Summer 2023

Modulation of eIF2α Signaling and the Integrated Stress Response by Targeted Therapeutic Attenuation of PKR Activity Contributes to Protection of DYT-*PRKRA* Cells From Apoptosis

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Modulation of eIF2α signaling and the Integrated Stress Response by targeted therapeutic attenuation of PKR activity contributes to protection of DYT-*PRKRA* cells from apoptosis

by

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Bachelor of Science

University of New Mexico, 2017

Submitted in Partial Fulfillment of the Requirements

For the Degree of Doctor of Philosophy in

Biological Sciences

College of Arts and Sciences

University of South Carolina

2023

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Dedication

This work is dedicated to my late grandfather, Woody Woodworth, along with my wife Shannon Frederick. Their unconditional, unrelenting support has meant everything to me and I wouldn't be here without them. I'm beyond grateful for their guidance and providing me incredibly valuable counsel on my scientific journey as I've developed into a professional scientist.

To Woody Woodworth for helping a young boy from a low socioeconomic upbringing find his curious mind. Our conversations on science and life as a whole helped shape me into who I am today. He showed me that science can be fun and carrying yourself with a certain standard can help elevate someone to living a life with deep purpose. His guidance and lessons will live on in me and I will pass them on to my daughter Stella.

To Shannon Frederick for her constant unconditional love and engaging in the type of truthful and constructive conversions that have helped me become a better husband, father, scientist, and man. I'm incredibly excited for our next journey and where we'll be.

Lastly, to my mother, Kathy Frederick, along with my late grandparents Irene Metarelis, Betty Hart, and Ken (T-Bone) Hart for their support and always wanting what was best for me.

Acknowledgments

I would initially like to thank my advisor, Dr. Rekha Patel, for her mentorship over the past 4 years. Her guidance and demand of greatness in regards to conceptual comprehension of science and tethering it to applying the knowledge to execute experiments at a high level has been integral to my development as a scientist. Her constructive criticism aided in enhancing my ability to understand and communicate translationally how the work that I do applies to helping others outside of the lab and clinic.

I would like to showcase appreciation to my dissertation committee, Drs. Caryn Outten, David Reisman, Alissa Armstrong, and Katie Kathrein for their guidance and helping me understand my projects on a fundamentally deeper level and how it carries over to helping members of the community. Each member took time to individually provide me professional development and I'm grateful to each of them.

Additionally, I need to express gratitude to Dr. Samuel Burnett for being an additional mentor teaching me technical skills, how to comprehend science on a fundamentally deeper level and helping facilitate my learning so that I could tether my work to a bigger picture. He helped me set my foundation for success in the Patel Lab.

To conclude, I'd like to thank Mrs. Indhira Handy for her generosity ranging from teaching me technical skills to setting up birthday celebrations along with simply being an overall kind and positive person who makes the lab that much brighter when she comes in for work. To Tricia Simon, it was tremendous getting to work with her and having the opportunity to mentor her. She also has been a close friend to my wife and I and we are indebted to the support that she has provided for us during times of personal struggles.

Abstract

Luteolin is a natural flavonoid present in vegetables, fruits, and medicinal herbs that possesses anti-oxidative, anti-cancer, and anti-inflammatory properties. In this study, we investigated the effect of luteolin on the integrated stress response (ISR), which is an evolutionarily conserved intracellular signaling network essential for adapting to environmental stresses to maintain healthy cells. Dysregulated ISR is involved in the etiology of many human diseases including the movement disorder DYT-*PRKRA*, which is caused by mutations in the *PRKRA* gene. *PRKRA* encodes for PACT, the **p**rotein **act**ivator of interferon-induced, double-stranded RNA (dsRNA)-activated protein kinase PKR. PACT-mediated PKR activation regulates the ISR via phosphorylation of the eukaryotic translation initiation factor eIF2α. A dysregulation of either the level or the kinetics of eIF2α phosphorylation in response to a stress signal can cause the normally pro-survival ISR to become pro-apoptotic. In DYT-*PRKRA* patient cells, the PRKRA mutations lead to an enhanced PACT-PKR interaction causing dysregulated of ISR and an increased sensitivity to apoptosis. We have previously identified luteolin as an inhibitor of the PACT-PKR interaction using high-throughput screening of chemical libraries. Our results presented in this study indicate that luteolin is markedly effective in disrupting the pathological PACT-PKR interactions to protect DYT-*PRKRA* cells against apoptosis. In addition to pinpointing how luteolin protects cells against maladaptive ISR, our

research also suggests a therapeutic option for using luteolin to treat several diseases that result from dysregulated ISR.

Our results indicate that disrupting PACT-PKR interactions in DYT-*PRKRA* patient cells restores the normal kinetics of PKR activation and eIF2α phosphorylation to reduce CHOP induction thereby preventing apoptosis in response to ER stress. These results also indicated that although luteolin disrupts the abnormally strong PACT-PKR interactions observed in patient cells in the absence of stress, it allows for the normal stress-induced and transient PACT-PKR interaction which is essential for protective functions of ISR. Our results in demonstrate that luteolin does not disrupt the transient stress-dependent interaction between phosphorylated PACT and PKR. Thus, luteolin selectively prevents pathological PACT-PKR interactions in DYT-*PRKRA* patient cells in the absence of stress while preserving the normal stress-induced PACT-PKR interactions to allow for a transient PKR activation during ISR.

We also describe the CReP inhibitor, Nelfinavir, on its ability to promote cellular recovery of DYT-*PRKRA* lymphoblasts in response to ER stress. CReP levels are significantly elevated within DYT-*PRKRA* patient lymphoblasts relative to wt cells both in the absence of stress and in response to ER stress-induction. Due to the reduction in CReP and increased eIF2α phosphorylation due to Nelfinavir, we next determined an enhanced ISR activity thereby leading DYT-*PRKRA* cells to have increased sensitivity to ER-stress induced apoptosis. Despite Nelfinavir inducing a robust ISR, this did not cause cellular protection

from ER stress-induced apoptosis. Of note, Nelfinavir induces apoptosis with wt and DYT-*PRKRA* cells even in the absence of ER stress-induction.

Next, we established that PKR is hyperactive and levels of eIF2α phosphorylation were increased within DYT-3 (XDP) neuronal progenitor cells (NPCs). To link the correlation between enhanced PKR activity and dysregulated eIF2α signaling to increased apoptosis, we investigated another target of active PKR, ATF3, which is a pro-apoptotic protein, to elucidate downstream effects further. We ascertained there is highly increased expression of ATF3 within XDP cells which buttresses the inference that dysfunctionality of the eIF2α axis and the ISR is a common causality that drives pathophysiology and disease progression within multiple dystonia types.

Lastly, we characterized three different PKRi compounds for their ability to promote cellular recovery of DYT-*PRKRA* lymphoblasts in response to ER stress. Two of the PKRi compounds, PKT-00888 and PKT-00954 did not protect DYT-*PRKRA* lymphoblasts from ER stress-induced apoptosis. Meanwhile, PKT-00941 did protect DYT-*PRKRA* cells from ER stress-induced apoptosis. Also, there were no cytotoxic effects seen with PKT-00941. PKT-00941 drastically diminishes p-PKR and p-eIF2α levels and duration in DYT-*PRKRA* cells. PKT-00941 significantly attenuates CHOP induction in response to ER stress in DYT-*PRKRA* cells. This disruption in CHOP induction highlights how PKT-00941 suppresses apoptosis thereby promoting cellular homeostasis as CHOP induction is essential for apoptosis after ER stress.

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LIST OF ABBREVIATIONS

2AP……..2-Aminopurine

3’UTR……..3’ Untranslated Region

5’UTR……...5’ Untranslated Region

ALS……Amyotrophic Lateral Sclerosis

AD……..……..Alzheimer’s Disease

ATF3……Activating Transcription Factor 3

ATF4……..Activating Transcription Factor 4

ATP……..Adenosine Triphosphate

ASD……Autism Spectrum Disorder

CHOP ……C/EBP Homologous Protein

CReP ……..Constitutive Regulator Of eIF2α Phosphorylation

dsRNA ……DOUBLE STRANDED RNA

dsRBM ……..DOUBLE STRANDED RNA BINDING MOTIF

DYT……..Dystonia

DYT-PRKRA……...Dystonia Subtype PRKRA

eIF2……..Eukaryotic Initiation Factor 2

eIF2α……..Eukaryotic Initiation Factor 2 α subunit

eIF2γ……...Eukaryotic Initiation Factor γ subunit

eIF2B ……..eIF2 Guanine Nucleotide Exchange Factor 2B

ER……..Endoplasmic Reticulum

FS ……..Frameshift

GADD34 ……..Growth Arrest and DNA Damage-inducible Protein 34

GCN2……...General Control Non-Derepressible 2

GDP……..Guanosine Diphosphate

GEF .....Guanine Nucleotide Exchange Factor xiii

GTP .....Guanosine Triphosphate

HRI.....Heme Regulated Inhibitor

HD……Huntington's Disease

IFN.....Interferon

IRAlu……Inverted Repeat Alu elements

ISG.....Interferon Stimulated Gene

ISR.....Integrated Stress Response

K296R PKR .....Dominant Negative PKR

KD.....Kinase Domain

lear-5J.....Little Ear 5J

LB……Lewy's Bodies

MPP+……*N*-methyl-4-phenyl pyridinium

mtRNA…….Mitochondrial RNA

NFT……Neurofibrillary Tangles

PACT .....Protein Activator of PKR

PD…….Parkinson's Disease

PAMP.....Pathogen Associated Molecular Pattern

PARP1.....Poly-ADP Ribose Polymerase 1

PBM.....PACT Binding Motif

PERK.....PKR-like ER Resident Kinase

PIC.....Pre-Initiation Complex

PKR .....Protein Kinase R

PKRi……Protein Kinase R Inhibitor

PP1.....Protein Phosphatase 1

PP1C .....Protein Phosphatase 1 Catalytic Subunit

PRR……Pathogen Recognition Receptor

RAX .....PKR Associated Protein X

ROS……Reactive Oxygen Species

TBI……Traumatic Brain Injury

TRBP ……TAR RNA Binding Protein

TC……Ternary Complex

uORF ……Upstream Open Reading Frame

UPR ……Unfolded Protein Response

WT ……Wild Type

Chapter 1:

Introduction

1.1 The double-stranded RNA activated protein kinase (PKR)

Interferons (IFNs) are a class of ubiquitously expressed cytokines that are essential for regulating optimal innate and adaptive immune responses in addition to their role in inflammatory signaling [8,9]. There are three classes of IFNs that are classified as Type I, Type II, and Type III respectively. Each class binds to a specific receptor and Type I interferons are the largest class, consisting of seven members: IFN-α, IFN-β, IFN-ε, IFN-κ, IFN-ω, IFN-δ, and IFN-τ [8, 9]. Each member of the Type I interferons serves as a ligand for the heterodimeric transmembrane receptor, IFNAR [8, 9]. A common stimulus for a type I IFN response begins when a pathogen associated molecular pattern (PAMP) is recognized by a pathogen recognition receptor (PRR) [8-11]. The PRRs initiate an intrinsic antiviral response as well as the synthesis and secretion of Type I IFNs, which generates an amplification loop [8, 9]. The IFNs produced by the initially virally infected cell are secreted and induction of over 300 interferon stimulated genes (ISGs) occurs which aids to blunt viral replication in the surrounding uninfected cells [8]. These ISGs confer viral resistance to cells by blocking viral transcription, degrading viral RNA, inhibiting viral translation, or modifying the intracellular proteosome [8-10]. IFNs and ISGs thus promote initial immunological protection as part of the innate immune system.

As an ISG, PKR expression is elevated during viral infections due to the elevated type I IFN production [12]. For this reason, PKR was initially characterized for its role in stimulating the integrated stress response (ISR) in response to viral infections by targeting its kinase activity to phosphorylate the α

subunit of eukaryotic initiation factor 2 (eIF2α) at serine 51 [1,2,12, 14, 15]. Although initially PKR was studied mostly within the context of viral infections, PKR also has been characterized to be an important regulator in signal transduction pathways involving inflammation, proliferation, differentiation, and apoptosis [39]. PKR activity has been directly involved in inducing activation of transcription factors such as p53, NF-κB, STAT1, and STAT3 via activation of downstream kinases such as p38 MAPK and JNK respectively [39,160]. PKR stays enzymatically latent in the absence of its activators which are either dsRNA or **p**rotein **act**ivator (PACT) [12,14,16,22,34,37,39,48,68,98]. Both dsRNA and PACT interact with PKR leading to PKR's homodimerization thereby causing an allosteric conformational change allowing exposure of each PKR monomer's ATP-binding site within its kinase domain (KD) to facilitate its trans-autophosphorylation at threonines 446 and 451 which causes PKR activation [Fig 1.2] [12,14,16,22,34,37,39,48,114]. dsRNA interaction with PKR occurs during viral infection and PACT-PKR interaction occurs in response to oxidative stress, ER stress, and serum deprivation [68,98,113]. Additionally, other molecules besides dsRNA and PACT can also activate PKR of which one class is IRAlus which reside in the 3' UTR of mRNAs [115-117]. When two Alu elements are in a reverse orientation within a single transcript, denoted as IRAlus, they can form an intramolecular dsRNA structure that binds to and activates PKR [115,118]. *Kim et al* determined that one molecular context that promotes binding between nuclear IRAlus and PKR is during mitosis [186]. Active PKR aids in regulating cell cycle signaling and progression by turning off certain G2-phase genes and inducing

mitosis-phase factors [186]. Like IRAlus, due to the bidirectional transcription of its circular genome, mitochondrial RNAs (mtRNAs) can form intramolecular dsRNAs which can activate PKR thereby showcasing PKR's involvement in mitochondrial signaling [187]. CUG expansion RNAs, which form imperfect hairpins and are among a class of mutant repeat expansion RNAs which are a common component found in repeat associated non-AUG (RAN) protein diseases can activate PKR as well [162].

In addition to the conserved carboxy-terminal catalytic domain (KD), PKR contains two highly conserved amino-terminal dsRNA binding motifs (dsRBMs) (Figure 1.2) [12, 19-21]. Structural studies have demonstrated that both dsRBMs have similar secondary structures consisting of a central hydrophobic core and form a dumbbell conformation flanking a 22-residue linker region [22]. These dsRBMs serve as important functional domains to mediate both PKR's dsRNA-binding as well as protein-protein interactions which includes interactions with PACT [15, 22-24,68]. Both dsRBM1 and dsRBM2 share high sequence and structural homology to other dsRNA binding proteins and include an α-β-β-β-α fold such that the α-helices are positioned on the face of a three-stranded antiparallel β-sheet [22]. Extensive mutation studies have been conducted to identify critical residues involved in mediating PKR-dsRNA interaction and have revealed highly conserved dsRNA binding sites within each motif [25-27]. These studies infer that the basic positively charged amino acids within dsRBM's 1 and 2 are the crucial residues for binding dsRNA [25-27].

This interaction is stabilized by the electrostatic interactions of the lysine residues within the dsRBMs with 2'-OH groups and the polyanionic phosphate backbone of the dsRNA [30,164]. Interestingly, *in vitro and in vivo* studies evaluating alternative polyanionic compounds' ability to activate PKR identified heparin, dextran sulfate, and chondroitin sulfate as effective PKR activators independent of dsRNA [26, 31, 32]. Further analysis of heparin mediated PKR activation suggests that it is facilitated through interactions with PKR's carboxy-terminal KD, as opposed to the dsRBMs [31].

Conserved hydrophobic residues within the amphipathic α-helices located in PKR's 2 dsRBMs have been shown to be critical for the inter- and intramolecular associations that are necessary to facilitate PKR activation [164]. Prior to interaction with one of its molecular activators, PKR exists in an inhibitory conformation such that PKR's carboxy-terminal KD is bound to dsRBM2 via intramolecular interactions [28]. Mechanistic studies have demonstrated that both dsRBM1 and dsRBM2 within PKR contribute to the molecular interactions with PKR activators. Interactions with these activators induce a conformational change in PKR such that the KD is released from dsRBM2 which then unveils the ATP binding site within the activation loop of the KD [14, 22]. PKR is then able to undergo trans-autophosphorylation to become catalytically active [29].

PACT activates PKR in a dsRNA-independent manner in response to stress signals other than a viral infection such as endoplasmic reticulum stress, oxidative stress, and serum deprivation [Fig. 1.3] [16, 24, 33-35,38,39,47,48,68]. Similar to PKR, PACT is also a dsRNA binding protein that is ubiquitously

expressed in all cell types but it is not an ISG product [24, 36]. PACT activates PKR via direct stress-dependent high affinity interaction with PKR [24, 33, 34, 36-38]. PACT contains three dsRBMs of which M1 and M2 are true dsRBMs because of their ability to interact with dsRNA [38,39]. PACT's M1 and M2 also facilitate interactions with PACT, PKR, and TRBP [38,39,47,48]. Although PACT's M3 is unable to bind dsRNA because it lacks the lysine residues essential for binding to dsRNA [38,48,165,166], it is essential for PACT-induced PKR activation as it interacts with the PACT-Binding Motif (PBM) within PKR's KD causing the activation of PKR [38,48,68,166]. Within PACT's M3, constitutive phosphorylation of serine 246 is a pre-requisite for stress-induced phosphorylation of serine 287 [38,39,68,98,166]. Stress-induced phosphorylation of PACT is necessary for PACT to be able to homodimerize and subsequently form a heteromeric complex with PKR to promote PKR activation [Fig. 1.3] [38,39,47,48,68,98,113].

Another dsRNA-binding protein, TRBP, regulates PKR activation negatively and also contains three dsRBMs of which M1 and M2 are true dsRBMs [38,39,47,48]. M3 does not bind dsRNA because it lacks lysine residues critical for binding to dsRNA [38,47,48,167-169]. M3 does aid TRBP's M1 and M2 in mediating protein-protein interactions with Merlin, Dicer, PACT, and PKR [38,39,47,48,167,168]. TRBP was initially identified because of its high binding affinity to trans-activating RNA (TAR) element of HIV-1 [43,170-171]. In addition, TRBP is a vital component of RNAi mediated gene silencing via its direct binding with RNA substrates as well as interactions with Dicer and Ago2 [167,172-179].

Despite PACT and TRBP being 40% homologous, they have contrasting effects on PKR activation [38,39,44,46,47,48,167]. In virally infected cells, TRBP inhibits PKR dimerization and activation by directly binding to it and forming heterodimers or by sequestering PKR's activator dsRNA [38,39,46]. Under normal conditions, TRBP inhibits PKR activation by direct binding as well as by forming heterodimers with PACT, thereby preventing PACT-PKR interactions [38,44,46,47,48]. However, stress signals cause PACT phosphorylation at serine 287 which leads to PACT-TRBP dissociation [38,39,46,47,48]. This promotes for PACT-PACT and PACT-PKR interactions [38,39,46-48]. Therefore, PACT and TRBP both play pivotal roles in regulation of PKR activity and the apoptotic cascade under conditions of cellular stress [38,39, 46-48,68]. Further studies have also shown that TRBP plays a critical role in the downregulation of PKR after a successful ISR through reassociation with PACT and PKR [47].

1.2 PACT: The Protein Activator of PKR

PACT is a 313 amino acid protein encoded for by the PRKRA gene which is expressed ubiquitously in various cell types but is most abundantly expressed in the placenta, colon, and testis [24,33,49]. PACT was initially identified through yeast two-hybrid screening of human placenta cDNA library for interaction with the trans-dominant negative PKR mutant which is catalytically inactive (K296R) [24]. The mouse homologue of PACT, RNA Binding Protein X (RAX), was subsequently identified and characterized showing similar molecular functions to its human homologue [33, 35]. Orthologs of PACT have since been identified

across numerous taxa including prominent model organisms such as, zebrafish, rat, and *Drosophila melanogaster*.

Unlike PKR, PRKRA gene is not induced by IFNs [49] but is constitutively expressed in cells and in addition to regulating PKR activation, PACT is also involved in PKR-independent cellular processes [180]. PACT participates in the RNA interference (RNAi) pathway via its interactions with Dicer and is a component of the RNA-induced silencing complex (RISC) [80]. Additionally, PACT is involved in the antiviral innate immune response via its regulatory interactions with retinoic acid-induced gene (RIG-I) and melanoma differentiation-associated protein (MDA-5) [80]. These heteromeric interactions promote type 1 IFN induction [80]. Interestingly, primer extension studies to map the transcription start site and analysis of the promoter region of the PRKRA gene revealed the absence of a conventional TATA box [49, 50]. These studies identified regulatory GC boxes which are known binding sites for the general transcription factor, Sp1[49, 50]. Six Sp1 binding sites were identified within the 300 base pairs (bp) upstream of the PRKRA transcriptional start site [49]. Through the generation of PRKRA nested promoter deletion constructs upstream of a firefly luciferase reporter gene, the minimal promoter was mapped within -101 bp to -1 bp, although all six GC boxes contribute to some extent for the maximal promoter activity [49]. A CCAAT box between positions -404 bp to -400 bp further boosted the promoter activity, but the effect of point mutations in this region was not studied and the transcription factor that binds at this site was also not identified [49].

The highest sequence conservation in PACT among various species is largely localized to the three dsRBMs that serve as important functional domains (Figure 1.2). PACT's two amino terminal dsRBMs, dsRBM1 and dsRBM2, are not only evolutionarily conserved across species, but also share sequence homology to the amino terminal dsRBMs found in both PKR and TRBP [24, 37, 38]. Additionally, PACT contains a comparatively less conserved third carboxy-terminal dsRBM, dsRBM3 [24, 37].

Further investigation into these functional motifs revealed that PACT's dsRNA binding and protein-protein interactions were largely attributed to the evolutionarily conserved amino acid residues within the dsRBMs [24, 36, 38, 39, 51]. In order to elucidate this further, a series of PACT deletion constructs were generated followed by co-immunoprecipitation and yeast two-hybrid studies to map out regions that were important for PACT's interactions with PKR and TRBP. These studies demonstrated that PACT's ability to interact with TRBP and PKR as well as PACT homodimerization require a cooperative effort between dsRBM1 and dsRBM2 while dsRBM3 was largely dispensable for these high affinity interactions despite being essential to induce PKR activation [36-39]. Additional studies confirmed that specific hydrophobic residues within dsRBM1 were most critical for PACT's protein-protein interactions, while dsRBM2 serves to stabilize and reinforce these interactions synergistically with dsRBM1 [38]. Notably, alanine 91 (A91), alanine 92 (A92), and leucine 99 (L99) of PACT's dsRBM1 were found to be critical for PACT-PACT homodimerization [38,51]. A91 and A92 were also found to be essential residues for PACT-PKR associations

while L99 was not [38]. Thus, to address the question if PACT homodimerization was required for PKR activation, the L99E PACT mutant was optimal. Even though this mutation had no consequence on PACT-PKR interactions, an *in-vitro* kinase assay indicated that L99E mutant was unable to activate PKR thus establishing the requirement of PACT-PACT interactions for PKR activation [51].

Characterization of the KD of PKR has revealed a short binding region spanning residues 326-337 that bind PACT's dsRBM3 with low affinity [28]. This interaction was demonstrated to be a product of five residues within PKR (D328, D333, D331, G329, Y332) and this has been termed the PACT binding motif (PBM) (Figure 1.2) [28]. Additionally, the PBM facilitates the intramolecular interaction between PKR's KD and dsRBM2 which keeps PKR in a closed conformation during normal cellular conditions [28]. Despite this association of dsRBM3 of PACT with the PBM, dsRBM1 and 2 are required for efficient PKR activation as they direct the high affinity PACT-PKR interaction [28,36-39,51].

While the mechanistic and biochemical studies outlined above gave tremendous insight into identifying the residues dictating PACT's interaction with PKR, the post-translational modifications that regulate the hetero- and homomeric interactions of PACT remains relatively less studied. Mutagenesis studies have given some insight to this question and identified two serine residues, S246 and S287, within PACT's dsRBM3 that serve as phosphorylation sites [16, 48, 52]. Results from these studies suggest that S246 is constitutively phosphorylated, whereas S287 is phosphorylated in a stress-dependent context. Utilizing phosphomimetic or phosphodefective mutations at each of these sites

demonstrates that the high affinity PACT-TRBP interactions occur only in the absence of the stress induced phosphorylation on S287 [48]. Additional studies have shown that phosphomimetic mutations at both serine residues significantly increase PACT's ability to homodimerize as well as to form PACT-PKR heterodimers [39,48,147].

1.3 The Integrated Stress Response

Eukaryotic cells have evolved a complex network of protective pathways called the integrated stress response (ISR) in order to regulate protein synthesis to maintain homeostasis in response to unfavorable environmental or intracellular stimuli [1, 2]. The primary node of this signaling network is the heterotrimeric eukaryotic translation initiation factor 2 (eIF2) [2]. This highly conserved factor consists of an α,β, and γ subunits with eIF2α being the most studied regulatory subunit during cellular stress [2]. To initiate mRNA translation, eIF2 forms the ternary complex (TC) with GTP and methionyl initiator tRNA (Met-tRNA$_i$) [2-4]. This TC then binds to the 40S ribosomal subunit along with two initiation factors, eIF1 and eIF1A, to form the 43S pre-initiation complex (PIC) [2-4]. Interactions between the PIC and other multi-protein complexes within the eukaryotic translation initiation factor family (eIF) facilitates translation through the recognition of and assembly at the 5'm^7-G-cap of mature mRNAs [2, 3, 4]. The ability of eIF2 to stay in its active form capable of initiating the formation of TC is dependent on the guanine exchange factor (GEF) activity of the eIF2B [3, 4]. Prior to the formation of the ternary complex, a GDP bound to the gamma subunit of eIF2 is substituted for a GTP through the enzymatic activity of eIF2B

[1, 2]. Binding of the TC to 5' cap on mRNAs requires activity of cap-binding initiation factor eIF4 complex and is the mechanism used by most cellular mRNAs [2].

In response to stress stimuli, cap-dependent protein translation is temporarily inhibited, which is accomplished by phosphorylating the α subunit of eukaryotic initiation factor 2 (eIF2) at serine 51 [1,2]. This post-translational modification prevents the formation of the ternary complex which is vital for translation initiation [1,5]. This strategy is a critical response to cellular stressors such as viral infections, misfolded protein accumulation, oxidative stress, and serum/growth factor deprivation [1]. There are a family of four serine/threonine kinases, double-stranded RNA activated protein kinase (PKR), PKR-like Endoplasmic Reticulum kinase (PERK), heme-regulated inhibitor (HRI), and gene control non-derepressible (GCN) kinase which phosphorylate eIF2α [Fig 1.1] [1,2]. While each of these kinases have an evolutionarily conserved kinase domain (KD), they respond to distinct stress signals sometimes in an overlapping manner [1,2]. The subsequent eIF2α phosphorylation inhibits the GEF activity of eIF2B [2, 5]. The inhibition of eIF2B's GEF activity ultimately blocks the formation of the TC because the amount of eIF2 is limiting in cells resulting in the attenuation of general protein synthesis while simultaneously promoting the translation of specific mRNAs whose protein products aid in recovery from stress (Figure 1.1) [2].

During general translational inhibition when eIF2α is phosphorylated, there are certain specific transcripts containing upstream open reading frames (ORFs)

in their 5'UTRs that are preferentially translated and the protein products of these mRNAs promote cellular recovery [1,2]. One of these mRNAs codes for ATF4, which acts as a master transcription factor during ISR and plays an instrumental role in determining cell fate [Fig 1.1] [2]. Depending on the stress severity and duration, ATF4 controls responses towards cell recovery or apoptosis [2]. Therefore, ATF4 function is important for regulating obesity, glucose metabolism, and neural plasticity [2]. ATF4 can form homodimers or heterodimers with other transcription factors such as its downstream target CHOP, which promotes cell death during ER stress [2]. CHOP has several mechanisms to promote apoptosis such as upregulating BH3-only pro-apoptotic BCL-2 family members and enhancing expression of DR5 and ATF5 [2]. During ER stress and amino acid depravation, ATF4 and/or CHOP preferentially bind to the promoter regions of ATF3 and GADD34 [2]. If eIF2α phosphorylation is sustained, pro-apoptotic transcripts are translated in a preferential manner to induce activation of caspases, PARP1 cleavage, and subsequent programmed cell death [1,2]. During the recovery phase, GADD34, which is a product of one such mRNAs translated under conditions of stress, interacts with the catalytic subunit of the serine/threonine protein phosphatase 1 (PP1C) to dephosphorylate eIF2α returning the cell to homeostasis [1,2].

1.4 PACT and Primary Early Onset Dystonia

The primary monogenic dystonias (DYT1-DYT26) are a group of heterogenous movement disorders directly linked to mutations in genes [58-60]. While there is variability between the age of onset and severity of the clinical

pathology between each DYT subtype, the patient populations commonly present with involuntary, sustained and often painful repetitive movements of the dystonic limb, twisted posture, and compromised gait which drastically inhibits their quality of life [58]. A key clinical evaluator in identifying dystonia is the constant lack of directionality of movements [58]. This consistency is due to the firing of both agonist and antagonist muscles simultaneously in response to a single stimulus [58]. Although secondary and pseudo forms of dystonia also occur, these forms of dystonia are symptomatic of either an alternative disease or a side effect of certain drugs [58]. Primary dystonia is distinguished from these alternatives by being the result of a specific genetic mutation and a primary symptom as opposed to being a symptom of an alternative disease [58].

DYT-*PRKRA* is an early onset generalized dystonia caused by mutations in the PRKRA gene which encodes PACT [Fig 1.4] [59,68,98,113]. It typically shows clinical manifestations early in childhood and can have a genetic inheritance pattern of both autosomal dominant and recessive [59]. DYT-*PRKRA* was first characterized by identification of a homozygous mutation in PACT utilizing whole exome sequencing in seven Brazilian patients from two unrelated families [61]. This missense PACT mutation substituted a proline residue for leucine at position 222 (P222L) [61]. A sequencing analysis of the unaffected family members led to the conclusion that they were heterozygous for the P222L mutation [61]. This initial study identifying the P222L mutation as a cause of dystonia led to additional identification of the P222L mutation in two Polish brothers [64]. A German dystonia patient with a frameshift (FS) mutation in PACT

was identified, which was the result of a two nucleotide deletion within PACT's first dsRBM leading to a premature stop codon [62]. The FS mutation causes a truncated PACT protein after 88 amino acids followed by 21 extraneous amino acids and a premature stop codon [62]. In the United States, a male patient was found to have a novel PACT mutation (C213R) on one allele while he inherited the P222L mutation from his mother as the other allele [63]. His father had no mutation in PACT therefore it was determined that the son's C213R mutation occurred de novo [63]. He began developing dystonia clinical symptoms as a toddler [63]. Another dystonia patient was discovered to have novel PACT mutations as well within both alleles which were two recessively inherited mutations (C77S and C213F) [65]. Two other dominantly inherited mutations were identified (N102S and T34S) subsequently and these are the only two substitution mutations characterized thus far with a dominant inheritance pattern [64]. Recently, an early onset DYT-*PRKRA* Italian patient was reported to be a compound heterozygote with a novel mutation (S265R) and a previously known mutation P222L [98].

Earlier studies from our lab, *Vaughn et al*. described that the lymphoblasts derived from patients homozygous for the most prevalent P222L mutation have dysregulated eIF2α stress response signaling and these cells are hypersensitive to ER stress [68]. They further demonstrated that as compared to wt PACT, the P222L mutation has a significantly higher affinity for both PKR and TRBP, while also exhibiting stronger PACT-PACT interactions. Finally, they demonstrated that the DYT-*PRKRA* patient cells show delayed but more prolonged PKR activation

and eIF2α phosphorylation in response to ER stress, thereby resulting in enhanced susceptibility to ER stress-induced apoptosis. [68]. Recently, *Vaughn et al.* highlighted that DYT-*PRKRA* augments RIG-I mediated IFN-β synthesis [180]. Both P222L homozygous and compound heterozygous P222L/C213R mutant patient cells have significantly enhanced basal levels of IFN-β and ISG relative to normal wt unaffected family member cells [180]. Additionally, in response to exposure to dsRNA, both DYT-*PRKRA* cells used in this study generated increased IFN-β production relative to wt lymphoblasts [180]. This study leads to the inference that DYT-*PRKRA* may be involved in dysregulating IFN signaling along with its established role in disrupting ISR signaling [180]. Dystonia has been a noted pathological symptom of interfernopathies, which results from an overproduction of type 1 IFNs [180].

In another study from our lab, *Burnett et al*. characterized that lymphoblasts derived from a compound heterozygous patient (P222L/C213R) are also hypersensitive to ER stress which was elucidated by showing that the cells have enhanced PACT-PKR heterodimer affinity, upregulated PKR kinase activity, dysregulated ISR axis signaling and enhanced apoptosis [113]. This study also elucidated that the two dominant mutations N102S and T34S lead to enhanced PACT-PACT and PACT-PKR affinity with heightened PKR activity and the recessive mutations C77S, C213R, C213F combined with the P222L mutation, also generated enhanced PACT-PACT homodimer affinity and elevated active PKR [113].

