak2[gene])%20AND%20(Mus%20musculus[orgn])%20AND%20alive[prop]%20NOT%20newentry[gene]&sort=weight)

3.3.1.5. NCBI Open Reading Frame (ORF) Finder

In order to find Open Reading Frame of interested kinase PKR for designing primers, NCBI ORF finder tool was used. (*Mus Musculus* PKR ORF length: 1547 bp, *Homo Sapiens* PKR ORF length: 1656 bp). (http://www.ncbi.nlm.nih.gov/projects/gorf/).

3.3.1.6. PrimerX Tool (For Site Directed Mutagenesis Primers Design)

Mouse PKR (M328A and M328G), Human PKR (M366A and M366G) primers were designed via using PrimerX Tool according to manufacturer's instructions. (http://www.bioinformatics.org/primerx/)

3.3.1.7. NEBcutter V2.0

NEBcutter tool was used in order to find restriction enzymes that do not cut open reading frame of PKR, to use them for cloning process.

3.3.2. Molecular Cloning Methods

3.3.2.1. Competent Cell Preparation

In order to make competent cells, at first, a BIOLABS NeB-5-alpha High efficiency competent cell was inoculated into (without antibiotic) 50 ml Luria-Bertani medium (LB) o/n in shaker incubator. (37°C, 225 rpm). Then 200 µl of this culture was incoulated in 100 ml

LB for dilution. After 1 hour growth, the optical density (OD) of the culture was measured via spectrophotometer as previously explained, and until the culture OD reached 0.345 OD. Then the culture was divided into 2x50 ml falcons on ice and then they were waited on ice for 10 minutes. After centrifugation at 1600 g (4°C) for 20 minutes, with ice cold $CaCl_2$ solution was resuspended. Then the suspension was centrifuged at 1100 g (4°C) for 10 minutes, then the pellet was resuspended in 10 ml ice-cold $CaCl_2$. After these steps, the solution was incubated on ice for 30 minutes, and centrifuged again at 1100 g (4°C) for 10 minutes. Finally the bacterial pellet was dissolved with 2 ml ice-cold $CaCl_2$ solution and divided in 1.5 ml prechilled labeled eppendorf tubes on ice (50 µl/ tubes). Then these eppendorf tubes with competent cells were frozen with liquid nitrogen and stored at -20 °C.

3.3.2.2. Transformation

In the beginning, 50 ul competent cell (1 eppendorf) was thawed on ice and 2 ul (<100 ng) plasmid was added gently in this eppendorf and tapped gently twice. Then, this was waited on ice for 30 minutes. After 30 minutes, heat shock was performed at 42 °C for 30 seconds, followed by 10 minutes incubation on ice. After this incubation, 200 µl LB was added in mixture near flame, and was put in shaker incubator for 1 hour. (at 37°C, 225 rpm). At the same time, Agar plates with Ampiccilin were pre-heated in bacteria incubator for 1 hour. After the incubation, the mixture was spreaded on two agar plates (150 µl and 50 µl), and left in bacteria incubator between 16-18 hours for colony growth.

3.3.2.3. Selection of Colonies From Agar Plate and Growth of them in LB for Midi-prep and Mini-prep

One single colony was chosen with 20 µl tip; was put in 10 ml LB in a falcon tube with 10 µl Ampiccilin for mini-prep, and was put in 100 ml LB in Erlenmeyer with 100 µl Ampiccilin

for midi-prep. Then the mixtures were grown overnight in shaker incubator (16 hours, 37 °C, 225 rpm).

3.3.2.4. Glycerol Stock Preparation

500 µl growth bacteria in LB with 1X Ampiccilin was mixed 500 µl with 50% filtered glycerol solution. Then it was frozen and was kept at -80°C for use.

3.3.2.5. Restriction Enzyme Digestion

mPKR WT plasmid insert was in PCMV-SPORT6 vector between NotI and SalI restriction. To confirm this insert which is approximately 2300 kb, approximately 1000 ng plasmid was cut with NotI and SalI enzymes sites for 4 hours at 37°C as recommended. After this experiment, samples were run on the 1% Agarose gel and visualized. Then the open reading frame of mPKR which is 1547 bp was cloned with KpnI and XhoI cloning primers in order to ligate them in mammalian expression vector pcDNA5F which has a FLAG tag. For this purpose cloning PCR was performed. After this cloning PCR, PCR purification step was applied, in order to obtain pure DNA from the PCR without chemical contaminations. After this process, restriction enzyme digestion was performed for both backbone vector (pcDNA5F) and mPKR insert. Finally, ligation, transformation and colony PCR were performed in order to confirm that mPKR insert was cloned in pcDNA5F successfully. For human PKR, insert is 1656 bp and it was in mammalian expression vector pcDNA6. In order to confirm that insert was in this plasmid, approximately 1000 ng plasmid was cut with BamHI-HF (High Fidelity) and XhoI (High Fidelity) enzymes from NEB Company for 4 hours. Then samples were run on 1 % Agarose gel, and the results were visualized. After it was confirmed that desired hPKR insert was in pcDNA6, this insert was cloned in pcDNA5F (FLAG) and pcDNA3.1 (Hygromycin Resistant) mammalian expression vectors with HindIII and XhoI enzymes.

3.3.2.6. Agarose Gel Electrophoresis

In order to visualize DNA samples and PCR products, Hoefer SUB10 Mini Plus submarine electrophoresis system (Holliston, MA, USA) and Hoefer PS300B 300 Volt Power Supply were used. For gel preparation 1X TAE buffer was used and gel concentration changed between 0.5%-1% depending on the size of DNA products and the aim of the experiment. For gel extraction process, 0.5 % gel was prepared and Agarose Reducta (instead of Basica) was used. To prepare the gel, adequate amount of agarose was dissolved in 1X TAE buffer and then boiled in microwave until agarose was completely dissolved. Then when the gel cooled down Ethidium Bromide was added in the ratio 10 mg/ ml. After that gel was poured and DNA samples were mixed with 10X Bromophenol Blue DNA Loading Dye (1:10 Ratio), and they were loaded into gel. Samples were run at room temperature for 1 hour at 90V, except for gel extraction which was at 100V for 40 minutes. Fermentas #SM0311 Gene Ruler 1 kb Ladder was used for visualization and size detection of DNA fragments. Finally the gels were visualized under UV light.

3.3.2.7. Site Directed Mutagenesis

For Site Directed Mutagenesis, first mutations sites (hPKR M366A, M366G and mPKR M328A, M328G) were determined via using PyMOL Software and they were program primers specific to mutagenesis site were designed as forward and reverse primers via using PrimerX tool. (http://www.bioinformatics.org/primerx/)

Table 3.3. Human PKR and Mouse PKR Primers.

Primer	Sequence	Tm
mPKR M328A Fwd	5’ GTGCCTCTTTATTCAAGCGGAATTCTGTGATAAAGG 3’	61.7
mPKR M328A Rev	5’ CCTTTATCACAGAATTCCGCTTGAATAAAG AGGCAC 3'	61.7
mPKR M328G Fwd	5’ GTGCCTCTTTATCAAGGGGAATTCTGTGATAAAGG 3’	61.7
mPKR M328G Rev	5' CCTTTATCACAGAATTCCCCTTGATAAAGAGGCAC 3'	61.7
hPKR M366A Fwd	5’ GTGCCTTTTCATCCAAGCGGAATTCTGTGATAAAGG 3’	63.2
hPKR M366A Rev	5’ CCTTTATCACAGAATTCCGCTTGGATGAAAAGGCAC 3’	63.2
hPKR M366G Fwd	5’ GTGCCTTTTCATCCAAGGGGAATTCTGTGATAAAGG 3’	63.2
hPKR M3666G Rev	5’ CCTTTATCACAGAATTCCCCTTGGATGAAAAGGCAC 3’	63.2

Site Directed Mutagenesis PCR reactions were first prepared in 10 µl total volume in order to confirm the reaction is working, then were prepared in 50 µl total volume. For 10 µl reactions, approximately 10 ng, for 50 µl reactions approximately 50 ng DNA template was used. The final concentration of site directed mutagenesis primers in reaction is 0.3 µM. 3% DMSO and 200 µM dNTP as well as 5x Phusion Buffer were added for each sample into reaction mix. Phusion Hot Start II, High Fidelity DNA polymerase enzyme, which has proofreading activity,

was used in these reactions, in order to reduce the risk of mutations occurring in the template DNA. (2 U/µl 0.3 µl→50 µl reaction) Reactions started with initial denaturation step 98°C for 5 minutes. Then 25 cycles of amplification was performed at 98°C 35 seconds, (61.5 °C for hPKR and 63°C for mPKR) for 30 seconds and 72°C for 4 minutes. (Approximately 30 s/kb). Final extension was at 72 °C for 10 minutes. After that, 5 µl of the reaction was run on gel, in order to confirm reaction worked and specific product was obtained.

3.3.2.8. DpnI Digestion

When it was confirmed that expected product was obtained with Site Directed Mutagenesis Reaction, 1 µl of DpnI enzyme (20u/ µl) purchased from NEB (Ipswich, MA) was added into 45 µl reaction in order to eliminate template DNA which was methylated via cutting it. This reaction took place at 37°C overnight in incubator. Then in order to inactivate the enzyme, heat inactivation process was performed via waiting the samples at 80°C for 20 minutes. Finally 2 µl of the reaction was transformed into competent cells.

3.3.2.9. PCR Purification

For mPKR experiments, after cloning PCR with KpnI Forward and XhoI reverse primers, before restriction enzyme digestion, PCR purification experiment was performed for DNA clean up from PCR. For this experiment PCR purification Kit from MO-BIO Company (CarlsbadCA, USA) was used and the experiment was performed according to manufacturer's instructions written on kit protocol.

3.3.2.10. Gel Extraction.

After PCR purification, cloned and purified mPKR DNA was cut with Restriction enzymes as well as vector backbone (pcDNA5F). Then they were run on 0.5% Agarose Reducta Gel for 40 minutes at 100V, after that insert and backbone band were sliced under UV light with razor

blades and Gel Extraction experiment was performed with ZymoClean Gel DNA Recovery kit according to manufacturer's instructions written on the kit protocol. Finally, samples were eluted in 8 µl nuclease free ddH_2O and their concentrations were measured with NanoDrop.

