Dysregulation of the Integrated Stress Response in Early Onset Dystonia (DYT16) Due to Mutations in Pact

Samuel B. Burnett

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DYSREGULATION OF THE INTEGRATED STRESS RESPONSE IN EARLY ONSET DYSTONIA (DYT16) DUE TO MUTATIONS IN PACT

by

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DEDICATION

This work is ultimately dedicated to my incredible wife and loving family, who have shaped me into the person I am today, while providing both encouragement and support in my pursuit of science.

To my late but unconditionally loving sister, Megan Danielle Burnett, I will always cherish our time together and will carry your example of authenticity and love for the rest of my life.

To the truly amazing person that is my wife, Dr. Crystal Hankin Burnett, who is the driving force in my life and the benchmark for what it means to be a genuine and loving person. I am beyond fortunate to call you my best friend and life partner. Without your steadfast love and support I would not be the man or scientist I am today.

Finally, to my parents, Paul and Kasey Burnett, my siblings, Megan and Asher Burnett, my grandmothers, Cherry Krest and Shirley Burnett, and all the members of my extended family, I am grateful for the influence each of you have had on my life. To my mother, Kasey, I am grateful for instilling in me the value of pursuing “a lifetime of learning” from a young age thus setting me on the path to pursue higher education.
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I am also grateful each member of the Patel Lab I have had the pleasure of working with during my time here. This truly talented group of scientists includes Dr. Evelyn Chukwurah who very patiently taught me many techniques while simultaneously becoming a surrogate sister within the lab. To my friends Benedicth “Bennie” Ukhueduan and Kenneth “Ken” Frederick, I am truly grateful
for the support and encouragement provided by you both on a daily basis.

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While I would consider myself rich in friends, the well that is my family is much deeper. I would like to thank all the extended members of both the Burnett and Krest families instilling values of hard work and discipline in me throughout my life.

Finally, I want to acknowledge my amazing wife and best friend, Dr. Crystal Hankin Burnett. I am so thankful for such a kind, loving, and intelligent person I get to spend every day of my life with. Thank you for the overwhelming love and support, without you I would not be where I am today.
ABSTRACT

Early onset primary dystonia 16 (DYT16) is a subtype of the neuromuscular movement disorder, dystonia. The resultant phenotypes of DYT16 consist of both agonist and antagonist muscles firing simultaneously, compromised posture and gait, as well as chronic repetitive movements. DYT16 has been shown to result from mutations in the protein activator of PKR, PACT. Under conditions of viral infection, ER stress, oxidative stress, and serum starvation, PKR phosphorylates eIF2α resulting in the attenuation of general protein synthesis. Transient eIF2α phosphorylation is favorable for cell survival, however, when prolonged leads to apoptosis. In the absence of stress, both PACT and PKR are in an inhibitory heterodimeric complex with TRBP thus preventing PKR’s activation. In response to stress stimuli, PKR is activated through the disassociation of these inhibitory heterodimers which promotes the formation of PACT homodimers, a critical intermediate for activating PKR, and PACT-PKR heterodimers that result in PKR activation. Here we describe how DYT16 mutations dysregulate eIF2α stress response signaling through PKR.

Our results indicate that a dominantly inherited DYT16 frameshift mutation truncating PACT within its first functional motif ablates PACT’s ability to bind dsRNA and interact with PKR. We then describe how this truncated protein not only retains its ability to interact with TRBP and PACT but is also capable of dissociating PACT-TRBP heterodimers. Furthermore, when expressing an N-
terminally tagged mCherry-FS fusion protein in mammalian cells we observe the accumulation of cytosolic protein aggregates, the induction of apoptosis through activation of caspases 3/7, and dysregulation in eIF2α stress signaling kinetics.

We also describe the effect of three recessive (C77S, C213F, C213R) and two dominant (N102S, T34S) DYT16 point mutations have on the biochemical properties of PACT. Our results reveal that PACT with dominant mutations interact more efficiently with PKR and all DYT16 mutations have an increased capacity to homodimerize resulting in their enhanced ability to activate PKR relative to wild type PACT. We then describe how DYT16 patient lymphoblasts form stronger PACT-PKR interactions, have high basal levels of active PKR, have dysregulated eIF2α stress response signaling, and heightened sensitivity to ER stress relative to control cells. We further show that the increased sensitivity to ER stress can be partially rescued by disrupting PACT-PKR interactions.