Seven reported DYT-*PRKRA* mutations lead to hyperactive PKR, dysregulated eIF2α signaling, and enhanced sensitivity to apoptosis in response to ER stress [68,98,113]. Dysregulated eIF2α signaling and a maladaptive ISR is a common link for pathophysiology of several inherited dystonia types which include DYT1, DYT3, DYT6, and DYT11 that has been inferred from multiple studies [73,121-124] in addition to our research on the disease etiology of DYT-*PRKRA* with homozygous P222L and heterozygous P222L/C213R patient cells [68,113]. Two independent studies substantiate the conclusion that dysfunctional eIF2α signaling plays a causative role in DYT1 synaptic defects [121,122]. Using an unbiased proteomics approach abnormal eIF2α signaling was observed in DYT1 mouse and rat brains which also matched with the results with human brain samples [122]. DYT1 is caused by mutations in the torsin A gene. Torsin A is an ER resident chaperone and the DYT1 mutations have been shown to chronically induce ER stress at low levels [71, 72]. This manifests in both the accumulation of misfolded proteins as well as secretory defects. Another study determined that pharmacological restoration of eIF2α signaling restored the cortico-striatal LTD in DYT1 knock-in mice. This study also observed that there were sequence variants in ATF4 amongst patients with focal cervical dystonia thereby indicating the involvement of defective eIF2α signaling in sporadic cervical dystonia [121]. The underlying genetic cause of DYT6 has been identified to be mutations in the gene coding for thanatos-associated domain-containing apoptosis associated protein-1 (THAP1) [59, 70]. THAP1 is a transcription factor with both cytosolic and nuclear localization. Interestingly,

THAP1 shares an amino terminal motif found in a protein inhibitor of PKR. While the mechanistic studies still need to be pursued, it is plausible that THAP1 induces low levels ER stress through the de-repression of PKR's kinase activity. eIF2α signaling was identified as one of the top dysregulated pathways within heterozygous DYT6 Thap1 mutant neonatal mouse striatum and cerebellum tissue which was analyzed utilizing RNA-Seq [123].

Multiple studies have directly tethered PKR-independently of PACT as an etiology of early onset dystonia due to hyperactive PKR, caused by PKR variants and increased eIF2α phosphorylation [120]. De novo missense mutations in PKR additionally cause a multifaceted neurodevelopmental condition that mirrors vanishing white matter disease. Recently, additional studies have linked newly discovered PKR missense variants (P31R, G130R, and G138A) which are autosomal dominant and one recessive PKR mutation (N32T) to patients who have dystonia [120,134-136]. These investigations emphasize PKR dysregulation as an essential catalyst for disease initiation and progression of dystonia.

1.5 PKR and eIF2α signaling dysregulation as an emerging molecular pathology of neurological diseases

The dysregulation of protein synthesis by affecting the eIF2 axis is quickly becoming an emerging theme in the pathology of movement disorders, neurodegenerative disorders, as well as intellectual disability disorders. Clinical studies have identified elevated disruption of eIF2α signaling in Alzheimer's disease (AD), ALS, and certain forms of autism [74-78]. Conversely, both *in-vivo* and *in-vitro* studies have implicated deficient phosphatase activity of PP1 in

severe neurodevelopment disorders [6, 7, 79, 80]. Patients and disease models deficient in the regulatory subunits of PP1 present with reduced body size, microcephaly, intellectual disability, and in some cases, AD [81, 82]. Most recently, mutations in the γ subunit of eIF2, a critical component in the formation of the ternary complex, have been identified to be a causative factor driving mental intellectual disability, epileptic seizures, hypogenitalism, microcephaly, and obesity (MEHMO) syndrome [83]. Samples taken from patients with MEHMO show signs of chronic stress markers as the causative mutations lead to a defect in ternary complex formation [83]. This ultimately results in the expression of stress response transcripts constitutively as ISR pathway is activated in these patients [83]. A noteworthy symptom that is present in a severe form of MEHMO syndrome is lower limb ataxia [83].

According to the World Health Organization, the number of elderly people will significantly increase over the next 40 years which will be a causality of a continual surge in incidence of morbidity and mortality due to age-related diseases [86]. Neurodegeneration is an example of age-related pathology of which the two most prevalent diseases are Alzheimer's and Parkinson's [86,87,88,89]. The pathophysiology of both diseases manifests in motor deficiencies of the limbic system such as tremor, bradykinesia, rigidity, and postural instability [89,93]. Also, there are cognitive symptoms present such as abnormalities in speech, behavior, mood, and memory deficits [89,93]. Although the pathophysiology of AD and Parkinson's Disease (PD) has not been fully elucidated, it is well known that neuronal dysfunction is correlated with neuronal

inflammation and oxidative stress [86,87,88,89]. The neuroinflammation leads to hyperactive microglia (resident macrophages of the central nervous system (CNS)) and hyperactive astrocytes which play an indispensable role in triggering neuronal apoptosis [93,95,96]. Both AD and PD contain dysregulated cholinergic functions and abnormal accumulations of misfolded proteins which are classified as tau and alpha-synuclein proteins respectively of which studies have determined that there is dysregulation of the proteasomal and autophagy-lysosomal degradation pathways, which clear the misfolded proteins from cells [89,90,92,94]. AD currently affects approximately 4 million Americans and is the third most expensive health care issue in the United States exceeded only by cancer and cardiovascular disease [90]. AD is characterized pathologically by intracellular neurofibrillary tangles (NFT) containing hyperphosphorylated tau protein fragments, insoluble extracellular amyloid-β plaques, and loss of cortical neurons and synapses [86,94]. PD is characterized by dopamine deficiency leading to a substantial apoptosis of dopaminergic neurons in the substantia nigra of the mesencephalon within the CNS [89,91,92]. Cell body of the nigrostriatal pathway resides in the substantia nigra where dopamine is synthesized to be supplied subsequently to striatum [92]. Depleted dopamine levels play a significant role in motor impairment of PD patients [92]. Loss of dopamine modifies neuronal metabolism towards excessive hydrogen peroxide production leading to abnormal levels of hydroxyl free radicals which cause oxidative stress [92]. Augmented reactive oxygen species (ROS) levels are tightly correlated with mitochondrial dysfunction [92]. Oxidative stress and

neuroinflammation most specifically activated microglia are hallmarks of nigrostriatal degeneration within PD patients [86,89,96]. Additional pathophysiology includes excessive accumulation of alpha-synuclein protein inclusions (Lewy bodies) in surviving neurons [89]. To optimize healthcare strategies, identifying and efficiently determining therapeutic efficacy and safety is paramount.

PKR has emerged as a major factor in several neurodegenerative diseases as aberrant heightened PKR activity is observed in post-mortem patient brains as well as in mouse models [112,125-126]. Increased PKR phosphorylation is reported in patients with AD, PD, Huntington's disease (HD), dementia, and prion disease [125,127-131]. Active PKR has been shown to be directly implicated in phosphorylation of tau proteins leading to tau aggregation which are indispensable in disease etiology of Alzheimer's [138]. Within PD and HD patients, strong induction of phosphorylated PKR was found within hippocampal neurons which ties PKR in as an etiological component of extrastriatal degeneration [56]. Hyperactive PKR's implication in elevating phosphorylated eIF2α, global protein attenuation and apoptosis are critical factors leading to degenerating neurons within AD, PD, and HD [139-141]. Activated PKR was also shown to be responsible for the behavioral and neuropathophysiological abnormalities in a mouse model of Down syndrome [132].

1.6 Current PKR and ISR targeted therapeutics

2-Aminopurine (2AP) inhibits PKR thereby diminishing phosphorylation of eIF2α in both *in vitro* and *in vivo* studies [146]. However, at the concentrations used in these studies off target effects were observed such as inhibition of other kinases [146]. The required concentrations essential to see PKR inhibition were in the millimolar range, making its clinical application difficult. The oxindole/imidazole compound, C16, is a small molecule that inhibits PKR thereby reversing the global protein synthesis attenuation induced by active PKR [145,146]. C16 is effective in inhibiting PKR in *In vitro* studies with neuronal cell lines and in *in vivo* studies in mice [145,146]. Additionally, investigations utilizing *in vivo* models of Alzheimer's disease, Hypoxia-Ischemia and Huntington's disease have confirmed that C16's inhibitory effect on PKR promotes neuronal cell recovery and attenuation of neuroinflammation [142,144-145]. However, C16 has additional molecular off-targets which includes cyclin dependent kinases [157], thereby questioning its clinical application. Optimization of C16 concentration, drug administration timeline, and elucidating pharmacokinetics for clinical relevance would need to be worked out. Also, further *in vivo* studies with C16 need to be done in mouse models to determine its suitability for Alzheimer's, Parkinson's, and Down Syndrome.

Small-molecule ISR inhibitor (ISRIB) has been used in multiple studies recently due to its pharmacological effect of rescuing protein synthesis even during elevated eIF2α phosphorylation [181]. ISRIB facilitates enhanced GEF activity thereby promoting formation of the ternary complex under conditions of

cellular stress [2,181]. ISRIB prevents the formation of stress granules in response to ER stress and oxidative stress *in vitro* [182]. *Krukowski et al* highlighted that ISRIB rescues ISR signaling, improves spatial learning and memory, blunts dendritic spine loss leading to enhanced hippocampal neuronal function in an age-related cognitive decline mouse model [183]. ISRIB promoted neurological protection in a prion disease mouse model and reversed neurological damage caused by traumatic brain injury (TBI) [181]. Additionally, within two different AD mouse models ISRIB rescued protein synthesis in the hippocampus, enhanced synaptic plasticity, and improved performance on memory-associated behavior tests [184]. However, ISRIB has been seen to be unable to attenuate ISR signaling when eIF2α phosphorylation exceeds a critical threshold level thereby showing that ISRIB has a narrow window of effect such as when eIF2α phosphorylation is low [181]. Other ISR modulators such as salubrinal and sephin1 affect eIF2α phosphorylation by inhibiting the GADD34-PP1C complex which promotes elevated phosphorylated eIF2α [2]. Salubrinal's showed antiviral activity by inhibiting replication of herpes simplex virus (HSV) both *in vitro* and *in vivo* [2]. In both AD and HD *in vitro* models, salubrinal was shown to reverse ER stress-induced cell death [2]. Sephin1 has been seen to attenuate toxic intracellular misfolded protein aggregation and motor neuron loss thereby blunting motor deficiencies in an ALS mouse model [185]. Collectively, these studies highlight the significant potential of targeted therapeutics that focus on the ISR signaling axis.

1.7 Luteolin as an emerging therapeutic for neurological diseases

Numerous studies have indicated the neuroprotective effects of natural polyphenols such as curcumin, quercetin, resveratrol, and luteolin due to their mechanism of action being directed at attenuating neuro-inflammation and reactive oxygen species (ROS) [86,87,88,89,90,91,92,93]. The polyphenol flavonoid luteolin (3',4',5,7-tetrahydroxy flavone) has shown significant potential in numerous studies designed to test its neuroprotective properties [84,85,86,87,88,89,90,91,92,93]. Luteolin is an abundant metabolite present in medicinal herbs, fruits such as oranges and apples, and vegetables such as broccoli and celery [84,86,91,92]. Structure and function analysis studies have shown that the presence of hydroxyl moieties at carbons 5,7, 3', and 4' positions and the presence of a 2-3 double bond are the primary properties that promote its multiple pharmacological effects [86,95] [Fig 1.6]. In plants luteolin is stored in its glycosylated form. Plants that contain high amounts of luteolin such as Chrysanthemum flowers have been utilized for neuroprotective therapy due to their anti-inflammatory and antioxidant properties in addition to their tumoricidal effects [84,86,92]. Luteolin improved cognitive abilities and hippocampal neurogenesis in a Down syndrome mouse model [85]. In a separate study, luteolin was given along with quercetin as a dietary supplement to children with Autism Spectrum Disorder (ASD) and was reported to promote attenuation of behavioral deficits and improve cognition with no major adverse effects reported [86]. Luteolin's antioxidant properties have been shown to ameliorate Aβ-induced cell death in murine cortical neurons along with improving spatial learning and

memory in an AD mouse model, which highlights luteolin's potential in being a neuroprotective agent against AD [86]. Additionally, luteolin has been seen to promote neuronal recovery in response to traumatic brain injuries (TBI) [86,91]. Luteolin has also displayed neuroprotection from *N*-methyl-4-phenyl pyridinium (MPP^+)- induced neurotoxicity in rat glial cells *in vitro*, which indicates luteolin as a possible PD therapeutic [91]. Previous studies have highlighted that luteolin aids in promoting neurological healing by suppressing inflammation through mechanisms such as attenuating macrophage and T cell presence in neuronal microenvironment along with pro-inflammatory cytokine levels such as TNF-α, IL-6 and IL-1β [84,86,91,93,97]. Also, luteolin blunts secretion of hydrogen peroxide and nitric oxide thereby highlighting its antioxidant properties [91,96,97]. On a molecular level, luteolin represses activity of NF-kB, STAT3, JNK, and TLR-4 pathways which are involved in activation of microglia and astrocytes generating neurological inflammation [84,86,88,91,95,96,97]. To substantiate its neuroprotective properties further, luteolin attenuates microglial and astrocyte activation both *in vitro* and *in vivo* thereby supporting an anti-inflammatory mechanism of action [86,91,93,97]. There has been a link between the inflammatory response of microglia and development of ASD which set the stage for a groundbreaking study showing that dietary luteolin supplementation in children led to a dramatic decrease in serum levels of pro-inflammatory cytokines TNF-α and IL-6 along with recovery of behavior deficits [97].

Dabo et al elucidated that luteolin decreases PACT-PKR interaction, which in turn inhibits PKR homodimerization and activation of its kinase activity [Fig 1.5]

[84]. They reported that in human THP1 macrophage cell line and murine primary macrophages, treatment with luteolin before induction of ER stress and oxidative stress caused reduction in the ISR and expression of pro-inflammatory cytokines IL-8 and IL-1β [Fig 1.5] [84]. In contrast, despite inhibiting the ISR and induction of pro-inflammatory cytokines in a PKR-dependent manner, luteolin caused an increase in activity of the NLRP3 inflammasome leading to an increase in caspase 1 activity in a PKR-independent manner upon induction of ER and oxidative stress [Fig 1.5] [84].

Due to our extensive research on multiple PACT mutations causing dysregulated PKR activity and eIF2α signaling in DYT-*PRKRA* cells, the heteromeric interaction between PACT and PKR is a logical therapeutic target for DYT-*PRKRA* [68,98,113]. Since previous results have highlighted the effect of luteolin on diminishing PACT-PKR affinity in macrophages after oxidative and ER stress, a part of this dissertation will address if luteolin is able to restore homeostasis in DYT-*PRKRA* cells in response to ER stress [84]. I also tested one additional drug, Nelfinavir, that targets ISR pathway by regulating the phosphatase that dephosphorylates eIF2α. Three novel small molecule inhibitors of PKR were also tested on DYT-*PRKRA* patient cells in collaboration with a biotech company. Additionally, I investigated the involvement of PKR activation in another type of monogenic inherited dystonia, X-linked dystonia parkinsonism (XDP). These results indicated PKR's involvement in XDP pathology and form the foundational work that will lead to more investigations in the future.

1.8 Structure of Dissertation

Chapter 2 of this dissertation presents results that establish that the disruption of interaction between PACT and PKR by luteolin restores dysregulated ISR in DYT-*PRKRA* patient cells.

Chapter 3 of this dissertation describes results that characterize the effect of a CReP inhibitor, Nelfinavir on restoration of cellular homeostasis in DYT-*PRKRA* patient cells.

Chapter 4 of this dissertation shows results that indicate the hyperactivity of PKR and subsequent enhanced apoptosis in XDP neural progenitor cells.

Chapter 5 of this dissertation presents results that establish that targeted pharmacological inhibition of PKR promotes cellular recovery in DYT-*PRKRA* patient cells after ER stress.

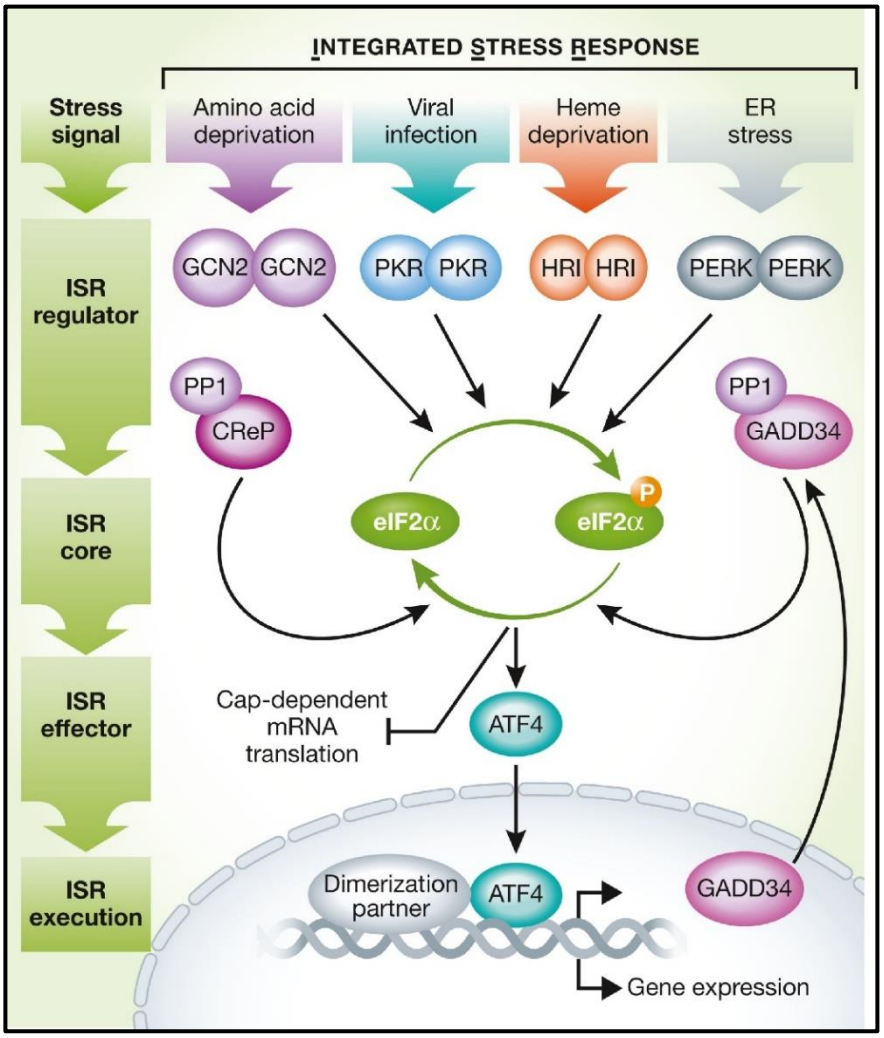

Figure 1.1 The Integrated Stress Response (ISR)

Four kinases (GCN2, HRI, PERK, and PKR) have evolved to attenuate general protein synthesis by phosphorylating the α subunit of the eukaryotic translation initiation factor 2 (eIF2α) in response to specific stress stimuli. This post translational modification blocks the formation of ternary complex, a critical component for the translation initiation. This results in a block in translation initiation from most cellular mRNAs. However, some specific mRNAs containing internal ribosomal entry sites (IRES) or upstream open reading frames (uORFs) such as ATF4 are preferentially translated under these conditions. These mRNAs code for proteins that will either ameliorate the stress or induce apoptosis depending on the level and duration of stress. The ISR is downregulated through the phosphatase activity of PP1. PP1's enzymatic activity on eIF2alpha is dependent on one of two regulatory subunits, CreP or GADD34. The PP1-CreP holophosphatase is critical for maintaining low basal levels of eIF2alpha phosphorylation in the absence of stress, while the PP1C GADD34 holophosphatase is mainly responsible for downregulating the ISR [Pakos-Zebrucka,K et al].

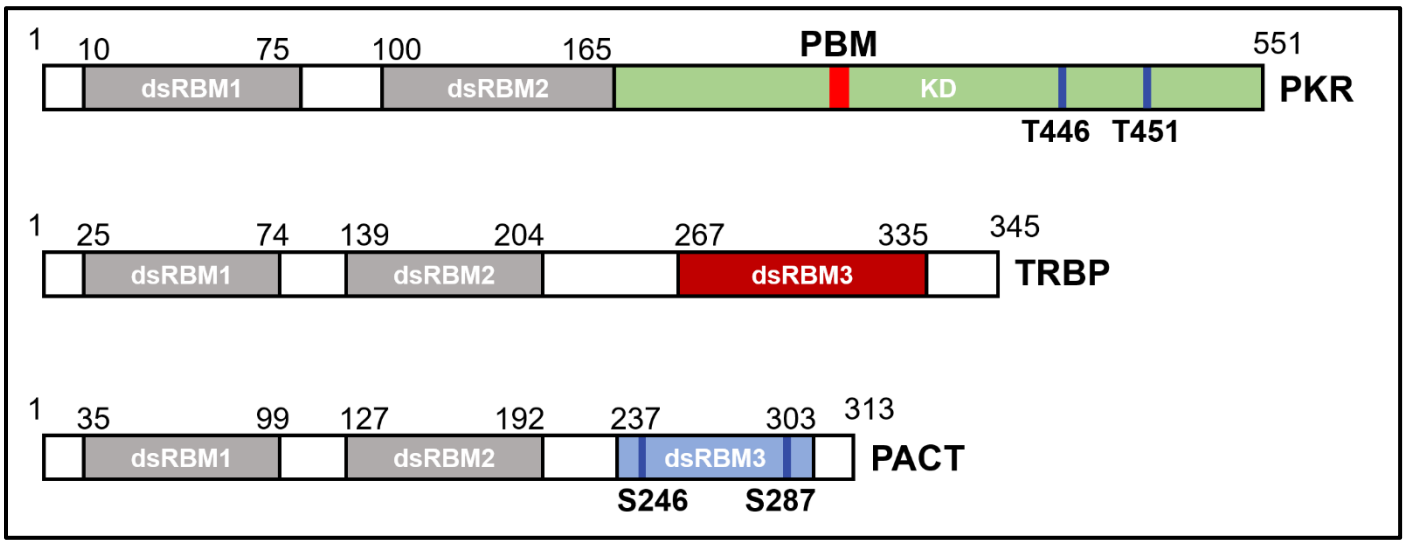

Figure 1.2 Schematic Representation of Domain Structures of PKR, TRBP, and PACT

PKR, TRBP, and PACT are members of a family of dsRNA binding proteins that regulate cell survival or death under conditions of cellular stress. All three proteins share the evolutionarily conserved amino terminal motifs, dsRBM1 and dsRBM2. These amino terminal motifs serve as functional domains mediating dsRNA binding and protein-protein interactions. Additionally, PKR contains a c-terminal catalytic domain containing a PACT-binding motif (PBM) and two critical threonine residues that serve as phosphorylation sites required to activate PKR. In the absence of stress, TRBP interacts with PACT and PKR via the shared amino terminal dsRBMs and keeps its kinase activity latent. Similar to TRBP, PACT also contains three copies of the dsRBMs. Under conditions of cellular stress, PACT dissociates from TRBP and undergoes homodimerization, which is required to bind PKR with higher affinity. PACT then activates PKR via the low-affinity interactions between the PBM in PKR's KD and PACT's dsRBM3. Finally, PACT's dsRBM3 contains two serine residues (S246 and S287) that serve as phosphorylation sites to cause a shift in the relative abundance of PACT-TRBP, PACT-PACT, and PACT-PKR interactions towards PKR activation.

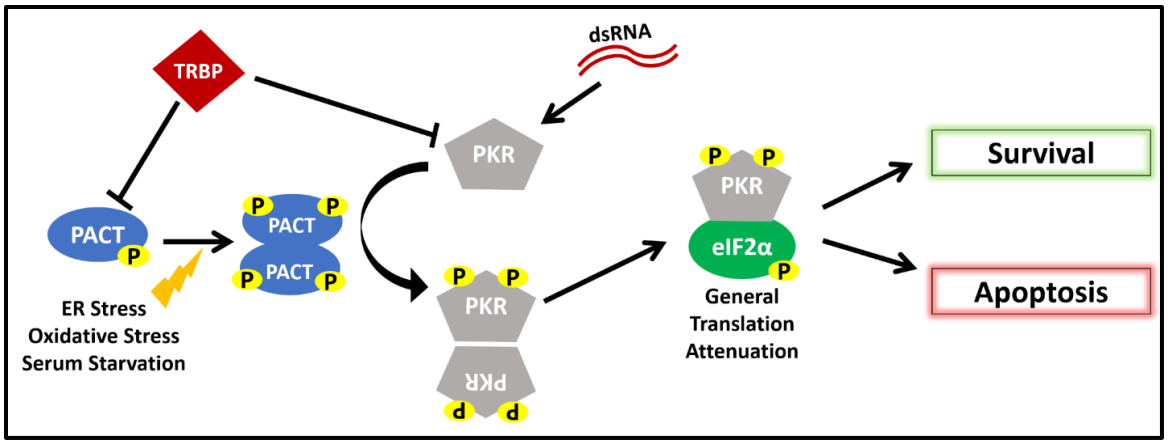

Figure 1.3 Schematic Representation of PKR Regulatory Mechanisms
Under homeostatic conditions, PKR's kinase activity remains latent due to the inhibitory effect of TRBP heterodimerization with PKR and PACT independently. In the absence of stress, PACT is constitutively phosphorylated on S246 which promotes the PACT-TRBP heterodimers. In response to viral stress, dsRNA outcompetes TRBP for PKR's amino terminal dsRBMs resulting in the activation of the kinase. Under conditions of ER stress, oxidative stress, or serum starvation, PACT gets phosphorylated on S287 resulting in the dissociation of the inhibitory PACT-TRBP heterodimers and simultaneously promoting PACT-PACT homodimers. PKR then binds the stress-induced PACT homodimers to facilitate catalytic activation. While PACT-PKR heterodimers are facilitated through the amino terminal motifs of each protein, PACT's c-terminal dsRBM3 is for activating PKR. Once activated, PKR then phosphorylates eIF2α on S51 resulting in the attenuation of cap-dependent general protein synthesis. If the stress stimulus persists or is too severe, the cell undergoes apoptosis. In response to weak or transient stress, however, the cell is able to initiate a successful ISR, reestablish homeostasis and survive.

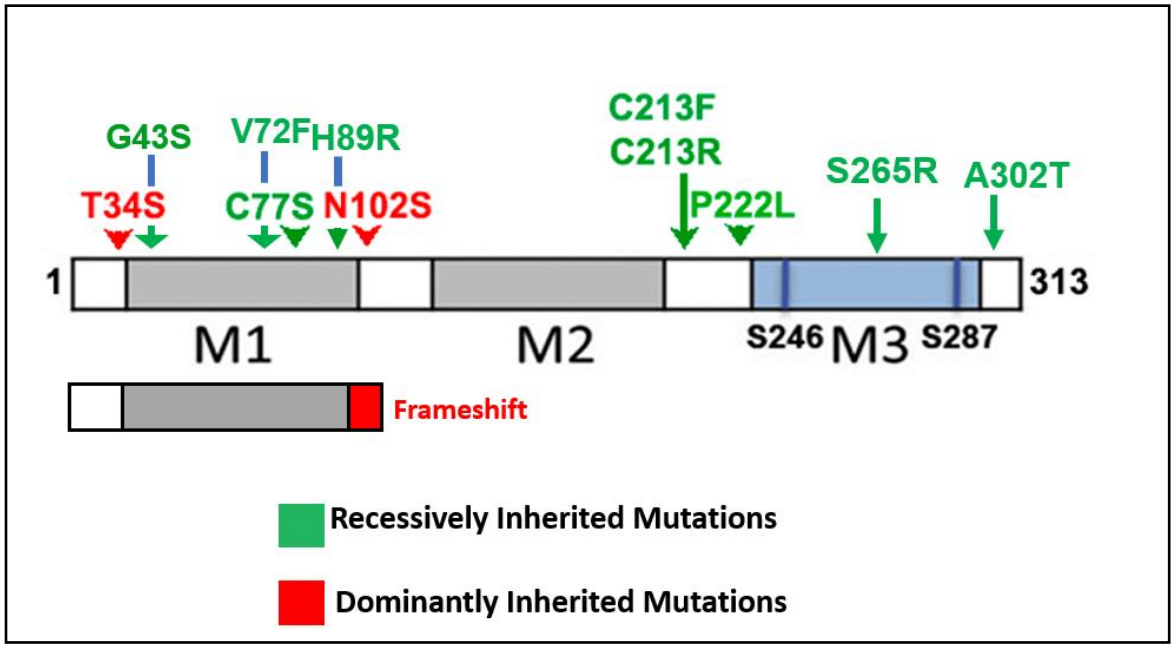

Figure 1.4 Schematic Representation of DYT-*PRKRA* mutations in PACT
Human genetic screenings have implicated mutations in the PRKRA gene which codes for the protein PACT to be the driving cause of early onset dystonia DYT-*PRKRA*. The recessively inherited mutations are indicated in green, and dominantly inherited mutations indicated in red.

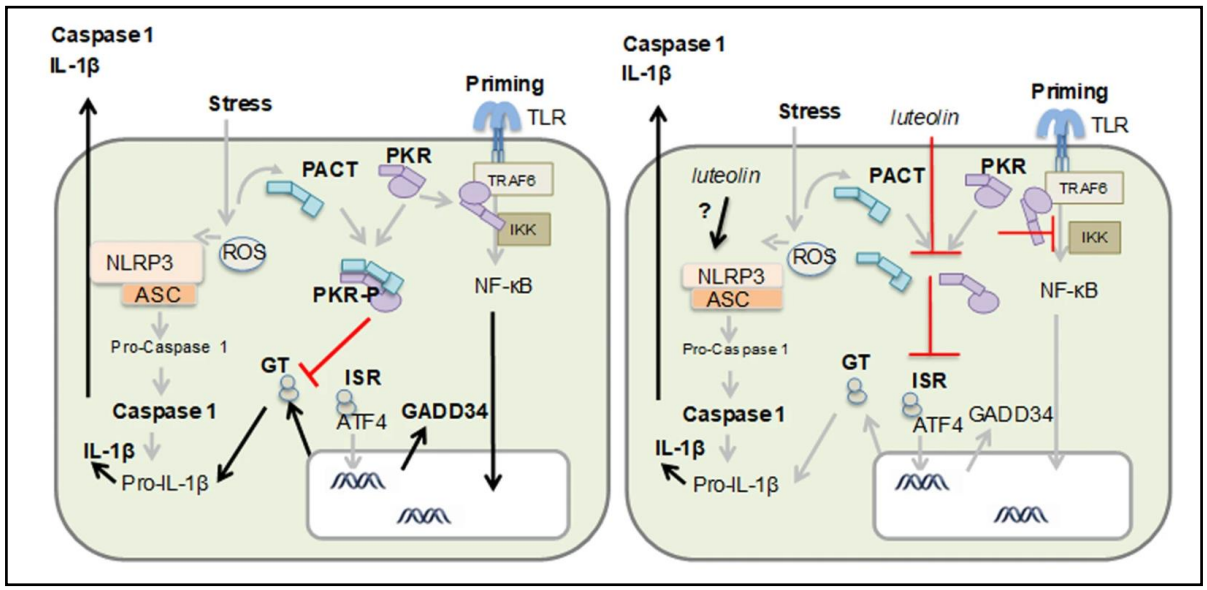

Figure 1.5: Schematic representation of activation of the integrated stress response (ISR) via PKR-dependent pathway and activation of the inflammasome via PKR-independent mechanism (left graphic). Luteolin disrupts PACT-PKR interaction (right graphic) via their double-stranded RNA binding motifs (DSRBMs) which inhibits PKR activation and subsequently diminishes the ISR. In addition, luteolin inhibits induction of pro-inflammatory cytokines. In contrast, luteolin activates the inflammasome in a PKR-independent manner (mechanism not fully elucidated). (Dabo, S., et al.)

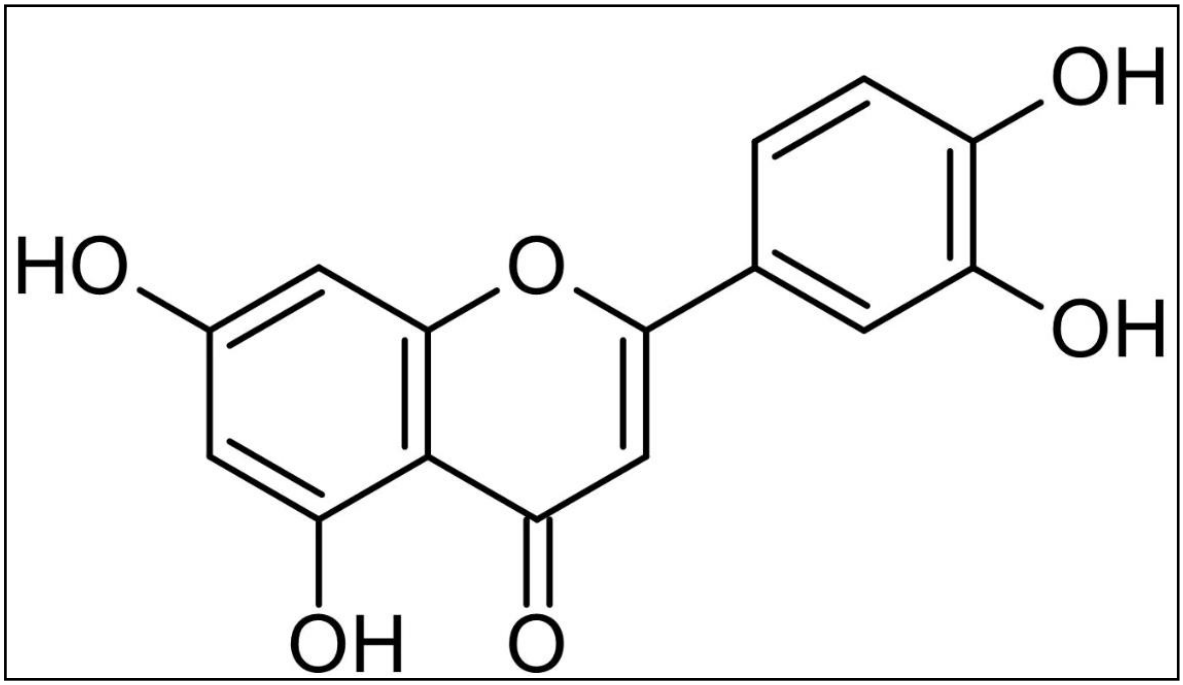

Figure 1.6 Chemical structure of luteolin

Chapter 2:

Luteolin protects DYT-*PRKRA* cells from apoptosis by suppressing PKR activation

Frederick, K., and Patel, R (2023) *Frontiers in Pharmacology*

doi: 10.3389/fphar.2023.1118725

2.1 Abstract

DYT-*PRKRA* is a movement disorder caused by mutations in the PRKRA gene, which encodes for PACT, the protein activator of interferon-induced, double-stranded RNA (dsRNA)-activated protein kinase PKR. PACT brings about PKR's catalytic activation by a direct binding in response to stress signals and activated PKR phosphorylates the translation initiation factor eIF2α. Phosphorylation of eIF2α is the central regulatory event that is part of the integrated stress response (ISR), an evolutionarily conserved intracellular signaling network essential for adapting to environmental stresses to maintain healthy cells. A dysregulation of either the level or the duration of eIF2α phosphorylation in response to stress signals causes the normally pro-survival ISR to become proapoptotic. Our research has established that the PRKRA mutations reported to cause DYT-*PRKRA* lead to enhanced PACT-PKR interactions causing a dysregulation of ISR and an increased sensitivity to apoptosis. We have previously identified luteolin, a plant flavonoid, as an inhibitor of the PACT-PKR interaction using high-throughput screening of chemical libraries. Our results presented in this study indicate that luteolin is markedly effective in disrupting the pathological PACT-PKR interactions to protect DYT-*PRKRA* cells against apoptosis, thus suggesting a therapeutic option for using luteolin to treat DYT-*PRKRA* and possibly other diseases resulting from enhanced PACT-PKR interactions.