3.3.2.11. Ligation

To ligate purified hPKR and mPKR inserts into mammalian expression vectors, first backbone and insert were cut with restriction enzymes for 4 hours at 37°C, gel extraction was performed. The inserts were ligated to backbone (1(plasmid): 7(insert)) ratio with T4 Ligase enzyme (Fermentas) (0.5 µl for 10 µl reaction), T4 Ligase Buffer (1 µl for 10 µl reaction) and nuclease free water (8 µl) and the samples were incubated overnight at 16- 18°C, next day they were transformed into competent cells.

3.3.2.12. Colony PCR

Single colonies were chosen with tips of pipettes from the transformation plates and then pcr tubes were swiped with these tips. After that these tips were put in LB, for further growth of the bacteria in order to make mini prep next day depending on Colony PCR results. PCR mixture was prepared as it follows: 1 µl of the 5X Phire enzyme buffer, 0.2 µl forward and reverse primers, 0.3 µl DMSO, 0.8 µl MgCl2, 0.2 µl dNTP, 0.2 µl Phire Hot Start Enzyme (Finnzymes) in nuclease free water (total reaction volume was 10 µl). As Cloning PCR primers were used in this reaction in order to confirm that insert was perfectly ligated in backbone, PCR program used in this reaction the was same program with the Cloning PCR program.

3.3.2.13. Miniprep

For miniprep, Invitrogen PureLink Quick Plasmid Miniprep Kit (Carlsbad, CA, USA) was used according to manufacturer's recommendations written on the kit protocol. In the elution

step, isolated DNA was dissolved in approximately 50 µl nuclease free water and its concentration was measured with NanoDrop.

3.3.2.14. Midiprep

For midiprep, PureLink® HiPure Plasmid Midiprep Kit (Invitrogen, Carlsbad,CA) was used according to manufacturer's recommendations written on the kit protocol. In the elution step, isolated DNA was dissolved with suitable volume of nuclease free water (100-200 µl) depending on pellet size and its concentration was measured with NanoDrop.

3.3.3. Tissue Culture Methods

3.3.3.1. Cell Lines Used in This Research and Their Growth Conditions

Cell culture mediums used for cell lines in this research were DMEM or RPMI. 293 Human Embryonic Kidney (293HEK) cells, $IRE^{-/-}$ and $PKR^{-/-}$ Mouse Embryonic Fibroblast (MEF) cell lines were grown with DMEM whereas THP1 and RAW cells were grown with RPMI. Both DMEM and RPMI mediums contained 10% Fetal Bovine Serum (FBS) and 1X-L-Glutamine. All cells were grown in 37°C incubators. They were checked regularly and they were seeded onto new plates when they reached high confluency.

3.3.3.2. Passage of the Cell Lines

First cells medium were sucked and they were washed with PBS. Then for 96 mm plate, 1 ml Trypsin-EDTA was added and the plate was put in incubator for 3-5 minutes. (10% of the total volume).When cells were detached, they were collected in complete medium (DMEM) with serological pipettes and were put in 15 ml or 50 ml falcons. Then cells were pipetted gently and they were re-seeded on new plates according to appropriate dilutions.

3.3.3.3. Freezing and Thawing of the Cells

When cells reached 70-80 % confluency in plates, first their mediums were sucked. Then they were washed with PBS and for detachment suitable volume of Trypsin-EDTA was added in these plates, then cells were collected in complete medium and were put in falcons. The collected cells were then centrifuged for 7 minutes at 1000 rpm. During this step, freezing mix which contains 50 % FBS, 40 % DMEM and 10 % DMSO was prepared. The medium of the centrifuged cells were sucked and the pellet was resuspended with this freezing medium. 1 ml cell-freezing mix solution was added in each cryovial tube and these cryovial tubes were put in -80°C freezer. One day later, one or two cryovials were placed in liquid nitrogen tank.

For thawing of the frozen cells, the vials that taken from -80°C were incubated in the water bath at 37°C for few minutes until they completely thawed. Then 1 ml pre-heated complete medium was added into each cryovial, meanwhile 5 ml preheated complete medium was poured in each 25 cm^2 flask that have filtered cap. Then this 2 ml cell and complete medium mixed solution rom each cryovial tube was added in each flask. (For PKR -/- MEFs, Complete medium with 20% FBS was used as these cells grew slowly.). Then each flask was placed in incubator and they were reseeded in 96 mm plates when they become confluent.

3.3.3.4. Transient Transfection of Cells

For transient transfection of 293HEK cells, Polyethylenimine (PEI) transfection was performed and for Mouse Embyonic Fibroblast Cells (MEFs) Electroporation method was performed.

(http://www.invitrogen.com/site/us/en/home/Products-and-Services/Applications/Cell-Culture/Transfection/Transfection___Selection-Misc/Neon-Transfection-System.html?CID=fl-neon)

3.3.3.4.1. Polyethylenimine (PEI) transfection

For transfection of plasmids into 293HEK cells Polyethylenimine (Cat #23966) from Polysciences. PEI (1ug/µl) was dissolved in %100 absolute EtOH to make a stock solution. For this type of transfection, cells were seeded in 6-well plates one day before transfection at 40% confluency. The next day, cells in these wells reached 80% confluency and they were used for transfection. For 6-well based transfection, 250 µl PEI-DNA mixtures (2 µg DNA and 4 µl PEI for each well (1:2 ratio DNA:PEI)) were prepared for each well. Then these mixtures were waited for 15 minutes at room temperature. After that step, 750 µl 2% DMEM were added on these 250 µl PEI-DNA mixtures to reach final total volume as 1 ml for each well. Finally, old mediums of these wells from 6-well plate were sucked and these 1 ml mixtures were added to each well. One day later, PEI transfection medium was replaced with fresh complete medium.

3.3.3.4.2. Transfection by the Electroporation Method

For this type of transfection method, Transfection Systems Pipette Station & Pipette and Transfection System 10ul and 100 ul Kit was purchased from NEON Transfection System Invitrogen (Carlsbad, CA, US) was used. In the beginning, optimization experiments (for 24 well plates) were performed in order to find the most appropriate voltage, pulse width and number for each cell lines in this research. In addition, the manufacturer's recommendations were also considered while searching for the suitable conditions. Finally the optimal conditions were determined for MEF cell lines as shown in Table 3.4.

Table 3.4. Optimal conditions for electroporation of plasmid DNA to MEFs with Neon Electroporation Kit.

The cell type	Pulse Voltage (V)	Pulse Width	Pulse Number	Cell Density	The amount of DNA	Tip Type	The amount of DNA	Tip Type	Media Type
MEFs	1050	30	2	5×10^5	2 µg	10 µl	4 µg	100 µl	DMEM

For electroporation experiment, at first one day before the electroporation cells were seeded in 96 mm plate at 40% confluency. Next day, their confluency was approximately ~%70-80 and they were ready for transfection. Then they were sucked, washed with PBS, were detached with Trypsin-EDTA and collected in complete medium and were quantified with hemocytometer in order to obtain 5×10^5 cells for each well. Then suitable amount of cells for transfection were collected with complete medium and were centrifuged at 500 rpm for 4 minutes. Then complete medium was sucked and cells were resuspended with PBS and cells were centrifuged at 500 rpm for 4 minutes. This cell washing step with PBS was performed twice. Finally the pellet was dissolved in HBS or R Buffer. (For each transfection 100 µl R buffer was used for 100 µl tip and 10 µl R buffer was used for 10 µl tip). Before starting the experiment, 2 ml complete medium was added in each well in 6 well plate and the plate was pre-heated in incubator during the experiment. In the end, electroporation device was used according to manufacturer's recommendations with suitable conditions described in the upper table. After electroporation, electroporated cells were divided into each well that had 2 ml preheated complete medium.

3.3.3.5. Treatment of Cells

During this research, Thapsigargin, Palmitate, NaPP1, 1-NMPP1, IRE1 RNAse inhibitor treatments were applied to 293HEK and MEF cell lines. For treatment, cells were seeded in plates according to experiment cell type, and then if it was needed, transient transfection was performed. For treatment process, cells' mediums were sucked and treatments were

performed in complete medium that contains suitable amount of chemicals such as thapsigargin, palmitate, and bulky ATP analogs which were used from fresh stocks and were diluted in complete medium. For negative controls (No treatment), same amount of solvents such as ethanol, DMSO and nuclease free water where chemicals were dissolved in were added in complete medium. Optimal conditions for treatments were listed in the table below next page.

Table 3.5. Optimized and used treatment conditions for cell lines used in this research.

	Amount (Molarity)	**The solvent for treatment**	**The solvent for dissolving**	**Treatment duration**	**Chemicals used for negative control**
Thapsigargin	600 nM (mutant) experiments 150 nM (in vitro kinase assay)	Complete DMEM	DMSO	1 hour	DMSO (600 nM)
Palmitate	1000 μM	RPMI with 1 % free fatty acid BSA	Absolute Ethanol	1 hour or 2 hour	Ethanol (1000 μM)
IRE RNAse inhibitor	300 μM	1% free fatty acid BSA RPMI	DMSO	2 hour pretreatment 1 and 2 hour with palmitate	DMSO and ethanol
NaPP1	40 μM	Complete DMEM	DMSO	Overnight after transfection 1 hour with Thapsigargin treatment	DMSO
1-NMPP1	40 μM	Complete DMEM	DMSO	Overnight after transfection 1 hour with Thapsigargin treatment	DMSO

3.3.4. Total Protein Isolation from Cultured Cells

In the beginning phospholysis buffer mix was prepared. 100X PMSF, 50X Na_3VO_4, 100X Protease and Phosphatase inhibitor was added in phsopholysis buffer and their final concentrations in phospholysis buffer was diluted to 1X.

Then, plates which were frozen with liquid nitrogen and then kept at -80°C freezer, were put on ice, and adequate volume of phospholysis buffer were added on them, then they were covered with aluminum folio and waited for 5 minutes, meanwhile they were shaken gently on ice. Then the plates were scraped with 1000 µl tip (for 6-well, 12-well plates) or with scraper (96 mm, 135 mm plates) and then collected in pre-chilled eppendorfs. Then these collected lysed cells were centrifuged at 13000 rpm at 4°C for 10 minutes. After this centrifugation step, supernatants were collected in another labeled eppendorfs and were kept in ice.