Finally, we investigate a novel DYT16 mouse model resulting from a frameshift mutation, Prkra\textsuperscript{ear-5J} (referred to as lear-5J), in the mouse homolog of PACT. We demonstrate that although the mRNA is being targeted for nonsense mediated decay, we still detect residual mRNA and the truncated lear-5J protein in the mouse brain, however, not in mouse embryonic fibroblasts. We show that lear-5J protein retains its ability to interact with PKR but is a less efficient activator PKR. We also identified defects in the development of the folia within the cerebellum and reduced levels of p-eIF2α in DYT16 mice. Finally, we further demonstrate dramatic defects in the arborization of the Purkinje neuron layer within the cerebellum of these mice.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>5'UTR</td>
<td>5' Untranslated Region</td>
</tr>
<tr>
<td>ALS</td>
<td>Amyotrophic Lateral Sclerosis</td>
</tr>
<tr>
<td>ATF4</td>
<td>Activating Transcription Factor 4</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine Triphosphate</td>
</tr>
<tr>
<td>CHOP</td>
<td>C/EBP Homologous Protein</td>
</tr>
<tr>
<td>CReP</td>
<td>Constitutive Regulator Of eIF2α Phosphorylation</td>
</tr>
<tr>
<td>DAPI</td>
<td>4,6-Diamidino-2-Phenylindole</td>
</tr>
<tr>
<td>dsRBM</td>
<td>dsRNA BINDING MOTIF</td>
</tr>
<tr>
<td>dsRNA</td>
<td>DOUBLE STRANDED RNA</td>
</tr>
<tr>
<td>DYT</td>
<td>Dystonia</td>
</tr>
<tr>
<td>DYT16</td>
<td>Dystonia Subtype 16</td>
</tr>
<tr>
<td>eIF2</td>
<td>Eukaryotic Initiation Factor 2</td>
</tr>
<tr>
<td>eIF2α</td>
<td>Eukaryotic Initiation Factor 2 α subunit</td>
</tr>
<tr>
<td>eIF2γ</td>
<td>Eukaryotic Initiation Factor γ subunit</td>
</tr>
<tr>
<td>eIF2B</td>
<td>eIF2 Guanine Nucleotide Exchange Factor 2B</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic Reticulum</td>
</tr>
<tr>
<td>FS</td>
<td>Frameshift</td>
</tr>
<tr>
<td>GADD34</td>
<td>Growth Arrest and DNA Damage-inducible Protein 34</td>
</tr>
<tr>
<td>GCN2</td>
<td>General Control Non-Derepressible 2</td>
</tr>
<tr>
<td>GDP</td>
<td>Guanosine Diphosphate</td>
</tr>
<tr>
<td>GEF</td>
<td>Guanine Nucleotide Exchange Factor</td>
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</tbody>
</table>
GF ................................................................. Green Fluorescent Protein
GTP .............................................................. Guanosine Triphosphate
HRI ............................................................... Heme Regulated Inhibitor
IFN ............................................................... Interferon
ISG ............................................................... Interferon Stimulated Gene
ISR ............................................................. Integrated Stress Response
K296R PKR ........................................................ Dominant Negative PKR
KD ................................................................. Kinase Domain
 Lear-5J ............................................................ Little Ear 5J
MEFs ............................................................. Mouse Embryonic Fibroblasts
PACT ............................................................. Protein Activator of PKR
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
PP1 ............................................................... Protein Phosphatase 1
PP1C ............................................................ Protein Phosphatase 1 Catalytic Subunit
TC ............................................................... Ternary Complex
TRBP .......................................................... TAR RNA Binding Protein
uORF ........................................................... Upstream Open Reading Frame
UPR ............................................................ Unfolded Protein Response
wt ............................................................... Wild Type
CHAPTER 1:
INTRODUCTION
1.1 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\textsuperscript{1,2}. The primary node of this signaling network is the heterotrimeric eukaryotic translation initiation factor 2 (eIF2) (Fig 1.1)\textsuperscript{2}. This highly conserved protein complex consists of an $\alpha$, $\beta$, and $\gamma$ subunit\textsuperscript{2}. As a mechanism to initiate general protein synthesis, eIF2 binds the methionyl charged initiator tRNA (Met-tRNA\textsubscript{i}) in order to form the ternary complex (TC)\textsuperscript{3,4}. This TC then directly interacts with the 40S ribosomal subunit to form the pre-initiation complex (PIC)\textsuperscript{3,4}.