2.2 Introduction

Dystonia is a diverse group of movement disorders that involve repetitive, often painful movements of affected body parts resulting in abnormal gaits and postures [58]. Several forms of inherited, monogenic dystonia have been characterized [197] and one such type is DYT-*PRKRA* (aka DYT16), caused by mutations in the *PRKRA* gene, which encodes the protein PACT [59,68,98,113]. DYT-*PRKRA* is a rare, childhood-onset dystonia that exhibits progressive limb, laryngeal, and oromandibular dystonia with features of parkinsonism [58-59,192]. Eleven mutations causing DYT-*PRKRA* have been identified thus far in the *PRKRA* gene (OMIM: DYT16, 612067) [61,62,63,64,65,67,158,198,199]. Although most *PRKRA* mutations causing dystonia are recessive, four dominantly inherited variants have also been reported so far [62,64].

PACT is an activator of protein kinase PKR in response to a variety of stress signals that include endoplasmic reticulum (ER) stress, oxidative stress, osmolarity changes, and serum deprivation [68,98,113,147]. PKR is a double-stranded RNA (dsRNA)-activated protein kinase, which is ubiquitously expressed, and its expression is induced by antiviral cytokine interferon (IFN) [1,2,12,14,15]. The kinase activity of PKR remains latent until it binds to an activator, which brings about a conformational change to expose the ATP-binding site and PKR's enzymatic activation [12,14,16,22,34,37,39,48,114]. In virus infected cells PKR is activated by direct interactions with dsRNA, a viral replication intermediate or virally encoded RNA with extensive ds structures [200]. However, in uninfected cells, stress signals activate PKR via its protein activator, PACT in a dsRNA-independent manner [24,34]. Once

activated, PKR phosphorylates the α subunit of the eukaryotic translation initiation factor 2 (eIF2α) on serine 51 resulting in a transient attenuation of general protein synthesis and this response is part of the integrated stress response (ISR) pathway [2,111]. ISR is an evolutionarily conserved pathway activated in eukaryotic cells by diverse stress signals that functions mainly to restore cellular homeostasis and recovery from stress [2]. One of the four serine/threonine kinases phosphorylate eIF2α and each one of these kinases responds to a specific stress signal sometimes acting in an overlapping manner [1,2]. Phosphorylation of eIF2α prevents the formation of the ternary complex required for translation initiation, leading to a significant decrease in general protein synthesis but at the same time promoting the selective translation of specific mRNAs encoding proteins that promote cellular recovery [2]. Although transient eIF2α phosphorylation promotes cellular survival, prolonged eIF2α phosphorylation induces apoptosis due to the transcriptional induction as well as preferential translation of pro-apoptotic transcripts [1,2]. Thus, the pro-survival ISR response can become pro-apoptotic after exposure to severe or chronic stress to regulate the cellular stress response depending on the duration or severity of the initiating stress signal [1,2].

Previously, our lab reported that four recessively inherited and two dominantly inherited PACT substitution mutations increase cell susceptibility to ER stress by hyperactivation of PKR and dysregulation of the eIF2α phosphorylation in DYT-*PRKRA* patient-derived lymphoblasts [68,113]. Furthermore, a truncated PACT protein resulting from a dominantly inherited frameshift mutation, increased PACT-mediated PKR activation, and an enhanced sensitivity to ER stress via dysregulation of the

eIF2α phosphorylation [98]. Based on these earlier studies, hyperactivation of PKR emerged as a common theme for the PACT mutations reported to cause DYT-*PRKRA*, thus indicating that inhibition of PKR hyperactivation may be able to restore normal ISR and protect against increased apoptosis in dystonia patient cells. Several hyperactive PKR mutations were also reported recently to cause early-onset dystonia especially after a febrile illness [120,134-136]. Based on our previous research on DYT-*PRKRA* and reports of hyperactive PKR in early onset dystonia, it is of interest to evaluate if inhibition of PKR can protect DYT-*PRKRA* cells from increased apoptosis after ER stress. In this study, we have used tunicamycin to induce ER stress and assess if PKR inhibition can protect the cells from apoptosis. A global inhibition of PKR by a chemical inhibitor could be detrimental in patients as PKR activation is an essential component of an innate antiviral response that is required to ward off severe consequences of viral infections [201-203]. Thus, a specific compound that could work by the disruption of PACT-PKR interaction may be best suited for clinical use. Our previous research has identified plant flavonoid luteolin as a compound that disrupts PACT-PKR interactions [84,113]. Thus, we investigated the effect of luteolin on DYT-PRKRA cells after ER stress and our results indicate that luteolin protects DYT-PRKRA patient cells after ER stress by disruption of pathological PACT-PKR interactions while allowing stress-induced transient PACT-PKR interactions to restore the normal, protective ISR response.

2.3 Methods:

Cell lines and antibodies: Both HeLaM and COS-1 cells were cultured Dulbecco's Modified Eagle's Medium (DMEM) containing 10% Fetal Bovine Serum and penicillin/streptomycin. wt and DYT-*PRKRA* Patient B-Lymphoblasts were cultured in RPMI 1640 medium containing 10% FBS and penicillin/streptomycin. Both wt and DYT-*PRKRA* patient lymphoblast cell lines were Epstein-Barr Virus-transformed to create stable cell lines as previously described by Dr. Nutan Sharma (Mass Gen. Hospital), who kindly provided them to us (29,40). All transfections were carried using Effectene transfection reagent (Qiagen) per manufacturer protocol. The antibodies used were as follows: PKR: anti-PKR (human) monoclonal (71/10, R&D Systems), P-PKR: anti-phospho-PKR (Thr-446) monoclonal (Abcam, [E120]), eIF2α: anti-eIF2α polyclonal (Invitrogen, AHO1182), p-eIF2α: anti-phospho-eIF2α (Ser-51) polyclonal (CST, #9721), PACT: Anti-PACT monoclonal (Abcam, ab75749), ATF4: Anti-ATF4 monoclonal (CST, #11815), CHOP: anti-CHOP monoclonal (CST, #2895), Cleaved PARP-1: anti-Cleaved-PARP monoclonal (CST, #32563), β-Actin: Anti- β-Actin-Peroxidase monoclonal (Sigma-Aldrich, A3854). Luteolin (sc-203119C) and tunicamycin (sc-203119C) was purchased from Santa Cruz Biotechnology.

PKR activity assays: HeLa M cells treated with IFN-*β* for 24-hours and harvested at 70% confluency, washed using ice-cold PBS and centrifuged at 600 g for 5-minutes. Cell were resuspended in lysis buffer (20 mM Tris–HCl pH 7.5, 5 mM MgCl2, 50 mM KCl, 400 mM NaCl, 2 mM DTT, 1% Triton X-100, 100 U/ml aprotinin, 0.2 mM PMSF, 20% glycerol) and incubated on ice for 5 minutes. Lysates were centrifuged at 10,000 g for an additional 5-minutes. PKR was immunoprecipitated from 100 µg of this protein

extract using anti-PKR monoclonal antibody (R&D Systems: MAB1980) in a high salt buffer (20 mM Tris–HCl pH 7.5, 50 mM KCl, 400 mM NaCl, 1 mM EDTA, 1 mM DTT, 100 U/ml aprotinin, 0.2 mM PMSF, 20% glycerol, 1% Triton X-100) at 4^{o}C on a rotating wheel for 30-minutes. We then added 10 µL of protein A-Sepharose beads to each immunoprecipitate followed by an additional 1 hour incubation under the same conditions. Protein A-Sepharose beads were washed 4 times in high salt buffer followed by an additional two washes in activity buffer (20 mM Tris–HCl pH 7.5, 50 mM KCl, 2 mM MgCl2, 2mM MnCl2, 100 U/ml aprotinin, 0.1 mM PMSF, 5%, glycerol). PKR activity assay using PKR bound to protein A-Sepharose beads was conducted by using 10µl activity buffer containing 0.1 mM ATP and 10 µCi of [γ-^{32}P] ATP. Either no activator, pure recombinant wt PACT (4 ng) or polyI:polyC dsRNA (400 pg) were used as the PKR activator and were added to the activity buffer befor the addition of ATP. Reaction was incubated at 30°C for 10 min and resolved on a 12% SDS-PAGE gel followed by phosphorimager analysis on Typhoon FLA7000.

Western blot analysis: Lymphoblasts derived from a compound heterozygous DYT-*PRKRA* patient containing both P222L and C213R mutations as independent alleles were cultured alongside lymphoblasts derived from a family member containing no mutations in PACT as our control wt cells. Cells were plated at a concentration of 300,000 cells/ml of RPMI media containing 10% fetal bovine serum and penicillin/streptomycin. To analyze cellular response to ER stress, we treated cells with 5 µg/ml of tunicamycin (Santa Cruz) over a 24-hour time course and harvested cells in RIPA (150 mM NaCl, 1.0% IGEPAL® CA-630, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0) buffer containing a 1:100 dilution of protease inhibitor

cocktail (Sigma) and phosphatase inhibitor (Sigma). Concentration of total protein extract was then determined using BCA assay and appropriate amounts of extracts were analyzed by western blot analyses using appropriate antibodies as indicated. When the cells were treated with luteolin prior to tunicamycin treatment, luteolin was added at 50 µM for 24 h.

Co-Immunoprecipitation assays with endogenous proteins: For Co-Immunoprecipitation (co-IP) of endogenous proteins DYT-*PRKRA* and wt lymphoblasts were seeded at a concentration of 300,000 cells/ml of RPMI complete media and either left untreated or treated with 50 µM of luteolin (Santa Cruz) for 24 h. When treated with tunicamycin for indicated time periods after luteolin treatment, tunicamycin was added at 5 µg/ml. Cells were harvested at indicated time points and whole cell extract was immunoprecipitated overnight at 4^{o}C on a rotating wheel in IP buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 20% Glycerol) using anti-PKR antibody (71/10, R&D Systems) and protein A sepharose beads (GE Healthcare). Immunoprecipitation was carried out using 100 ng of anti-PKR antibody and 10 µl of protein A sepharose beads slurry per immunoprecipitation. Immunoprecipitates were washed 3 times in 500 µl of IP buffer followed by resuspension and boiling for 5 minutes in 1X Laemmli buffer (150 mM Tris–HCl pH 6.8, 5% SDS, 5% β-mercaptoethanol, 20% glycerol). Samples were resolved on 10% SDS-PAGE denaturing gel and probed with anti-PACT antibody to determine co-IP efficiency and anti-PKR antibody to determine equal amounts of PKR were immunoprecipitated in each sample. Input blots of whole cell extract without immunoprecipitation are shown to indicate equal amounts of protein in each sample.

***Mammalian 2-hybrid* interaction assays**: In all cases, wt PACT, P222L, C213R, DD (S246D, S287D) mutant PACT, or PKR ORFs were sub-cloned into both pSG424 expression vector such that it created an in-frame fusion to a GAL4 DNA binding domain (GAL4-DBD), and pVP16AASV19N expression vector such that it maintains an in-frame fusion to the activation domain of the herpes simplex virus protein VP16 (VP16-AD). All of these plasmids have been described in our earlier publications. COS-1 cells were then transfected with: (i) 250 ng each of the GAL4-DBD and the VP16-AD constructs, (ii) 50 ng of pG5LUC a firefly luciferase reporter construct, and (iii) 1 ng of pRLNull plasmid (Promega), to normalize for transfection efficiencies. Cells were then harvested 24-hours post transfection and assayed for both firefly and renilla luciferase activities using Dual Luciferase® Reporter Assay System (Promega). Fusion proteins were assayed for interaction in all combinations.

Caspase 3/7 activity assays: Both wt and patient derived lymphoblasts were seeded at a concentration of 300,000 cells/ml of RPMI complete medium and treated with a concentration of 5 µg/mL of tunicamycin for 24 hours. Samples were collected at indicated time points and mixed with equal parts Promega Caspase-Glo 3/7 reagent (Promega G8090) and incubated for 45 minutes. Luciferase activity was measured and compared to cell culture medium alone and untreated cells as the negative controls. To address the effect of inhibiting PACT-PKR interaction on cell viability, we cultured wt and patient lymphoblasts as described above in 50 µM of luteolin for 24 hours followed by treatment with 5 µg/ml of tunicamycin in luteolin free media over the same 24 hours.

RNA isolation and qRT-PCR: Total RNA was isolated from lymphoblasts using RNAzol RT (Sigma-Aldrich). After two washes with ice-cold PBS, 250 µL of RNAZol RT was added and total RNA was isolated as per the manufacturer's instructions. For each sample we reverse transcribed with 800 ng of RNA using kit iScript™ Reverse Transcription Supermix for RT-qPCR (Bio-Rad, Hercules, CA, United States). The expression analysis of ATF4, CHOP and GAPDH was performed using the following primers.

ATF4 (Origene): Forward 5′-TTCTCCAGCGACAAGGCT AAGG-3'

Reverse 5′-CTCCAACATCCAATCTGTCCCG-3'.

CHOP (Origene): Forward 5′-GGTATGAGGACCTGCAAG AGGT-3'

Reverse 5′-CTTGTGACCTCTGCTGGTTCTG-3'.

GAPDH (Origene): Forward 5′-GTCTCCTCTGACTTCAAC AGCG-3'

Reverse 5′-ACCACCCTGTTGCTGTAGCCAA-3'

TaqMan Universal PCR Master Mix (Applied Biosystems), and cDNA derived from 40 ng total RNA was used. All reactions were run on a BioRad CFX96 Real-Time System C1000 thermal cycler machine using the conditions recommended for the primer sets (Origene). For each treated sample, relative quantification (RQ) ($2^{-\Delta\Delta CT}$) (Pfaffl, 2001), i.e., the normalized fold change relative to the mean of each of the controls, was calculated.

Data Sharing: All data is contained within this manuscript. Data sharing is not relevant for this work.

2.4 Results

PKR is hyperactive in DYT-*PRKRA* cells sensitizing them to ER stress

Previously, our research established that DYT-*PRKRA* patient lymphoblasts are more susceptible to ER stress compared to the unaffected, wild type (wt) lymphoblasts [68,113]. To investigate if this susceptibility to apoptosis results from higher levels of PKR's kinase activity, we performed a PKR activity assay to measure active kinase levels and a western blot analysis to compare levels of the phosphorylated form of PKR (p-PKR) in wt and patient cells in the absence of any stress. As seen in Figure 2.1A, the DYT-*PRKRA* patient cells show about 5-fold higher levels of PKR kinase activity (orange bar) compared to the wt cells (blue bar) in the absence of ER stress. The higher levels of active PKR were further supported by the western blot analysis with an antibody specific for p-PKR. These results demonstrate that DYT-*PRKRA* cells exhibit constitutive activation of PKR in the absence of ER stress. As PKR like endoplasmic reticulum resident kinase (PERK) is the other kinase that is activated in response to ER stress, we investigated if the total expression levels of PERK or phosphorylated active PERK were more in DYT-PRKRA cells. The levels of total PERK and phosphorylated form of PERK are similar in wt and DYT-*PRKRA* cells. As seen in Figure 2.1B, when subjected to ER stressor tunicamycin, the levels of eIF2α phosphorylation rise within 1 h in both wt and patient lymphoblasts. However, the patient lymphoblasts show significantly higher levels of eIF2α phosphorylation which also persists at 8 h after tunicamycin treatment whereas in wt cells there is a decrease in eIF2α phosphorylation at 8 h. PKR activation and levels of phosphorylated PKR also rise at 1 h after tunicamycin treatment and start to decline at 8 h after treatment in

wt cells. In contrast, the levels of phosphorylated PKR are significantly high in the absence of treatment in the DYT-*PRKRA* patient cells with barely a detectable increase after tunicamycin treatment as analyzed by western blot analysis. The levels of GADD34, which is the regulatory subunit of protein phosphatase 1 (PP1) whose expression is induced in response to ER stress and acts to regulate the dephosphorylation of eIF2α and return cells to homeostasis were also compared in the wt and DYT-*PRKRA* cells. As seen, GADD34 is induced at higher levels in DYT-*PRKRA* cells as compared to wt cells. However, this increased expression of GADD34 is not sufficient to reduce the eIF2α phosphorylation that results from PKR activity remaining high at 8 h after ER stress in DYT-*PRKRA* cells. To confirm PKR activation in response to tunicamycin in DYT-*PRKRA* patient cells, we next performed PKR activity assays, which are more quantifiable and sensitive than the western blot analysis to detect a tunicamycin-induced increase in PKR activity above the high constitutive levels of activated PKR. As seen in Figure 2.1C, there is an increase in PKR activity following tunicamycin treatment in both wt and DYT-*PRKRA* patient lymphoblasts and the patient lymphoblasts have about 5-fold higher PKR activity as compared to wt lymphoblasts both with and without tunicamycin treatment. The elevated PKR kinase activity predisposes the DYT-*PRKRA* lymphoblasts to apoptosis as seen in Figures 2.1D, E. The levels of cleaved PARP1, which is a marker for apoptosis, are significantly higher in DYT-PRKRA patient lymphoblasts as compared to the wt lymphoblasts at 8–12 h after tunicamycin treatment. The levels of caspase 3/7 activity, another marker for apoptosis, are also significantly higher in DYT-*PRKRA* patient lymphoblasts in untreated as well as at 24 h after tunicamycin treatment

(orange bars). These results thus indicate that the DYT-*PRKRA* lymphoblasts have elevated levels of active PKR at basal levels which increase further after ER stress.

Inhibition of PKR protects DYT-*PRKRA* cells against ER stress-induced apoptosis

To test if inhibition of PKR activity can protect the DYT-*PRKRA* cells from ER stress-induced apoptosis, we used an established PKR inhibitor C16 [144-146]. Our previous results established that the PACT mutations in DYT-*PRKRA* patients cause enhanced association of PACT with PKR in the absence of stress and result in elevated PKR activation [113]. The enhanced PKR activation observed in DYT-PRKRA lymphoblasts (Figure 2.1) thus results from PACT-mediated PKR activation, making it important to determine that C16 inhibits PKR when activated by PACT. Previously, C16 was reported to inhibit PKR when activated by PACT [221-222] and thus we first confirmed this in DYT-PRKRA cells. As seen in Figure 2.2A, in the absence of an activator, PKR activity is barely detectable (lane 1) and dsRNA (lane 2), as well as PACT (lane 3), both activate PKR robustly. When added in the presence of dsRNA or PACT, C16 inhibits PKR significantly at both concentrations tested (lanes 4–7). Next, we tested the actions of C16 on PKR activity in wt and DYT-*PRKRA* patient lymphoblasts. As seen in Figure 2.2B, tunicamycin treatment activated PKR strongly in wt cells (lane 2) and this activation is inhibited significantly at 0.1 μM and almost completely at 0.5 μM of C16 (lanes 3 and 4). Similarly, in the DYT-*PRKRA* patient lymphoblasts, PKR activity is partially inhibited at 0.1 μM and almost completely at 0.5 μM of C16 (lanes 7 and 8). The effect of C16 on eIF2α phosphorylation seems less pronounced compared to its effect on PKR, possibly

because C16 does not inhibit PERK. The eIF2α phosphorylation is significantly reduced in both wt and DYT-PRKRA cells by 0.5 μM of C16 (lanes 4 and 8). To investigate the effect of C16 on apoptosis induced by tunicamycin, we used both the cleaved PARP1 and caspase assays. As seen in Figure 2.2C, in wt lymphoblasts, C16 inhibited PARP1 cleavage significantly at both 0.1 and 0.5 μM concentrations (lanes 3 and 4). In DYT-*PRKRA* patient lymphoblasts, C16 inhibited PARP1 cleavage partially at 0.1 μM (lane 7) and almost completely at 0.5 μM (lane 8). To further confirm that C16 can inhibit apoptosis, we used a caspase 3/ 7 assay. As seen in Figure 2.2D, DYT-*PRKRA* patient lymphoblasts (orange bars) show a higher level of caspase activity without any ER stress and this basal caspase activity is inhibited by C16. At 24 h after tunicamycin treatment, the caspase activity increases about 6-fold in wt (blue bars) and about 4.5-fold in DYT-*PRKRA* cells. C16 inhibits this increase significantly in both wt and DYT-*PRKRA* cells with about 70% decrease in wt (blue bars) and about 80% decrease in DYT-*PRKRA* cells (orange bars). These results establish that inhibition of PKR protects both wt and DYT-*PRKRA* cells after ER stress.

Luteolin disrupts the stronger PACT-PKR interaction in DYT-*PRKRA* cells

Previously, we have established that luteolin, a plant flavonoid, disrupts the interaction between PACT and PKR [84,113]. In human THP-1 macrophages, luteolin inhibits PKR phosphorylation and the induction of pro-inflammatory cytokines in response to oxidative stress and toll-like receptor (TLR) agonist lipopolysaccharide [84]. The ISR induced by oxidative stress or ER stressor thapsigargin was only partially blocked by luteolin treatment in this study, which was attributed to the activity of PERK remaining unaffected by luteolin. In our DYT-*PRKRA* cells, we wanted to

characterize if luteolin can effectively disrupt the enhanced interaction between PACT mutant P222L and PKR. To determine this, we used coimmunoprecipitation analysis with wt and DYT-*PRKRA* patient lymphoblasts that are homozygous for P222L mutation [68]. We have established previously that luteolin disrupts the PACT-PKR interaction in compound heterozygous DYT-*PRKRA* lymphoblasts carrying P222L and C213R mutations [113]. We used the P222L homozygous lymphoblasts in the coimmunoprecipitation analysis because we have previously established that in compound heterozygous patient cells, only the P222L-PKR interaction is enhanced but the C213R-PKR interaction has similar affinity as the wt PACT-PKR interaction [113]. Both P222L homozygous and compound heterozygous DYT-*PRKRA* cells undergo enhanced apoptosis in response to ER stress [68,113] and thus the P222L homozygous cells are better suited for coimmunoprecipitation analysis without any interference from the C213R mutant that would occur in the compound heterozygous patient cells. As seen in Figure 2.3A, in the absence of any ER stress, the wt lymphoblasts show very slight interaction between PACT and PKR (lane 2, co-IP panel), which is characteristic in the absence of a stress signal and in accordance with previous research [68]. However, the DYT-*PRKRA* cells homozygous for P222L mutation show markedly enhanced interaction between PKR and PACT (lane 5, co-IP panel) even in the absence of ER stress. When treated with luteolin for 24 h, the interaction between PACT and PKR in wt lymphoblasts is undetectable (lane 3, co-IP panel), and the interaction between P222L mutant and PKR is markedly reduced (lane 6, co-IP panel) indicating that luteolin disrupts the enhanced interaction between P222L mutant and PKR. The IP panel shows that an equal amount of PKR was

immunoprecipitated in all samples except for antibody-negative controls (lanes 1 and 4). The input panel shows that equal amounts of PACT were present in all samples. These results establish that a 24 h treatment with luteolin disrupts the PACT-PKR interaction in DYT-*PRKRA* patient cells. To confirm these results further, we tested the interaction between PACT and PKR using mammalian two-hybrid analysis. We have previously used such analyses to establish that the DYT-*PRKRA* mutations result in enhanced interactions between PACT and PKR in intact mammalian cells in the absence of a stress signal [68,113]. As seen in Figure 2.3B, the PKR interaction with wt PACT is detectable at basal levels in the absence of ER stress in this system and luteolin treatment disrupts this interaction significantly (white bars). As compared to this, the interaction between P222L mutant and PKR is about 3-fold stronger at basal levels in the absence of stress and luteolin can disrupt the interaction markedly. The C213R-PKR interaction is comparable to the wt PACT-PKR interaction as expected based on our previous research (Burnett et al., 2020) and is also disrupted efficiently by luteolin. These results establish that luteolin disrupts the stronger interaction between DYT-*PRKRA* mutant P222L and PKR and indicated that luteolin may potentially be a good candidate to test for protecting the DYT-PRKRA cells from ER stress-induced apoptosis.

Luteolin protects DYT-*PRKRA* cells from ER stress-induced apoptosis

We next tested the ability of luteolin to protect DYT-*PRKRA* cells from ER stress-induced apoptosis using PARP1 cleavage and caspase 3/7 activity as apoptosis markers. As seen in Figure 2.4A, in the absence of luteolin pre-treatment, there are significant amounts of cleaved PARP1 at 12 and 24 h after tunicamycin

treatment in wt cells (lanes 3 and 4), which is markedly reduced by luteolin pretreatment (lanes 7 and 8). In contrast to wt cells, the DYT-*PRKRA* cells show markedly increased cleaved PARP1 at 8, 12, and 24 h after tunicamycin treatment (lanes 10–12) and luteolin pre-treatment significantly reduces the amount of cleaved PARP1 at all these time points after tunicamycin treatment (lanes 14–16). In agreement with this, as seen in Figure 2.4B, there is a significant reduction of caspase 3/ 7 activity after ER stress in luteolin pre-treated cells. The wt cells show about 7.5-fold induction of caspase 3/7 activity at 24 h after tunicamycin treatment, and luteolin pre-treatment shows about 64% repression (blue bars). Compared to wt cells, the DYT-*PRKRA* patient cells, there is about 4-fold higher level of caspase 3/ 7 activity in the absence of any stressor, and luteolin can repress about 60% of this basal activity (orange bars). The DYT-*PRKRA* patient cells show about 4.5-fold induction of caspase 3/7 activity 24 after tunicamycin treatment and luteolin pre-treatment shows about 70% reduction, thus supporting the PARP1 cleavage results in Figure 2.4A. Luteolin is thus effective in protecting both the higher basal level of apoptosis in DYT-PRKRA cells as well as tunicamycin-induced apoptosis in both wt and DYT-PRKRA cells.

Luteolin suppresses higher PKR and eIF2α hyperphosphorylation in DYT-*PRKRA* cells

To further assess the effect of luteolin on the PKR activation and eIF2α phosphorylation and understand the mechanism for the protection from apoptosis offered by luteolin, we pre-treated the wt and DYT-*PRKRA* patient cells with luteolin for 24 h and then treated with tunicamycin for various time intervals to compare their

response. As seen in Figure 2.5A, tunicamycin treatment induced significant PKR phosphorylation at 2, 4, and 8 h after the treatment in wt cells (-lut panel, lanes 4–6) and the luteolin pretreatment reduced both the level of PKR phosphorylation and the duration (+lut panel, lanes 4–6). In DYT-*PRKRA* cells, tunicamycin treatment induced a detectable PKR phosphorylation above the high basal level (-lut panel, lanes 10–14), and the luteolin pre-treatment reduced both the level of PKR phosphorylation and the duration (+lut panel, lanes 10–14). In agreement with this, the eIF2α phosphorylation levels and duration are also significantly reduced after luteolin treatment in both wt and DYT-*PRKRA* cells (compare p-eIF2α: − lut and +lut panels, lanes 2–6 and lanes 10–14). We quantified the band intensities of p-eIF2α and total eIF2α from four independent experiments and calculated the ratio of p-eIF2α to total eIF2α, which is represented in a graphical format in Figure 2.5B. The results indicate that disrupting the interaction between PACT and PKR blunts the level and duration of both PKR and eIF2α phosphorylation in wt and DYT-*PRKRA* patient cells. This indicates that luteolin-mediated protection of the DYT-*PRKRA* cells after ER stress could result from inhibition of PKR activity. Thus, the excessive or prolonged phosphorylation of PKR and eIF2α is prevented by luteolin and this may be one of the reasons for restoration of homeostasis after ER stress.

Luteolin inhibits ER stress-induced expression of ATF4 and CHOP

We next examined if the ER stress-dependent induction of transcription factors ATF4 and CHOP also reflect a similar reduction after luteolin treatment. As seen in Figure 2.6A, the DYT-*PRKRA* lymphoblasts induced ATF4 and CHOP at higher levels (lanes 9–12) as compared to wt lymphoblasts (lanes 1–4). Luteolin treatment

attenuated both ATF4 and CHOP induction significantly in wt lymphoblasts (lanes 5–8) as well as in DYT-*PRKRA* lymphoblasts (lanes 13–16). While there was almost a complete block of CHOP induction in luteolin treated cells, ATF4 induction was significantly reduced by luteolin treatment. There was also a corresponding reduction in the mRNA levels of ATF4 and CHOP as seen in Figure 2.6B. This can partly explain the protection from apoptosis seen in Figure 4 as CHOP is known to contribute to apoptosis after ER stress (Silva et al., 2005; Sano and Reed, 2013). CHOP has been shown to induce genes involved in protein synthesis (Han et al., 2013) and high rates of protein synthesis leads to ATP depletion, oxidative stress, and cell death, thus high levels of expression of CHOP are known to be harmful for cellular recovery and homeostasis after ER stress.

Luteolin inhibits the persistent PACT-PKR interaction at later time points in DYT-*PRKRA* cells while allowing transient PACT-PKR interaction at earlier time points after ER stress

Our previous work established that PACT is phosphorylated in response to stress signals and the phosphorylated PACT associates with PKR at a higher affinity thereby inducing PKR activation [16,34,39,48]. As luteolin disrupts the interaction between PACT and PKR, we reasoned that luteolin may be able to disrupt the enhanced interaction between mutant PACT and PKR present in DYT-*PRKRA* cells in the absence of ER stress while permitting a transient interaction of phosphorylated mutant PACT with PKR at early time points after ER stress. This would explain why PKR can still show activation after ER stress in the presence of luteolin (Figure 2.5). We tested this using coimmunoprecipitation and a mammalian two-hybrid analysis. As

seen in Figure 2.7A, in DYT-*PRKRA* cells, mutant PACT coimmunoprecipitates with PKR efficiently in the absence of ER stress (lane 2). A luteolin treatment for 24 h results in a complete disruption of PACT-PKR interaction and no coimmunoprecipitation of mutant PACT can be detected (lane 3). The PACT-PKR interaction is maintained after tunicamycin treatment in the absence of luteolin (lanes 4–6). Interestingly, when treated with tunicamycin to induce ER stress after a 24 h pretreatment with luteolin, mutant PACT co-immunoprecipitates with PKR is at 2 h and 4 h after tunicamycin treatment (lanes 7 and 8) but not at 8 h after tunicamycin treatment (lane 9). As 24 h after luteolin treatment PACT-PKR interaction is significantly disrupted (lane 3), these results indicate that early phosphorylation of PACT after tunicamycin treatment [16] allows for PACT-PKR interaction in the presence of luteolin but at later time points when PACT-PKR interaction is disrupted. These results demonstrate that luteolin disrupts the high PACT-PKR interaction very efficiently in the absence of ER stress when mutant PACT is not phosphorylated. However, once phosphorylated after ER stress [16], the stronger interaction between phosphorylated mutant PACT and PKR can occur in the presence of luteolin only while PACT stays phosphorylated (lane 9). This was further tested using a mammalian two-hybrid interaction assay and a phosphomimic mutant of PACT where we replaced the two serines at 246 and 287 that are phosphorylated in after stress signals with aspartic acids (S246D, S287D or DD mutant). This mutant has been used previously in several studies by us and other labs as it is established that the phosphorylation of serines 246 and 287 results in enhanced interaction between PACT and PKR after cellular stress [39,48]. As seen in Figure 2.7B, PKR and wt PACT interact at a

detectable level in this assay and the interaction is enhanced more than 2-fold between the phosphomimic mutant PACT (DD PACT) as indicated by the black bars. When treated with luteolin, the interaction between PKR and wt PACT is barely detectable above the negative controls but the interaction between DD PACT and PKR is still detectable (white bars), although reduced compared to the interaction in the absence of luteolin. These results indicate that luteolin prevents the enhanced PACT-PKR interactions in DYT-*PRKRA* cells at the late adaptive phase of ISR and allows restoration of cellular homeostasis while maintaining the PKR and eIF2α phosphorylation at the earlier time points after ER stress.

2.5 Discussion

To develop effective therapeutic strategies for dystonia, it is essential to understand the underlying molecular mechanisms that lead to this movement disorder. Aiming to elucidate the possible pathological mechanisms, our previous work on DYT-*PRKRA* focused on studying how the mutations reported in DYT-PRKRA patients affect the biological PKR activation function of PACT [68,98,113]. As enhanced PKR activation due to stronger PACT-PKR interactions emerged as a common theme for DYT-*PRKRA*, in this study we examined the effect of disrupting PACT-PKR interactions using luteolin. Luteolin is a natural flavonoid that exhibits beneficial effects on human health, which have been described in several traditional medicines that make therapeutic use of natural plants, fruits, and herbs [84,86,91,92,205]. With the availability of modern analytical biochemical and molecular techniques, luteolin's effects on a variety of cellular responses have been studied and documented and currently luteolin is being explored for its beneficial activity in treating various human ailments [204]. Among the diverse health benefits of luteolin, its anti-cancer, anti-microbial, anti-inflammatory, antioxidant, and anti-diabetic effects have been studied in detail in various cell types and mouse models [205]. Additionally, luteolin is blood-brain barrier permeable and is reported to have a neuroprotective effect in cell culture and animal models of Alzheimer's [206], Parkinson's [207], and Huntington's [208] disease. A combination of luteolin and quercetin also proved effective in reducing symptoms of autism spectrum disorders (ASD) [209]. The exact mechanisms by which luteolin exerts these effects remains poorly characterized and the effects are often thought to be pleiotropic. We previously identified luteolin as an inhibitor of the PKR-

PACT interaction using high-throughput screening of chemical libraries [84]. Thus, our current study to test therapeutic potential of luteolin for DYT-*PRKRA* stemmed from the prior extensive biochemical and molecular knowledge about the disease mechanisms operative in DYT-PRKRA and the demonstrated ability of luteolin to disrupt PACT-PKR interactions.