For Bradford measurements, 49 µl nuclease free water was added in each 2-ml eppendorf and 1 µl protein sample was added into these eppendorfs. For blank measurement, 1 µl phospholysis buffer was added into 49 µl nuclease free water added eppendorf. Then 450 µl Bradford Solution (SIGMA-ALDRICH (St.Louis, MO, USA)), was added into each eppendorf. Then absorbances of protein samples were determined with spectrophotometer at 595 nm and concentrations were calculated with help of BSA curve. Dilutions were done according to calculations and finally 5X SDS Loading Dye were added to each sample, ratio being 1X in total volume. Finally samples were vortexed and boiled for 5 minutes at 95°C. They were spinned down and kept at -20°C.

3.3.5. Western Blot

3.3.5.1. Tris-glycine SDS-Polyacrylamide Gel Electrophoresis

Samples were taken from -20°C and were boiled for 5 minutes at 95°C before loading. 40 to 80 µg protein was loaded into each well in gel depending on experiment type. 10 µl Protein ladder was loaded into one well in order to detect and analyze the expected protein due to its size. Tris-glycine SDS-Polyacrylamide Gel Electrophoresis method was used for running samples on gel with 1X Running Buffer. Gel concentration depended on size of proteins, in this research generally, 10% resolving and 5% stacking gel were used, in some experiments 12% resolving gel was also used. The samples were run on gel for approximately 35 minutes at 80V until they pass to resolving gel from stacking gel and then they were run at 120V until the loading dye disappears, and the bands of the ladder opens.

3.3.5.2. Transfer of Proteins from SDS-Polyacrylamide Gels to Solid Supports

After running process completed, proteins on gel were transferred onto Thermo Scientific PVDF Transfer membranes. Wet Transfer method was used during this step. First membranes were soaked in methanol for 10 seconds for activation, and then they were incubated in 1X Transfer Buffer. 1X Transfer Buffer was prepared from 5X Transfer Buffer Stock. 600 ml ddH_2O, 200 ml methanol and 200 ml 5X Transfer Buffer were mixed in order to dilute 5X stock to 1X working transfer buffer. Then 3 sponges, 4 filter papers were soaked in 1X Transfer Buffer for each sandwich.

The order of sandwich:

Black side of the sandwich was down.

Onto black side, in order:

•2 sponge

•1 Whatman

•SDS Gel

•PVDF Membrane (The protein will be in the back face) before usage soak into methanol for 10 sec to be activated.

•1 Whatman

•1Sponge

were placed. Then the sandwich apparatus was closed. Ice packet was placed in tank with ransfer cassette, tank was filled with transfer buffer and was run at 95 V for 2 hours in 4°C room.

3.3.5.3. Blocking, Washing and Binding Antibodies

After transfer ended, protein transferred membranes were washed with TBS-T quickly for few minutes. Then blocking step overnight or 1 hour with 5% TBS-T BSA or 5% TBS-T milk was performed. Primary Antibody was prepared in suitable dilutions in 5%TBS-T BSA or 5% TBS-T milk (2 ml for each membrane) were put on plate which was surrounded with wet towels and left at 4°C overnight or 1 hour at room temperature. For washing, membranes were washed with TBS-T (%0.5 tween) 3 times 5,15,5 minutes on shaker in order. Afterward, horseradish peroxidase (HRP) conjugated antibodies; anti-mouse or anti-rabbit were used as secondary antibody and ratio was determined according to primary antibody efficiency. (between 1:5000-1:10000). After 1 hour incubation, membranes were washed with TBS-T (%0.5 tween) on shaker for 4 times in order 5,15,5,5.

3.3.5.4. Detection of Proteins Immobilized on Membranes

For Detection ECL-Prime (Amersham, UK) was used in 1:1 ratio. 1 ml solution A and 1 ml solution B was mixed for each membrane and were put on membrane side that has proteins on. Then the membrane was kept on dark for 5 minutes, and developed with X-ray films and Developer device. X-ray films were exposed to emitted chemiluminiscent light that came

from the reaction of HRP and this reaction led to detection of proteins on X-ray film. Exposure duration of X-ray films to membrane depends on primary and secondary antibody efficiency and working dilutions of them used during the experiment.

3.3.6. *In Vitro* Kinase Assay

3.3.6.1. Immunoprecipitation

After cells were lysed with phospholysis buffer, more than 250 µg protein containing cell lysates were aliquoted in each tube. Their volumes are adjusted to 500 µl with phospholysis buffer. Cell lysate was incubated with 13 µl total PKR antibody. (Santa Cruz B-10, sc6282. Dallas. USA). Then cell lysate was immunoprecipitated with antibody for 16 hours (overnight) at 4 ^{o}C on tube turner. 50 µL of agarose beads suspension were added in each tube and these tubes were incubated for 1 hour at 4 ^{o}C on tube turner. Then the complexes were washed with phospholysis buffer and were centrifuged at 4000 rpm for 3 minutes twice. The complexes were then washed with phospholysis buffer three times at 4 ^{o}C.

3.3.6.2. *In Vitro* Kinase Assay

The supernatants were removed by aspiration. 50 µl PKR kinase buffer was added in each tube. The tubes were incubated at 30 ^{o}C for 30 minutes. 20 mM EDTA was added to each tube. 2.5 mM PNBM was added in appropriate controls. Then tubes were incubated at room temperature for 1-2 hour on rotator. 5X SDS loading dye (Sample Buffer) were added in each tube to stop the reaction. Total volume became approximately 60 µl. Then they were boiled at 95 ^{o}C for 5 minutes. Samples were spinned down and were loaded on 10% SDS-gel. 30 µl of IP samples were loaded for Western Blot. Then transfer-western protocol was followed with Thiophopshate Ester Specific antibody.

3.3.7. Total RNA isolation from Cultured Cells

1 ml Trizol (Trisure) was added to each well of 6 well plate. Then the mixture was pipetted up-down, and collected into 1.5 ml eppendorf tube. Then 200 µl chloroform was added in the tube, shaked vigorously for 15 seconds, and waited for 3 minutes at room temperature. Then the mixture was centrifuged at 14 000 rpm, for 15 minutes at 4°C. After phase separation line was detected, the supernatant was taken into another tube. 500 µl isopropanol was added in the tube, mixed vigorously, waited for 10 minutes at room temperature, then centrifuged at 14 000 rpm at 4°C, then isopropanol was sucked carefully not touching to the pellet. Then 1 ml 75% ethanol was added into the tube for first wash of the pellet. The tube was then centrifuged at 8000 rpm for 8 minutes at 4°C. Then the 75% ethanol was sucked and 1 ml 99.8% ethanol was added to the pellet for second washing and centrifuged at 8000 rpm for 8 minutes at 4°C.then the supernatant was removed and the pellet was dried under laminar flow for few minutes. Then the pellet was dissolved with 20 µl nuclease free water. Then the RNA values were measure with nanodrop.

3.3.8. First Strand c-DNA Synthesis

First strand cDNAs were synthesized through using Fermentas RevertAid cDNA synthesis Kit (GMBH Fermentas, Germany). Then the instructions were followed according to manufacturer's instructions. First RNAs were diluted for cDNA synthesis, then The oligo $(dT)_{18}$ primer, nuclease free water was added and the total volume was obtained up to 10 µl. After that the sample was gently mixed, followed by incubation at 65°C for 5 minutes. After that 2 µl reaction buffer, 1 µl dNTP mix,0.5 µl RNAase inhibitor,0.5 µl Revert aid M-Mul V Reverse Transcriptase (200u/µl) and DNA/RNAase free water up to 10 µl, then the samples were mixed gently, and another PCR program was run. In this program samples was

incubated for 60 minutes at 42^{o}C, then heat inactivation step was performed at 70 ^{o}C for 5 minutes.

3.3.9. Semi Quantitative RT-PCR

After cDNA was obtained, then semi quantitative PCR was performed for analysis of XBP1 splicing during palmitate induced ER stress. For semi quantitative RT-PCR, for each reaction, 2 µl 5x Phire Buffer, 0.5 µl 10 mM dNTP, 0.75 µl $MgCl_2$, 0.5 µl human spliced XBP1 forward primer, 0.5 µl human spliced XBP1 reverse primer, 4.5 µl nuclease free water and 0.2 µl Phire enzyme were mixed, making volume approximately 10 µl. PCR program started with initial denaturation step at 95 °C for 3 minutes. Then 35 cycles of amplification step was performed at 95 °C for 30 seconds, 60 °C for 30 seconds, 72 °C for 45 seconds. Final extension was 72 °C for 10 minutes. Then the product was run on 3 % gel for 90 minutes and analyzed with UV transilluminator.

4. RESULTS

4.1. Generation of Kinase Switch Mutant of PKR

Interferon inducible double stranded RNA (dsRNA)-activated Protein Kinase R (PKR), also known as eukaryotic initation factor02 alpha kinase-2 (EIF2AK2), is a serine/threonine kinase that modulates protein translation through phosphorylating and inhibiting eukaryotic initiation factor 2 α (eIF2α)[43]. Two different isoforms of this gene are encoded by three transcript variants. [Provided by RefSeq, Oct 2011]. The transcripts with the accession number (NM_001135651.2 and NM_002759.3) encode the longest same transcript (isoform a), whereas the transcript with the accession number (NM_001135652.2) encodes the alternate in-frame exon deficient transcript (isoform b) (Figure 4.1. PKR's kinase domain shares similarities with three other eIF2α phosphorylating serine/threonine kinases, namely the heme-regulated eIF2α kinase (HRI), the amino acid deprivation-activated general control nondepressible kinase 2 (GCN2) and the PKR-like eukaryotic initiation factor 2α kinase, PERK [117].

In NCBI web site, PKR's gene identification (ID) is 5610. It locates on short 2p22-p21 arm of Chromosome 2: NC_000002.11 between sulfotransferase family cytosolic 6B member 1 (SULT6B1) gene and G patch domain containing 11 (GPATCH11) gene (Figure 4.2). The entire gene is approximately 50 kb in length and contains 17 exons[134]. Translation starts at exon 3 and ends at exon 17[134]. The open reading frame of this gene is 1656 bp length and encodes 551 aminoacid PKR protein. PKR contains two dsRNA binding domains, one serine/threonine kinase catalytic domain (S_TKc) and a protein kinase catalytic domain (PK_c like superfamily) (Figure 4.3) (http://www.ncbi.nlm.nih.gov/gene/5610)

Figure 4.1. Genomic context and transcripts of PKR.