Interactions between the PIC and other multi-protein complexes within the eukaryotic translation initiation factor family (eIF) stimulate translation through the recognition of and assembly at the 5’-G-cap of mature mRNAs\textsuperscript{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\textsuperscript{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\textsuperscript{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 the majority of cellular mRNAs.

In response to stress stimuli, the translation of cap-dependent transcripts is negatively regulated by a family of four serine/threonine protein kinases by directing their enzymatic activity to the alpha subunit of eIF2 (Fig 1.1)\textsuperscript{1,2}. This family of kinases consists of Heme Regulated Inhibitor (HRI), double-stranded
RNA activated Protein Kinase (PKR), PKR-like Endoplasmic Reticulum Kinase (PERK), and the General Control Non-derepressible 2 (GCN2) (Fig 1.1)\(^1,2\).

Although these kinases share a substrate and a very conserved kinase domain (KD), they have been well characterized for their role in responding to specific stress stimuli (Fig 1.1)\(^1\). The subsequent eIF2α phosphorylation (p-eIF2α) event serves two functions: (i) stimulates the binding of eIF2B to p-eIF2α, (ii) inhibits the GEF activity of eIF2B\(^2,5,6\). The inhibition of eIF2B’s GEF activity ultimately blocks the formation of the TC resulting in the attenuation of general protein synthesis while simultaneously promoting the translation of stress specific mRNAs (Fig 1.1)\(^2\).

The first responder to conditions of cellular stress is the activating transcription factor 4 (ATF4), which is constitutively expressed in cells. The ATF4 mRNA is kept untranslated until cells encounter a stress signal which activates its preferential translation when eIF2α is phosphorylated (Fig 1.1)\(^2\). This preferential translation is facilitated by the recruitment of ribosomes to an internal ribosome entry site (IRES) and a short upstream open reading frame (uORF) in the 5’ untranslated region (5’UTR) in the ATF4 mRNA\(^2\). Under conditions of acute or moderate stress, ATF4 is responsible for the induction of many stress response genes necessary to ameliorate the assault\(^1,2\). The resultant stress response transcripts are then preferentially translated in the 5’m\(^7\)-G-cap independent manner described above\(^2\). One such ATF4 induced gene is a regulatory subunit of protein phosphatase 1 (PP1), GADD34 (Fig 1.1)\(^7,8\). The GADD34-PP1 holoenzyme functions to restore eIF2α function after a successful
ISR by dephosphorylating eIF2α (Fig 1.1)\textsuperscript{7,8}. Under conditions of severe or chronic stress, however, the bioenergetic demands required of the cell to recover are too great and ATF4 induces the expression of pro-apoptotic transcripts such as CHOP\textsuperscript{2}. Ultimately, the phosphorylation state of eIF2α plays a seminal role in cell fate decisions through the regulation of protein synthesis and either promoting recovery and homeostasis or the accumulation of pro-apoptotic transcripts and cell death.

1.2 THE DOUBLE-STRANDED RNA ACTIVATED PROTEIN KINASE (PKR)

Interferons (IFNs) are a family of ubiquitously expressed cytokines that play a critical component in both innate and adaptive immune response as well as inflammatory signaling. There are three classes of IFNs (Type I, Type II, and Type III). 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\textsuperscript{9,10}. Each member of the Type I interferons serves as a ligand for the heterodimeric transmembrane receptor, IFNAR\textsuperscript{9,10}. A common stimulus for a Type I IFN response begins when a pathogen (usually a virus) associated molecular pattern (PAMP) is recognized by a pathogen recognition receptor (PRR) as well as through cytokine signaling\textsuperscript{9-12}. The PRRs stimulate an intrinsic antiviral response as well as the production and secretion of Type I IFNs, thus creating an amplification loop via this signaling cascade\textsuperscript{9,10}. The IFNs produced by the initial virus infected cell are secreted and stimulate the expression of over 300 interferon stimulated genes (ISGs) in surrounding uninfected cells\textsuperscript{9}. These ISGs confer viral resistance to cells by enabling them to
successfully combat either being infected or inhibit the virus replication. IFNs and ISGs thus form the first line of defense as part of our innate immune system.