Our results presented in this study indicate that disrupting PACT-PKR interactions in DYT-PRKRA patient cells represses PKR activation, eIF2α phosphorylation, ATF4 induction as well as CHOP induction in response to ER stress. Luteolin also prevented the higher levels of apoptosis seen in DYT-*PRKRA* cells in response to ER stress. Our results indicated that although luteolin disrupts the strong PACT-PKR interactions observed in patient cells in the absence of stress, it allows for the stress-induced and transient PACT-PKR interaction. PACT is phosphorylated constitutively at serine 246 in the absence of stress and is rapidly phosphorylated at serine 287 in response to cellular stress [34,39,48]. Our results in Figure 2.9 demonstrated that luteolin does not disrupt the transient stress-dependent interaction between PACT and PKR. Thus, luteolin prevents enhanced PACT-PKR interactions in DYT-*PRKRA* patient cells in the absence of stress while preserving the normal stress-induced transient PACT-PKR interactions to allow for a transient PKR activation during ISR (Figure 2.8). This potentially indicates that the interaction between phosphorylated PACT and PKR has higher affinity than the affinity between DYT-*PRKRA* PACT mutants and PKR.

It is interesting that disrupting PACT-PKR interaction with luteolin almost completely prevents induction of CHOP, a transcription factor that contributes at least

in part to apoptosis after ER stress [210-213]. Although the repression of CHOP induction after luteolin treatment supports previous research that reported an essential role of PACT-mediated PKR activation in ER stress-induced apoptosis [16,214], it is possible that the antioxidant actions of luteolin contribute to its protective effects after ER stress. Previously it has been observed that an antioxidant treatment and CHOP deletion act through a common mechanism to suppress apoptosis after ER stress [215]. In future, the contribution of antioxidant actions of luteolin towards protection from apoptosis after ER stress needs to be examined by comparing the actions of luteolin with other antioxidants that have no effect on PACT-PKR interaction. Additionally, transcriptional induction by ATF4 and CHOP has also been shown to increase protein synthesis leading to oxidative stress and cell death [216], thus indicating that CHOP may contribute to apoptosis via induction of oxidative stress. However, in our study, we observe the protective actions of luteolin in the absence of CHOP, as the CHOP induction is almost completely blocked after luteolin treatment. Any contribution of luteolin's antioxidant actions towards CHOP induction after ER stress can be investigated in the future studies to understand the contribution of oxidative stress for CHOP induction. Furthermore, in our current study we did not investigate the effects of luteolin under conditions of chronic stress, which is likely to be present in DYT-*PRKRA* cells at basal low levels. As ATF4 and CHOP has been shown to contribute to a coordinated stress-induced transcriptional reprograming that prevents cell death under conditions of chronic ER stress [217], in future studies, luteolin's effects on possible reprograming in DYT-*PRKRA* cells can offer mechanistic insights.

We have previously shown that PACT-induced PKR activation is essential for tunicamycin-induced apoptosis and PACT as well as PKR null cells are markedly resistant to apoptosis, show defective eIF2α phosphorylation and compromised CHOP induction [16]. A reconstitution of PKR and PACT expression in the respective null cells rendered them sensitive to tunicamycin, thus establishing that PACT-induced PKR activation plays an essential function in induction of apoptosis. Additionally, when overexpression of the trans-dominant negative, catalytically inactive mutant K296R was used to inhibit PKR in neuroblastoma cells, it protected the cells from undergoing apoptosis [137]. K296R overexpressing cells showed defective PKR activation, delayed eIF2α phosphorylation, compromised CHOP expression, and reduced caspase-3 activation.

Our approach of inhibiting the heightened PKR activation observed in DYT-*PRKRA* with luteolin while preserving a transient PKR activation under conditions of stress could be helpful for treatment of diseases that involve overactive PKR [218]. Higher levels of activated PKR are noted in post-mortem patient studies as well as in mouse models of neurodegenerative conditions [112,125-126]. Increased levels of phosphorylated PKR have been reported in the brains of Alzheimer's disease (AD) patients [127], Parkinson's disease, Huntington's disease [128-129], dementia [130], and prion disease [131]. Inhibiting PKR has proven to be effective in rescuing synaptic and learning deficits in two different AD mouse models [133]. In the context of these neurodegenerative diseases, it will be essential to investigate PACT's involvement in activating PKR. Currently PACT-mediated PKR activation has been reported only in the case of Alzheimer's patient brains and mouse models [219]. Activated PKR could

also contribute to the behavioral and neurophysiological abnormalities in Down syndrome as PKR inhibitory drugs were able to partially rescue the synaptic plasticity and long-term memory deficits in a mouse model [132]. Thus, our results presented here possibly have broader implications beyond DYT-*PRKRA*. Luteolin may also be useful for treating diseases triggered by inflammation where involvement of PACT-PKR pathway has been established such as in hepatic stellate cells, which are major contributors for the progression of hepatic fibrosis [220]. Additionally, luteolin could also be effective against inflammatory conditions such as colitis in which the involvement of PACT-PKR pathway is established [221-223]. PKR has also been shown to be an important regulator of hematopoietic stem/progenitor cell fate and proliferation and is thought to play a role in bone marrow failure conditions including myelodysplastic syndrome [224]. The involvement of PACT in hematopoietic lineages has not been investigated in depth and it could be interesting area for future investigation to evaluate if luteolin affects hematopoietic stem/ progenitor cell fate. Other flavonoids such as quercetin are also known to reduce ISR and ATF4 expression in Alzheimer's mouse models and improve memory [225]. In our previous study with flavonoids, quercetin showed ability to disrupt PACT-PKR interaction and to inhibit PKR activation under conditions of oxidative stress and inflammation [84]. Our research thus opens a new area of investigation to evaluate the suitability of luteolin and other flavonoids in treating DYT-*PRKRA* and possibly other neurodegenerative and inflammatory conditions.

In the context of DYT-*PRKRA*, the patient cells exhibit enhanced interactions between mutant PACT and PKR even in the absence of ER stress. Consequently, the

levels of p-PKR are about 5-fold higher (Figure 2.1) in patient cells in the absence of ER stress. Moreover, the patient cells from compound heterozygous individual carrying P222L and C213R mutations used in this study as well as previously used P222L homozygous patient cells exhibit higher level of apoptosis in the absence of cellular stress. Thus, neurodegeneration in DYT-*PRKRA* patients can be expected as a long-term outcome of the increased level of apoptosis in the absence of cellular stress. A limited number of imaging studies for the compound heterozygous patient carrying P222L and C213R mutant alleles used in the current study have indications of some neuronal apoptosis. Brain imaging performed at different ages indicated progressive MRI abnormalities with significant bilateral volume loss in the basal ganglia [63,226], which could have resulted from enhanced apoptosis. This individual also developed dystonia after a febrile illness, which could have been a possible cellular stress event triggering hyperactivation of PACT-PKR pathway and progressive neuronal dysfunction or loss. Additionally, in accordance with our earlier in vitro studies with lymphoblasts from three Brazilian P222L homozygous patients that showed enhanced apoptosis [68], the imaging studies on one Portuguese P222L homozygous patient showed significant bilateral loss of striatal presynaptic dopamine transporters, suggesting nigrostriatal neurodegeneration [156]. Recently, Masnada et al. [158] also reported bilateral striatal degeneration in two non-related DYT-*PRKRA* patients with two compound heterozygous patients. One of these patients had P222L and G43S mutations and presented dystonia at 30 months and the other had C213F and V72F mutations and presented at 14 months of life. Both patients showed recurrent fever-induced episodes of acute encephalopathy resulting in cognitive

impairment, and generalized dystonia, among other symptoms. Evidence of cerebellar atrophy was also documented in one of these patients. A DYT-*PRKRA* patient homozygous for G43C mutation also showed MRI abnormalities with mild cerebral atrophy [227]. Additionally, there is evidence of neuronal apoptosis in lear-5J mice which carry a spontaneously arisen PRKRA frameshift mutation that truncates PACT protein. Homozygous lear-5J mice exhibit progressive dystonia, kinked tails, and mortality and apoptosis in the dorsal root ganglia and the trigeminal ganglion [66].

As the PACT-PKR stress response pathway functions similarly in all cell types including neuronal cells and PACT mediated PKR activation and its involvement in neurodegeneration has been noted in Alzheimer's patients and mouse models [137,219,228], it is important to study the ISR dysregulation in DYT-*PRKRA* neurons. Currently no DYT-*PRKRA* neurons are available and our studies on patient lymphoblasts indicate that considerable efforts involved in undertaking in-depth studies using DYT-*PRKRA* patient-derived neurons from induced pluripotent stem cells (iPSCs) would be worthwhile in future. Our results thus open a new area of investigation to evaluate the suitability of luteolin in treating DYT-*PRKRA* and possibly other neurodegenerative conditions. The lear5J mouse model [66] of DYT-*PRKRA* will be very useful for characterizing the contribution of ISR dysregulation to dystonia phenotype, evaluating luteolin as a therapeutic agent, and determining therapeutic windows in which luteolin mediated ISR modulation could prove beneficial.

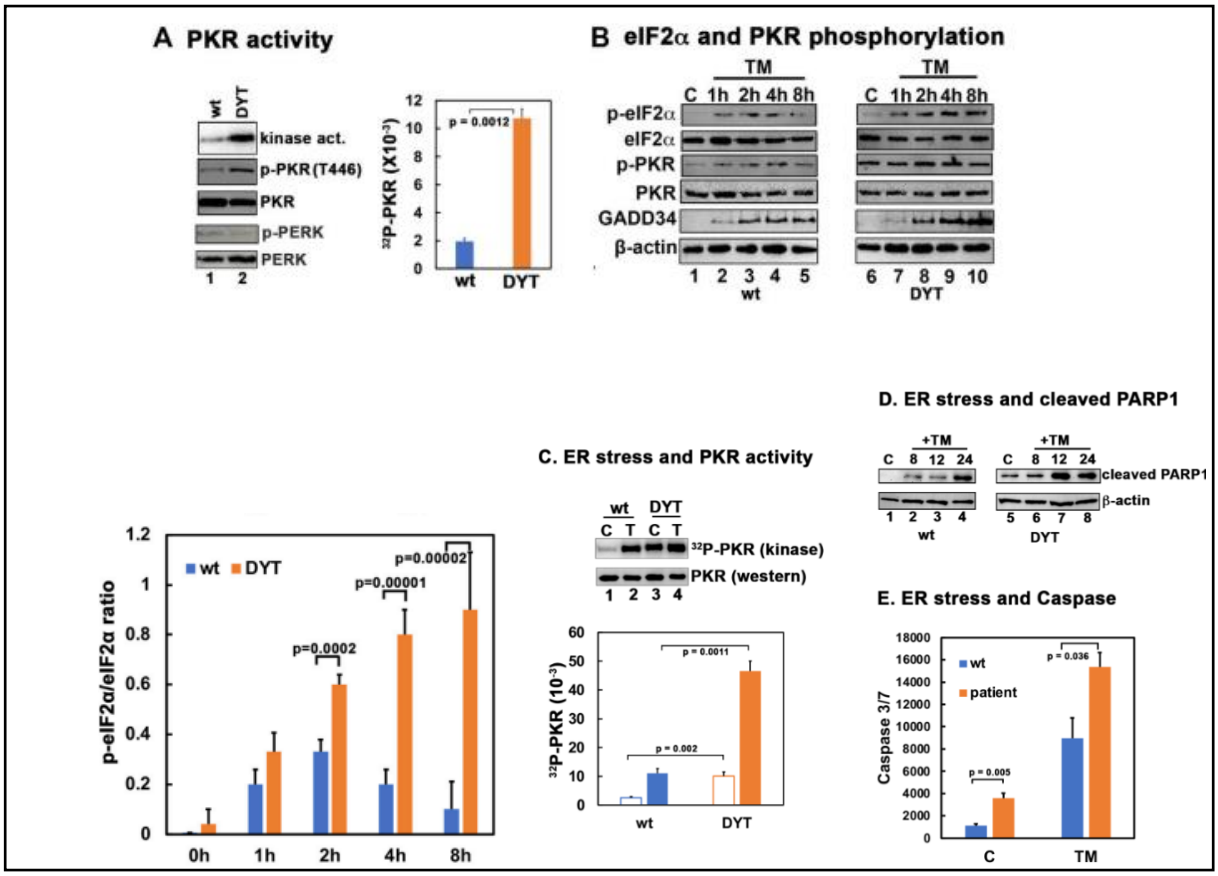

Figure 2.1: (A) PKR is hyperactive in DYT-*PRKRA* patient lymphoblasts. PKR activity assay and western blot analysis for p-PKR and total PKR. PKR kinase activity assay was performed using PKR immunoprecipitated from wt and DYT-PRKRA lymphoblast extracts using a monoclonal PKR antibody (R&D Systems) and protein A-sepharose beads. PKR activity was assessed without any externally added activator and the bands represent endogenous activity levels of PKR. The PKR band intensities were quantified using Imagequant TL (Cytiva), and the bar graph shows data from 3 independent experiments and the p values are as indicated. Blue bar: wt and orange bar: DYT-*PRKRA*. Whole cell extracts from normal (wt) and DYT-PRKRA patient derived lymphoblasts were analyzed. Blots were probed for p-PKR, total PKR, p-PERK, and total PERK. Best of three representative blots are shown. **(B) Western blot analysis for p-PKR, p-eIF2α and GADD34**. Normal (wt) and DYT-*PRKRA* patient derived lymphoblasts treated with 5 µg/ml of tunicamycin (TM) and cell extracts were prepared at various time points as indicated above the lanes after treatment and from untreated cells. Western blot analysis was performed with the indicated antibodies. The signal intensities of p-eIF2α and total eIF2α bands were quantified

using Imagequant TL (Cytiva) and the ratio p-eIF2α/eIF2α was calculated for each time point using three separate experiments. The p values are as indicated. Blue bar: wt and orange bar: DYT-PRKRA **C. PKR activity in wt and DYT-*PRKRA* cells after ER stress.** Lymphoblast lines established from wt and DYT-*PRKRA* patient were treated with 5 µg/ml tunicamycin and cells extracts were prepared for PKR kinase activity assay and western blot analysis 2 h after the treatment. PKR kinase activity **Figure 2.1 (Continued):** assay was performed using immunoprecipitated PKR as in part A. The bar graph shows data from 3 independent experiments and the p values are as indicated. Blue bar: wt and orange bar: DYT-*PRKRA*. Whole cell extracts from normal (wt) and DYT-*PRKRA* patient derived lymphoblasts were analyzed for total PKR. **D. Western blot analysis for cleaved PARP1.** Whole cell extracts from normal (wt) and DYT-*PRKRA* patient derived lymphoblasts treated with 5 µg/ml of tunicamycin (TM) were analyzed at indicated time points using anti-cleaved PARP1 and anti-β-actin antibodies. **E. Caspase-Glo 3/7 activity.** Lymphoblast lines established from wt and DYT-*PRKRA* patient were treated with 5 µg/ml tunicamycin and the caspase 3/7 activities were measured at 0 h and 24h. Blue bars: wt cells, and orange bars: DYT-*PRKRA* cells. The data is an average of three independent experiments and the p values are as indicated.

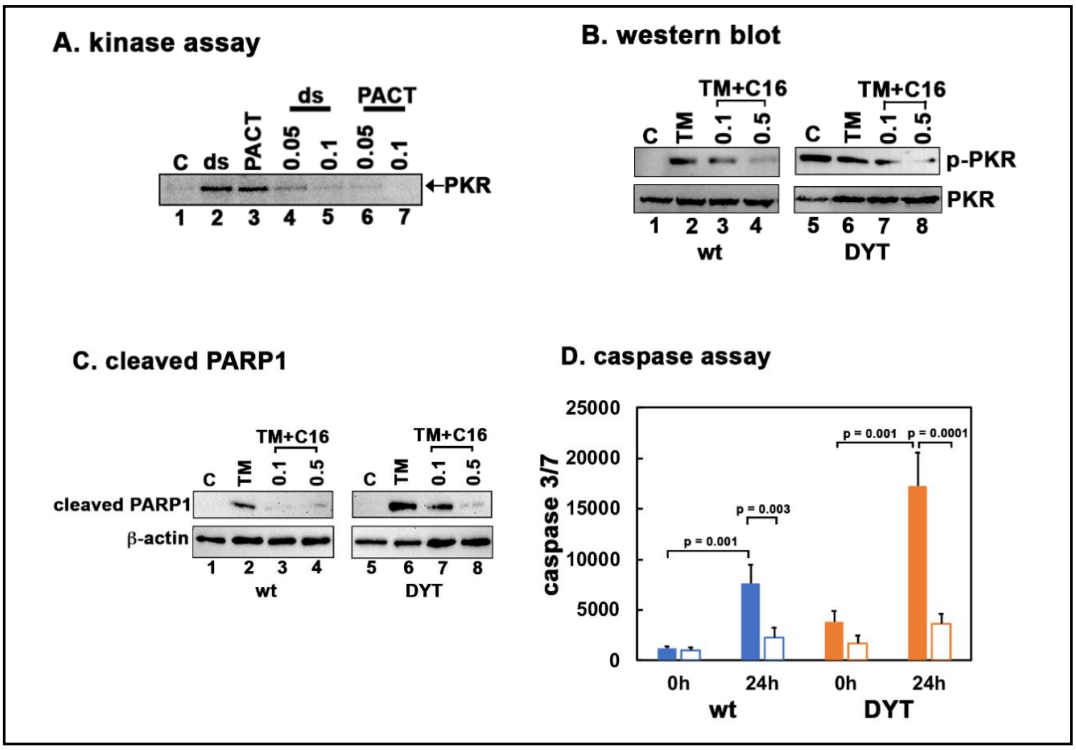

Figure 2.2: A. PKR inhibition by C16. Kinase activity assay was performed using PKR immunoprecipitated from HeLa cell extracts using a monoclonal PKR antibody (R&D Systems) and protein A-sepharose beads. Either 1μg/ml polyI:polyC (lanes 2, 4, 5) or 4 ng recombinant wt PACT (lanes 3,6,7) were used as PKR activators. C16 was added either at 0.05 μM (lanes 4 and 6) or 0.1μM (lanes 5 and 7) as indicated on the top of the lanes. Lanes 1: no activator added. **B. Inhibition of PKR activation in lymphoblasts by C16.** The normal (wt) and DYT-*PRKRA* patient derived lymphoblasts were treated with either 5 μg/ml of tunicamycin (TM), TM + 0.1 μM C16 or TM + 0.5 μM C16 for 2h. Whole cell extracts were prepared at 2h after the treatments and were analyzed by western blot analysis. Blots were probed for p-PKR, and total PKR. Best of four representative blots are shown. **C. Western blot analysis for cleaved PARP1.** The normal (wt) and DYT-*PRKRA* patient derived lymphoblasts were treated with either 5 μg/ml of tunicamycin (TM), TM + 0.1 μM C16 or TM + 0.5 μM C16 for 24 h. Whole cell extracts prepared at 24h after treatments were analyzed using anti-cleaved PARP1 and anti-β-actin antibodies. **D. Caspase-Glo 3/7 activity.** Lymphoblast lines established from wt and DYT-*PRKRA* patient were treated either with 5 μg/ml tunicamycin or with tunicamycin and 0.5 μM C16 for 24 h. The caspase 3/7 activities were measured at 0 h and 24 h. Blue bars: wt cells, and orange bars: DYT-*PRKRA* cells, filled bars: tunicamycin treated and unfilled bars: tunicamycin and C16 treated. The data is an average of three independent experiments and the p values are as indicated.

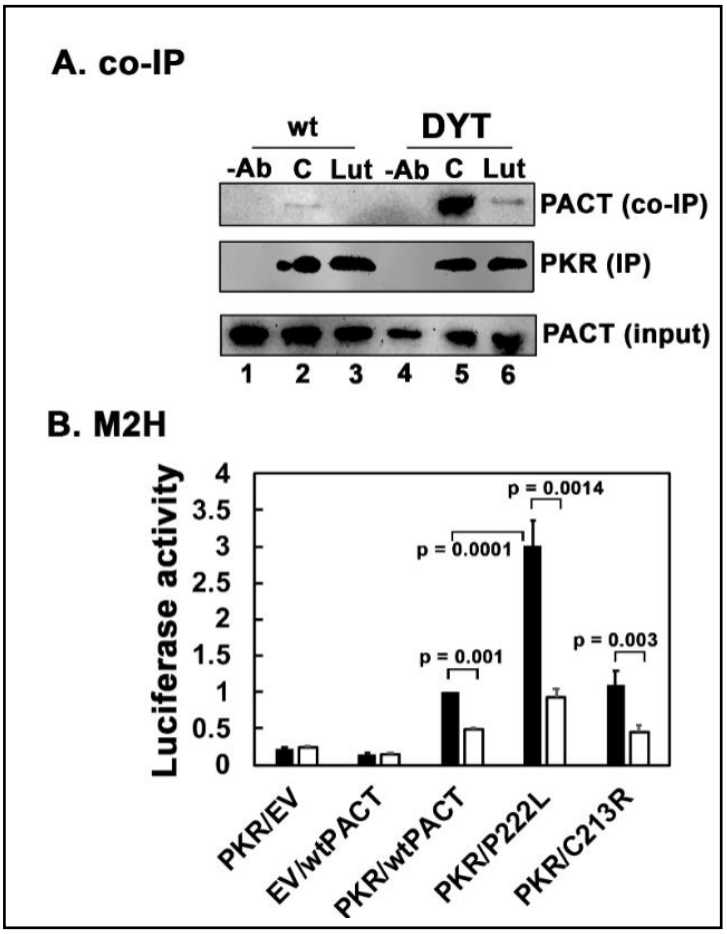

Figure 2.3: A. Luteolin disrupts the interaction between PKR and PACT. A. Co-IP of endogenous PKR and PACT proteins. Lymphoblasts from unaffected family member (wt) or DYT-*PRKRA* patient (patient) were treated with 50 μM luteolin. The cell extracts were prepared 24h after the treatment, and endogenous PKR protein was immunoprecipitated using anti-PKR mAb and protein A-sepharose, which immunoprecipitates total PKR. The immunoprecipitates were analyzed by western blot analysis with anti-PACT monoclonal antibody (Co-IP panel). The blot was stripped and re-probed with anti-PKR mAb to ascertain an equal amount of PKR was immunoprecipitated in each lane (IP panel). Input blot: Western blot analysis of total proteins in the extract with anti-PACT mAb showing equal amount of PACT in all samples. **B. Mammalian two-hybrid analysis.** HeLa cells were transfected with 250 ng of each of the two test plasmids encoding proteins to be tested for interaction, 50 ng of the reporter plasmid pG5Luc, and 1 ng of plasmid pRL-Null to normalize transfection efficiency. 2 h after transfection, one set of samples were left untreated, and one set was treated with 50μg/ml luteolin. Cells were harvested 24 h after luteolin treatment, and cell extracts were assayed for luciferase activity. The plasmid combinations are as indicated, PKR was expressed as a GAL4 DNA-binding domain fusion protein (bait) and all PACT proteins were expressed as VP16-activation domain fusion proteins (preys). The experiment was repeated twice with each sample in triplicate, and the averages with standard error bars are presented. The p values are as indicated. RLU, relative luciferase units.

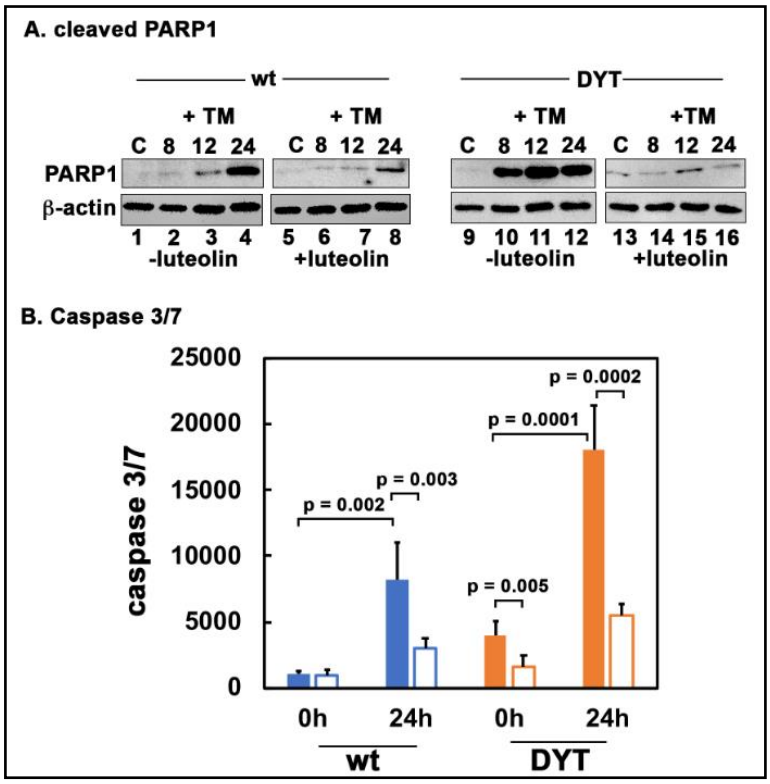

Figure 2.4: Luteolin protects DYT-*PRKRA* cells from apoptosis in response to ER stress. A. Western blot analysis for cleaved PARP1. The normal (wt) and DYT-*PRKRA* patient derived lymphoblasts were treated with 5 µg/ml of tunicamycin (TM) either without pretreatment with luteolin or with 50 µM luteolin pretreatment for 24 h. Whole cell extracts prepared at the indicated time points after TM treatment were analyzed using anti-cleaved PARP1 and anti-β-actin antibodies. **B. Caspase-Glo 3/7 activity.** The normal (wt) and DYT-*PRKRA* patient derived lymphoblasts were treated with 5 µg/ml of tunicamycin (TM) either without pretreatment with luteolin or with 50 µM luteolin pretreatment for 24 h. The caspase 3/7 activities were measured at 0 h and 24 h. Blue bars: wt cells, and orange bars: DYT-*PRKRA* cells, filled bars: tunicamycin treated and unfilled bars: luteolin and tunicamycin treated. The data is an average of three independent experiments and the p values are as indicated.

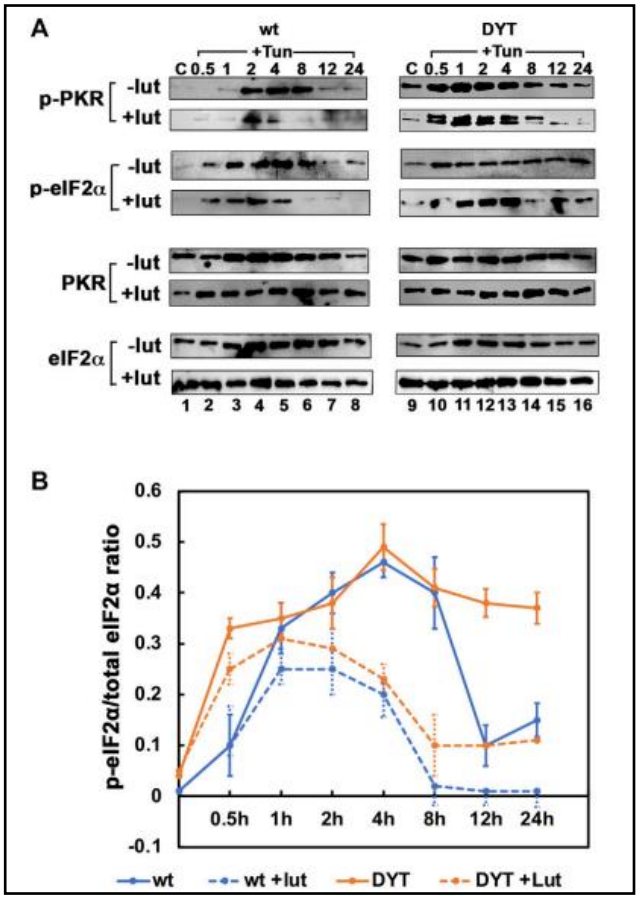

Figure 2.5: (A) Effect of luteolin on PKR activation and ISR in response to tunicamycin in normal and DYT-*PRKRA* patient lymphoblasts: western blot analysis for p-PKR and p-eIF2α. Whole cell extracts from normal (wt) and DYT-*PRKRA* patient derived lymphoblasts treated with 5 µg/ml of tunicamycin (TM) without any luteolin pretreatment or after 24 h pretreatment with 50 µM luteolin were analyzed at indicated time points. Blots were probed for p-eIF2α, total eIF2α, p-PKR, and total PKR. Best of four representative blots are shown. **(B)** The signal intensities of p-eIF2α and total eIF2α bands were quantified using Imagequant TL (Cytiva) and the ratio p-eIF2α/eIF2α was calculated for each time point using four separate experiments. The p values for differences between − lut and + lut for both wt and DYT-PRKRA cells were all below 0.001. Blue lines: wt and orange lines: DYT-*PRKRA*. Solid lines: without luteolin and dotted lines: with luteolin.

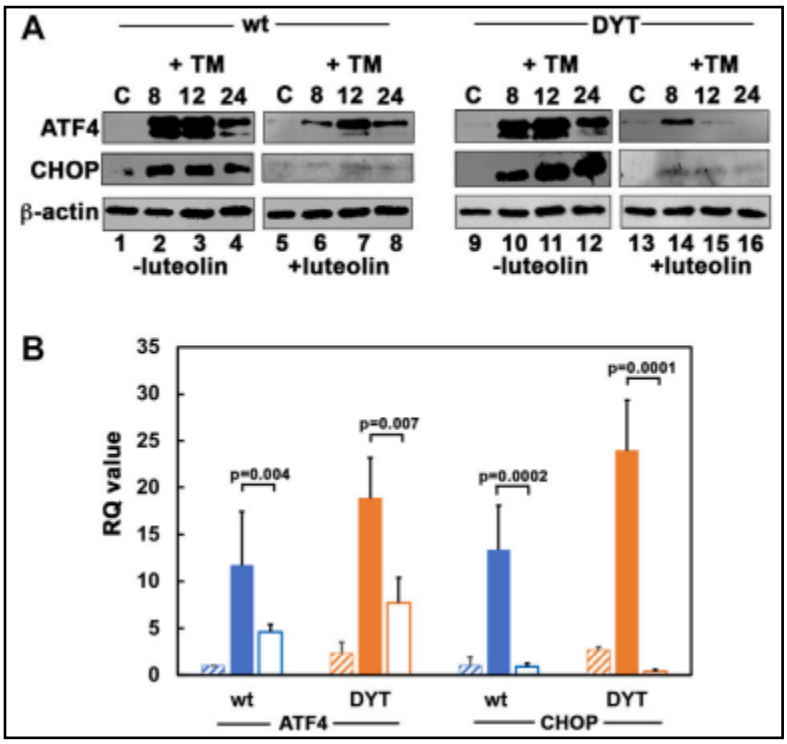

Figure 2.6: (A) Effect of luteolin on PKR activation and ISR in response to tunicamycin in normal and DYT-*PRKRA* patient lymphoblasts: western blot analysis for ATF4 and CHOP. Whole cell extracts from normal (wt) and DYT-*PRKRA* patient derived lymphoblasts treated with 5 µg/ ml of tunicamycin (TM) without any luteolin pretreatment or after 24 h pretreatment with 50 µM luteolin were analyzed at indicated time points. Blots were probed for ATF4, and CHOP. Best of four representative blots are shown. β-actin was used as a loading control to ensure equal amounts of protein was loaded in each lane. **(B) DYT-PRKRA patient lymphoblasts express higher levels of ATF4 and CHOP mRNAs in response to tunicamycin and luteolin treatment downregulates ATF4 and CHOP induction**. Quantitative RT-PCR of ATF4 and CHOP in wt (blue bars) and DYT-*PRKRA* (orange bars) lymphoblasts. The hatched bars indicate untreated control values, solid filled bars indicate tunicamycin treated values, and unfilled bars indicate luteolin and tunicamycin treated values. The RQ values indicate that ATF4 and CHOP expression was upregulated in response to tunicamycin and this upregulation was suppressed by luteolin pretreatment in both wt and DYT-*PRKRA* cells. Data from 3 separate experiments was analyzed and the p values are as indicated.