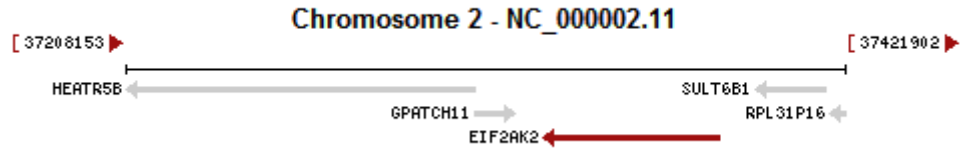


Figure 4.2. Chromosomal location and genomic context of PKR (EIF2AK2) gene in Homo Sapiens.

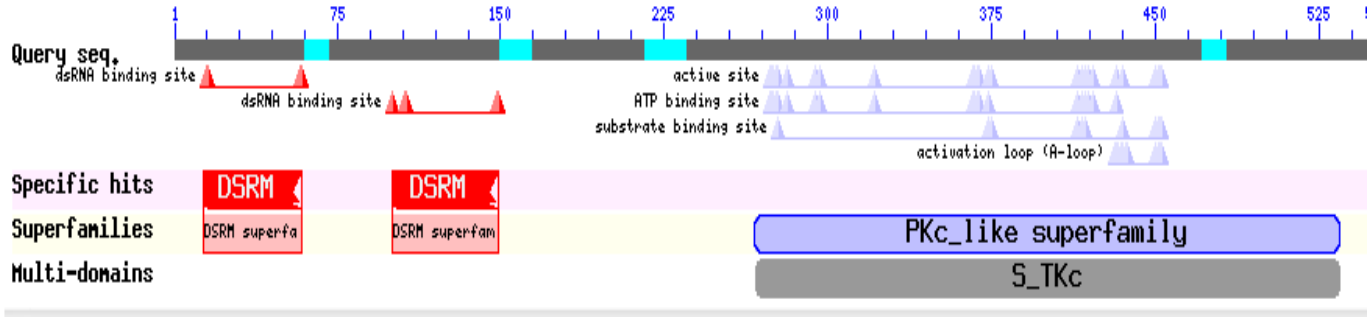


Figure 4.3. The conserved domains of PKR.

Only the crystal structure of Saccharomyces Cerevisiae PKR's kinase domain has been resolved to date[135]. Padyana and colleagues resolved the crystal structure of one of the homologous eIF2α phosphorylating kinases, namely GCN2, in 2005[141]. Since the ATP binding pockets of eIF2α phosphorylating kinases are highly conserved among themselves and with other kinases, the aformentioned crystal structure was used in this thesis study to identify the gatekeeper residues of PKR suitable for chemical-genetic approach. For this purpose, PKR amino acid sequence was aligned with other kinases using the ClustalW2 and PyMol Software programs. Next, the gate keeper residue in PKR suitable was determined based on earlier published gate-keeper mutants in other kinases. (Figure 4.4). The 366th

Methionine (M) residue on human PKR was identified as ATP binding gate keeper residue to be mutated to a smaller side-chain amino acid in order to generate a kinase-switch mutant of PKR (Figure 4.4).

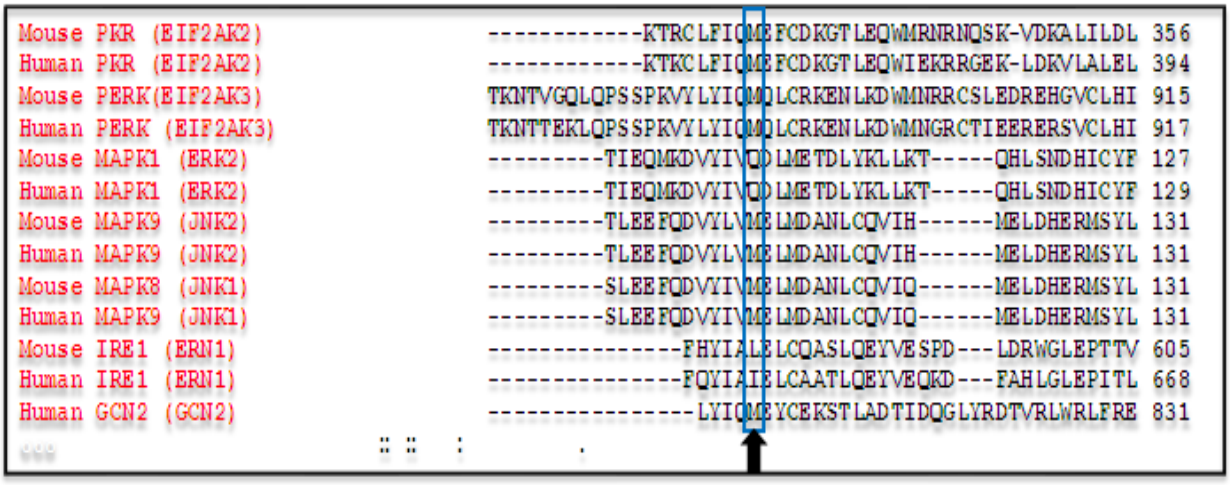


Figure 4.4. Multiple sequence alignment of kinases in order to identify specific gatekeeper residue in PKR via using Clustal OMEGA.

Next hypothetical three dimensional (3D) structure of human PKR WT kinase was formed with the aid of SwissProt website and mutations were displayed in the PyMoL software. (Figure 4.5).

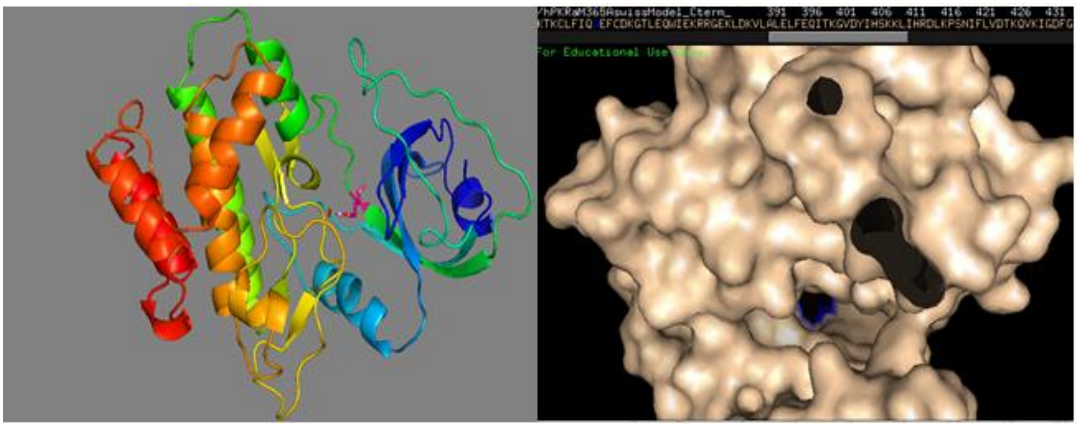


Figure 4.5. The hypothetical three dimensional (3D) structure of human PKR WT.

Left: The gate keeper mutation site, Methionine at 366th position, is depicted with hot pink, in sticks in pretty model. Right: The gatekeeper mutation site, Methionine at 366th position is depicted with blue in this surface model.

To confirm the gate keeper position is correct, wild type human PKR protein was aligned with human GCN2 and TgCDPK1 whose crystal structures were resolved in complex with ATP or 1NM-PP1 (ATP analog) (at 2.00 Å resolution (PDBID:1ZY5); [141]and http://www.rcsb.org/pdb/explore/explore.do?structureId=1ZY5) in complex with ATP or 1NM-PP1 (ATP analog).via using PyMoL software (Figure 4.6).

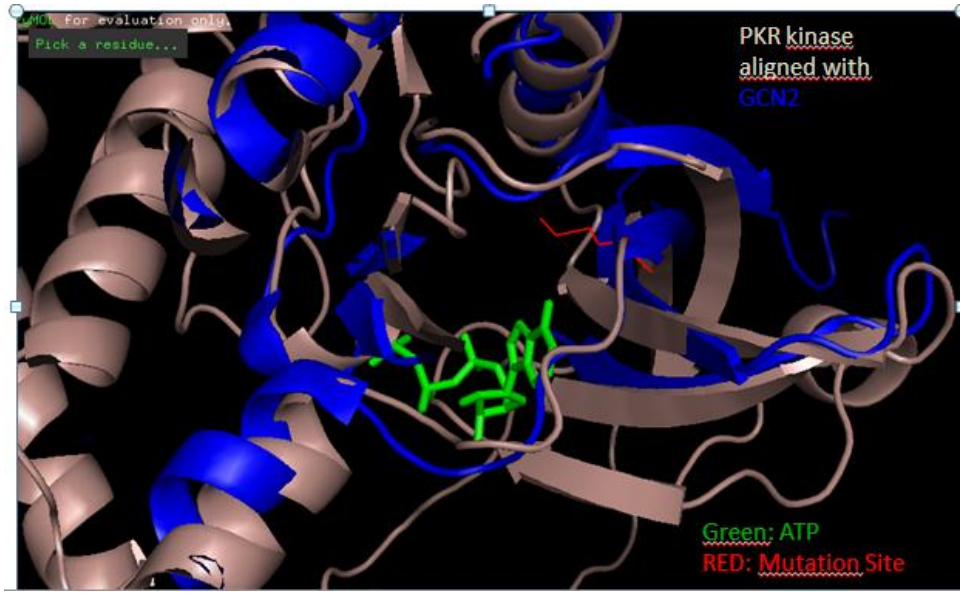


Figure 4.6. The hypothetical three dimensional (3D) model of PKR aligned with GCN2-ATP complex.
The hypothetical three dimensional (3D) model of PKR aligned with GCN2-ATP complex. In this figure, the alignment was carried out in the PyMoL Software at 2.00 Å resolution for PKR and GCN2 (PDBI ID of GCN2: 1ZY5)[141]. Red depicts the gate keeper mutation site, green depicts the ATP, and grey is for PKR kinase and blue is for GCN2.

As observed above, ATP fits the gate keeper residue of wild type human PKR (Figure 4.6). However, the bulky ATP ortholog 1NM-PP1 does not (Figure 4.7). This modeling then confirms the exact site of the gatekeeper residue that needs to be mutated to build an ASKA mutant of PKR.