During viral infections, increased interferon levels lead to enhanced expression of ISGs, which have established anti-viral activity\(^8,11\). As an ISG, PKR expression is enhanced during viral infections due to the elevated type I IFN production\(^13\). Although PKR expression is stimulated in by IFNs, PKR's kinase activity stays latent until it is bound by double-stranded (ds) RNA, a replication intermediate of many viruses\(^13\)-\(^15\). For this reason, PKR was initially characterized for its role in stimulating the ISR in response to viral infections by targeting its kinase activity to eIF2\(\alpha\)\(^13,15,16\). Since the original identification and characterization of PKR, its kinase activity has also been shown to be stimulated by a wide variety of stress signals including heat shock, endoplasmic reticulum (ER) stress, oxidative stress, and serum starvation in a dsRNA independent manner through interactions with its protein activator (PACT)\(^1,17-19\).

**Structure of PKR**

In addition to the conserved carboxy-terminal KD, PKR contains two highly conserved amino-terminal dsRNA binding motifs (dsRBMs): dsRBM1 (amino acid residues 10-72) and dsRBM2 (amino acid residues 100-165) (Fig 1.2)\(^13,20-22\). Structural studies have demonstrated both dsRBMs have similar secondary structures consisting of a central hydrophobic core and form a dumbbell conformation flanking a 22-residue linker region\(^23\). These dsRBMs serve as important functional domains to mediate PKR's protein-dsRNA interactions as well as protein-protein interactions\(^16,23-25\). Both dsRBM1 and dsRBM2 share high
sequence and structural homology to other dsRNA binding proteins and include an α-β-β-α fold such that the α-helices are positioned on the face of a three-stranded antiparallel β-sheet\(^\text{23}\). 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\(^\text{26-28}\). These studies suggest a partially positive charge within dsRBM1 located on the PKR-dsRNA binding interface. Certain hydrophobic residues within the amphipathic α-helices located in these dsRBMs have been shown to be indispensable for the inter- and intramolecular interactions that are necessary to facilitate PKR activation.

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 the amino terminal dsRBM2 via intramolecular interactions\(^\text{29}\). Mechanistic studies have demonstrated that both dsRBM1 and dsRBM2 within PKR contribute to the molecular interactions. Interactions with these activators to 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\(^\text{15,23}\). PKR is then able to undergo a trans-autophosphorylation to become catalytically active\(^\text{30}\).

**Activation of PKR**

One of the most potent and well characterized activators of PKR is dsRNA\(^\text{1,26,27}\). DsRNA binds PKR via high affinity interactions facilitated by the amino terminal dsRBMs. While dsRBM1 is indispensable for PKR’s ability to bind
dsRNA, both dsRBM1 and dsRBM2 are necessary for optimal binding of PKR to dsRNA and induce an allosteric change and trans autophosphorylation required to activate PKR\textsuperscript{15,23}. Collectively, representative data from biochemical and structural studies point to a mechanism where the dsRBMs of PKR wrap around the dsRNA duplex\textsuperscript{15,23}. This interaction is stabilized by the electrostatic interactions of the dsRBMs with 2’-OH groups and the polyanionic phosphate backbone of the dsRNA\textsuperscript{31}. Interestingly, \textit{in vitro} and \textit{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\textsuperscript{27,32,33}. Of these compounds, heparin was the most potent activator. Further analysis of heparin mediated PKR activation in our lab suggest that it is facilitated through interactions with PKR’s carboxy-terminal KD, as opposed to the dsRBMs\textsuperscript{32}.

PACT activates PKR in a dsRNA-independent manner in response to stress signals other than a virus infection\textsuperscript{17,25,34-36}. Similar to PKR, PACT is also a dsRNA binding protein that is ubiquitously expressed in all cell types, however, it is not an ISG product\textsuperscript{25,37}. PACT activates PKR via direct stress-dependent high affinity interaction with PKR\textsuperscript{25,34,35,37-39}. These protein-protein interactions are mediated through amino terminal dsRBMs located on each protein. A critical intermediate step for PACT’s ability to interact with and activate PKR is formation of PACT-PACT homodimers\textsuperscript{40}. PKR then binds these stress induced PACT homodimers to facilitate the catalytic activation described above.
**Downregulation of PKR**