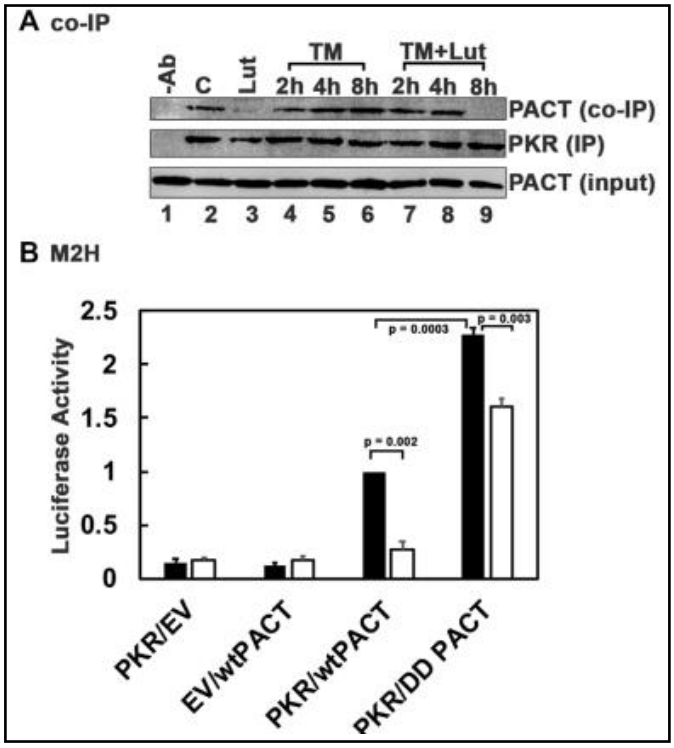

Figure 2.7: Luteolin allows for a transient PACT-PKR interaction after ER stress. **(A) Co-IP of endogenous PKR and PACT proteins**. The DYT-*PRKRA* lymphoblasts were treated with 5 µg/ml tunicamycin (TM) either with or without luteolin pre-treatment for 24 h. The cell extracts were prepared at indicated time points after the tunicamycin treatment, and endogenous PKR protein was immunoprecipitated using anti-PKR mAb and protein A-sepharose, which immunoprecipitates total PKR. The immunoprecipitates were analyzed by western blot analysis with anti-PACT monoclonal antibody (Co-IP panel). The blot was stripped and re-probed with anti-PKR mAb to ascertain an equal amount of PKR was immunoprecipitated in each lane (IP panel). Input blot: Western blot analysis of total proteins in the extract with anti-PACT mAb showing equal amount of PACT in all samples. **(B) Mammalian two-hybrid analysis**. HeLa cells were transfected with 250 ng of each of the two test plasmids encoding proteins to be tested for interaction, 50 ng of the reporter plasmid pG5Luc, and 1 ng of plasmid pRL-Null to normalize transfection efficiency. 2 h after transfection, one set of samples were left untreated and one set was treated with 50 µM luteolin. Cells were harvested 24 h after luteolin treatment, and cell extracts were assayed for luciferase activity. The plasmid combinations are as indicated, PKR was expressed as a GAL4 DNA-binding domain fusion protein (bait) and all PACT proteins were expressed as VP16-activation domain fusion proteins (preys). The experiment was repeated twice with each sample in triplicate, and the averages with standard error bars are presented. The p values are as indicated. RLU, relative luciferase units.

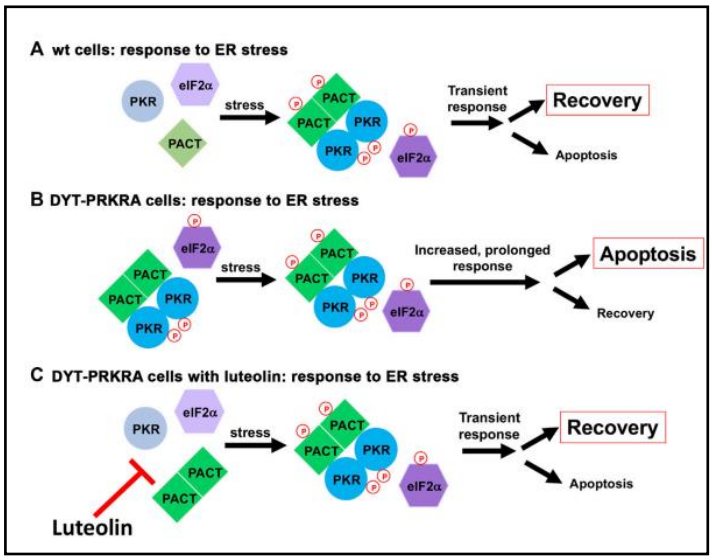

Figure 2.8: A schematic model for ER stress response in wt and DYT-*PRKRA* cells. **(A) ER stress response in wt cells**. In the absence of stress, PACT is not phosphorylated and PKR is not activated. After ER stress, PACT is phosphorylated and PACT-PACT and PACT-PKR interactions are enhanced thereby causing a transient PKR activation and eIF2α phosphorylation. This response leads to restoration of homeostasis promoting survival. **(B) ER stress response in DYT-*PRKRA* cells**. In the absence of stress, mutant PACT is not phosphorylated but forms strong PACT-PACT as well as PACT-PKR interactions and PKR is activated. After ER stress, PACT is phosphorylated and PACT-PACT and PACT-PKR interactions are further enhanced thereby causing a persistent PKR activation and eIF2α phosphorylation promoting apoptosis. **(C) ER stress response in DYT-*PRKRA* cells in the presence of luteolin.** In the absence of stress, mutant PACT is not phosphorylated and the PACT-PKR interactions are disrupted by luteolin and PKR is not activated. After ER stress, PACT is phosphorylated and PACT-PACT and PACT-PKR interactions are enhanced thereby causing a transient PKR activation and eIF2α phosphorylation. This transient response leads to restoration of homeostasis promoting survival.

Chapter 3:

Characterizing the effect of Nelfinavir on restoration of cellular homeostasis in DYT-*PRKRA* cells

Kenneth Frederick and Rekha Patel. To be submitted 2023

3.1 Abstract

DYT-*PRKRA* is an early onset generalized dystonia caused by mutations in the PRKRA gene which encodes PACT, the protein activator of the interferon-induced, double-stranded RNA (dsRNA)-activated protein kinase PKR. It typically shows clinical manifestations early in childhood and can have a genetic inheritance pattern of both autosomal dominant and recessive. PACT activates PKR in a dsRNA-independent manner in response to stress signals such as endoplasmic reticulum stress, oxidative stress, and serum deprivation. Activated PKR subsequently phosphorylates the translation initiation factor eIF2α. eIF2α phosphorylation is an essential regulatory step to the integrated stress response (ISR) in which eukaryotic cells utilize a complex network of protective pathways in order to regulate protein synthesis to maintain homeostasis in response to unfavorable environmental or intracellular stimuli. During general translational inhibition when eIF2α is phosphorylated, there are certain specific transcripts that are preferentially translated. One of these mRNAs is ATF4, which is a transcription factor that plays a central role in determining cell fate during cell stress. Depending on stress severity and duration, eIF2α phosphorylation and ATF4 have molecular adaptability to tailor responses towards apoptosis. Our previous research has elucidated that DYT-*PRKRA* mutations lead to enhanced PACT-PKR interactions thereby increasing eIF2α phosphorylation causing dysregulated ISR signaling which promotes an increased sensitivity to apoptosis. Nelfinavir is an aspartyl protease inhibitor (HIV-PI) which also has an off-target effect that downregulates expression of the eIF2α dephosphorylation regulator

CReP. Thus, our scope was to determine if an increase in eIF2α phosphorylation and ISR activation would promote DYT-*PRKRA* cell desensitization to apoptosis. Our results presented in this study determined that while Nelfinavir does induce increased eIF2α phosphorylation and a robust ISR, this promoted elevated apoptosis within DYT-*PRKRA* cells in both the absence of stress and in response to ER-stress induction thereby suggesting that Nelfinavir is not an effective therapeutic for DYT-*PRKRA.*

3.2 Introduction

Eukaryotic cells have evolved a complex network of protective pathways called the integrated stress response (ISR) in order to regulate protein synthesis to maintain homeostasis in response to unfavorable environmental or intracellular stimuli [1, 2]. The primary node of this signaling network is the heterotrimeric eukaryotic translation initiation factor 2 (eIF2) (Figure 1.1) [2]. This highly conserved protein complex consists of an α, β, and γ subunit with eIF2α being the main regulatory subunit because its activity is inhibited by stress-induced phosphorylation [2]. To initiate mRNA translation, eIF2 forms the ternary complex (TC) with GTP and methionyl-initiator tRNA (Met-tRNA$_i$) [2-4]. This TC directly interacts with the 40S ribosomal subunit along with two initiation factors, eIF1 and eIF1A, to form the 43S pre-initiation complex (PIC) [2-4]. Interactions between the PIC and other multi-protein complexes within the eukaryotic translation initiation factor family (eIF) facilitates translation through the recognition of and assembly at the 5' m^7-G-cap of mature mRNAs [2, 3, 4]. The ability of eIF2 to stay in its active form capable of initiating the formation of TC is dependent on the guanine exchange factor (GEF) activity of the eIF2B [3, 4]. Prior to the formation of the TC, a GDP molecule bound to the gamma subunit of eIF2 has to be exchanged for a GTP molecule through the enzymatic activity of eIF2B [1, 2]. Binding of the TC to 5' cap on mRNAs requires activity of cap-binding initiation factor eIF4 complex and is the mechanism used for translation initiation of the majority of cellular mRNAs [2].

In response to stress stimuli, diminishment of cap-dependent protein translation is accomplished by phosphorylation of the α subunit of eukaryotic initiation factor 2 (eIF2α) at serine 51 [1,2]. This post-translational modification stops the formation of the ternary complex which is vital for translation initiation [1,5]. This strategy is a critical response to restore cell homeostasis after exposure to cellular stressors such as viral infections, misfolded protein accumulation, oxidative stress, and serum deprivation [1]. There are a family of four serine/threonine kinases, double-stranded RNA activated protein kinase (PKR), PKR-like Endoplasmic Reticulum kinase (PERK), heme-regulated inhibitor (HRI), and gene control non-derepressible (GCN) kinase which phosphorylate eIF2α [Fig 1.1] [1,2]. While each of these kinases share an evolutionarily conserved, homologous kinase domain (KD), they respond to distinct stress signals [1,2]. The subsequent eIF2α phosphorylation. When eIF2 binds to eIF2B, phosphorylated eIF2α prevents GEF activity of eIF2B [2, 5] and the inhibition of eIF2B's GEF activity ultimately leads to an inhibition of the formation of the TC resulting in the attenuation of general protein synthesis while simultaneously promoting the translation of some specific mRNAs (Figure 1.1) [2].

During general translational inhibition when eIF2α is phosphorylated, there are certain specific transcripts containing upstream open reading frames (ORFs) in their 5'UTRs that are translated and the protein products of these mRNAs promote and optimize cellular recovery [1,2]. One of these mRNAs is ATF4, which is a transcription factor that plays a central role in determining cell fate

during cell stress [Fig 1.1] [2]. Depending on stress severity and duration, ATF4 has molecular adaptability to tailor responses towards cell recovery or apoptosis [2]. ATF4 is thus indispensable in regulating many diseases and disorders arising from maladaptive stress responses [2]. ATF4 can form homodimers or heterodimers with other transcription factors such as its downstream target CHOP which promotes cell death [2]. CHOP promotes apoptosis by upregulating BH3-only pro-apoptotic BCL-2 family members and enhancing expression of DR5 and ATF5 [2]. During ER stress and amino acid depravation, ATF4 and/or CHOP preferentially bind to the promoter regions of ATF3 and GADD34 [2]. If eIF2α phosphorylation is sustained, pro-apoptotic transcripts are translated in a similar manner to induce activation of caspases, PARP1 cleavage, and subsequent programmed cell death [1,2]. Dephosphorylation of eIF2α and subsequent restoration of global protein translation is an essential step that regulates recovery from stress and termination of the ISR [1,2,190]. Two cellular regulatory subunits of the serine/threonine protein phosphatase 1 (PP1) complex aid in dephosphorylating eIF2α [1,2,190]. CReP is constitutively active and expressed in unstressed cells which is essential to maintain a low basal eIF2α phosphorylation [1,2,190]. GADD34, induced in response to cellular stress, is a product of an mRNA specifically translated under conditions of stress. GADD34 interacts with the catalytic subunit of the serine/threonine protein phosphatase 1 (PP1C) to dephosphorylate eIF2α thereby terminating the ISR and returning the cell to homeostasis [1,2,190]. Mechanisms that modulate eIF2α

dephosphorylation are gaining more momentum as promising targets for therapeutics in neurodegenerative diseases [2,190].

Dystonias (DYT1-DYT26) are a group of movement disorders that are characterized by sustained involuntary postures/or slow twisting movements that generate motor disability and pain [58-60,192]. Clinical manifestations vary from focal dystonia, affecting single limb or other body part, to generalized dystonia where most of the body is involved in abnormal posturing/or slow uncontrolled twisting movements [192]. While there is variability between age of onset, once symptoms begin, they usually endure throughout an individual's lifetime leading to severe disability and pain [58,192]. Dystonia is the third most common movement disorder after Parkinson's disease and essential tremor [192]. Dystonia causes range from sporadic and inherited forms which are classified as primary dystonia to those that occur in association as a secondary pathology to a traumatic brain injury, stroke, metabolic disorder, or antipsychotic medication use [58,192].

DYT-*PRKRA* is an early onset generalized dystonia caused by mutations in the PRKRA gene which encodes PACT [Fig 1.4] [59,68,98,113]. It typically shows clinical manifestations early in childhood and can have a genetic inheritance pattern of both autosomal dominant and recessive [59]. DYT-*PRKRA* was first reported due to a homozygous PRKRA mutation utilizing whole exome sequencing in seven Brazilian patients from two unrelated families [61]. This missense PACT mutation substituted a leucine residue for proline at position 222 (P222L) [61]. After the initial study identifying P222L mutation in DYT-*PRKRA*,

the P222L mutation was found in two Polish brothers [64]. A German DYT-*PRKRA* patient with a frameshift (FS) mutation within PACT was also identified, which was the result of a two nucleotide deletion within PACT's first dsRBM leading to a premature stop codon [62]. The FS mutation causes a truncation of PACT protein after 88 amino acids followed by 21 extraneous amino acids and a premature stop codon [62]. In the United States, a male patient was found to have a novel PACT mutation (C213R) on one allele which occurred de novo while he inherited the P222L mutation from his mother as the other allele [63]. He began developing dystonia clinical symptoms at an early age [63]. Another dystonia patient was discovered to have novel PACT mutations within both alleles which were two recessively inherited mutations (C77S and C213F) [65]. Two other dominantly inherited mutations were identified of which two were located within PACT's coding region like the other mutations (N102S and T34S) [64]. Recently, three novel DYT-*PRKRA* mutations (G43S, V72F, and S265R) have been discovered in patients adding to the growing evidence implicating PACT in disease etiology of dystonia [98,158].

In their 2020 study, *Burnett et al*. characterized that lymphoblasts derived from a compound heterozygous patient (P222L/C213R) are also hypersensitive to ER stress due to enhanced PACT-PKR heterodimer affinity, upregulated PKR, and dysregulated ISR signaling. [113]. This work was built on *Vaughn et al.'s* work that described that the lymphoblasts derived from patients homozygous for the most prevalent P222L mutation have enhanced PKR activity and dysregulated eIF2α stress response signaling which enhanced cellular sensitivity

to ER stress [68]. Seven reported DYT-PRKRA mutations lead to hyperactive PKR, dysregulated eIF2α signaling, and enhancing cell sensitivity to apoptosis in response to ER stress [68,98,113]. Maladaptive eIF2α signaling and an aberrant ISR is a common link for pathophysiology of several dystonias which include DYT1, DYT3, DYT6, and DYT11 [73,121-124] in addition to our research on the disease etiology of DYT-*PRKRA* with homozygous P222L and heterozygous P222L/C213R patient cells [68,113].

Multiple studies have directly tethered PKR-independently of PACT as an etiology of early onset dystonia due to hyperactive PKR, caused by PKR variants and increased eIF2α phosphorylation [120]. De novo missense mutations in PKR additionally cause a multifaceted neurodevelopmental condition that mirrors vanishing white matter disease. Recently, additional studies have linked newly discovered PKR missense variants (P31R, G130R, and G138A) which are autosomal dominant and one recessive PKR mutation (N32T) to patients who have dystonia [120,134-136]. These investigations emphasize PKR as an essential catalyst for disease initiation and progression within dystonia.

The current therapeutic regimens for dystonia patients are limited. Current mainstream medications include anticholinergic drugs, benzodiazepines, and muscle relaxants [192]. While these medications attenuate symptoms, they do not eliminate them thereby leaving patients with a poor quality of life [192]. Injectable botulinum toxin is utilized as well, but it has major limitations such as only being effective for focal dystonia patients, inconvenience, cost, and the necessity for patients to still be able to maintain normal muscle movements [192].

Nelfinavir is an aspartyl protease inhibitor (HIV-PI) which was approved by the FDA in 1997 for initial use to treat HIV patients [190,191] as it inhibits the HIV protease activity. Its antiretroviral properties showed significant suppression of HIV replication in patients [190]. One of the off-target effects of Nelfinavir is downregulation of CReP expression. As CReP is the constitutively expressed regulatory subunit of protein phosphatase PP1, a decrease in CReP levels leads to decreased association between the CReP-PP1C complex and eIF2α [2]. Thus, Nelfinavir promotes an increase in eIF2α phosphorylation and ISR activation by inhibition of PP1 activity [2]. eIF2α dephosphorylation via CReP or through stress dependent PP1C regulatory subunit GADD34 acts a mechanism to regulate intensity and duration of cellular stress response [2,190]. Nelfinavir has been reported to induce ATF4 expression and a robust ISR both *in vitro* and *in vivo* [190]. Additionally, ongoing clinical trials to elucidate Nelfinavir's potential as a cancer therapeutic have shown promising initial results [190,191]. Along with promoting ATF4 expression, Nelfinavir's induction of the ISR diminishes cellular protein synthesis, which promotes cellular proteostasis through improved protein folding or protein degradation [2,190].

Recently, *Caffall et al* showed that the HIV protease inhibitor, Ritonavir, induces enhanced ATF4 levels and attenuates DYT1 hTorsinA protein mislocalization *in vitro* [192]. Also, in a DYT1 mouse model, Ritonavir rescued DYT1 associated pathology of dopamine modulation within striatal cholinergic interneurons and caused long-lasting normalizing changes to brain microstructural defects associated with the DYT-1 genotype [192]. Collectively,

these results highlight that inducing a robust ISR could be of therapeutic value across multiple dystonia subtypes. With the numerous studies implicating misfolded protein accumulations generating ER stress and subsequent neurological disease pathology, characterizing the effects of a CReP-targeted therapeutic will provide significant mechanistic insight about the relationship between modulation of eIF2α phosphorylation restoration of cellular homeostasis within DYT-*PRKRA* patient cells. In this study, we tested if Nelfinavir induces CReP downregulation to protect the DYT-*PRKRA* cells from the enhanced apoptosis seen in response to ER stress. Our results indicate that Nelfinavir downregulates CReP expression and upregulates eIF2α phosphorylation confirming earlier reports. However, contrary to our expectation, Nelfinavir is unable to restore homeostasis after ER stress and is detrimental to cell survival and recovery in both wt and DYT-*PRKRA* cells.

3.3 Materials and Methods:

Cell lines and antibodies

Wt and DYT-*PRKRA* Patient B-Lymphoblasts were cultured in RPMI 1640 medium containing 10% percent FBS and penicillin/streptomycin. Both wt and DYT-*PRKRA* patient lymphoblast cell lines were Epstein-Barr Virus-transformed to create stable cell lines as previously described [68]. The antibodies used were as follows: PKR: anti-PKR(human) monoclonal (71/10, R&D Systems), P-PKR: anti-phospho-PKR(Thr-446) monoclonal (Abcam, {E120}), eIF2α: anti-eIF2α polyclonal (Invitrogen, AHO1182), p-eIF2α: anti-phospho-eIF2α(Ser-51) polyclonal (CST, #9721), ATF4: anti-ATF4 monoclonal (CST, #11815), CHOP: anti-CHOP monoclonal (CST, #2895), Cleaved PARP: anti-Cleaved PARP monoclonal (CST, #32563), β-Actin: anti-β-Actin-Peroxidase monoclonal (Sigma-Aldrich, A3854), CReP: anti-PPP1R15B polyclonal (Proteintech, #14634-1-AP), GAPDH, anti-GAPDH-Peroxidase monoclonal (Sigma-Aldrich, G9295)

Western blot analysis

Lymphoblasts derived from a homozygous recessive DYT-*PRKRA* patient containing P222L mutations on both alleles were cultured alongside lymphoblasts derived from a family member containing no mutations in PACT as our control cells. Cells were plated at a concentration of 300,000 cells/ml of RPMI media containing 10% percent fetal bovine serum and penicillin/streptomycin. To analyze cellular response to ER stress, we treated cells with 5 um/ml of Tunicamycin (Santa Cruz) over the indicated time points and harvested cells in RIPA buffer (150 mM NaCl, 1.0% IGEPAL CA-630, 0.5% sodium deoxycholate,

0.1% SDS, 50 mM Tris, pH 8.0) containing 1:100 dilution of protease inhibitor cocktail (Sigma) and phosphatase inhibitor (Sigma). Concentration of total protein extract was then determined using BCA assay and appropriate amounts of extracts were analyzed by western blot analyses using appropriate antibodies as indicated. To analyze cellular response to Nelfinavir treatment, cells were treated with Nelfinavir (40μM) (Fisher Scientific) for the indicated time points and cells were harvested, total protein extract concentration determined and western blot analyses methods were done identically to methods stated above. To address the effect of Nelfinavir or Luteolin on cellular response to ER stress-induction cultured wt and patient lymphoblasts as described above were either treated with Nelfinavir (40μM) for 6 hours before Tunicamycin treatment or Luteolin (50μM) (Santa Cruz) for 24 hours prior to Tunicamycin treatment.

3.4 Results

CReP is expressed at higher levels in DYT-*PRKRA* patient lymphoblasts

Our previous research has established that DYT-*PRKRA* patient lymphoblasts have maladaptive ISR signaling thereby enhancing sensitivity to ER stress-induced apoptosis relative to the unaffected, wild type (wt) lymphoblasts [68,98,113]. To understand the mechanism promoting heightened sensitivity to apoptosis, we investigated the possibility if the expression levels of CReP in wt and DYT-*PRKRA* cells are different. CReP is the constitutively expressed regulatory subunit of protein phosphatase 1 that is also induced under conditions of ER stress. Constitutive expression levels of CReP without any ER stress were examined by western blot analysis in wt and DYT-*PRKRA* patient lymphoblasts. As seen in Fig 3.1A, CReP levels are significantly elevated in DYT-*PRKRA* patient lymphoblasts compared to wt cells. These results indicate that DYT-*PRKRA* cells constitutively upregulated expression of CReP in the absence of ER stress.

We next determined CReP levels in response to ER stress induced by tunicamycin in both wt and DYT-*PRKRA* patients cells. As shown in Fig 3.1B, in wt lymphoblasts there are low basal levels of CReP in the untreated cells (left panel, lane 1), followed by increased CReP levels at 2, 4, 6, 8, and 12 hours after treatment (left panel, lanes 2-6). In DYT-*PRKRA* cells, CReP is significantly increased at basal levels relative to wt cells (right panel, lane 8), and no induction in CReP levels is detectable after ER stress, which could be a limitation of the western blot technique. The elevated CReP levels seem to be sustained

throughout the entire time course even at 24 hours post-treatment (right panel, lanes 9-14). These results indicate that the DYT-*PRKRA* patient cells express CReP at higher levels both in the absence of ER stress and induction of ER stress as compared to wt cells.

Nelfinavir, a drug that reduces the expression of CReP promotes increased eIF2α phosphorylation in DYT-*PRKRA* lymphoblasts in the absence of ER stress

As seen in Fig 3.2, Nelfinavir treatment significantly reduces CReP levels after 6 hours in both wt (lane 5) and DYT-PRKRA cells (lane 10). These results confirm previous published work from another lab that Nelfinavir downregulates CReP [2]. Previous studies have indicated that Nelfinavir promotes decreased association between the CReP-PP1C phosphatase holocomplex and eIF2α thereby enhancing eIF2α phosphorylation and promoting ISR activation [2]. As shown in Fig. 3.2, wt cells have low basal eIF2α (Lane 1) and these levels increase at 4 and 6 hours post-treatment with Nelfinavir (Lane 4 and 5). In DYT-PRKRA cells, phosphorylated eIF2α is low at basal levels (Fig 3.2, Lane 6) which seems to be slightly higher than basal levels in wt cells (Fig 3.2, Lane 1). The levels of phosphorylated eIF2α significantly increase beginning at 2 hours after Nelfinavir treatment (Lane 8) and continue to rise at 4 and 6 hours post-treatment with Nelfinavir (Lanes 9-10). Collectively, these results indicate that Nelfinavir downregulates CReP levels thereby increasing eIF2α phosphorylation without ER stress.

Nelfinavir promotes ISR signaling and apoptosis in both wt and DYT-*PRKRA* lymphoblasts

Due to the reduction in CReP expression and increased eIF2α phosphorylation, we sought to determine if Nelfinavir would trigger an ISR. Induction of ISR response in many types of pathological conditions is documented to have a protective effect on cell survival [190,192]. We began with western blot analysis investigating levels of cleaved PARP1 to compare apoptosis in wt and DYT-*PRKRA* patient lymphoblasts treated with Nelfinavir for 6 hours then treated with tunicamycin for various time intervals. As shown in Fig 3.3, wt cells exhibit no basal apoptosis and treatment with tunicamycin only induces minimal PARP1 cleavage after 24 hours (Lanes 1-4). In contrast, treatment with Nelfinavir alone induces significant cleaved PARP1 levels in wt cells relative to treatment with tunicamycin alone (Lane 5). Additionally, Nelfinavir augments PARP1 cleavage in wt cells in response to tunicamycin treatment at 8, 12, and 24 hours (Fig 4.3, Lanes 6-8). Similarly, in DYT-*PRKRA* lymphoblasts, Nelfinavir alone (Fig 3.3, Lane 13) causes drastically increased levels of PARP1 cleavage relative to tunicamycin treatment alone (Lanes 10-12). Also, Nelfinavir pre-treatment maintains steady elevated cleaved PARP1 after ER stress compared to tunicamycin treatment alone (Lanes 14-16). These results clearly indicate that Nelfinavir is not a suitable therapeutic for reducing higher levels of apoptosis seen in DYT-*PRKRA*. Next, we examined the effects of Nelfinavir on ATF4 induction, which is an established marker for the ISR activation. As seen in Fig 3.3, wt cells treated with Nelfinavir alone have significantly increased ATF4

levels relative to basal levels of ATF4 in untreated cells (Lanes 1 and 5). Notably, Nelfinavir treatment alone induced ATF4 levels similar to tunicamycin treatment in wt cells (Lanes 2-5). Additionally, in wt cells, Nelfinavir augments ATF4 levels in response to tunicamycin treatment at 8 and 12 hours (Lanes 6-7). Like wt cells, DYT-*PRKRA* cells treated with Nelfinavir alone have significantly elevated ATF4 levels relative to untreated DYT-*PRKRA* cells as well as DYT-*PRKRA* cells treated with tunicamycin alone (Fig 3.3, Lanes 9-13). Nelfinavir pre-treatment followed by ER stress-induction augments ATF4 levels in DYT-*PRKRA* cells relative to tunicamycin treatment alone (Fig 3.3, Lanes 10-12 and 14-16). Collectively, this data indicates that despite a robust ISR induction in both wt and DYT-*PRKRA* lymphoblasts, Nelfinavir not only does not protect cells from ER stress-induced apoptosis, in fact it has a detrimental effect on cell survival.

Luteolin protects against apoptosis in response to ER stress by restoring homeostasis in DYT-*PRKRA* cells

We next tested if luteolin, a plant flavonoid, which dissociates the PACT-PKR interaction can protect DYT-*PRKRA* cells from ER stress-induced apoptosis. As shown in Fig 3.4A, wt cells have significantly reduced cleaved PARP1 levels in response to tunicamycin treatment when pre-treated with luteolin relative to cells treated with tunicamycin alone (Lanes 2-4 and 6-8). Additionally, luteolin promotes cellular recovery after ER stress-induction in DYT-*PRKRA* cells as seen by a marked reduction in PARP1 cleavage levels at 8,12, and 24 hours (Fig 3.4A, Lanes 10-12 and 14-16). It is worth noting that even in the absence of stress, cleaved PARP1 levels are significantly elevated in DYT-*PRKRA* patient

cells and treatment with luteolin significantly attenuates apoptosis (Fig 3.4A, Lanes 9 and 13). Next, we examined the effects of luteolin on ISR in the context of ER stress by doing western blot analysis to analyze levels of two ISR transcription factors ATF4 and CHOP. As seen in Fig 3.4B, luteolin pre-treatment diminishes ATF4 levels in wt cells in response to tunicamycin treatment at 8, 12, and 24 hours when compared to tunicamycin treatment alone (Lanes 2-4 and 6-8). In DYT-*PRKRA* cells, luteolin leads to significantly decreased ATF4 levels at 8, 12, and 24 hours (Lanes 14-16) relative to cells treated with tunicamycin alone (Lanes 10-12). Luteolin drastically attenuates CHOP levels in both wt and DYT-*PRKRA* patient cells (Fig 3.4B, Lanes 6-8 and 14-16) relative to wt and DYT-*PRKRA* cells treated with tunicamycin alone (Lanes 2-4 and 10-12). This repression of CHOP and ATF4 induction indicates how luteolin may rescue the ER stress-induced apoptosis after tunicamycin treatment to promote cellular survival.

Luteolin inhibits enhanced PKR and eIF2α phosphorylation after ER stress in DYT-*PRKRA* lymphoblasts

Due to the reduction in apoptosis in response to ER stress with luteolin, we sought to identify an upstream mechanism of the ISR that was responsible for the decreased sensitivity to tunicamycin treatment in DYT-*PRKRA* lymphoblasts. We began with western blot analysis investigating levels of p-PKR (activated PKR) in wt and DYT-*PRKRA* cells pre-treated with Luteolin for 24 hours then treated with tunicamycin for various time intervals to compare the levels and kinetics. As the major difference in luteolin treated cells is seen later time points

after ER stress based on our previous work, we looked at the time points later than 4 hours for evaluating p-PKR and p-eIF2α levels. As shown in Fig 3.5, tunicamycin induced PKR phosphorylation at 4h after treatment in wt cells that drops to basal levels at 8 hours and later (-lut, Lanes 1-5) and luteolin pre-treatment significantly reduced the levels of PKR phosphorylation both in the absence of ER stress and after ER stress-induction (+lut, Lanes 1-5). In DYT-*PRKRA* cells, PKR phosphorylation is high at basal levels and tunicamycin treatment augments these levels at 4 h and the levels remain elevated through 12 hours (Fig 3.5, -lut, lanes 7-9). In response to luteolin pre-treatment, PKR phosphorylation levels were significantly diminished in DYT-*PRKRA* cells (Fig 3.5, +lut, lane 6) and the duration for the presence of p-PKR was also shortened (compare p-PKR: -lut and +lut panels, lane 7-10). Next, we determined levels of p-eIF2α in response to luteolin treatment and found that it significantly blunts the p-eIF2α levels in wt cells along with reducing its duration (Fig 3.5, compare p-eIF2α: -lut and +lut panels, lanes 2-5). These effects are also seen in DYT-*PRKRA* cells treated with luteolin. As seen in Fig 3.5, in the absence of luteolin, DYT-*PRKRA* cells treated with tunicamycin maintain significantly elevated p-eIF2α levels for a long duration of time through 24 hours (-lut panel, lanes 7-12). Meanwhile, pre-treatment with luteolin drastically reduces p-eIF2α levels beginning at 8 hours restoring them to levels similar to basal and maintaining that through the duration of the time course (Fig 3.5, +lut panel, lanes 8-10). These results indicate that the inhibition of PACT-PKR interaction, blunts the level and duration of both PKR phosphorylation which attenuates eIF2α phosphorylation in

wt and DYT-*PRKRA* lymphoblasts which suggests how luteolin protects DYT-*PRKRA* cells from ER stress.

3.5 Discussion:

Dystonias (DYT1-DYT26) are a group of movement disorders characterized by sustained involuntary postures/or slow twisting movements that generate motor disability and pain [58-60,192]. Clinical manifestations vary from focal dystonias to generalized dystonias where most of the body is involved in abnormal posturing/or slow uncontrolled twisting movements while symptoms generally remain throughout an individual's lifetime leading to a drastically diminished quality of life [58,192]. Dystonia etiology can range from sporadic, idiopathic or inherited to those that occur as a secondary pathology [58,192].

DYT-*PRKRA* is an early onset primary generalized dystonia caused by mutations in the *PRKRA* gene which encodes PACT [Fig 1.4] [59,68,98,113]. It typically shows clinical manifestations early in childhood and can have a genetic inheritance pattern of both autosomal dominant and recessive [59]. DYT-*PRKRA* was first characterized by identification of a homozygous mutation in seven Brazilian patients from two unrelated families [61]. This missense PACT mutation substituted a proline residue for leucine at position 222 (P222L) [61]. After the initial study identifying a causative mutation in PACT leading to dystonia, nine other mutations in PACT have been discovered [62-65,98,158].

In their 2020 study, *Burnett et al*. characterized that lymphoblasts derived from a compound heterozygous patient (P222L/C213R) are hypersensitive to ER stress and exhibit enhanced eIF2α phosphorylation, dysregulated integrated

stress response (ISR) and increased apoptosis [113]. This work was built on *Vaughn et al.'s* work that described that the lymphoblasts derived from patients homozygous for the most prevalent P222L mutation have enhanced PKR activity and dysregulated eIF2α stress response signaling which increased cellular sensitivity to ER stress-induced apoptosis [68].

Seven of the ten reported DYT-*PRKRA* mutations lead to hyperactive PKR and dysregulated eIF2α signaling thereby enhancing cell sensitivity to apoptosis in response to ER stress [68,98,113]. Maladaptive eIF2α signaling and an aberrant ISR is observed in several dystonias which include DYT1, DYT3, DYT6, and DYT11 [73,121-124] in addition to our research on the disease etiology of DYT-*PRKRA* with homozygous P222L and heterozygous P222L/C213R patient cells [68,113].