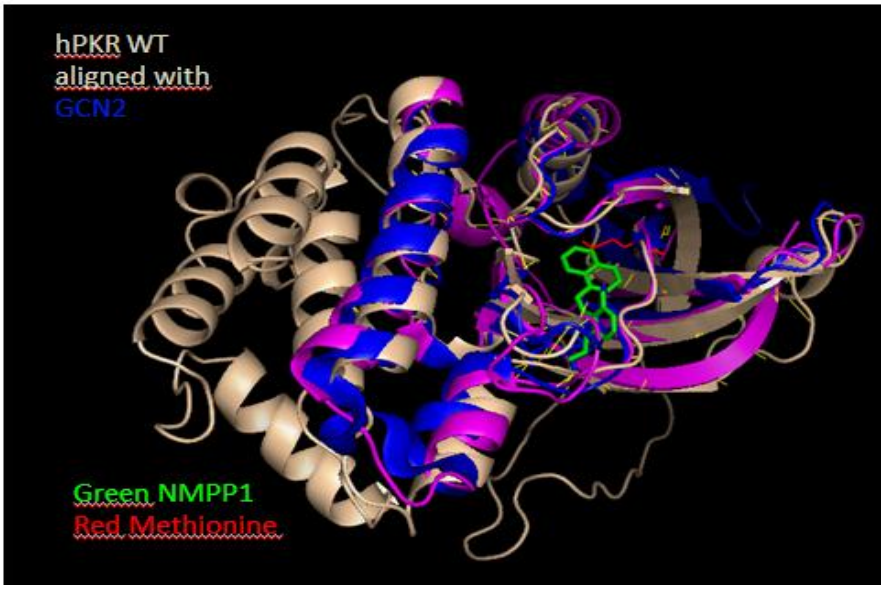
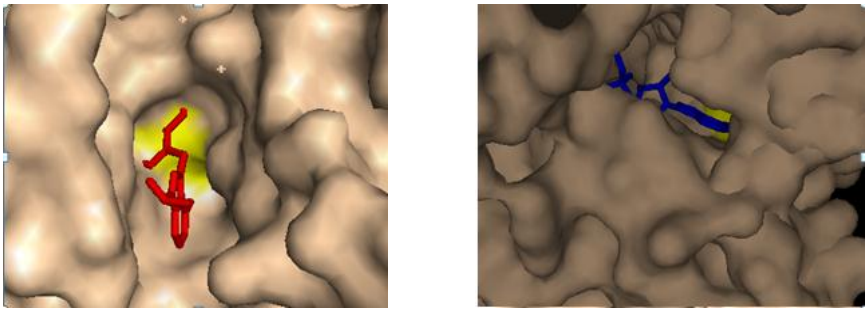


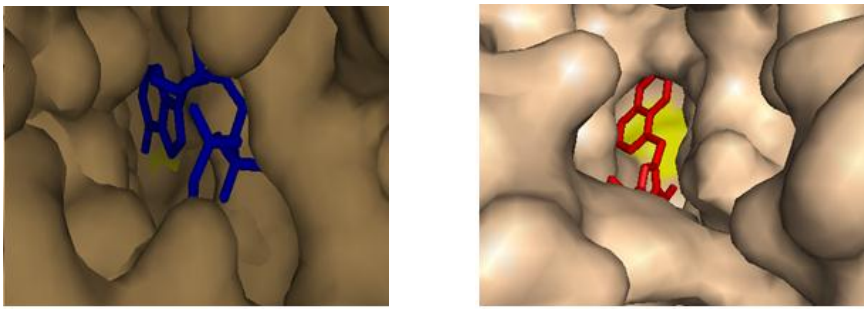
Figure 4.7. The hypothetical 3D model of PKR aligned with GCN2-1NM-PP1 complex.
The alignment was carried out in the PyMoL Software at 2.00 Å resolution. The mutation site is depicted in red, 1NM-PP1 in green, GCN2 in blue and wild type human PKR in grey.

These results demonstrated that potential gatekeeper site for site directed mutagenesis is the methionine at 366th position. Since a bulky ATP analog should be accommodated by a larger ATP binding cavity, I decided to pursue the mutation at methionine 366 to either alanine or glycine. The site of the gatekeeper mutation was further modeled in the PyMoL software, where the interaction between the ATP analog (1NM-PP1) or ATP to the mutated kinase and WT kinase were visualized. As shown in next page, 1NM-PP1 cannot fit the wild type PKR ATP binding cavity, while it is perfectly in fit with the slightly larger, mutated cavity (M366A mutation) of ASKA PKR mutant (Figure 4.8 and 4.9). Similar gatekeeper mutations were also identified for mPKR as M328G and M328A site (Fig. 4.4).



hPKR WT and 1NM-PP1 **hPKR WT and ATP**

Figure 4.8. Wild type human PKR in complex with ATP and 1NM-PP1.
The gate keeper mutation site is depicted with yellow, 1NM-PP1 in red, ATP in blue, and wild type human PKR (PKR-WT) in grey. ATP perfectly fits PKR-WT while inhibitory ATP analog 1NM-PP1 does not.



hPKR M366A and ATP **hPKR M366A and 1NM-PP1**

Figure 4.9. Human PKR kinase-switch mutant in complex with 1NM-PP1 or ATP.
The gate-keeper mutation is depicted in yellow, 1NM-PP1 in red, ATP in blue and human PKR kinase switch mutant (M366A) is shown in grey. 1NM-PP1 perfectly fits the enlarged cavity of the M366A mutant.

4.2. Generation of Kinase Switch Mutants of Human PKR by Site Directed Mutagenesis

First, I proceed to mutate the mouse PKR plasmid, which was purchased from Openbiosystems. (Figure 4.10). When the plasmid was received, I confirmed the insert by restriction enzyme digestion (the PKR cDNA insert was cloned between NotI and SalI restriction sites in the PCMV-SPORT6 vector). Then site directed mutagenesis was performed to generate the point mutations for M328A and M328G by a PCR based amplification approach. The mutated plasmids were confirmed by sequencing (Figure 4.11). Next, they

were transfected into cells to observe expression; however, antibodies specifically raised against phosphorylated form of the human PKR did not recognize phosphorylated form of the mouse PKR protein. This meant that we would not be able to assess PKR kinase activity change by western blotting approach that is practical As a result; I decided to switch to human PKR for mutagenesis and chemical genetics approaches since the anti-phospho-PKR antibody can recognize this. For this purpose, we obtained the wild type human PKR (in pcDNA6 plasmid) as a generous gift from Prof. Charles Samuel (University of California at Santa Barbara). Human PKR cDNA (1656 bp) was first cut out and subcloned into a mammalian expression plasmid carrying a FLAG epitope tag (pCDNA-5F) using the 5' BamHI and 3' Xho1 restriction sites available on the receiving plasmid. The success of the insertion was verified by restriction enzyme digestion of the insert using BamHI and XhoI. Next, site directed mutagenesis was performed to generate the point mutations for PKR M366A and M366G and the mutations were confirmed by sequencing.

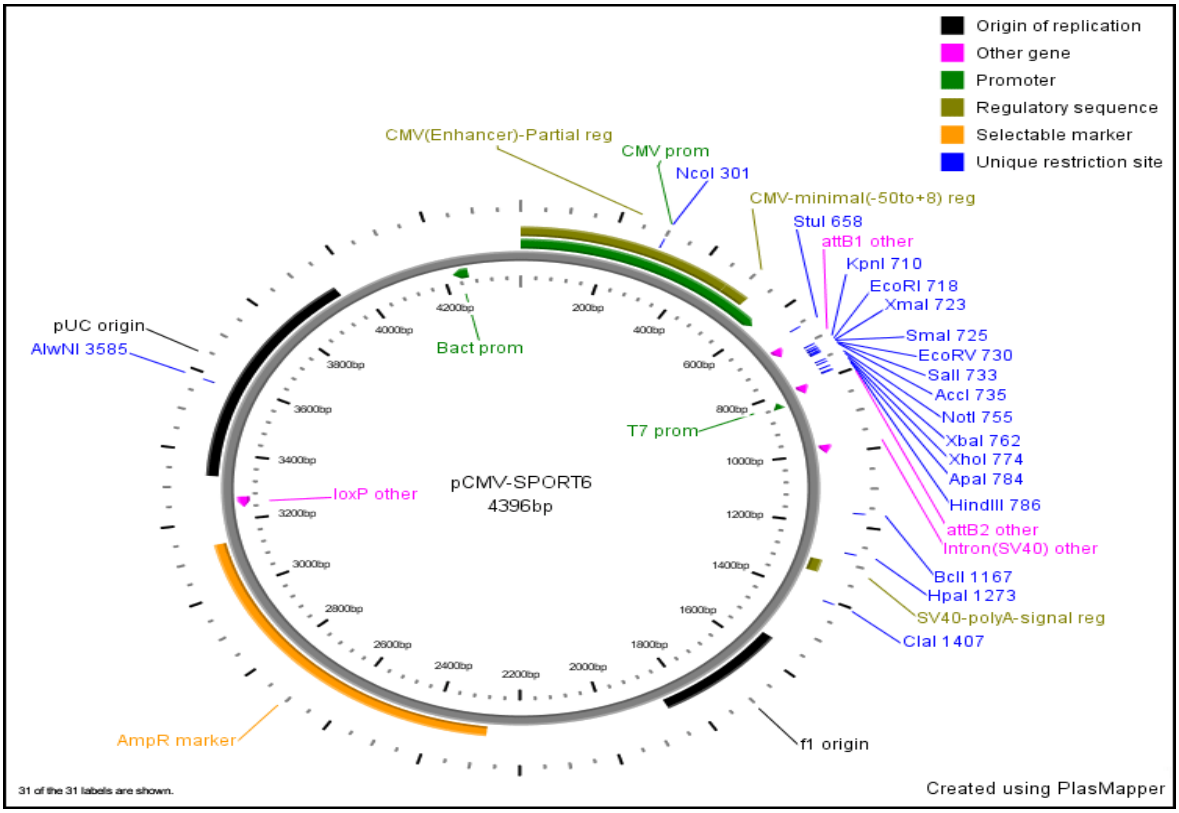


Figure 4.10. The map of mouse PKR plasmid bought from Openbiosystems.
Mouse PKR cDNA is cloned between NotI and SalI Restriction Sites.