While a significant effort has been made to understand the mechanisms behind how PKR is activated, the mechanisms behind the downregulation of PKR’s kinase activity are poorly understood. Interestingly, *Tan et al.* demonstrated the catalytic subunit of PP1 (PP1C) both directly interacts with and dephosphorylates PKR returning the kinase to its inactive basal state\(^{41}\). This interaction was facilitated via a short PP1C binding motif that overlaps with end of PKR’s dsRBM2 (residues 164-167). As PP1C regulates a wide array of cellular processes, the substrate specificity of this enzyme is facilitated by various regulatory subunits. Interactions between PP1C and a regulatory subunit results in the formation of the highly specific functional holoenzyme. While the inhibitory nature of the PP1C-PKR interactions is clear, the cellular context under which this negative regulation operates is not, and whether or not any regulatory subunits of PP1C are required remains unknown.

The trans-activation response (TAR) RNA binding protein (TRBP), which was first identified in the context of HIV infected cells, is a strong inhibitor of PKR\(^{18,42,43}\). Similar to PKR, TRBP contains two amino terminal dsRBMs (dsRBM1 and dsRBM2) that facilitate interactions with both dsRNA and other proteins containing such motifs (Fig 1.2)\(^{42,44,45}\). Additionally, TRBP also contains a third carboxy-terminal dsRBM (dsRBM3) that does not bind dsRNA but mediates the protein-protein interactions with merlin, dicer, and PACT and thus is termed the medipal domain\(^{46}\). TRBP inhibits PKR is by the sequestration of its cellular activators, dsRNA and PACT, as well as through direct interaction with
PKR\textsuperscript{43,44,47}. Mechanistic biochemical studies indicate that in the absence of stress, the basal state of PKR activation remains low due to the prevalence of both TRBP-PACT and TRBP-PKR inhibitory heterodimers\textsuperscript{48}. Stress signals result in the dissociation of PACT-TRBP as well as PKR-TRBP heterodimers thereby allowing PKR activation. 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\textsuperscript{48}.

1.3 PACT: THE PROTEIN ACTIVATOR OF PKR

Identification and Transcriptional Regulation

PACT is a 313 amino acid protein encoded for by the PRKRA gene (Fig 1.2)\textsuperscript{25}. PACT was initially identified through yeast two-hybrid screening of human placenta cDNA library for interaction with the trans-dominant negative PKR (K296R)\textsuperscript{25}. Further studies on the physiological functions of PACT demonstrated through a series of \textit{in vivo} and \textit{in vitro} studies that PACT directly interacts with PKR via the amino terminal dsRBMs of PKR and activates its kinase activity\textsuperscript{25,34,35,37,38,49}. The mouse homologue of PACT was subsequently identified and characterized in a similar series of assays\textsuperscript{34,36}. These proteins all share an exceptional degree of conservation as they differ only in six amino acid residues of which four are conservative replacements. Orthologs of PACT have since been identified across numerous taxa including prominent model organisms such as, \textit{Danio rerio}, \textit{Drosophila melanogaster}, rat, mouse, and monkey. The extensively high degree of conservation of PACT between species indicates it
could be playing a universal role in development and the maintenance of cellular homeostasis.

Unlike PKR, the PRKRA gene is not induced by IFNs. 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\(^{50,51}\). These studies identified regulatory GC boxes which are known binding sites for the general transcription factor, Sp1\(^{50,51}\). Six Sp1 binding sites were identified within the 300 base pairs (bp) upstream of the PRKRA transcriptional start site\(^{50}\). 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\(^{50}\). 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\(^{50}\). Finally, these studies not only characterized PRKRA promoter, they also demonstrated that PRKRA is most abundantly expressed in the placenta, colon, and testis\(^{25,34,50}\).

*Domain Structure of PACT*

The extensive degree of sequence conservation observed in PACT among various species is largely localized to the three dsRBMs that serve as important functional domains (Fig 1.2). PACT's two amino terminal dsRBMs, dsRBM1 and dsRBM2, are not only evolutionarily conserved among species within the PACT protein, but also share sequence homology to the amino terminal dsRBMs found
in both PKR and TRBP. Additionally, PACT contains a comparatively less conserved third carboxy-terminal dsRBM, dsRBM3. This dsRBM3 is incapable of binding dsRNA, however, is indispensable for PACT’s ability to activate PKR.