The dysregulation of protein synthesis by affecting the eIF2 phosphorylation status is quickly becoming an emerging theme in the pathology of other neurodegenerative disorders, as well as intellectual disability disorders, cancer, diabetes, and obesity [74-78,190]. Clinical studies have identified elevated eIF2α phosphorylation in Alzheimer's disease (AD), ALS, and certain forms of autism [74-78]. Conversely, both *in vivo* and *in vitro* studies have implicated deficient phosphatase activity of PP1, which is the phosphatase responsible for dephosphorylation of eIF2α, in severe neurodevelopment disorders [6, 7, 79, 80]. Patients and mouse models deficient in the regulatory subunits of PP1 present with reduced body size, microcephaly, intellectual disability, and in some cases, AD [81, 82]. Most recently, mutations in the γ subunit of eIF2, a critical

component in the formation of the ternary complex, have been identified to be a causative factor driving mental intellectual disability, epileptic seizures, hypogenitalism, microcephaly, and obesity (MEHMO) syndrome [83]. Samples taken from patients with MEHMO show signs of chronic stress markers as the causative mutations lead to a defect in ternary complex formation [83]. A noteworthy symptom that presence in a severe form of MEHMO syndrome is lower limb ataxia [83]. This ultimately results in the expression of stress response transcripts and constitutively stimulates the ISR in these patients [83].

During general translational inhibition when eIF2α is phosphorylated, there are certain specific transcripts that are preferentially translated to optimize cellular recovery [1,2]. One of these transcripts is ATF4 which acts as a transcription factor during the ISR and plays an indispensable role in determining cell fate during cell stress [Fig 1.1] [2]. Depending on stress severity and duration, ATF4 tailors responses towards cell recovery or apoptosis [2]. ATF4 has been shown to maintain pancreatic β-cell homeostasis during ER stress *in vivo* [163]. *Galehdar et* al showed that ER stress-induced apoptosis in mouse cortical neurons is driven in an ATF4-CHOP dependent manner [196]. Additionally, increased ATF4 expression was shown to be a mediator in the pathology of retinal degeneration, Alzheimer's Disease, and Parkinson's Disease in mouse models [153]. This underscores the importance of ATF4 in promoting homeostasis in pancreatic β-cells and neurons [150-152,163]. If eIF2α phosphorylation is sustained, ATF4's molecular function pivots towards promoting apoptosis via mediating enhanced expression of pro-apoptotic

transcripts such as CHOP, ATF3, ATF5, and Noxa [1,2,190,191]. Proteins encoded by these pro-apoptotic transcripts cause activation of caspases, PARP1 cleavage, and subsequent programmed cell death [1,2]. However, one of the ISR transcripts, GADD34, a regulatory subunit of the PP1 holocomplex aids in dephosphorylation of eIF2α and subsequent restoration of global protein translation which is an essential step that regulates recovery from stress and termination of the ISR [1,2,190]. CReP is a constitutively active regulatory subunit of PP1C and is expressed in unstressed cells, which maintain very low basal eIF2α phosphorylation [1,2,190]. Molecules that regulate eIF2α dephosphorylation and the ISR are showing potential as a targeted therapeutic in neurodegenerative diseases [2,190].

Nelfinavir is an aspartyl protease inhibitor (HIV-PI) which was initially utilized for HIV therapy [190-191]. Nelfinavir treatment shows off-target effects as well by downregulation of the protein phosphatase CReP levels which leads to decreased association between the CReP-PP1C complex and eIF2α [2,190]. Thus, Nelfinavir promotes an increase in eIF2α phosphorylation and ISR activation [2,190]. Nelfinavir's effect on eIF2α phosphorylation is downstream of the four eIF2α kinases but there is a dependency of the enzymatic rate of each kinase due to their ability to augment phosphorylation of eIF2α [2]. Nelfinavir has been seen to induce enhanced ATF4 levels and a robust ISR both *in vitro* and *in vivo* [190]. Along with promoting ATF4 expression, Nelfinavir's induction of the ISR diminishes cellular protein synthesis, which allows for cellular proteostasis to occur through improved protein folding or protein degradation [2,190].

Additionally, ongoing clinical trials to elucidate Nelfinavir's potential as a cancer therapeutic have shown promising initial results [190,191]. Strategies aimed at enhancing eIF2α phosphorylation have been identified to limit cancer cell proliferation and tumor growth [190,193-195]. Another CReP inhibitor, Salubrinal, has shown pro-apoptotic/anti-cancer properties similar to Nelfinavir by enhanced expression of ATF4 and CHOP [195]. Thus, there is accumulating evidence that fine-tuning of eIF2α phosphorylation via pharmacological activation of the ISR is tilting cellular fate towards apoptosis.

In the present study, we investigated the CReP inhibitor, Nelfinavir, on its ability to promote cellular recovery of DYT-*PRKRA* lymphoblasts in response to ER stress. CReP levels are significantly elevated within DYT-*PRKRA* patient lymphoblasts relative to wt cells both in the absence of stress and in response to ER stress-induction (Fig 3.1). As enhancing eIF2α phosphorylation is protective for cells and DYT-*PRKRA* cells are more sensitive to apoptosis, based on our previous research we tested if Nelfinavir mediated down regulation of CReP and consequent upregulation of eIF2α phosphorylation is protective to DYT-*PRKRA* cells. As seen in Fig 3.2, Nelfinavir significantly diminishes CReP levels after 6 hours of treatment in both wt and DYT-*PRKRA* cells. This correlates with drastically increased eIF2α phosphorylation after 6 hours of Nelfinavir treatment in both wt and DYT-*PRKRA* patient cells (Fig 3.2). Due to the reduction in CReP and increased eIF2α phosphorylation, we sought to determine if Nelfinavir would generate enhanced ISR activity thereby rescuing DYT-*PRKRA* cells from increased sensitivity to ER-stress induced apoptosis. Nelfinavir treatment alone

significantly increases ATF4 levels in both wt and DYT-PRKRA cells (Fig 3.3). Additionally, in both wt and DYT-*PRKRA* lymphoblasts, pre-treatment with Nelfinavir followed by tunicamycin treatment enhanced ATF4 levels relative to tunicamycin treatment alone (Fig 3.3). Despite Nelfinavir inducing a robust ISR, this did not cause cellular protection from ER stress-induced apoptosis. As shown in Fig 3.3, in both wt and DYT-*PRKRA* cells, Nelfinavir enhances cleaved PARP1 levels in response tunicamycin treatment. Also, Nelfinavir treatment alone significantly increases PARP1 cleavage in both wt and DYT-*PRKRA* cells relative to tunicamycin treatment alone (Fig 3.3).

As Nelfinavir treatment did not offer any protection from ER stress-induced apoptosis, we tested the effect of luteolin, a polyphenol flavonoid, which previously showed protection in compound heterozygous DYT-*PRKRA* cells carrying P222L and C213R mutations. The polyphenol flavonoid, Luteolin, does protect both wt and DYT-*PRKRA* cells from ER stress-induced apoptosis (Fig 3.4). Additionally, DYT-*PRKRA* cells have significantly elevated basal cleaved PARP1 levels and treatment with luteolin decreases these thus demonstrating attenuation of apoptosis in the absence of ER stress (Fig 3.4). Next, we characterized the effects of luteolin on the ISR signaling axis within the context of ER stress-induction and determined that luteolin significantly blunts induction of both ATF4 and CHOP in response to tunicamycin in both wt and DYT-*PRKRA* cells (Fig 3.4). Luteolin drastically diminishes p-PKR levels both in the absence of stress and after ER stress in wt cells (Fig 3.5). In DYT-*PRKRA* cells, Luteolin also significantly decreased PKR phosphorylation levels and shortened p-PKR

duration in response to tunicamycin treatment (Fig 3.5). Additionally, Luteolin pre-treatment displayed effectiveness is attenuating p-eIF2α levels in wt and DYT-*PRKRA* cells in response to tunicamycin treatment (Fig 3.5). Collectively, these results indicate that a targeted therapeutic that decreases p-eIF2α levels and diminishes the ISR generates cellular protection from ER stress. This data supports previous research that concluded targeted therapeutics affecting the ISR signaling axis are ineffective when eIF2α phosphorylation exceeds a critical threshold level thereby showing that cellular recovery occurs in response to stress when eIF2α phosphorylation is low [181]. Additionally, previous studies demonstrated that inhibiting eIF2α-specific phosphatase cofactor activity, thereby prolonging attenuation of global protein synthesis promotes elevated CHOP expression in an ATF4-dependent manner which highlights that Nelfinavir does not promote cellular recovery from stress and actually generates enhanced apoptosis even in the absence of stress [181,190,191].

Numerous studies have indicated the neuroprotective effects of natural polyphenols such as Quercetin and Luteolin due to their mechanism of action being directed at attenuating neuro-inflammation and reactive oxygen species (ROS) [86,87,88,89,90,91,92,93]. Luteolin is an abundant metabolite present in medicinal herbs, fruits such as oranges and apples, and vegetables such as broccoli and celery [84,86,91,92]. Luteolin promoted behavior performance and hippocampal neurogenesis in a Down syndrome mouse model [85]. In a separate study, luteolin was given along with quercetin as a dietary supplement in children with Autism Spectrum Disorder (ASD) which promoted attenuation of aberrant

behavior with no major adverse effects reported [86]. There has been a link between the inflammatory response of microglia and development of ASD which set the stage for a groundbreaking study showing that dietary luteolin supplementation in children led to a dramatic decrease in serum levels of pro-inflammatory cytokines TNF-α and IL-6 along with recovery of behavior deficits [97]. Luteolin's antioxidant properties have been shown to ameliorate Aβ-induced cell death within murine cortical neurons along with improving spatial learning and memory within an AD mouse model which highlights luteolin's potential in being a neuroprotective agent against AD [86]. Additionally, luteolin has been seen to promote neuronal cellular recovery in response to traumatic brain injuries (TBI) [86,91]. At a molecular level, luteolin has been shown to blunt microglia and astrocyte activation both *in vitro* and *in vivo* by subsiding activity of NF-kB, STAT3, JNK, and TLR-4 pathways which are involved in activation of microglia and astrocytes generating neurological inflammation [84,86,88,91,93,95,96,97]. *Dabo et al* elucidated that luteolin inhibits PKR homodimerization and subsequent activation [Fig 1.5] [84]. They showed that in THP1 macrophages and murine primary macrophages, treatment with luteolin before induction of ER stress and oxidative stress caused reduction in the ISR and expression of pro-inflammatory cytokines IL-8 and IL-1β [Fig 1.5] [84]. This study highlights that targeted therapeutics that maintaining low levels of eIF2α phosphorylation and ATF4 thereby promoting moderate ISR signaling after ER stress is a strategy to promote cellular homeostasis in DYT-*PRKRA* cells.

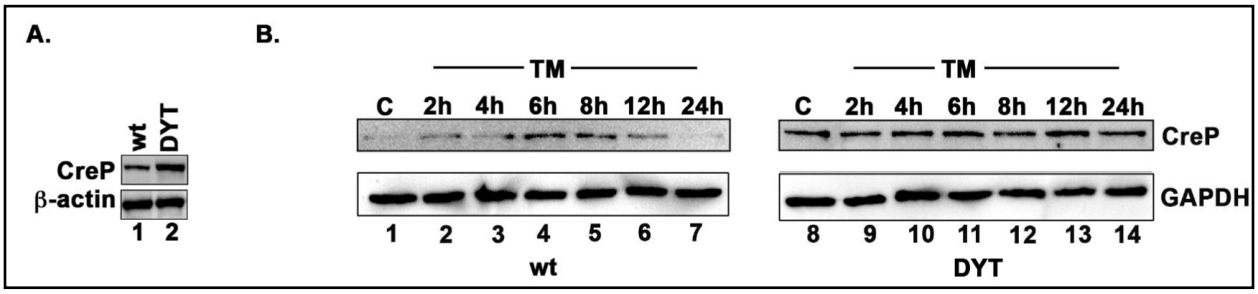

Figure 3.1: Effect of DYT-*PRKRA* mutation on CReP levels in DYT-*PRKRA* patient lymphoblasts. (A) Western blot analysis for CReP basal levels was done using whole cell extracts from normal (wt) and DYT-*PRKRA* patient derived lymphoblasts. Blots were probed for CReP. β-actin was used as a loading control to ensure equal amounts of protein were loaded in each lane. **(B)** Western blot analysis for CReP in response to tunicamycin treatment in normal (wt) and DYT-*PRKRA* patient lymphoblasts. Whole cell extracts from normal (wt) and DYT-PRKRA patient derived lymphoblasts were treated with 5 µg/ml tunicamycin (TM) for the indicated time points. Blots were probed for CReP. GAPDH was used as a loading control to ensure equal amounts of protein were loaded in each lane.

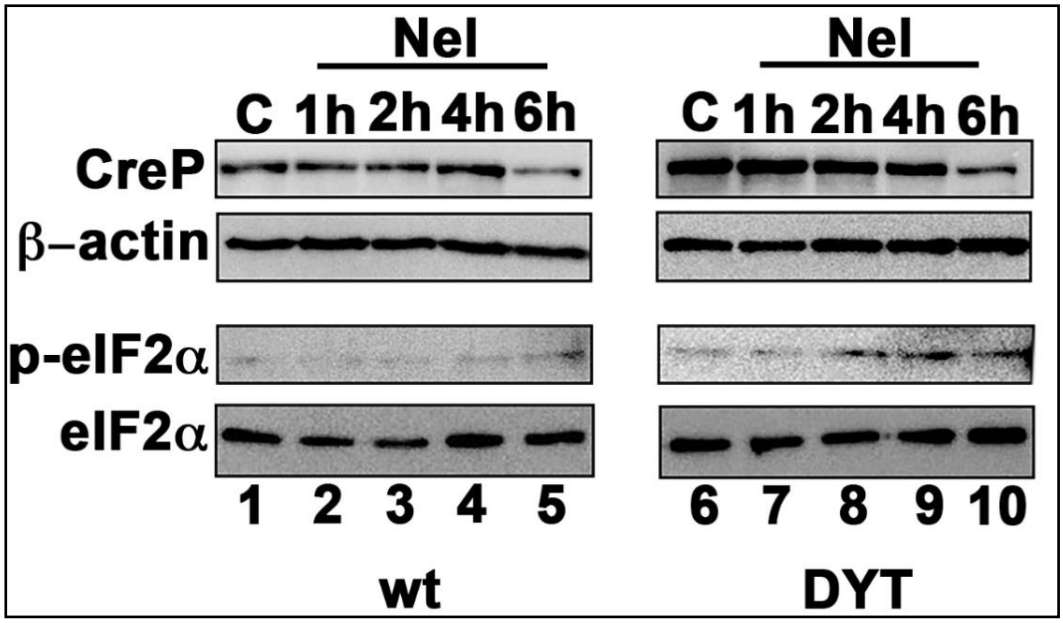

Figure 3.2: Effect of Nelfinavir on CReP and eIF2α phosphorylation in DYT-*PRKRA* patient lymphoblasts. (A) Western blot analysis for CReP. Whole cell extracts from normal (wt) and DYT-*PRKRA* patient derived lymphoblasts were treated with 40 μM Nelfinavir for the indicated time points. Blots were probed for CReP. β-actin was used as a loading control to ensure equal amounts of protein were loaded in each lane. Western blot analysis for p-eIF2α. Whole cell extracts from normal (wt) and DYT-*PRKRA* patient derived lymphoblasts were treated with 40 μM Nelfinavir for the indicated time points. Blot were probed for p-eIF2α and total eIF2α.

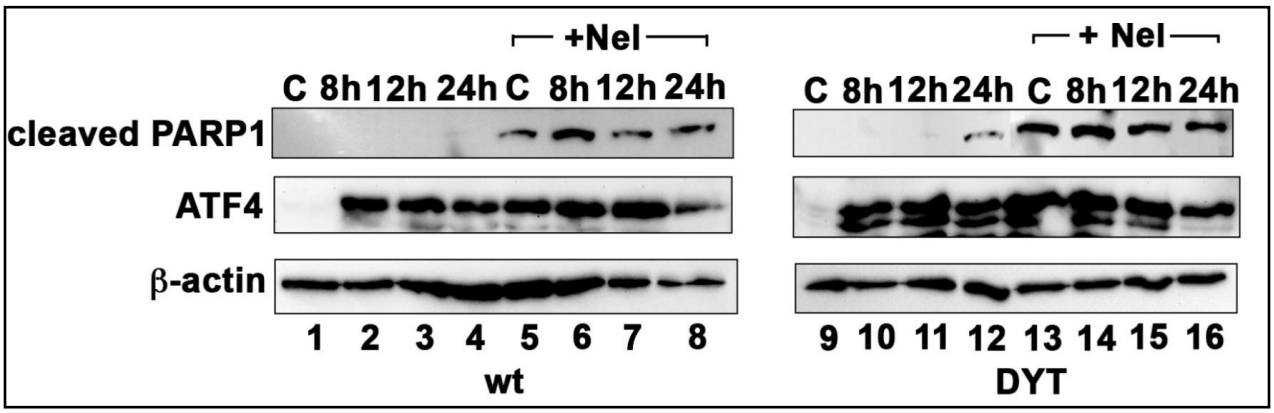

Figure 3.3 PARP1 cleavage and ATF4 in response to Nelfinavir treatment followed by tunicamycin in DYT-*PRKRA* patient lymphoblasts. Western blot analysis for cleaved PARP1 and ATF4. Whole cell extracts from normal (wt) and DYT-*PRKRA* patient derived lymphoblasts treated with 40 μM Nelfinavir for 6 hrs followed by treatment with 5 μg/ml tunicamycin (TM) were analyzed at indicated time points. Blots were probed for cleaved PARP1 and ATF4. β-actin was used as a loading control to ensure equal amounts of protein were loaded in each lane.

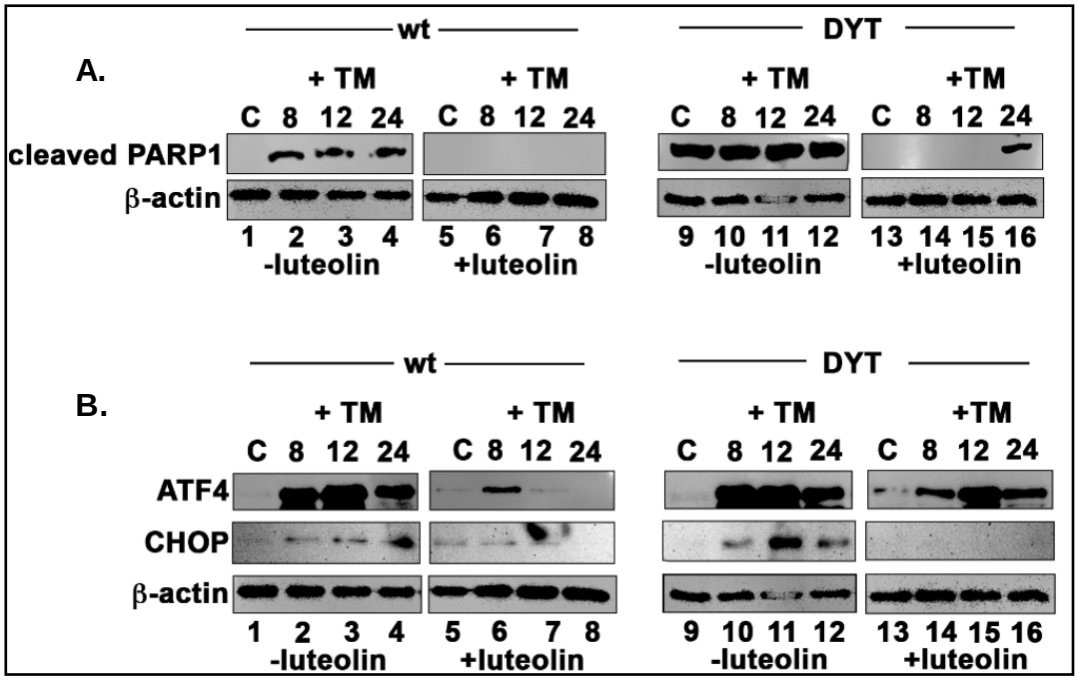

Figure 3.4: PARP1 cleavage and ISR in response to Luteolin treatment followed by tunicamycin in DYT-*PRKRA* patient lymphoblasts. (A) Western blot analysis for cleaved PARP1. Whole cell extracts from normal (wt) and DYT-*PRKRA* patient derived lymphoblasts treated with 50 μM Luteolin for 24 hrs followed by treatment with 5 μg/ml tunicamycin (TM) were analyzed at indicated time points. Blots were probed for cleaved PARP1. β-actin was used as a loading control to ensure equal amounts of protein were loaded in each lane. **(B)** Western blot analysis for ATF4 and CHOP. Whole cell extracts from normal (wt) and DYT-*PRKRA* patient derived lymphoblasts treated with 50 μM Luteolin for 24 hrs followed by treatment with 5 μg/ml tunicamycin (TM) were analyzed at indicated time points. Blots were probed for ATF4 and CHOP. β-actin was used as a loading control to ensure equal amounts of protein were loaded in each lane.

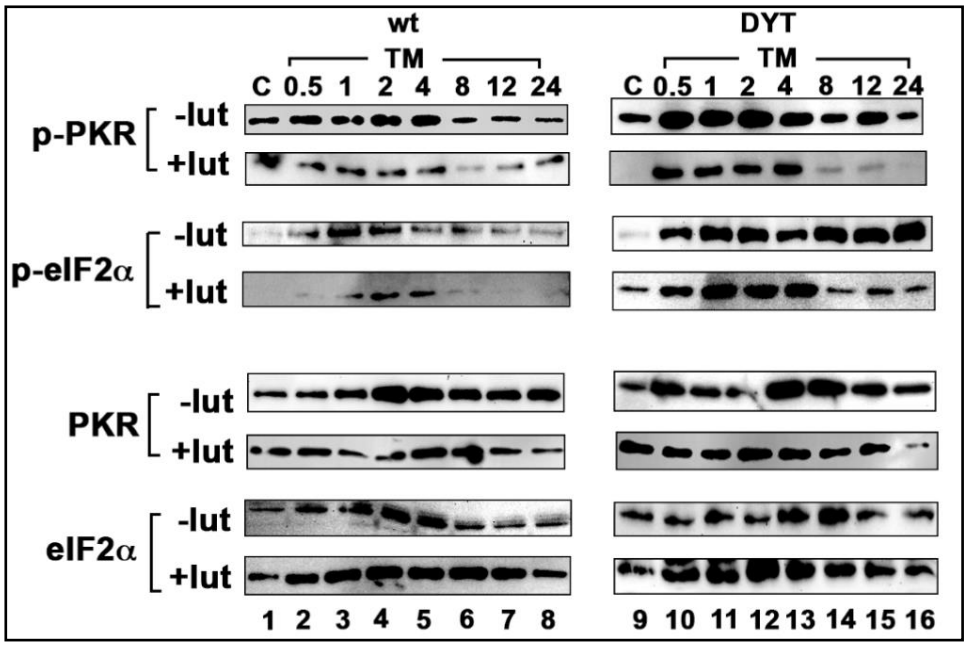

Figure 3.5: PKR activation and eIF2α phosphorylation in response to Luteolin treatment followed by tunicamycin in DYT-*PRKRA* patient lymphoblasts. Western blot analysis for p-PKR and p-eIF2α. Whole cell extracts from normal (wt) and DYT-*PRKRA* patient derived lymphoblasts treated with 50 µM Luteolin for 24 hrs followed by treatment with 5 µg/ml tunicamycin (TM) were analyzed at indicated time points. Blots were probed for p-eIF2α, total eIF2α, p-PKR, and total PKR.

Chapter: 4

Hyper-activity of PKR and subsequent enhanced apoptosis within XDP neural progenitor cells

4.1 Introduction:

The X-linked Dystonia Parkinsonism (XDP, DYT3) is a progressive neurodegenerative disease endemic to the island of Panay, Philippines [99]. XDP exhibits dystonic symptoms at early disease stages that shift over time towards a more parkinsonism phenotype [99,100]. Neuropathological studies show that XDP involves a progressive loss of medium spiny neurons (MSNs) in the striatum and decreased numbers of neural progenitor cells (NPCs) within the subventricular zone [101,102,103]. The TATA-Binding Protein-Associated Factor-1 (TAF1) gene is responsible for XDP pathogenesis and encodes a transcription factor (TAF1, formerly known as TAFII250), which is part of the transcription factor IID (TFIID) complex involved in RNA Polymerase II-mediated transcription [104,105]. Previously, an XDP-specific insertion of an SVA (SINE-VNTR-Alu) type retrotransposon in intron 32 of TAF1, and a neural-specific TAF1 isoform (N-TAF1), which showed decreased expression in post-mortem XDP brains was characterized [106]. Since these initial studies, the new studies have elucidated that XDP is caused by a DNA repeat expansion within an intronic SVA retrotransposon insertion in TAF1 [107-110]. The number of the CCCTCT hexameric repeats in SVA was reported to range from 35-52 and showed a highly significant inverse correlation to the age of disease onset [107]. This represents a direct link between sequence variation in XDP probands and disease manifestation, thereby establishing a role as a causality for SVA in disease pathogenesis [110]. This correlation has since been confirmed in an independent XDP cohort, while also observing that hexamer length may modify

the dystonic vs parkinsonian characteristics [110]. PKR is a ubiquitously expressed interferon (IFN)-induced and RNA-activated, serine/threonine kinase that mediates antiviral actions and regulates apoptosis [111,112]. PKR is expressed at basal levels in all cells and within the context of viral infections it is transcriptionally induced via IFNs [111]. PKR stays enzymatically latent in the absence of its activators which are either dsRNA or PACT of which these interactions occur via facilitation of PKR's dsRBMs leading to unmasking of PKR's ATP-binding site which causes PKR activation [Fig 4.1] [12,14,16,22,34,37,39,48,114]. Additionally, other structures besides dsRNA and PACT can activate PKR of which one class is IRAlu's which resides in the 3' UTR of mRNAs [115-117]. More critically, when an Alu element in reverse orientation within a single transcript, denoted as IRAlu, the two Alu elements can form an intramolecular dsRNA that binds to and activates PKR [115,118]. Even though the presence of mRNAs containing IRAlu sequences in XDP patients has not been reported, it is rational to infer that RNAs originating within the multiple transcript system contain sequences with extensive stem loops and ds regions. It is also possible that a promoter within or near the SVA insertion site within the TAF1 intron 32 gives rise to sufficient amounts of SVA-derived RNA in XDP patients. As extremely tiny amounts of dsRNA can achieve PKR activation it can feasibly cause chronic low-level PKR activity sensitizing cells to apoptosis [Fig 4.1] [112,119]. In XDP, expression of ATF3, a pro-apoptotic protein and established downstream target of PKR has been reported to be upregulated [109]. The most direct evidence for hyperactive PKR causing dystonia comes

from the studies by Kuipers *et al* [120]. Their research determined hyperactive PKR variants and enhanced eIF2α phosphorylation in early-onset dystonia [120]. Previous research has elucidated the enhanced PKR activity and dysregulated eIF2α signaling as the causality of increased sensitivity to apoptosis within DYT-*PRKRA* cells [68,98,113]. DYT-*PRKRA* involves mutations in the protein associated activator of PKR (PACT) [68,98,113]. For DYT-*PRKRA*, a dominant frameshift mutation reported in a German patient and five different missense mutations each cause excessive PKR activation and disrupted eIF2α signaling [98,113]. Stress-induced phosphorylation of PACT at serine 287 is critical for PACT's interaction with PKR which subsequently leads to PKR activation and downstream phosphorylation of eIF2α leading to translation inhibition [16,34,39,48]. Dysregulated eIF2α signaling as a common link for pathophysiology of several dystonias which include DYT1, DYT6, and DYT11 has been inferred from multiple studies [121-124]. PKR has emerged as a major factor in several neurodegenerative diseases as aberrant heightened PKR activity is observed in post-mortem patient brains as well as in mouse models [112,125-126]. Increased PKR phosphorylation is reported in patients with Alzheimer's disease, Parkinson's disease, Huntington's disease, dementia, and prion disease [127-131]. Activated PKR was shown to be indispensable for the behavioral and neuropathophysiological abnormalities in a mouse model of Down syndrome and PKR inhibitory drugs partially rescued the synaptic plasticity and long-term memory deficits [132]. Drugs that target the eIF2α signaling pathway have also shown to be therapeutic in mouse models for neurodegenerative

diseases and inhibiting PKR has been proven to be effective in the recovery of synaptic connections and learning deficits in two different Alzheimer's disease mouse models [133]. With the molecular pathophysiological overlaps with DYT-*PRKRA* and XDP, this leads to the rationale for this chapter that has an objective to analyze active PKR levels and the effect on its downstream targets within XDP NPCs.

4.2 Results:

Active PKR and its downstream targets are dysregulated in XDP NPCs

In order to examine if PKR is hyperactive in XDP relative to wt cells, we investigated the levels of p-PKR (activated PKR) in XDP and control neural progenitor cells (NPCs) using western blot analysis (Figure 2). As seen in (Fig 4.2, lanes 7-12) all six XDP samples have elevated p-PKR relative to control samples (Fig 4.2, lanes 1-6). Due to the heightened active PKR within XDP samples, we next determined levels of p-eIF2α in order to gain some insight into possible dysregulation of the integrated stress response pathway (ISR). As shown in (Fig 4.3, lanes 1-6) all six wt NPCs have low basal levels of phosphorylated eIF2α which is expected, while in five of the six XDP samples (Fig 4.3, lanes 8-12) there is elevated eIF2α phosphorylation. As we noted the differences in phosphorylation levels of PKR and eIF2α between wt and XDP NPCs, we probed another target of active PKR, ATF3, which is a pro-apoptotic protein, to elucidate downstream effects further. In all of the XDP samples (Fig 4.4, lanes 7-12), there is highly increased expression of ATF3 relative to wt controls (Fig 4.4, lanes 1-6). Collectively these results demonstrate hyperactive PKR leading to enhanced phosphorylation of eIF2α along with upregulation of pro-apoptotic marker ATF3 within XDP NPCs.

4.3 Discussion:

XDP, (DYT3) is a progressive neurodegenerative disease [99] which exhibit dystonic symptoms at early disease stages that shift over time towards a more parkinsonism phenotype [99,100]. Neuropathological studies show that XDP involves a progressive loss of MSNs in the striatum and decreased numbers of NPCs within the subventricular zone [101,102,103]. New studies have elucidated that XDP is caused by a DNA repeat expansion within an intronic SVA retrotransposon insertion in TAF1 [107-110]. This represents a direct link between sequence variation in XDP probands and disease manifestation, thereby establishing a role as a causality for SVA in disease pathogenesis [110]. PKR is a ubiquitously expressed interferon (IFN)-induced and RNA-activated, serine/threonine kinase that mediates antiviral actions and regulates apoptosis [111,112]. PKR is expressed at basal levels in all cells and within the context of viral infections it is transcriptionally induced via IFNs [113]. PKR stays enzymatically latent in the absence of its activators which are either dsRNA or PACT of which these interactions occur via facilitation of PKR's dsRBMs leading to unmasking of PKR's ATP-binding site which causes PKR activation [Figs 1.3 and 4.1] [12,14,16,22,34,37,39,48,114]. Even though the presence of mRNAs containing IRAlu sequences in XDP patients has not been reported, it is rational to infer that RNAs originating within the multiple transcript system contain sequences with extensive stem loops and ds regions which would be PKR activators [Fig 4.1]. The most direct evidence for hyperactive PKR causing dystonia comes from the studies by Kuipers *et al* [120]. Their research

determined hyperactive PKR variants and enhanced eIF2α phosphorylation in early-onset dystonia [120]. Previous research has elucidated the enhanced PKR activity and dysregulated eIF2α signaling as the causality of increased sensitivity to apoptosis within DYT-*PRKRA* cells [68,98,113]. DYT-*PRKRA* involves mutations in PACT [68,98,113]. For DYT-*PRKRA*, a dominant frameshift mutation reported in a German patient and five different missense mutations each cause excessive PKR activation and disrupted eIF2α signaling [98,113]. Stress-induced phosphorylation of PACT at serine 287 is critical for PACT's interaction with PKR which subsequently leads to PKR activation and downstream phosphorylation of eIF2α leading to translation inhibition [16,34,39,48]. Stress-induced eIF2α phosphorylation by any of the ISR kinases causes attenuation of general protein synthesis but simultaneously selectively enhances translation of ISR specific mRNAs with long 5'-UTRs that have at least one short upstream open reading frame (uORF) [2, Fig.1]. These ISR transcripts are preferentially translated which optimizes cellular energy conservation while also allowing for restoration of cellular homeostasis [2]. A couple of these preferentially translated transcripts are ATF4 and CHOP during eIF2α phosphorylation [2, Fig.1]. These induce the transcription of other genes coding for ER enzymes and chaperones in order to attenuate the accumulation of unfolded proteins in the ER or trigger apoptosis if cellular recovery cannot be achieved due to stress severity or duration [2]. The results in this study add to an increasing amount of evidence highlighting maladaptive ISR signaling within multiple dystonia types and that the ISR is a critical axis for normal functioning

neurons. eIF2α phosphorylation driven translational modifications are an indispensable feature of normal neuronal functions in the absence of stress and all four eIF2α kinases help in regulation either individually or synergistically [113]. eIF2α phosphorylation dependent translation regulation allows neurons to rapidly change protein compositions at the synapse in a stimulus-dependent manner which is an essential process for neuron homeostasis [113]. In the present study, we establish PKR is hyperactive and levels of p-eIF2α are elevated in XDP cells [Fig 4.2 and Fig 4.3]. This provides some insight into possible dysregulation of the ISR which is a common mechanistic feature of DYT-*PRKRA*. To link the correlation between enhanced PKR activity and dysregulated eIF2α signaling to increased apoptosis which occurs within DYT-*PRKRA* cells, we investigated another target of active PKR, ATF3, which is a pro-apoptotic protein, to elucidate downstream effects further. We ascertained there is highly increased expression of ATF3 within XDP cells [Fig 4.4] which buttresses the inference that dysfunctionality of the eIF2α axis and the ISR is a common causality that drives pathophysiology and disease progression within multiple dystonia types. Dysregulated eIF2α signaling and a maladaptive ISR as a common link for pathophysiology of several dystonias which include DYT1, DYT6, and DYT11 has been inferred from multiple studies [121-124] in addition to our research on the disease etiology of DYT-*PRKRA* with homozygous P222L and heterozygous P222L/C213R patients [68,113]. Two independent studies substantiate the conclusion that dysfunctional eIF2α signaling plays a causative role in DYT1 synaptic defects [121,122]. Using an unbiased proteomics approach abnormal

eIF2α signaling occurred within DYT1 mouse and rat brains which overlapped with eIF2α activation results with human brain samples [122]. Another study determined that pharmacological restoration of eIF2α signaling restored the cortico-striatal LTD in DYT1 knock-in mice along with discovering that there were sequence variants in ATF4 amongst patients with focal cervical dystonia [121]. eIF2α signaling was identified as one of the top dysregulated pathways within heterozygous DYT6 Thap1 mutant neonatal mouse striatum and cerebellum tissue which was analyzed utilizing RNA-Seq [123]. The work done in this study within the scope of DYT3 adds a new layer for dystonia research and allows for targeted therapeutics work done focusing on the PKR- eIF2α signaling axis to be able to be done to treat several dystonia types.