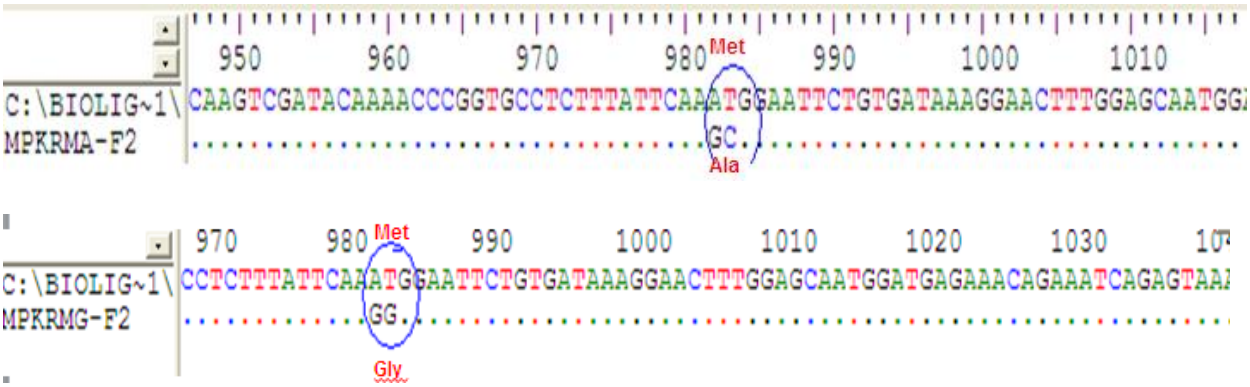


Figure 4.11. The sequencing result for M328A and mPKR M328G mutation.
According to sequencing results, Methionine residue at 328th position of mPKR plasmid was successfully mutated to either Alanine (top panel) or Glycine (bottom panel).

4.3. The expression of wild type and ASKA mutant of PKR in mammalian cells.

In the chemical-genetics approach, a specific kinase is mutated at a gatekeeper amino asid residue in its ATP binding cavity in order to accommodate a bulky ATP analog that is not an effective substrate for the wild type kinase. Thus, only the mutated kinase can be followed by the activating or inhibiting bulky ATP analogs. A kinase that is regulated in such a manner is called the ATP analog sensitive kinase (ASKA)[140]. This ASKA provides a useful tool for dissecting the role of specific kinases in signal transduction and biological processes.

It is known that PKR has several substrates, but its diverse biological roles, especially the newly recognized metabolic functions, are not fully explained by the limited number of downstream players known. When activated autophosphorylates itself. The most well-known substrate of PKR is the eukaryotic initiation factor 2 alpha (eIF2α) [128]. PKR also directly interacts and phosphorylates IRS1 at 307th Serine residue leading to insulin resistance. Finding and targeting other substrates of PKR during metabolic stress can be a novel therapeutic approach for metabolic diseases such as obesity, diabetes and insulin resistance[128]. Therefore in this thesis study, human M366A and M366G ASKA mutants were generated in order to investigate other substrates of PKR during metabolic stress. Once the gatekeeper human PKR M366A and M366G mutants were generated with site directed mutagenesis and confirmed by sequencing, I transfected them into human embryonic kidney cell lines (HEK293) in order to verify their expression (Figure 4.12).

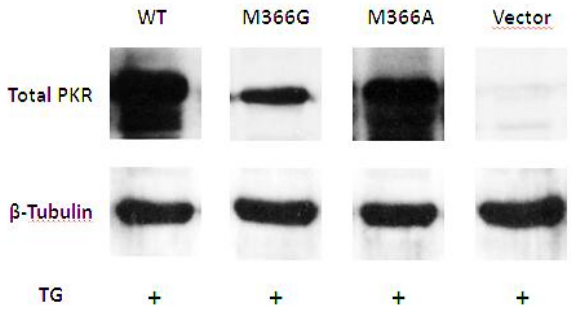


Figure 4.12. PKR expression in HEK cells.
2 µg hPKR WT, hPKR M366A and hPKR M366G and empty mammalian expression vector pcDNA3.1 plasmid DNA were transfected to HEK cells by PEI method. They were treated with thapsigargin to induce ER stress. PKR Protein levels were detected with total PKR antibody.

As can be observed from the figure 4.12, PKR protein expression and phosphorylation was many folds more above that seen in empty vector transfected HEK cells (negative control) upon treatment with the ER stress inducer thapsigargin. In summary these results confirm the expression of these PKR mutants in mammalian cells.

4.4. Confirming the Inhibition of PKR ASKA Mutant by the Bulky ATP Inhibitors in Mammalian Cells

After confirming the expression of the PKR mutants, I set forth to determine whether these gatekeeper PKR mutants could indeed be inhibited with a bulky ATP inhibitor, NaPP1, and therefore, utilized for future applications in signal transduction studies in mammalian cells. PKR knockout mouse embryonic fibroblasts ($PKR^{-/-}$ MEFs) were transfected with 4 µg hPKR M366A, hPKR M366G, hPKR WT plasmids or an empty vector (pcDNA3.1) by electroporation method. 4 hours after transfection, 40 µM NaPP1 and 40 µM 1NM-PP1 were directly given to cells for 16 hours. Then cells were treated with 600nM Thapsigargin to induce ER stress for 1 hour. After treatments, cells were lysed, run on SDS protein gels, followed by detection of PKR phosphorylation in Western blots (Figure 4.13, 4.14 and 4.15).

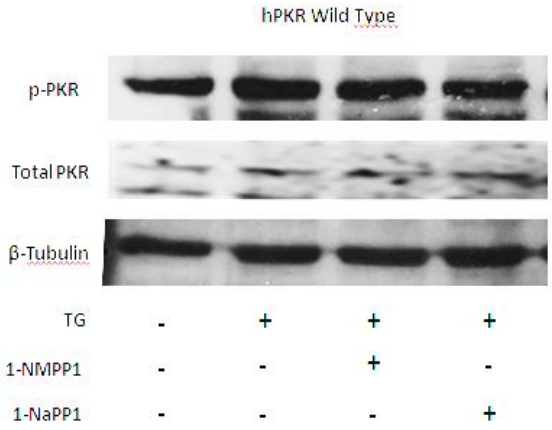


Figure 4.13. Bulky ATP analogs NaPP1 and 1NM-PP1 cannot inhibit wild type hPKR.
4 µg hPKR WT plasmids were transfected to $PKR^{-/-}$ MEF cells with electroporation method. Then, the cells were treated with 40 µM NaPP1 and 1NM-PP1, inhibitory ATP analogs 4 hours after transfection and incubated overnight. After this incubation, 600 nM Thapsigargin was given to cells for 1 hour. Then cells were lysed and PKR phosphorylation, total PKR and Beta Tubulin levels were analyzed from the lysates in Western blot via using specific antibodies.

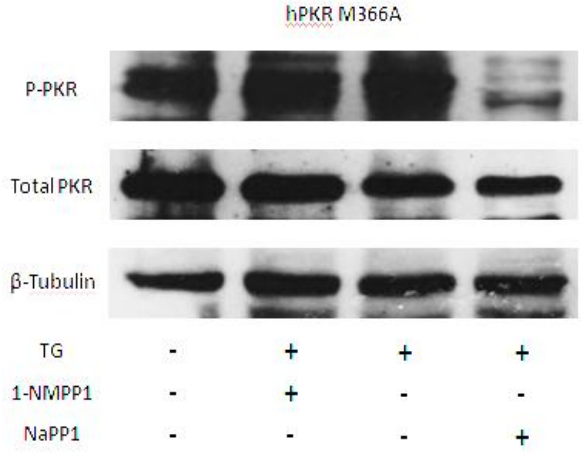


Figure 4.14. NaPP1 inhibits hPKR M366A ASKA mutant, whereas 1NM-PP1 cannot inhibit hPKR M366A mutant expressed in PKR-/- MEF cells.
4 µg hPKR M366A plasmids were transfected to $PKR^{-/-}$ MEF cells by electroporation method. Then, the cells were treated with 40 µM NaPP1 or 1NM-PP1 for 4 hours after transfection and incubated overnight. After this incubation, 600 nM Thapsigargin (TG) was given to cells for 1 hour. Cells were lysed and PKR phosphorylation, total PKR and beta-tubulin levels were analyzed from the lysates in Western blots by using specific antibodies.

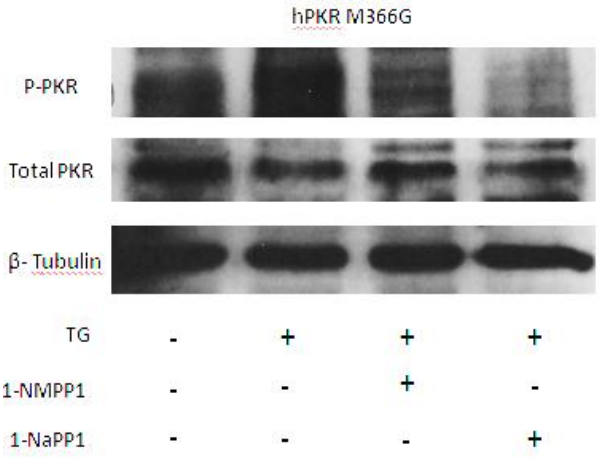


Figure 4.15. NaPP1 inhibits hPKR M366G ASKA mutant, whereas 1NM-PP1 slightly reduces kinase activity of hPKR M366G mutant expressed in PKR-/- MEF cells.
4 µg hPKR M366G plasmids were transfected to $PKR^{-/-}$ MEF cells by electroporation method.Then, the cells were treated with 40 µM NaPP1 or 1NM-PP1 for 4 hours after transfection and incubated overnight. After this incubation, 600 nM Thapsigargin (TG) was given to cells for 1 hour. Cells were lysed and PKR phosphorylation, total PKR and beta-tubulin levels were analyzed from the lysates in Western blots by using specific antibodies.

The results show that NaPP1 treatment extremely decreased phosphorylation level of PKR to in hPKR M366A and hPKR M3666G mutant overexpressed PKR -/- cells. However with the same treatment, there was no change in the phosphorylation of hPKR in hPKR WT overexpressing PKR -/- cells. 1NM-PP1 did not have an effect on the phosphorylation of neither hPKR WT nor hPKR M366A ASKA mutant, but slightly reduces the phosphorylation of hPKR M366G mutant in PKR -/- overexpression experiments.