Further investigation into these functional motifs revealed that PACT’s dsRNA binding and protein-protein interactions were largely attributed to the amino terminal dsRBMs. In order to investigate this question, a series of PACT deletion constructs was generated and followed by co-immunoprecipitation and yeast two-hybrid studies. The studies demonstrated that PACT’s ability to interact with TRBP, PACT homodimerization, and PKR interactions were resultant of a cooperative effort between dsRBM1 and dsRBM2 while dsRBM3 was largely dispensable for these high affinity interactions. Following these results, the contribution of individual hydrophobic amino acids within each domain was evaluated. Through mutagenetic studies and a similar series of assays, it was determined that specific hydrophobic residues within dsRBM1 were most critical for PACT’s protein-protein interactions, while dsRBM2 serves to stabilize and enhance these interactions. Of particular note, the leucine at position 99 (L99) of PACT’s dsRBM1 was found to be indispensable for PACT-PACT homodimerization. To address the question if PACT homodimerization was required for PKR activation, site specific mutagenesis substituting this leucine for glutamate (L99E) was utilized to generate a PACT construct incapable of homodimerizing. Surprisingly, this mutation had no consequence on PACT-PKR interactions, but was completely inactive for PKR activation in in vitro kinase
assays thus indicating the requirement for PACT-PACT interactions for PKR activation.

The PACT deletion constructs outlined above describe how the two amino terminal dsRBMs are responsible for the formation of PACT-PKR heterodimers. Further analysis into these functional domains revealed that while dsRBM1 and dsRBM2 are required for the interaction, deletion constructs lacking PACT’s dsRBM3 were incapable of activating PKR. Furthermore, PACT’s dsRBM3 alone is capable of activating PKR albeit moderately and only at high concentrations because this domain interacts within PKR’s KD to bring about PKR activation. Investigation into the KD of PKR has revealed a short binding region spanning residues 326-337 in PKR that bind PACT’s dsRBM3 with low affinity. This low affinity 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) (Fig 1.2). However, interaction of dsRBM3 with the PBM is fairly weak and the dsRBM1 and 2 are required for efficient PKR activation in cells and in vitro as they direct PACT-PKR interaction even at low PACT concentrations. Interestingly, additional studies utilizing chimeric proteins where the carboxy-terminal dsRBM3 of PACT was linked to the two amino terminal dsRBD of TRBP showed that the chimeric protein was capable of stimulating PKR’s kinase activity in vitro.

While the mechanistic and biochemical studies outlined above gave tremendous insight into the underlying properties dictating PACT’s interaction with PKR, the post-translational modifications that regulate the hetero- and
homomeric interactions of PACT remains relatively less studied. Mutagenic 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. Results from these studies suggest that S246 is constitutively phosphorylated, whereas S287 is phosphorylated in a stress-dependent context. Utilizing phosphomimetic or phosphodeficienct mutant constructs at each of the sites demonstrates that the high affinity PACT-TRBP interactions occur only in the absence of the stress induced phosphorylation on S287. In agreement with this data, these studies have shown that phosphomimetic mutations at both of these serine residues significantly increase PACT’s ability to homodimerize as well as to form PACT-PKR heterodimers.