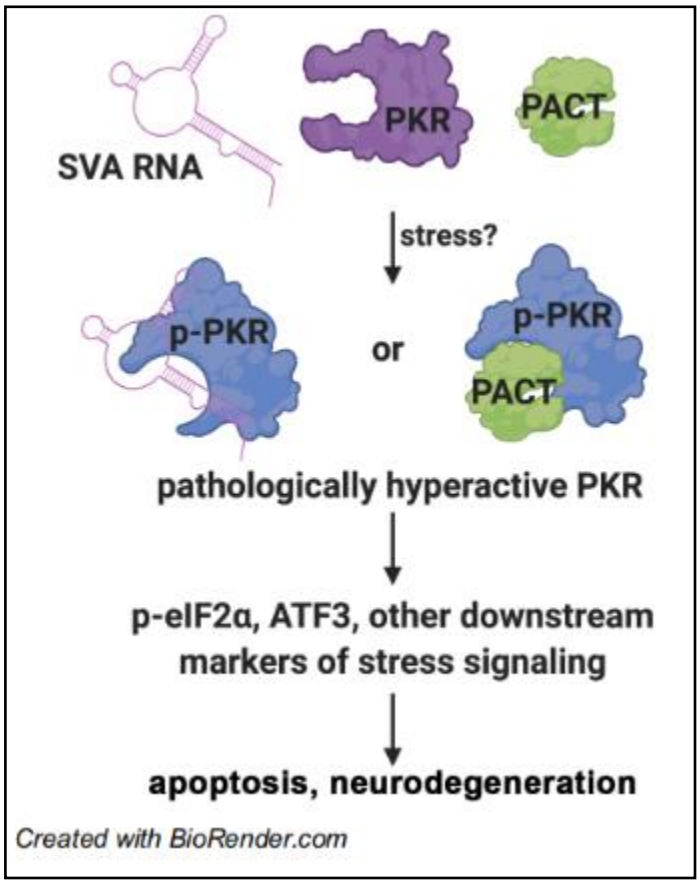

Figure 4.1: Schematic model depicting PKR activation and its downstream effects. dsRNA and PACT are known activators of PKR. Active PKR causes phosphorylation of eIF2α and upregulation of ATF3 amongst other downstream markers of cellular stress signaling. Chronic PKR activity sensitizes cells to apoptosis and subsequent neurodegeneration.

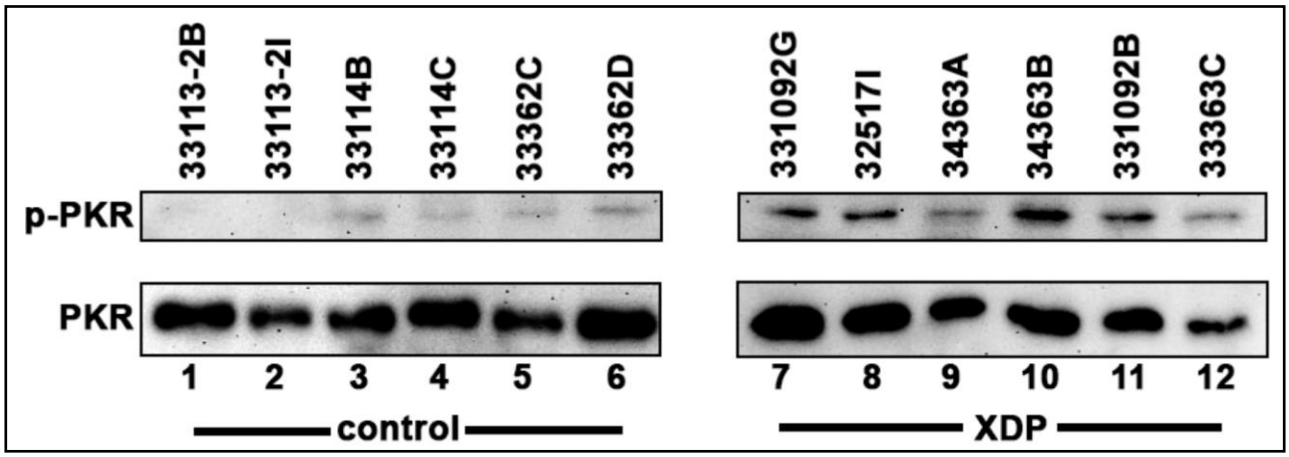

Figure 4.2: PKR activation in XDP NPCs. Western blot analysis for p-PKR. Whole NPC extracts were prepared from six control and six XDP samples. Blots were probed for p-PKR (active PKR) and total PKR. Total PKR was used as a loading control to ensure equal amounts of protein were loaded in each lane. Lanes 1-6: unaffected control samples and lanes 7-12: XDP samples. The numbers above lanes correspond to cell line numbers assigned by the stock center (Collaborative Center for XDP, Massachusetts General Hospital and Harvard Medical School).

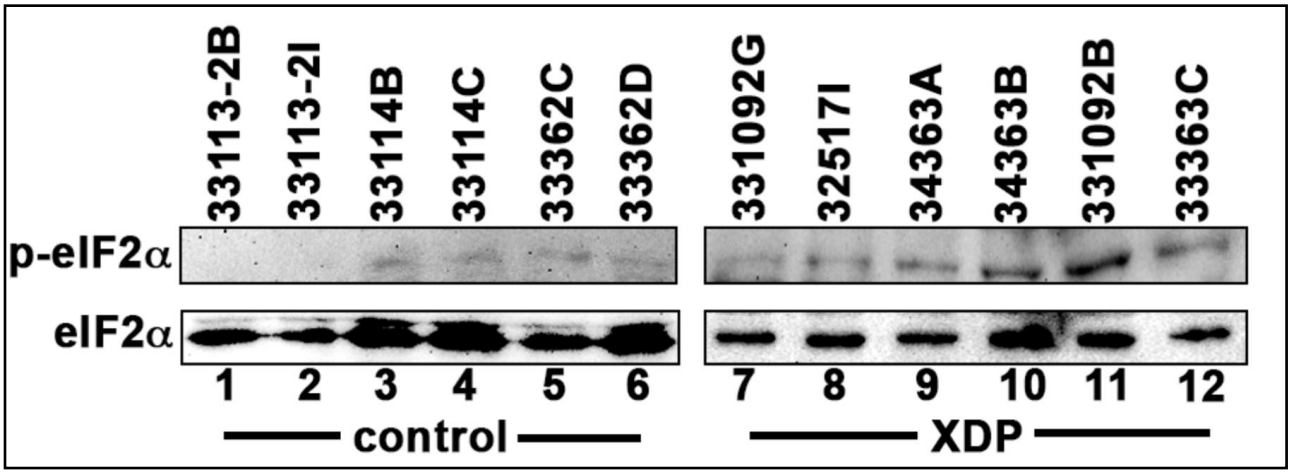

Figure 4.3: eIF2α phosphorylation in XDP NPCs. NPC extracts prepared from control and XDP samples were tested by western blot analysis with anti-p-eIF2α and anti-eIF2α (total eIF2α) antibodies. Total eIF2α was used as a loading control to ensure equal amounts of protein were loaded in each lane. Lanes 1-6: unaffected control samples and lanes 7-12: XDP samples. The numbers above lanes correspond to cell line numbers assigned by the stock center (Collaborative Center for XDP, Massachusetts General Hospital and Harvard Medical School).

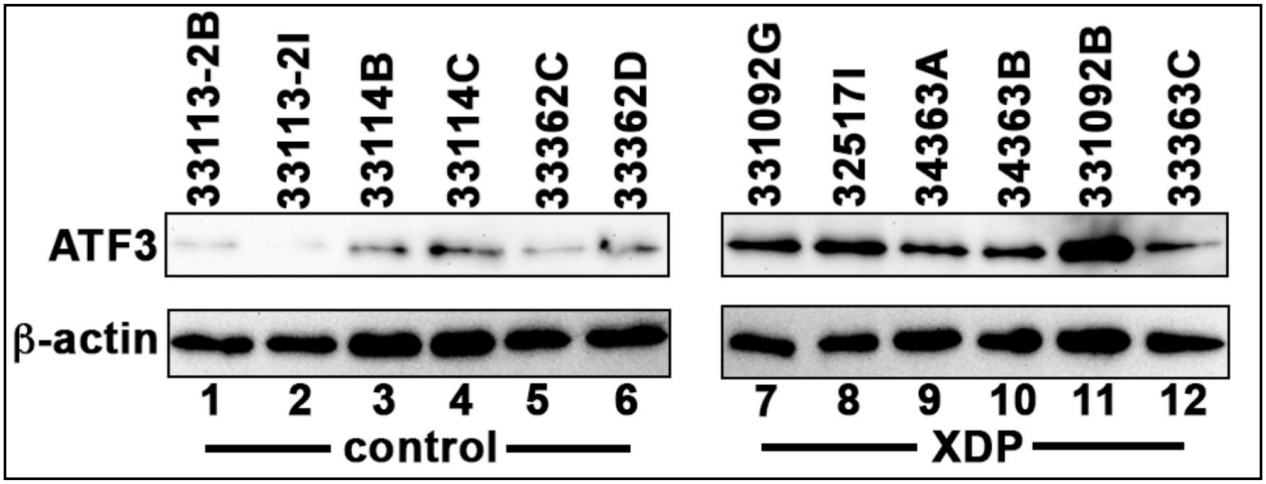

Figure 4.4: Analysis of ATF3 expression in XDP NPCs. NPC extracts prepared from control and XDP samples were tested by western blot analysis with anti-ATF3 and anti-β-actin antibodies. β-actin was used as a loading control to ensure equal amounts of protein were loaded in each lane. Lanes 1-6: unaffected control samples and lanes 7-12: XDP samples. The numbers above lanes correspond to cell line numbers assigned by the stock center (Collaborative Center for XDP, Massachusetts General Hospital and Harvard Medical School).

Chapter 5:

Targeted pharmacological inhibition of PKR promotes ISR restoration and subsequent

cellular recovery in DYT-*PRKRA* cells

5.1 Abstract:

DYT-*PRKRA* is a movement disorder caused by mutations in the PRKRA gene, which encodes for PACT, the protein activator of interferon-induced, double-stranded RNA (dsRNA)-activated protein kinase PKR. PACT through its heteromeric interactions with PKR facilitate PKR's autophosphorylation and subsequent activation in response to certain stress signals. Active PKR phosphorylates the translation initiation factor eIF2α. eIF2α phosphorylation is a critical step that regulates an evolutionarily conserved intracellular signaling network called the integrated stress response (ISR) which is indispensable for molecular adaptions to environmental stresses to promote homeostasis. Maladaptive eIF2α signaling or ISR axis in response to stress signals causes the normally pro-survival ISR to become pro-apoptotic. Our previous research has established that DYT-*PRKRA* mutations lead to hyperactive PKR, increased eIF2α phosphorylation and aberrant ISR signaling which enhances DYT-*PRKRA* cellular sensitivity to apoptosis. In the present study, we characterized the effects of 3 novel proprietary PKR inhibitors (PKRi) on their ability to promote cellular recovery within DYT-*PRKRA* cells in response to ER-stress induction. Our results determined that one of the PKRi compounds (PKT-00941), desensitized DYT-*PRKRA* cells from ER stress-induced apoptosis through diminishing levels of eIF2α phosphorylation along with decreasing the ISR signaling axis. This work suggests that a PKR-centered targeted therapeutic could be an effective option to treat DYT-*PRKRA* and possibly other diseases resulting from enhanced PKR activation.

5.2 Introduction:

PKR is a ubiquitously expressed interferon (IFN)-induced and double-stranded (ds)RNA-activated, serine/threonine kinase that mediates antiviral actions and regulates apoptosis [111,112]. PKR is expressed at basal levels in all cells and within the context of viral infections it is transcriptionally induced via IFNs [111]. PKR stays enzymatically latent in the absence of its activators which are either dsRNA or PACT. Both dsRNA and PACT interact with PKR via PKR's two double-stranded RNA binding motifs (dsRBMs) leading to PKR's homodimerization thereby causing an allosteric conformational change allowing exposure of each PKR monomer's ATP-binding site within its kinase domain (KD) to facilitate its trans-autophosphorylation at threonines 446 and 451 which causes PKR activation [Fig 1.3] [12,14,16,22,34,37,39,48,114]. dsRNA interaction with PKR occurs during viral infection and PACT-PKR interaction occurs within the context of oxidative stress, ER stress, and serum deprivation [68,98,113]. Stress-induced phosphorylation of PACT at serine 287 is critical for PACT's heterodimeric interaction with PKR which subsequently leads to PKR activation. [16,34,39,48].

Active PKR then acts by diminishing cap-dependent protein translation which is accomplished by phosphorylating the α subunit of eukaryotic initiation factor 2 (eIF2) at serine 51 [1,2]. This post-translational modification stops the formation of the ternary complex which is vital for translation initiation [1,5]. This strategy is a critical response to cellular stressors such as viral infections, misfolded protein accumulation, oxidative stress, and serum deprivation [1].

There are a family of four serine/threonine kinases, double-stranded RNA activated protein kinase (PKR), PKR-like Endoplasmic Reticulum kinase (PERK), heme-regulated inhibitor (HRI), and gene control non-derepressible (GCN) kinase which phosphorylate eIF2α [Fig 1.1][1,2]. While each of these kinases have an evolutionarily conserved KD, they respond to distinct stress signals [1,2]. During general translational inhibition when eIF2α is phosphorylated, there are certain specific transcripts containing upstream open reading frames (ORFs) in their 5'UTRs that are translated and the protein products of these mRNAs promote and optimize cellular recovery [1,2]. One of these examples is ATF4 which acts as a master transcription factor during the ISR and plays an instrumental role in determining cell fate during cell stress [Fig 1.1] [2]. Depending on stress severity and duration, ATF4 has molecular adaptability to tailor responses towards cell recovery or apoptosis [2]. ATF4 can form homodimers or heterodimers with other transcription factors such as downstream target CHOP which promotes cell death during ER stress [2]. CHOP has several mechanism to promote apoptosis such as upregulating BH3-only pro-apoptotic BCL-2 family members and enhancing expression of DR5 and ATF5 [2]. If eIF2α phosphorylation is sustained, pro-apoptotic transcripts are then translated in a similar manner to induce activation of caspases, PARP1 cleavage, and subsequent programmed cell death [1,2]. During the recovery phase, GADD34, which is a product of one such mRNA translated under conditions of stress, interacts with the catalytic subunit of the serine/threonine protein phosphatase 1 (PP1C) to dephosphorylate eIF2α returning the cell to homeostasis [1,2].

DYT-*PRKRA* is an early onset generalized dystonia caused by mutations in the PRKRA gene which encodes PACT [Fig 1.4] [59,68,98,113]. It typically shows clinical manifestations early in childhood and can have a genetic inheritance pattern of both autosomal dominant and recessive [59]. DYT-PRKRA was first characterized by identification of a homozygous mutation in PACT utilizing whole exome sequencing in seven Brazilian patients from two unrelated families [61]. This missense PACT mutation substituted a proline residue for leucine at position 222 (P222L) [61]. Upon analyzing the unaffected family members of this research group determined that they were heterozygous for the P222L mutation [61]. The initial study identifying causation in a mutation of PACT generating dystonia, worldwide research accelerated leading to the discovery of the P222L mutation in two Polish brothers [64]. Within a German dystonia patient, a frameshift (FS) mutation within PACT was identified which was the result of a two nucleotide deletion within PACT's first dsRBM leading to a premature stop codon [62]. The FS mutation causes a truncated PACT protein after 88 amino acids followed by 21 extraneous amino acids and a premature stop codon [62]. In the United States, a male patient was found to have a novel PACT mutation (C213R) on one allele while he inherited the P222L mutation from his mother as the other allele [63]. His father had no mutation in PACT therefore it was determined that the son's C213R mutation occurred de novo [63]. He began developing dystonia clinical symptoms at an early age [63]. Another dystonia patient was discovered to have novel PACT mutations as well within both alleles which were two recessively inherited mutations (C77S and

C213F) [65]. Two other dominantly inherited mutations were identified of which two were located within PACT's coding region like the other mutations (N102S and T34S) [64]. Recently, an early onset DYT-*PRKRA* Italian patient was reported to be a compound heterozygote with a novel mutation (S265R) with a previously known mutation P222L [98].

In their 2015 study, *Vaughn et al.* describe that the lymphoblasts derived from patients homozygous for the most prevalent P222L mutation have dysregulated eIF2α stress response signaling and these cells are hypersensitive to ER stress [68]. They further demonstrated that as compared to wt PACT, the P222L mutation has a significantly higher affinity for both PKR and TRBP, while also exhibiting stronger PACT-PACT interactions. Finally, they demonstrated that the DYT-*PRKRA* patient cells show delayed but more prolonged PKR activation and eIF2α phosphorylation in response to ER stress, thereby resulting in enhanced susceptibility to ER stress-induced apoptosis. [68].

In their 2020 study, *Burnett et al*. characterized that lymphoblasts derived from a compound heterozygous patient (P222L/C213R) are also hypersensitive to ER stress which was elucidated by showing that the cells have enhanced PACT-PKR heterodimer affinity, upregulated PKR, dysregulated ISR axis signaling and enhanced apoptosis [113]. This study also elucidated that the two dominant mutations N102S and T34S lead to enhanced PACT-PACT and PACT-PKR affinity with heightened PKR activity as while the recessive mutations C77S, C213R, C213F, and P222L additionally generated enhanced PACT-PACT homodimer affinity and elevated active PKR [113].

Seven reported DYT-*PRKRA* mutations lead to hyper-active PKR, dysregulated eIF2α signaling, and enhancing cell sensitivity to apoptosis in response to ER stress [68,98,113]. Research on the disease etiology of DYT-*PRKRA* has led to testing for maladaptive eIF2α axis signaling within other dystonia types of which have been confirmed in DYT1, DYT3, DYT6, DYT11 and sporadic cervical dystonia [109,121-124]

Kuipers et al elucidates vividly direct causation of early onset dystonia due to hyperactive PKR, who identified hyperactive PKR variants and increased eIF2α phosphorylation [120]. Initiation of dystonia or neuronal deterioration in the context of febrile illness or general anesthesia was noted in some patients [120]. De novo missense mutations in PKR additionally cause a multifaceted neurodevelopmental condition that mirrors vanishing white matter disease. Of the eight patients within the cohort, five of them presented dystonia clinical symptoms while all eight exhibited neurological regression in the setting of a febrile illness [120]. It's pragmatic to infer that onset of dystonia following a febrile illness arose due to hyperactive PKR as the causative agent. Recently, multiple studies have linked newly discovered PKR missense variants (P31R, G130R, and G138A) which are autosomal dominant and one recessive PKR mutation (N32T) to patients who have dystonia [120,134-136].

PKR has emerged as a major factor in several other neurodegenerative diseases as aberrant heightened PKR activity is observed in post-mortem patient brains as well as in mouse models [112,125-126]. Increased PKR phosphorylation is reported in patients with Alzheimer's disease (AD),

Parkinson's disease (PD), Huntington's disease (HD), dementia, and prion disease [125,127-131]. Active PKR has been shown to be directly implicated in phosphorylation of tau proteins leading to tau aggregation which are indispensable in disease etiology of Alzheimer's [138]. Within PD and HD patients, strong induction of phosphorylated PKR was found within hippocampal neurons which ties PKR in as an etiological component of extrastriatal degeneration [56]. Hyperactive PKR's implication in elevating phosphorylated eIF2α, global protein attenuation and apoptosis are critical factors leading to degenerating neurons within AD, PD, and HD [139-141]. Activated PKR was shown to be indispensable for the behavioral and neuropathophysiological abnormalities in a mouse model of Down syndrome and PKR inhibitory drugs partially rescued the synaptic plasticity and long-term memory deficits [132]. Drugs that target the eIF2α signaling pathway have also shown to be therapeutic in mouse models for neurodegenerative diseases and inhibiting PKR has been proven to be effective in the recovery of synaptic connections and learning deficits in three different Alzheimer's disease mouse models [133,143].

Frederick et al. 2023, characterized the effect of luteolin, a flavonoid compound that targets the heteromeric interaction between PACT-PKR, and determined that it attenuates hyperactive PKR within DYT-*PRKRA* cells after ER stress induction [147]. Additionally, luteolin restores ISR signaling axis levels and kinetics thereby causing a significant reduction in apoptosis. This work builds on the *Vaughn et al*. 2014 study that highlighted when neuroblastoma cells express a catalytically inactive form of PKR (K296R), these cells have defective PKR

activation, diminished ATF4 levels and downregulated CHOP expression which causes protection from ER stress induced apoptosis [137]. The studies done to date have clearly shown that pursuing a targeted PKR therapeutic can become an effective treatment option for early intervention within dystonia patients and potentially patients suffering from other neurological diseases.

2-Aminopurine (2AP) inhibits PKR thereby diminishing active PKR and subsequent phosphorylation of eIF2α in both *in-vitro* and *in-vivo* studies [146]. However, the concentrations used in these studies to identify these effects were too high leading to inhibition of other kinases [146]. Being that treatments were done in millimolar concentrations, this lacks physiological clinical application. The oxindole/imidazole compound, C16, is a small molecule that inhibits the autophosphorylation of PKR thereby reversing the global protein synthesis attenuation induced by active PKR [145,146]. *In-vitro* studies with neuronal cell lines and *in-vivo* studies have determined C16's effectiveness in reducing active PKR [145,146]. Additionally, investigations utilizing *in-vivo* models of Alzheimer's disease, Hypoxia-Ischemia and Huntington's disease have confirmed that C16's inhibitory effect on PKR promotes neuronal cell recovery and attenuation of neuroinflammation [142,144-145]. Hurdles do remain regarding long-term clinical efficacy of C16. Broad scale kinomics mapping needs to be investigated to identify if C16 has additional molecular off-targets besides cyclin dependent kinases [157]. Optimization of C16 concentration, drug administration timeline, and elucidating pharmacokinetics for clinical relevance still needs to be worked through. Also, further *in-vivo* studies with C16 need to be done to determine

initial viability with Alzheimer's, Parkinson's, and Down Syndrome mouse models.

ProteKt, a biotechnology company based in Israel, developed novel PKR targeted drugs and testing was done by us to determine *in-vitro* pre-clinical effectiveness. Our in-vitro system is ideal for testing these compounds because we have two DYT-*PRKRA* patient cell lines which we have mapped out the molecular pathophysiology involving hyperactive PKR leading to disrupted eIF2α signaling axis. This system allows for a definitive study to establish a cause and effect relationship between hyperactive PKR and enhanced apoptosis to determine if these compounds are potential candidates for *in-vivo* and possibly clinical studies.

With the extensive number of studies done highlighting the indispensable role of hyperactive PKR on disease etiology and progression of general onset dystonia, AD, PD, and HD optimizing a tailored drug to target PKR with minimal off target effects would be a translational medicinal breakthrough. Building on work done with other PKR inhibitors such as luteolin and C16, this will also provide a mechanistic insight into focusing a therapeutic to inhibit PKR's kinase activity via either the regulatory dsRBMs or the KD region that contains the ATP binding site.

5.3 Materials and Methods:

Cell lines and antibodies

Wt and DYT-*PRKRA* Patient B-Lymphoblasts were cultured in RPMI 1640 medium containing 10% percent FBS and penicillin/streptomycin. Both wt and DYT-*PRKRA* patient lymphoblast cell lines were Epstein-Barr Virus-transformed to create stable cell lines as previously described [68]. The antibodies used were as follows: PKR: anti-PKR(human) monoclonal (71/10, R&D Systems), P-PKR: anti-phospho-PKR(Thr-446) monoclonal (Abcam, {E120}), eIF2α: anti-eIF2α polyclonal (Invitrogen, AHO1182), p-eIF2α: anti-phospho-eIF2α(Ser-51) polyclonal (CST, #9721), ATF4: anti-ATF4 monoclonal (CST, #11815), CHOP: anti-CHOP monoclonal (CST, #2895), Cleaved PARP: anti-Cleaved PARP monoclonal (CST, #32563), and β-Actin: anti-β-Actin-Peroxidase monoclonal (Sigma-Aldrich, A3854).

Western blot analysis

Lymphoblasts derived from a compound heterozygous DYT-*PRKRA* patient containing a P222L mutation on one allele and a C213R mutation on the other allele were cultured alongside lymphoblasts derived from a family member containing no mutations in PACT as our control cells. Cells were plated at a concentration of 300,000 cells/ml of RPMI media containing 10% percent fetal bovine serum and penicillin/streptomycin. To analyze cellular response to ER stress, we treated cells with 5 ug/ml of Tunicamycin (Santa Cruz) over 24 hours and harvested cells in RIPA buffer (150 mM NaCl, 1.0% IGEPAL CA-630, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0) containing 1:100 dilution

of protease inhibitor cocktail (Sigma) and phosphatase inhibitor (Sigma). Concentration of total protein extract was then determined using BCA assay and appropriate amounts of extracts were analyzed by western blot analyses using appropriate antibodies as indicated. To analyze cellular response to the 3 different PKRi treatments, cells were treated with either PKT-00888, PKT-00954, or PKT-00941 (ProteKt) for the indicated concentrations for 24 hours before Tunicamycin treatment and cells were harvested, total protein extract concentration determined and western blot analyses methods were done identically to methods stated above.

Caspase 3/7 activity assays: Both wt and patient derived lymphoblasts were seeded at a concentration of 300,000 cells/ml of RPMI complete medium and treated with a concentration of 5 µg/mL of tunicamycin for 24 hours. Samples were collected at indicated time points and mixed with equal parts Promega Caspase-Glo 3/7 reagent (Promega G8090) and incubated for 45 minutes. Luciferase activity was measured and compared to cell culture medium alone and untreated cells as the negative controls. To address the effect of inhibiting PKR activation on cell viability, we cultured wt and patient lymphoblasts as described above in 0.5 µM of PKT-00941 for 24 hours followed by treatment with 5 µg/ml of tunicamycin over the same 24 hours.

5.4 Results:

PKRi compounds PKT-00888 and PKT-00954 do not protect against apoptosis in response to ER stress

Our previous research has established that DYT-*PRKRA* patient lymphoblasts are more susceptible to ER stress-induced apoptosis relative to the unaffected, wild type (wt) lymphoblasts [68,98,113]. Additionally, we've determined that a compound that can diminish hyper-active PKR leads to a restoration of normal ISR signaling and protection from ER stress-induced apoptosis [147]. We received three different PKR inhibitor (PKRi) compounds from ProteKt and our initial aim was to examine if each of these compounds can promote cellular recovery of DYT-*PRKRA* lymphoblasts in response to ER stress. We investigated the effect of PKRi compound PKT-00888 on apoptosis induced by tunicamycin, which blocks protein glycosylation resulting in an accumulation of misfolded proteins in the ER and thereby causes ER stress. Cleaved PARP1 levels were examined by western blot analysis to determine PKT-00888's effect on both wt and DYT-*PRKRA* cells. As seen in Fig 5.1, in wt lymphoblasts, PKT-00888 does not inhibit PARP1 cleavage at 1 µM, 5 µM, or 10 µM after ER stress (lanes 6-8). Additionally, the drug shows cytotoxic effects in wt cells with enhanced PARP1 cleavage even in the absence of ER stress (Fig 5.1, Lane 4). In DYT-*PRKRA* patient lymphoblasts, PKT-00888 has no effect on reducing apoptosis after ER stress (Fig 5.1, lanes 14-16) and similar to the wt cells, actually induces apoptosis within DYT-*PRKRA* cells at 10 µM without tunicamycin treatment (lane 12). We next determined cleaved PARP1 levels in wt

and patient cells using the PKRi drug PKT-00954 in the context of ER stress. As shown in Fig 5.2, PKT-00954 augments PARP1 cleavage in response to tunicamycin treatment in wt cells in a concentration dependent manner (lanes 3-5). PKT-00954 does not reduce apoptosis in response to ER stress in DYT-*PRKRA* cells either (Fig 5.2, lanes 11-12) but on the contrary, enhances cleaved PARP1 levels at 5 µM (lane 13). Collectively, these results determine that neither of these PKRi compounds are viable therapeutics for reducing ER stress-induced apoptosis.

PKRi compound PKT-00941 protects against apoptosis in response to ER stress

As seen in Fig 5.3A, wt cells have significantly reduced cleaved PARP1 levels in response to tunicamycin treatment when pre-treated with PKT-00941 (lanes 6-7). These two effective concentrations, 0.5 µM and 1 µM, also do not show cytotoxic effects (lanes 2-3). Additionally, PKT-00941 promotes cellular recovery after ER stress-induction in DYT-*PRKRA* cells as seen by a marked reduction in cleaved PARP1 (Fig 5.3A, lanes 14-15). Like wt cells, 0.5 µM and 1 µM PKT-00941 does not enhance PARP1 cleavage in the absence of tunicamycin treatment (Fig 5.3A, 10-11). It is worth noting that at 5 µM, PKT-00941 induces apoptosis in the absence of tunicamycin (lanes 5 and 12) and enhances apoptosis in the tunicamycin treated cells (lanes 8 and 16) in both wt as well as DYT-*PRKRA* cells. To confirm our findings further that PKT-00941 can inhibit apoptosis, we used a caspase 3/7 assay. As seen in Fig 5.3B, the DYT-*PRKRA* cells have about 5-fold higher level of caspase 3/7 activity at basal levels without

any tunicamycin treatment (blue bars). At 24 hours after tunicamycin treatment, the caspase activity increases approximately 7-fold in wt and about 2.5-fold in DYT-PRKRA cells (orange bars). The increase in DYT-*PRKRA* cells is smaller than that in wt cells because the DYT-*PRKRA* cells have a higher basal level of apoptosis without any ER stress. PKT-00941 significantly decreases caspase activity in response to ER stress in both wt and DYT-*PRKRA* cells with approximately 50% reduction in wt and DYT-*PRKRA* cells respectively (green bars). These results further establish that inhibition of PKR activity after ER stress can protect cells from apoptosis.

PKRi compound PKT-00941 inhibits PKR and eIF2α phosphorylation which promotes normal ISR mediated homeostasis in DYT-*PRKRA* lymphoblasts

Due to the reduction in apoptosis in response to ER stress with PKT-00941, we sought to identify a mechanism that was responsible for the decreased sensitivity to tunicamycin treatment in DYT-*PRKRA* lymphoblasts. We began with western blot analysis investigating levels of p-PKR (activated PKR) in wt and DYT-*PRKRA* cells pre-treated with PKT-00941 for 24 hours then treated with tunicamycin for various time intervals to compare the levels and kinetics. As shown in Fig 5.4, tunicamycin induced significant PKR phosphorylation at 2, 4, and 8 hours after treatment in wt cells (-941 panel, lanes 4-6) and the PKT-00941 pre-treatment significantly reduced the levels of PKR phosphorylation (+941 panel, lanes 4-6). In DYT-*PRKRA* cells, PKR phosphorylation is high at basal levels and tunicamycin treatment augments these levels significantly beginning at 30 minutes through 8 hours (Fig 5.4, -941 panel, lanes 9-14). In response to

PKT-00941 pre-treatment, PKR phosphorylation levels were significantly diminished in DYT-PRKRA cells (Fig 5.4, +941 panel, lanes 10-12) and the duration for the presence of p-PKR was also shortened (compare p-PKR: -941 and +941 panels, lane 16). Next, we determined levels of p-eIF2α in response to PKT-00941 treatment and found that it significantly blunts the p-eIF2α levels in wt cells along with reducing its duration (Fig 5.4, compare p-eIF2α: -941 and +941 panels, lanes 2-8). These effects are also seen in DYT-*PRKRA* cells treated with PKT-00941. As seen in Fig 5.4, in the absence of PKT-00941, DYT-*PRKRA* cells treated with tunicamycin maintain significantly elevated p-eIF2α levels for a long duration of time (-941 panel, lanes 10-16). Meanwhile, pre-treatment with PKT-00941 drastically reduces p-eIF2α levels beginning at 30 minutes and shortens the duration for the presence of p-eIF2α by returning to basal levels at 12 hours (Fig 5.4, +941 panel, lanes 9-15). These results indicate that the inhibition of PKR's kinase activity blunts the level and duration of both PKR and eIF2α phosphorylation in wt and DYT-*PRKRA* lymphoblasts which highlights how PKT-00941 protects DYT-*PRKRA* cells from ER stress.