4.5. Determining Substrates of Human PKR through In Vitro Kinase Assay

After confirming expression of hPKR ASKA mutant, I set forth to search for potential PKR substrates by using ATPγS in PKR in vitro kinase assays. HEK cells were transfected with hPKR M366A mutant via the PEI method. Next, *in vitro* kinase assay was performed (using ATPγS and PNMB for thiophosphateester modification of substrates) followed by Western Blot detection of the potential substrates by an anti-thiophosphate ester specific antibodies. After stimulation of ER stress with 150 nm thapsigargin treatment for half an hour, cells were lysed followed by immunoprecipitation of PKR (using anti-PKR antibody and protein G

beads) and in vitro kinase assay. As a result of these procedures, potential substrates were detected from the lysates by immunoblotting with the anti- thiophosphate ester specific antibody (Figure 4.16).

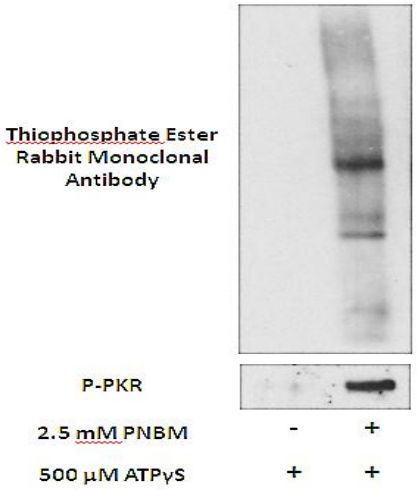


Figure 4.16. Detection of PKR substrates with in vitro kinase assay.
After cells were lysed with phospholysis buffer immunoprecipitation was performed with total PKR antibody, followed by in vitro kinase assay protocol. 2.5 mM PNBM and 500 µM ATPγS were used in the experiment and the substrates were detected through using Thiophosphate Ester Rabbit monoclonal antibody, as ATPγS thiophosphorylates ASKA mutants' substrates and PNBM alkylation yields thiophosphate esters on substrates providing robust and accurate detection of specific substrates of PKR under ER stress. The second lane on the figure was the negative control showing that PNBM alkylation is necessary for thiophosphate ester specific antibody's recognition of PKR substrates The same membrane was blotted with P-PKR antibody in order to confirm that immunoprecipitation with PKR was successful.

4.6. The Role of IRE1's Endoribonuclease Function in the Activation of PKR by Lipid-induced ER Stress

When ER stress is induced, ER-resident transmebrane Inositol Requiring Enzyme 1 (IRE1) is oligomerizes and becomes highly activated through autophosphorylation. Besides its kinase activity, IRE1 also possesses an RNAse activity. It splices X- box binding protein 1 (Xbp1) mRNA leading to its activation[50,28]. IRE1 can also nonspecifically cleave some mRNAs on the ER membrane, which serves to reduce protein load on the ER and is known as the IRE1 dependent mRNA decay pathway (RIDD))[50]. PKR is an interferon inducible, double strand RNA activated kinase and it can also be activated by ER stress as shown by increased phosphorylation activity upon treatment with ER stress inducers Thapsigargin and

Palmitate[128]. In this study, we hypothesized that the RNA sensing PKR may be coupled to IRE1's RNase function by ER stress. To test this hypothesis, I utilized a specific IRE1 RNAse domain inhibitor to uncouple IRE1's endoribonuclease function from kinase activity and determined its impact of PKR activation by ER stress specifically induced by saturated lipids. Based on several optimization trials, I determined 300 μM IRE1 RNAse inhibitor was optimal to inhibit IRE1's RNAse function without changes in the kinase activity. This inhbitor was given to HEK cells stressed with 500 μM or 1000 μM palmitate for 2 hours in order to induce lipotoxic ER stress response. The lysates were analyzed by immunoblotting with anti phopsho-PKR antibody to determine the impact of loss of IRE1 RNAse activity in ER stress conditions.

The results of my experiments showed that the loss of IRE1 RNAse activity significantly blocks PKR activation by lipids as demonstrated by reduced phosphorylation of PKR in these blots. In addition to these Western Blot experiments, RNA was isolated and Xbp1 mRNA splicing was detected by a PCR-based approach. This showed that the inhibitor completely blocked IRE1's RNAse activity at the given doses (Figure 4.17B, 4.17D). Importantly, the functional connection between IRE1 and PKR was dependent on IRE1's endoribonuclease activity. Furthermore, IRE1's kinase activity was not sufficient to induce PKR autophosphorylation (Figure 4.17A, 4.17C).

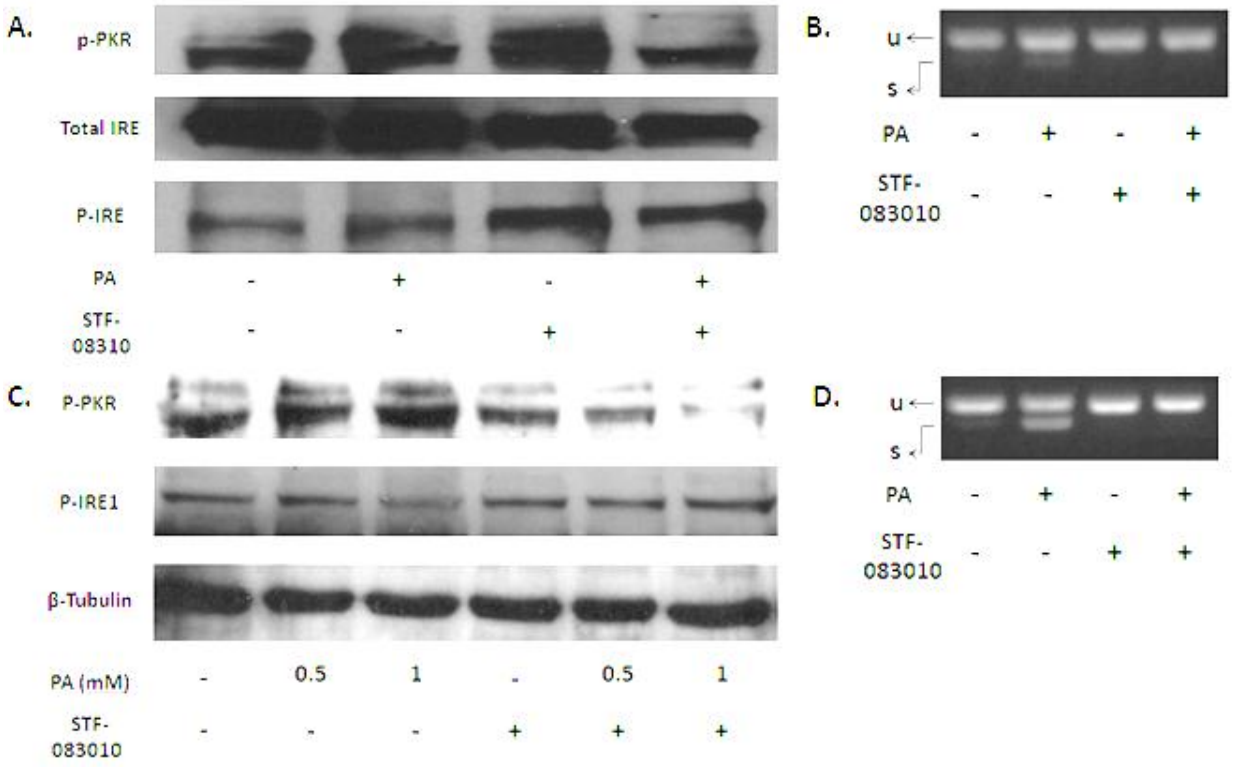


Figure 4.17. The loss of IRE1 RNAse activity blocks PKR activation by lipids.
HEK293 cells were pre-treated (for 2 hours) with 300 µM IRE1 RNAse inhibitor before the addition of varying doses of palmitate (with 1% BSA in 10% RPMI) for 2 hours. (A) The lysates from these cells were analyzed in Western blots with anti-phospho-PKR antibody. The same membrane was blotted with anti-phospho-IRE antibody in order to demonstrate IRE1 kinase activity does not reduce by the inhibitor treatment. Anti-beta tubulin antibody was used to blot for tubulin to demonstrate equal loading. (B) Next, Xbp1 mRNA splicing was detected by PCR in order to confirm that palmitate induced ER stress leads to IRE1 RNAse activation and that the inhibitor can completely block this. C) Same experiment was repeated in HEK293 cells with 1mM palmitate and 300 µM IRE1 RNAse inhibitor treatment for 2 hours and again the same result was obtained. The same membrane was blotted with total IRE antibody for equal loading and with P-IRE antibody in order to confirm that STF's inhibitory activity is specific for IRE1 RNAse activity, not leading to decrease in P-IRE levels. D) Xbp1 mRNA splicing was detected by PCR in order to confirm that palmitate induced ER stress leads to IRE1 RNAse activation and that the inhibitor can completely block this. This time treatment was for 6 hours, in order to completely activate IRE1 RNAse activity with palmitate. (PA: Palmitate, STF-08310: IRE1 RNAse Inhibitor).

5. DISCUSSION AND CONCLUSION

The focus of this thesis study is primarily on the role of PKR in mediating the lipid-induced ER stress. In order to achieve this goal, I first generated a chemical-genetic tool to modulate PKR kinase activity, namely the ATP analog sensitive kinase allele mutant of PKR. My goal has been to use this mutant to dissect the specific role and downstream targets of PKR in lipid-induced ER stress. In order to begin discovering the PKR substrates in lipid-induced ER stress, the PKR ASKA mutant was optimized for in vitro kinase assays and for the first time, several downstream substrates of PKR were documented by my initial experiments. Furthermore, upstream of PKR, I analyzed a unique relationship between PKR and IRE1 RNAse activity that are coupled by lipid-induced ER stress in macrophages.

5.1. Creating an ATP Analog Sensitive Mutant of PKR

Chemical genetics is a powerful approach, which can be used for dissecting the role of a kinase in biological or pathological processes and also for identifying novel kinase substrates. The discovery of new substrates, can pave the way to new therapeutical approaches to be developed targeting these in the future. In addition, turning on or off the activity of a kinase by the addition of a small chemical can also be useful in analyzing signal transduction pathways in different cellular or metabolic states. To modulate the kinase activity for discovery of specific substrates, a semisynthetic reaction is established. In this reaction, an analog sensitive kinase allele (ASKA) mutant is created that can utilize bulky inhibitory ATP inhibitors such as NAPP1 or NMPP1 (for blocking kinase activity) as well as bulky ATP analogs like N6-ATPγS for thio-phosphorylation reactions. Following thio-phosphorylation, potential substrates are further alkylated by reacting with p-nitrobenzyl mesylate (PNBM), creating thiophosphate ester or a “semisynthetic epitope” on these modified substrates that can later be recognized by a highly specific rabbit monoclonal antibody against

thiophoshateesters[137]. In this thesis, I used a site-directed mutagenesis approach to generate an analog sensitive kinase allele mutant of PKR that can accommodate bulky ATP analogs for activation or inhibition. The future perspective of this study is to utilize this mutant to dissect PKR's role in lipid-induced ER stress response and to discover novel substrates of PKR that relay the ER stress response to cellular outcomes like inflammation and cell death.

In the beginning of this thesis, I first identified the potential gatekeeper residues of PKR to create suitable mutations that would slightly expand the ATP binding cavity in order to accommodate bulky ATP analogs. Since the ATP binding cavities of kinases are higly conserved, PKR's ATP binding cavity sequence was aligned with other known ASKA mutants and the mutation was structurally verified to interact with NMPP1 in 3D modeling in PYMOL software (Figure 4.6 and Figure 4.7). These mutation sites were identified as M366A and M366G on human PKR and M328A and M328G on mouse PKR. After these mutations, it was confirmed by in vitro and in vivo reactions that the kinase activity of these mutants remained intact (Figure 4.12). In addition, the results of my experiments showed that hPKR M366A and M3666G mutants' kinase activity can be inhibited by NaPP1 (Figure 4.14 and 4.15) while wild type hPKR kinase activity remained unchanged (Figure 4.13). This result implied that NaPP1 as a bulky inhibitory ATP analog perfectly fits into the ATP binding cavity of hPKR M366A and hPKR M366G ASKA mutant, leading to its kinase activity to be inhibited. In the future aims of this project, this mutant can be utilized for studying the impact of PKR in lipid-indcued ER stress response (via inhibition by NAPP1) and also for identifying potential substrates of PKR during lipid induced ER stress using the bulky ATP analog (via reacting with N6benzylATP γS) in kinase assay coupled with mass spectrometric analysis of the thiophosphorylated substrates.

5.2. Searching Substrates of Human PKR Using the Chemical-Genetic Approach

To begin optimizing the kinase assays for substrate discovery, in vitro kinase assay were optimized for human PKR M366A mutant. In this experiment, HEK cells were transfected with hPKR M366A ASKA mutant, followed by thapsigargin treatment to induce a state of ER stress. From these lysates, PKR was immunoprecipitated and used for vitro kinase assay in reaction with ATPγS. After this, thiophosphorylatied PKR substrates were further alkylated with PNBM, yielding thiophosphate esters on the potential substrates. In order to detect the novel, thiophosphoesterified substrates, the kinase reactions were run on SDS gels, and blotted with a highly specific, rabbit monoclonal anti-thiophosphoester antibody in Western Blot experiments (Figure 4.16). For these experiments, PNBM absent kinase assay sample were used as a negative control to show that PNBM alkylation is necessary for yielding of thiophosphate esters on substrates and recognition by the rabbit monoclonal anti-thiophosphateester specific antibody. Furthermore, IgG alone control immunoprecipitates demonstrated the specificity of the reaction of PKR. The results of these early experiments showed for the first time that there are potentially multiple PKR substrates in lipid-induced ER stress conditions. In the future, as a continuation of the findings in this thesis, similar kinase assay experiments will be coupled with mass-spectroscopic identification of the thiophosphateesterified substrates. Furthermore, to demonstrate the newly identified proteins are real subtrates of PKR, they will be reacted with PKR in in vitro and in vivo kinase reactions.

5.3. Coupling of IRE1 Endoribonuclease Activity with PKR by Lipid-induced ER Stress

IRE1 is historically the first discovered and only conserved arm of the UPR from yeast to mammals. It has a major role in controlling the expression of key ER chaperone proteins and activatites by splicing XBP1 mRNA to generate an active transcription factor[28]. In my

preliminary observations, I noted a marked difference in the activating PKR phosphoryation at 446th threonine site between IRE$^{-/-}$ mouse embryonic fibroblasts (MEF) stably expressing either wild type or RNAse inactive PKR mutant (PKR_K907A). However, since the commercially designed antibodies against phospho-PKR (P-PKR) poorly recognize the mouse form, in my follow up experiments I had to revert to overexpression of the human PKR plasmid in mouse embryonic fibroblasts. But, the transfection or electroporation of plasmids into stable cell lines proved to be extremely difficult with very slight expression of hPKR protein that could be recognized weakly by the commercial antibodies. Because of these technical challenges, I changed my experimental approach to study IRE1's impact on PKR and decided to utilize a specific and widely used IRE1 RNAse inhibitor in my cellular experiments. I first defined the experimental conditions for optimal the inhbition of IRE1's RNAse activity in the human embyonic kidney cell line, HEK293 cells. Inhibition of IRE1 RNAse activity by the inhibitor resulted in a significant block in lipid's ability to induce PKR phosphorylation and thereby it's kinase activity (determined by autophosporylation of PKR) (Fig. 4.17). Together with my early observations in IRE$^{-/-}$ MEF cell lines, this result strongly argues that IRE1's RNAse activity provides an essential input for PKR to respond to lipid stress.

The outcome of the above studies are important, because they show that in ER stress response to lipids, lots of kinases such as IRE and PKR are coordinated and interact, and moreover, they can regulate each other's activities. It is also interesting that IRE1 RNAse activity is required for PKR activity and suggests an RNA connection between IRE1 and PKR in lipid-induced ER stress. Very recently, Papa et al showed that IRE1α RNAse domain leads to fast decay of pre-miRNAs 17, 34, 96 and 125, the mature forms of which suppress Caspase-2 mRNA translation and thereby, apopotosis[58]. Therefore, in the light of these recent findings

and my findings, future effort should be concentrated on defining potential noncoding RNA targets of IRE1 that couple it to PKR activation in lipid-induced ER stress.

In conclusion, the experimental tools generated during this thesis study were instrumental in discovering there are multiple substrates of PKR in lipid induced ER stress. The future studies stemming from my findings should be able to determine the identities of these new PKR targets by coupling chemical-genetics with proteomic methods. Potential novel substrates of PKR can evolve into specific therapeutic targets against metabolic and inflammatory diseases such as diabetes, insulin resistance and obesity. In addition, the results of my thesis study unraveled a surprising function of IRE1 RNAse domain in activating the PKR kinase activity. The future perspective of these studies includes the identification of RNA species regulated by IRE1's RNAse output and that can bind and regulate PKR or its partners. Ultimately, the discovery of specific miRNAs that can be targeted to uncouple IRE1 from PKR may become another novel therapeutic approach in metabolic diseases.

6. FUTURE PERSPECTIVES

In this thesis study, I generated a functional ASKA mutant of human PKR that is suitable for chemical-genetics approach to delineate signal transduction pathways. As a result of my studies, I successfully created two ASKA mutants of human PKR, M366A and M3666G, and demonstrated temporal and specific inhibition of their kinase activity with bulky ATP inihibitors. These mutants can be used in in vitro kinase and in vivo kinase assay experiments coupled to proteomics for substrate discovery. After identification of potential substrates of PKR, their function and role in ER-stress signal transduction pathways should be analyzed through further in vitro and in vivo kinase assays using the potential substrate. Moreover, creation of a transgenic mouse model with the ASKA mutant of PKR could be valuable to dissect its role in metabolic disease. Other studies showed it is possible to administer NaPP1 (subcutaneously, intraperitoneally and orally) to mice for temporal and specific control of the kinase activity in vivo. This could become a useful tool in studying the specific function of PKR in lipid induced ER stress in chronic metabolic diseases, especially because two separate partial knock-outs of PKR show opposing metabolic profiles128. Furthermore, by analyzing PKR's specific substrate preferences during metabolic disease pathogenesis, new and more specific therapeutical approaches may be generated against these diseases.

Secondly, the outcome of my studies illuminates a unique relationship between IRE1 and PKR for the first time. By specifically inhbiting the IRE1's RNAse activity with an established IRE1 RNAse inhibitor, I showed that IRE1's RNAse activity is required for PKR kinase activation by lipids, this function of IRE1 RNAse domain was previously unsuspected and should be validated through several approaches. For instance, IRE1 ASKA mutant can be transfected to HEK cells with IRE WT, and its kinase and RNAse activity can be uncoupled by the addition of 1-NMPP1 bulky ATP analog, that inhibits the kinase domain while

specifically activating the endoribonuclease domain. In these cells, a comparison of PKR autophosphorylation will tell us whether IRE1's kinase domain is required and whether RNAse domain is sufficient in activation of PKR. Furthermore, since PKR was recently shown to be associated with the inflammasome and lipids can induce both ER stress and PKR, the IRE1 RNAse inhibitor (or IRE1 specific siRNA validated in our laboratory) can also be utilized to dissect the role of PKR in IRE1 induced inflammasome activation. For instance, experiments can be designed so that IRE1 dependent inflammasome activity can be analyzed after treatment with IRE1 RNAse inhibitor in primary bone marrow derived mouse macrophages induced by lipids. Alternatively, the ASKA mutant of IRE1 can be used with NMPP1 to induce its RNAse activity and together with LPS to stimulate the inflammasome in cells where PKR is inhibited via siRNA-induced knock down. Moreover, the overexpession of IRE1 ASKA mutant in PKR-ASKA primary bone marrow derived macrophages (obtained from a future PKR ASKA transgenic mouse) where NMPP1 will be used to turn on IRE1 activity but turn of both IRE1 and PKR kinase activities can be instrumental to dissect PKR's role in LPS and IRE1 induced inflammasome activation. Many more experiments can be envisioned using one or a combination of these mutants and tools to dissect complex interactions in ER stress response that are tied to cellular outcomes like inflammation and death. Through targeting the specific molecular interaction points between these two ER stress dependent kinases, it may be possible in the future to design new therapeutical approaches against metabolic diseases such as insulin resistance, fatty liver, diabetes, atherosclerosis and obesity.

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163

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