To expand upon the mechanistic insight into how PKT-00941 restores cellular homeostasis, we examined downstream effects of eIF2α phosphorylation which involves two ISR transcription factors ATF4 and CHOP. In response to ER stress, DYT-*PRKRA* cells induce drastically elevated levels of ATF4 and CHOP (Fig 5.5, lanes 10-12) compared to wt lymphoblasts (lanes 2-4). As seen in Fig 5.5, PKT-00941 pre-treatment diminishes ATF4 levels in wt cells in response to tunicamycin treatment at 8 and 12 hours (lanes 6-7), while surprisingly ATF4

increase significantly at 24 hours (lane 8). Meanwhile, in DYT-*PRKRA* cells, PKT-00941 leads to elevated ATF4 levels at 8 hours (lane 14) but then by 24 hours ATF4 levels significantly decrease (lanes 15-16) relative to DYT-*PRKRA* cells treated with tunicamycin alone (lanes 10-12). PKT-00941 significantly attenuates CHOP levels in both wt and DYT-*PRKRA* lymphoblasts (Fig 5.5, lanes 6-8 and 14-16). This disruption in CHOP induction elucidates how PKT-00941 rescues ER stress-induced apoptosis after tunicamycin treatment and promotes cellular survival.

5.5 Discussion:

PKR is a ubiquitously expressed IFN-induced and (ds)RNA-activated, serine/threonine kinase that regulates the antiviral innate immune response, inflammatory signaling, and stress-induced apoptosis [111,112]. PKR is expressed at a low basal level in all cells and during viral infections it is transcriptionally induced via IFNs [111]. PKR stays enzymatically latent in the absence of its activators which are either dsRNA or PACT. Both dsRNA and PACT interact with PKR via PKR's two dsRBMs leading to PKR's homodimerization thereby causing an allosteric conformational change allowing exposure of each PKR monomer's ATP-binding site within its KD to facilitate its trans-autophosphorylation at threonines 446 and 451 and activation of its kinase activity [Fig 1.2] [12,14,16,22,34,37,39,48,114]. dsRNA interaction with PKR occurs during viral infections and PACT-PKR interaction occurs in the context of oxidative stress, ER stress, and serum deprivation [68,98,113]. Additionally, other molecules besides dsRNA and PACT can activate PKR of which one class is inverted repeat Alu sequences (IRAlus) which reside in the 3' UTR of some mRNAs [115-117]. More critically, when an Alu element in reverse orientation is present within a single transcript, denoted as IRAlu, the two Alu elements can form an intramolecular dsRNA that binds to and activates PKR [115,118]. *Kim et al* determined that one molecular context that promotes binding between nuclear IRAlus and PKR is during mitosis when the nuclear membrane is absent and the nuclear RNAs can interact with cytoplasmic PKR protein [186]. Like IRAlus, due to the bidirectional transcription of its circular genome, mitochondrial RNAs (mtRNAs) can form intramolecular dsRNAs which can activate PKR thereby

allowing PKR's involvement in signaling compromised mitochondrial integrity under conditions of cellular stress [187]. CUG expansion RNAs, which form imperfect hairpins and are among a class of repeat expansion RNAs which are a common component found in repeat associated non-AUG (RAN) protein diseases such as amyotrophic lateral sclerosis (ALS) can also activate PKR [162]. Active PKR then acts by attenuating general protein synthesis which is accomplished by phosphorylating eIF2α [1,2]. This is primarily a protective response to restore cellular homeostasis and is activated only transiently after a stress signal [1,2]. However, if PKR remains active for an extended period, it triggers apoptosis [1,2].

DYT-*PRKRA* is an early onset generalized dystonia caused by mutations in the PRKRA gene which encodes PACT [Fig 1.4] [59,68,98,113]. It typically shows clinical manifestations early in childhood which includes repetitive, often painful movements of affected body parts such as limbs, laryngeal, and oromandibular regions with features of parkinsonism leading to atypical gaits and postures [113,147]. DYT-*PRKRA* can have a genetic inheritance pattern of both autosomal dominant and recessive despite originally being characterized as only having an autosomal recessive inheritance lineage which highlights the extensive research being accomplished recently around dystonia [59,68,113,147]. DYT-*PRKRA* was first characterized by identification of a homozygous missense mutation in PACT (P222L) utilizing whole exome sequencing in seven Brazilian patients from two unrelated families [61]. After the initial study identifying P222L

mutation of PACT leading to dystonia, worldwide studies have led to the discovery of nine more mutations affecting PRKRA [62,63,64,65,98,158].

Kuipers et al highlights direct involvement of early onset dystonia due to hyperactive PKR, who identified hyperactive PKR variants and increased eIF2α phosphorylation [120]. Initiation of dystonia or neuronal deterioration in the context of febrile illness or general anesthesia was noted in some patients [120]. *De novo* missense mutations in PKR additionally cause a multifaceted neurodevelopmental condition that mirrors vanishing white matter disease. It's logical to infer that onset of dystonia following a febrile illness arose due to hyperactive PKR as the causative agent. Recently, multiple studies have linked newly discovered PKR missense variants (P31R, G130R, and G138A) which are autosomal dominant and one recessive PKR mutation (N32T) in patients who have dystonia [120,134-136].

In addition to dystonia, PKR has emerged as a major component in multiple other neurodegenerative diseases as abnormally heightened PKR activity is observed in post-mortem patient brains as well as in mouse models [112,125-126]. Increased PKR phosphorylation is reported in patients with AD, PD, HD, dementia, and prion disease [127-131]. Active PKR has been shown to be directly involved in phosphorylation of tau proteins leading to tau aggregation which is critical in disease etiology of Alzheimer's [138]. Within PD and HD patients, strong induction of phosphorylated PKR was found within hippocampal neurons which ties PKR in as an etiological component of extrastriatal degeneration [56]. Hyperactive PKR's implication in elevating phosphorylated

eIF2α, global protein attenuation and apoptosis are critical factors leading to degenerating neurons within AD, PD, and HD [139-141]. Activated PKR was shown to be indispensable for the behavioral and neuropathophysiological abnormalities in a mouse model of Down syndrome and PKR inhibitory drugs partially rescued the synaptic plasticity and long-term memory deficits [132]. In a separate study, metformin inhibits PKR activity leading to attenuated RAN protein levels, diminished neuroinflammation, and improved behavior within an ALS mouse model [162]. Drugs that target PKR have been proven to be effective in the recovery of synaptic connections and learning deficits in three different Alzheimer's disease mouse models [133,143].

The presence hyperactive PKR in DYT-*PRKRA* cells is well established and thus these cells serve as a good cell culture model system to evaluate PKR inhibitors and characterize their effect of cell survival after ER stress [68,98,113]. Our previous work on DYT-*PRKRA* was focused on studying how the mutations reported in DYT-*PRKRA* patients affect the activity of PKR. Seven of the reported DYT-*PRKRA* mutations lead to hyper-active PKR, dysregulated eIF2α signaling, and enhancing cell sensitivity to apoptosis in response to ER stress [68,98,113]. Our previous research characterizing molecular pathology of lymphoblasts derived from patients homozygous for the most prevalent P222L mutation have dysregulated eIF2α stress response signaling and these cells are hypersensitive to ER stress [68]. We further demonstrated that as compared to wt PACT, the P222L mutation has a significantly higher affinity for PKR, while also exhibiting stronger PACT-PACT interactions. Finally, the DYT-*PRKRA* patient cells show

delayed but more prolonged PKR activation and eIF2α phosphorylation in response to ER stress, thereby resulting in enhanced susceptibility to ER stress-induced apoptosis [68]. Additionally, building on that work, we characterized that lymphoblasts derived from a compound heterozygous patient (P222L/C213R) are also hypersensitive to ER stress due to enhanced PACT-PKR heterodimer affinity, elevated PKR kinase activity, dysregulated ISR axis signaling and increased apoptosis [113].

In the present study, we investigated three different PKRi compounds for their ability to promote cellular recovery of DYT-*PRKRA* lymphoblasts in response to ER stress. Two of the PKRi compounds, PKT-00888 and PKT-00954 did not protect DYT-*PRKRA* lymphoblasts from ER stress-induced apoptosis (Figs 5.1-5.2). Additionally, PKT-00888 induced apoptosis independent of ER stress in wt and DYT-*PRKRA* cells while PKT-00954 enhanced apoptosis in both wt and DYT-*PRKRA* lymphoblasts in response to ER stress (Figs 5.1-5.2). Alternatively, the third PKRi compound, PKT-00941 did protect both wt and DYT-PRKRA cells from ER stress-induced apoptosis. As shown in Fig 5.3A, two different concentrations of PKT-00941 significantly decreased PARP1 cleavage levels after ER stress in DYT-*PRKRA* cells. Also, there were no cytotoxic effects seen (Fig 5.3). PKRi compound PKT-00941 drastically diminishes p-PKR and p-eIF2α levels and duration in both wt and DYT-*PRKRA* cells (Fig 5.4). PKT-00941 also significantly attenuates CHOP induction in response to ER stress in wt and DYT-*PRKRA* cells (Fig 5.5). This disruption in CHOP induction highlights how PKT-00941 suppresses apoptosis thereby promoting cellular homeostasis as

CHOP induction is essential for apoptosis after ER stress. Collectively, these results indicate that the disruption of PKR activation by PKT-00941 desensitizes DYT-*PRKRA* cells to ER stress. This study strengthens our previous research that elucidated when neuroblastoma cells express a catalytically inactive form of PKR (K296R), these cells have defective PKR activation, diminished ATF4 levels and downregulated CHOP expression which causes protection from ER stress induced apoptosis [137]. Dysregulated eIF2α signaling and a maladaptive ISR as a common link for pathophysiology of several dystonias which include DYT1, DYT3, DYT6, DYT11, and sporadic cervical dystonia has been inferred from multiple studies [109,121-124] in addition to our research on the disease etiology of DYT-*PRKRA* [68,98,113,147]. The studies done to date have clearly shown that pursuing a PKR-centered therapeutic can become an effective treatment option for early intervention within dystonia patients.

Current PKR-targeted therapeutics include 2AP and C16. 2AP has shown to decrease active PKR and subsequent phosphorylation of eIF2α in both *in-vitro* and *in-vivo* studies [146]. However, the concentrations used in these studies were out of range of being physiologically clinically applicable along with having promiscuous effects on other kinases [146]. There have been similar issues with C16. C16 in multiple studies has been seen to inhibit the activation of PKR both *in-vitro* and *in-vivo* [145,146]. Additionally, investigations utilizing *in-vivo* models of Alzheimer's disease, Hypoxia-Ischemia and Huntington's disease have confirmed that C16's inhibitory effect on PKR promotes neuronal cell recovery and attenuation of neuroinflammation [142,144-145]. However, C16 has been

shown to have additional molecular binding partners such as CDKs [157]. With the extensive number of studies highlighting the indispensable role of hyperactive PKR on disease etiology and progression of dystonia, AD, PD, and HD; a specific drug to target PKR with minimal off target effects would be a significant milestone in translational research. This made our collaboration with the biotechnology company ProteKt, a pragmatic opportunity to test their novel PKRi compounds developed by them. Our *in-vitro* system is ideal because using our two DYT-*PRKRA* patient cell lines we have established the pathological role of hyperactive PKR. This allowed for a conclusive investigation to establish a cause-and-effect relationship between aberrant PKR activity and enhanced apoptosis. Based on our results with PKT-00941, this compound is a viable candidate for *in-vivo* and potentially clinical studies. Additional future studies elucidating the biochemical interactions between PKT-00941 and PKR will provide a mechanistic insight into how to optimize a therapeutic to inhibit PKR's kinase activity via either the regulatory dsRBMs or the KD region that contains the ATP binding site.

In addition to neurodegenerative diseases, PKR also plays a significant role in diabetes mellitus [159]. PKR acts as a central integrator for the inflammatory component of metabolic syndrome by linking nutrient- and pathogen-sensing pathways [160]. PKR is activated in response to free fatty acid-induced metabolic stress and phosphorylates the insulin receptor substrate 1(IRS1) [159]. Long term dysregulation of this signaling axis results in cellular resistance to insulin and IGF1 [159]. This is a defining pathophysiological

mechanism of type 2 diabetes mellitus. Chronic hyperactive PKR is also seen in obese patients which present a diabetes mellitus co-morbidity [159]. Another molecular pathology that overlaps with obesity and/or diabetes mellitus is chronic inflammation [159-160]. PKR's involvement in inflammation progression has been studied extensively with PKR being shown to directly interact with two known inflammatory regulators, c-Jun N-terminal kinase (JNK) and IκB kinase (IKK) [160]. JNK has been a key component of ER stress-induced pathology of insulin resistance and diabetes [160]. *Nakamura et al* showed that inhibition of PKR activity with C16 led to drastically decreased active JNK within white adipose tissue of obese mice along with diminished IRS1 phosphorylation both *in-vitro* and *in-vivo* [161]. Additionally, the ISR via ATF4 has been shown to modulate maintenance of pancreatic β-cell homeostasis in response to ER stress-induction *in-vivo* [163]. This tethers the importance of ATF4 within diabetes similar to its indispensability in promoting neuronal homeostasis [150-152,163]. ATF4 is a well-established regulator of the ISR which plays a critical role in determining cell fate during cell stress [2]. Stress severity and duration drive ATF4 molecular function towards either cell recovery or apoptosis [2]. PKR activity has been directly involved in inducing expression of transcription factors such as p53, NF-κB, STAT1, and STAT3 along with having critical roles in activation of downstream kinases such as p38 MAPK, ASK1, GSK3, and JNK respectively which highlights PKR's pathological role in multiple diseases showcasing that identifying an optimal PKRi compound is worthwhile [39,160,188,189].

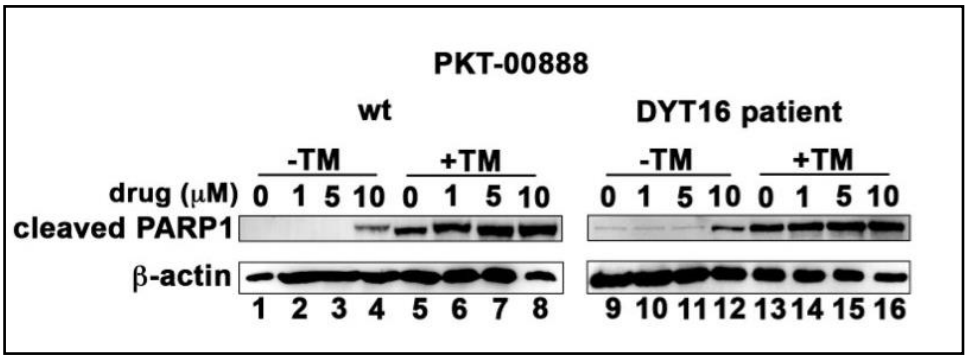

Figure 5.1: Effect of PKRi compound PKT-00888 on PARP1 cleavage in response to tunicamycin in DYT-*PRKRA* patient lymphoblasts. Western blot analysis for cleaved PARP1. Whole cell extracts from normal (wt) and DYT-*PRKRA* patient derived lymphoblasts were treated with 1 μM, 5 μM, or 10 μM of PKT-00888 for 24hrs followed by treatment with 5 μg/ml tunicamycin (TM) for 24 hrs. Blots were probed for cleaved PARP1. β-actin was used as a loading control to ensure equal amounts of protein were loaded in each lane.

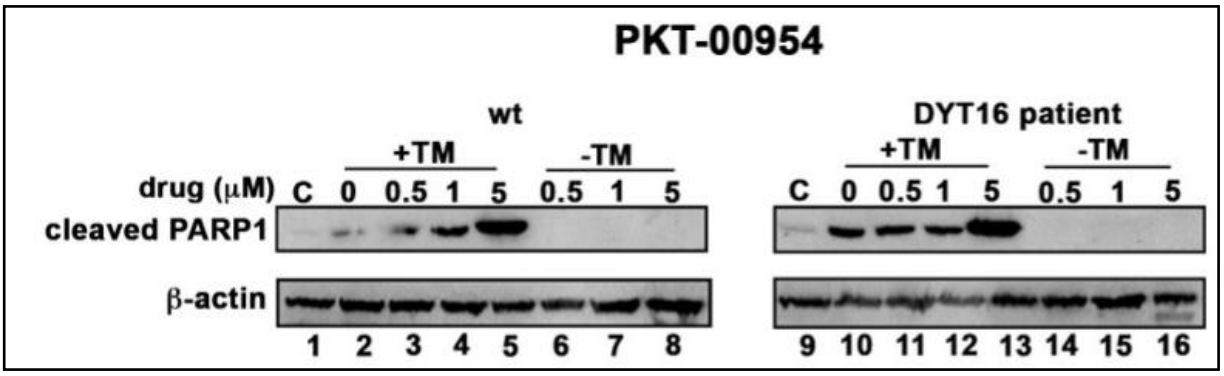

Figure 5.2: Effect of PKRi compounds PKT-00954 on PARP1 cleavage in response to tunicamycin in DYT-*PRKRA* patient lymphoblasts. Western blot analysis for cleaved PARP1. Whole cell extracts from normal (wt) and DYT-*PRKRA* patient derived lymphoblasts were treated with 0.5 μM, 1 μM, or 5 μM of PKT-00954 for 24hrs followed by treatment with 5 μg/ml tunicamycin (TM) for 24 hrs. Blots were probed for cleaved PARP1. β-actin was used as a loading control to ensure equal amounts of protein were loaded in each lane.

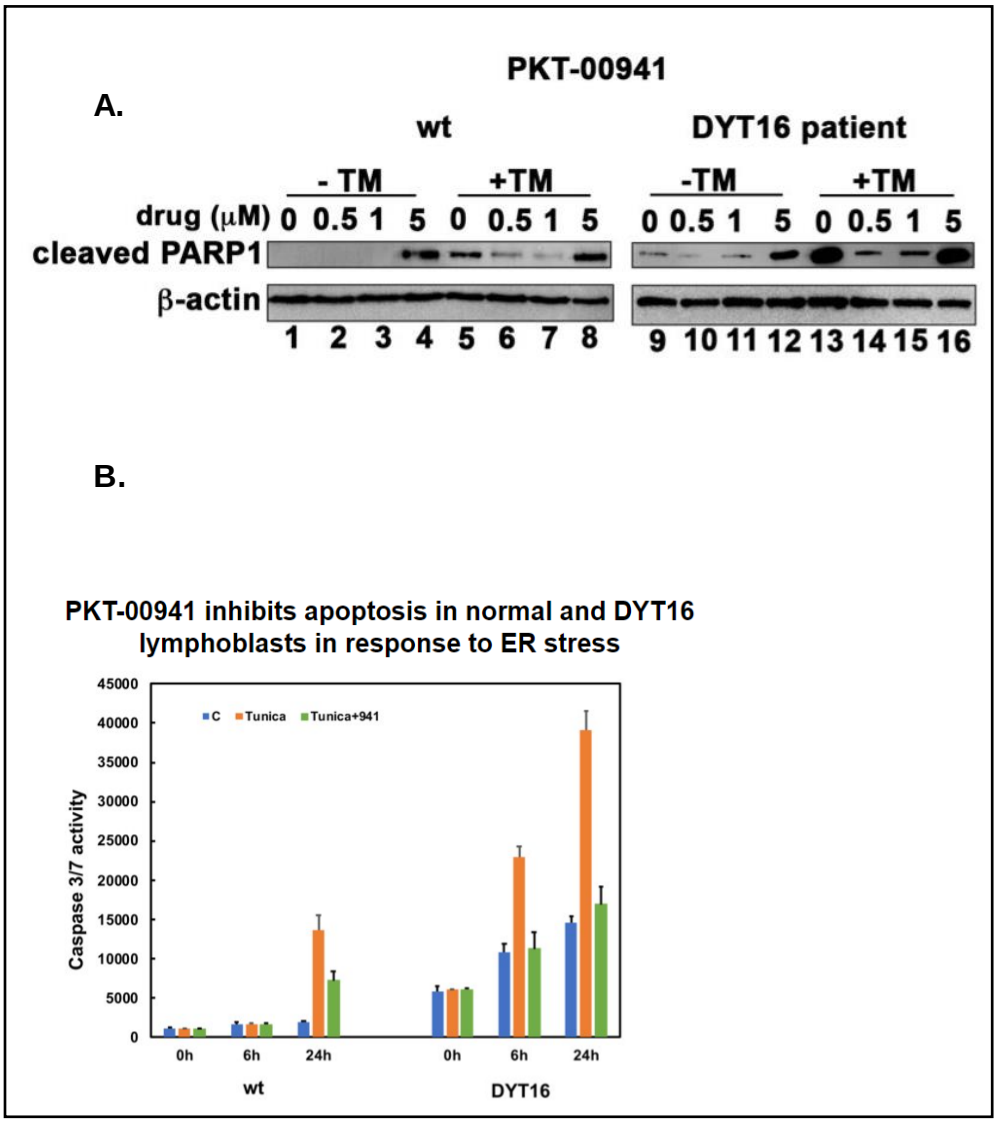

Figure 5.3: Effect of PKRi compound PKT-00941 on PARP1 cleavage in response to tunicamycin in DYT-*PRKRA* patient lymphoblasts. (A) Western blot analysis for cleaved PARP1. Whole cell extracts from normal (wt) and DYT-*PRKRA* patient derived lymphoblasts were treated with 0.5 μM, 1 μM, or 5 μM of PKT-00941 for 24hrs followed by treatment with 5 μg/ml tunicamycin (TM) for 24 hrs. Blots were probed for cleaved PARP1. β-actin was used as a loading control to ensure equal amounts of protein were loaded in each lane. **(B)** Effect of PKT-00941 on caspase 3/7 activity in lymphoblasts. Lymphoblasts from normal (wt) and DYT-*PRKRA* were treated for 24hrs with 1 μM PKT-00941 (green) or left untreated (orange) followed by treatment with 5 μg/ml tunicamycin (TM). Lymphoblasts left untreated for PKT-00941 and TM (blue) were used an internal control. Caspase 3/7 activity was measured at indicated time points after TM treatment. The p values are as indicated.

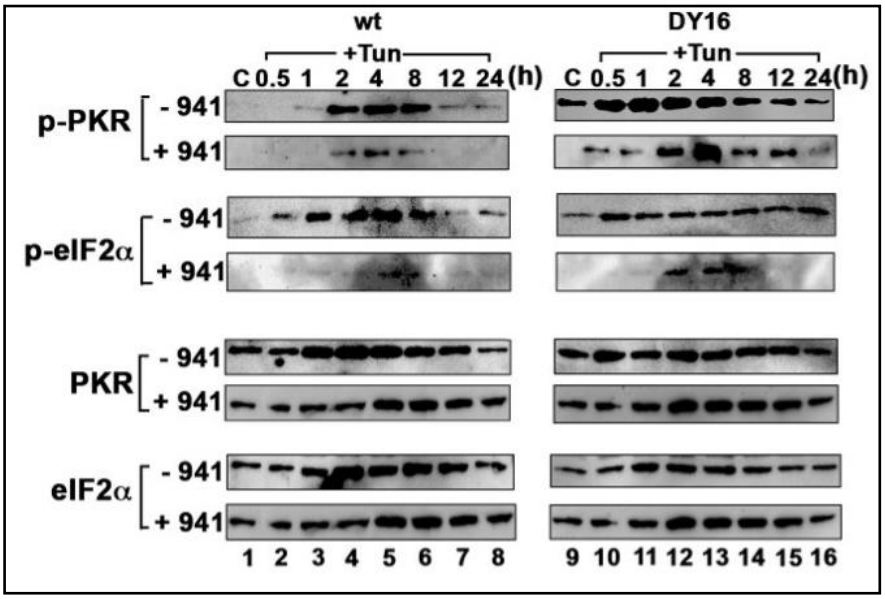

Figure 5.4: PKR activation and eIF2α phosphorylation in response to PKRi compound PKT-00941 treatment followed by tunicamycin in DYT-*PRKRA* patient lymphoblasts. Western blot analysis for p-PKR and p-eIF2α. Whole cell extracts from normal (wt) and DYT-*PRKRA* patient derived lymphoblasts treated with 1 µM PKT-00941 for 24hrs followed by treatment with 5 µg/ml tunicamycin (TM) were analyzed at indicated time points. Blots were probed for p-eIF2α, total eIF2α, p-PKR, and total PKR.

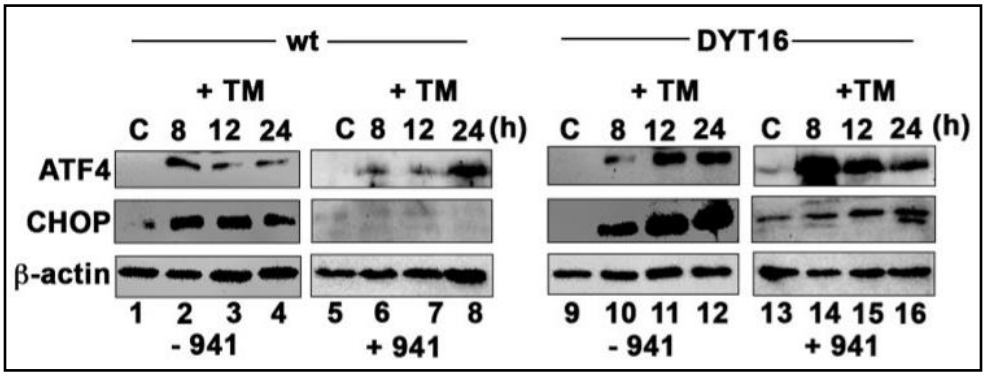

Figure 5.5: ISR signaling in response to PKRi compound PKT-00941 treatment followed by tunicamycin in DYT-*PRKRA* patient lymphoblasts. Western blot analysis for ATF4 and CHOP. Whole cell extracts from normal (wt) and DYT-*PRKRA* patient derived lymphoblasts treated with 1 µM PKT-00941 for 24hrs followed by treatment with 5 µg/ml tunicamycin (TM) were analyzed at indicated time points. Blots were probed for ATF4 and CHOP. β-actin was used as a loading control to ensure equal amounts of protein were loaded in each lane.

Chapter 6:

General Discussion

The focus of this dissertation is on elucidating how targeted therapeutics effecting eIF2α signaling and the ISR axis can promote cellular homeostasis in DYT-*PRKRA*. This work builds on previous studies that mapped out molecular pathology associated with early onset dystonia, DYT-*PRKRA*. This work demonstrates how modifying PKR phosphorylation, eIF2α phosphorylation, and ATF4 expression tailors cellular fate decisions towards either homeostasis or apoptosis. Collectively, these results establish that targeted therapeutics attenuating the abnormally high PKR activity lowers levels of phosphorylated eIF2α leading to a moderate ISR which steers the cellular fate towards recovery in response to ER stress. This work highlights a therapeutic option that can be explored further to continue drug development strategies that can become a part of a comprehensive clinical plan for early intervention for DYT-*PRKRA* patients as well as potentially other primary and secondary dystonia patients due to the known convergence of disease etiology across the dystonia spectrum.

In chapter 2 of this dissertation, we characterized the effects of the plant flavonoid polyphenol, Luteolin, on rescuing DYT-*PRKRA* patient lymphoblasts from ER stress-induced apoptosis. Luteolin accomplishes this by decreasing the heteromeric interaction between PACT and PKR. Our results indicate that disrupting PACT-PKR interactions in DYT-*PRKRA* patient cells restores the normal kinetics of PKR activation and eIF2α phosphorylation to reduce ATF4 and CHOP induction thereby preventing apoptosis in response to ER stress. These results also indicated that although luteolin disrupts the abnormally strong PACT-PKR interactions observed in patient cells in the absence of stress, it allows for the normal stress-induced and

transient PACT-PKR interaction which is essential for protective functions of ISR. PACT is phosphorylated constitutively at serine 246 in the absence of stress and is rapidly phosphorylated at serine 287 in response to cellular stress. Our results demonstrate that luteolin does not disrupt the transient stress-dependent interaction between phosphorylated PACT and PKR. Thus, luteolin selectively prevents pathological PACT-PKR interactions in DYT-*PRKRA* patient cells in the absence of stress while preserving the normal stress-induced PACT-PKR interactions to allow for a transient PKR activation during ISR. This potentially indicates that the interaction between phosphorylated PACT and PKR has higher affinity than the affinity between DYT-*PRKRA* PACT mutants and PKR.

Additionally, there is evidence of neuronal apoptosis in *lear-5J* mice which carry a spontaneously arisen *PRKRA* frameshift mutation that synthesizes a truncated PACT protein. Homozygous *lear-5J* mice exhibit progressive dystonia, kinked tails, mortality and apoptosis in the dorsal root ganglia and the trigeminal ganglion. The *lear-5J* mouse model of DYT-*PRKRA* will be very useful for characterizing the contribution of ISR dysregulation to dystonia phenotype, as well as for evaluating luteolin as a therapeutic agent, and determining therapeutic windows in which luteolin mediated ISR modulation could prove beneficial.

As dystonia pathology primarily originates in neuronal cell types especially in the basal ganglia, it is important to study the ISR dysregulation in DYT-*PRKRA* neurons. Currently no DYT-*PRKRA* neurons are available and our studies on patient lymphoblasts indicate that considerable efforts involved in undertaking in-depth studies using DYT-*PRKRA* patient-derived neurons from induced pluripotent stem

cells (iPSCs) would be pragmatic in the future. Our results thus open a new area of investigation to evaluate the suitability of luteolin in treating DYT-*PRKRA* and possibly other neurodegenerative conditions where abnormal PKR activation has been documented.

There are other factors such as drug administration, absorption, bioavailability, and potential unfavorable molecular interactions that need to be considered when utilizing luteolin therapy *in-vivo* and for clinical trials [92]. Luteolin crosses the blood brain barrier and is thus suited for reaching effectively in brain. However, luteolin's solubility in aqueous fluids is limited, thereby limiting its bioavailability. Effective dosage of luteolin for clinical trials for long-term administration will need to be determined [92]. However, strategies that can be implemented to tackle these hurdles include acetylation and esterification to enhance luteolin's uptake into cells, increase bioavailability and improve lipophilicity [92]. An alternative plan of attack can be encapsulation technologies in the form nanoparticles to create a more optimized delivery vehicle for luteolin [92]. Enhancing the delivery of luteolin molecules specifically to the brain can also reduce the concentration of luteolin needed to be administered to provide significant efficacy.

Chapter 3 of this dissertation provided insight into the efficacy of another targeted therapeutic, the CReP inhibitor Nelfinavir, on restoring cellular homeostasis within DYT-*PRKRA* cells in response to ER stress-induction. We mapped out Nelfinavir's effect on eIF2α phosphorylation and ISR signaling. Our results indicate that Nelfinavir enhances eIF2α phosphorylation leading to a

significant increase in ATF4 levels within DYT-*PRKRA* patient lymphoblasts relative to untreated patient cells. Interestingly, Nelfinavir treatment led to a significant increase in apoptosis in DYT-*PRKRA* cells both in the absence of stress and after ER stress-induction. In contrast, when DYT-*PRKRA* lymphoblasts were treated with luteolin before ER stress-induction, eIF2α phosphorylation levels decreased along with a significant reduction in expression of ATF4 and CHOP leading to protection from apoptosis. Consequently, these results indicate that a prolonged and more robust ISR does not protect cells from apoptosis but actually enhances apoptosis. *Galehdar et* al showed that ER stress-induced apoptosis in mouse cortical neurons is driven in an ATF4-CHOP dependent manner [196]. Additionally, increased ATF4 expression was shown to be a mediator in the pathology of retinal degeneration, Alzheimer's Disease, and Parkinson's Disease in mouse models [153]. Collectively, these results indicate that a targeted therapeutic that decreases p-eIF2α levels and diminishes the ISR provides cellular protection in response to ER stress. This data supports previous research that concluded targeted therapeutics affecting the ISR signaling axis are ineffective when eIF2α phosphorylation exceeds a critical threshold level thereby showing that cellular recovery occurs in response to stress when eIF2α phosphorylation is low [181]. Additionally, previous studies demonstrated that inhibiting eIF2α-specific phosphatase cofactor activity, thereby prolonging attenuation of global protein synthesis promotes elevated CHOP expression in an ATF4-dependent manner which supports our results that Nelfinavir does not

promote cellular recovery from stress and generates enhanced apoptosis even in the absence of stress [181,190,191].

In chapter 4, we investigated a possible pathomolecular mechanism for DYT3 (XDP) neuronal progenitor cells (NPCs) to identify possible overlaps with DYT-*PRKRA*. Our results determined that DYT3 cells exhibit elevated PKR activity along with enhanced eIF2α phosphorylation. Lastly, we determined expression of a known downstream PKR target that is involved with apoptosis regulation, ATF3 in DYT3 cells. ATF3 levels were significantly increased in DYT3 NPCs relative to wt cells. The results in this study add to an increasing amount of evidence highlighting maladaptive ISR signaling within multiple dystonia types, thereby indicating that ISR may be critical for normal functioning of neurons. The eIF2α phosphorylation driven translational changes are an indispensable feature of normal neuronal functions in the absence of stress and all four eIF2α kinases help in regulation either individually or synergistically [113]. eIF2α phosphorylation dependent translation regulation allows neurons to rapidly change protein compositions at the synapse in a stimulus-dependent manner which is an essential process for neuron homeostasis [113]. Collectively, this work highlights that dysregulated PKR activation along with aberrant eIF2α signaling occurs in another type of dystonia adding to the growing number of evidence indicating that there may be a pathological overlap between multiple types of dystonia.

In chapter 5, we tested the efficacy of three novel PKR inhibitor (PKRi) compounds for rescuing DYT-*PRKRA* cells from ER stress-induced apoptosis.

One of these compounds, PKT-00941, diminished the sensitivity to ER stress-induced apoptosis in *DYT-PRKRA* cells. Next, we outlined mechanistically how PKT-00941 protects cells from ER stress-induced apoptosis beginning with PKR and eIF2α phosphorylation and analyzing key ISR regulators, ATF4 and CHOP. We determined that PKT-00941 reduced phosphorylated eIF2α along and decreased levels of ATF4 and CHOP. This work elucidates some therapeutic possibilities for future drug development to reduce apoptosis in DYT-*PRKRA*. Taking into consideration the pathological role of PKR in onset and progression for multiple diseases, identifying an optimal PKRi compound is worthwhile and can possibly lead to attenuating disease pathology for several diseases.

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