**PACT: A Stress Dependent Negative Regulator of Translation**

The studies outlined above describe the mechanism whereby PACT mediates PKR activation in a dsRNA independent manner (Fig 1.3). These studies demonstrate that in the absence of stress, PACT is constitutively phosphorylated on S246 and is in an inhibitory heterodimeric complex with TRBP. In response to stress stimuli, PACT is phosphorylated by a yet to be characterized kinase on S287 resulting in the dissociation of PACT-TRBP heterodimers while simultaneously promoting the formation of PACT-PACT homodimers. These PACT-PACT homodimers then interact with PKR via the highly conserved amino terminal dsRBMs on each protein. We speculate that the resultant conformational change induced in PKR may then be stabilized through low affinity interactions between the PBM in PKR’s KD with PACT’s dsRBM3. Once activated, PKR’s
enzymatic activity is then directed toward eIF2α resulting in the attenuation of
general protein synthesis while promoting the preferential translation of a subset
of transcripts whose protein products are crucial in promoting cellular recovery
(Fig 1.1, Fig 1.2). In response to chronic or severe stress, eIF2α phosphorylation
is prolonged and the cell begins to undergo apoptosis as more pro-apoptotic
transcripts and proteins are synthesized. For this reason, both PACT-PACT
homodimers and PACT-PKR heterodimers are considered pro-apoptotic while
TRBP-PACT and TRBP-PKR heterodimers are considered to favor cell survival.
PACT mediated PKR activation has been observed in response to the
accumulation of misfolded proteins in the endoplasmic reticulum (ER) stress,
oxidative stress, starvation, and heat shock\textsuperscript{17,43,49}. PKR phosphorylation has also
been identified in the pathology of many neurodegenerative disorders including,
Huntington’s disease, Parkinson’s disease, and Alzheimer’s disease\textsuperscript{54-58}. While
dimeric and phosphorylation state of PACT in these diseases remains an open
question, it is highly plausible PACT is mediating PKR’s activation in these
diseases.

1.4 PACT AND PRIMARY EARLY ONSET DYSTONIA

*Primary Dystonia*

The dystonias (DYT1-DYT26) are a group of heterogenous movement
disorders directly linked to mutations in 25 genes\textsuperscript{59-61}. While the age of onset and
severity of the symptoms vary 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 mobility\textsuperscript{59}. A hallmark in identifying primary dystonia is the consistent directionality of the associated movements. This consistency is due to the firing of both agonist and antagonist muscles simultaneously in response to a single stimulus\textsuperscript{59}. Although secondary and pseudo forms of dystonia often present in the clinic, these forms of dystonia are symptomatic of either an alternative disease or a side effect of certain drugs\textsuperscript{59}. Primary dystonia, however, is distinguished from these forms of dystonia by being the result of a specific genetic mutation and a primary symptom as opposed to being a symptom of an alternative disease\textsuperscript{59}.

\textit{PACT in Early Onset Dystonia and Parkinsonism (DYT16)}

Early onset dystonia and parkinsonism subtype 16 (DYT16) is a severe form of primary dystonia which typically presents in patients during early childhood or adolescence and follows both autosomal recessive as well as dominant inheritance patterns\textsuperscript{60}. DYT16 was first described by Camargos and colleagues who identified a homozygous mutation in PACT as the causative mutation through whole exome sequencing in seven Brazilian patients\textsuperscript{62}. This was a missense mutation in PACT resulting in the substitution of a proline residue at position 222 for leucine (P222L), located between PACT’s dsRBM2 and dsRBM3 (Fig 1.4). The cohort in which the P222L mutation was identified consisted of seven individuals from two unrelated Brazilian families. The analysis of asymptomatic family members of individuals within this cohort revealed those who are heterozygous for the P222L mutation were unaffected.
After the initial identification of a causative mutation in the PRKRA gene leading to DYT16 dystonia, further investigations were carried out worldwide in evaluating primary dystonia patients. This led to the discovery of the homozygous P222L mutation in two Polish brothers and the novel discovery of a dominantly inherited frameshift mutation in one German patient\textsuperscript{63-65}. This frameshift (FS) mutation was resultant from the deletion of two nucleotides within PACT’s first dsRBM1 (c.266_267delAT) which introduced a premature stop codon into the reading frame of the PRKRA mRNA (Fig 1.4). The FS mutation truncates the protein after 88 amino acids immediately followed by 21 extraneous amino acids before a premature stop codon.

The identification of new causative DYT16 mutations in PACT continues to increase. The identification of a novel \textit{de novo} C213R mutation in PACT in a male patient in USA was recently identified\textsuperscript{66}. While the mother of this patient carries the P222L mutation, his father showed no mutation in PACT. Thus, the patient inherited the P222L mutation from his mother and a \textit{de novo} C213R mutation that occurred in the proband manifesting in DYT16 symptoms and development of dystonia at a very early age. Two recessively inherited mutations were identified in a compound heterozygous patient such that one allele had a C77S mutation and the other allele had a C213F mutation\textsuperscript{66}. Additionally, three more dominantly inherited mutations were identified such that two were located within PACT’s coding region (N102S and T34S), while one was in the 5' UTR (-14A>G)\textsuperscript{65}, which is likely to change the coding region in the longer isoform of PACT that has not been studied so far but has been described to exist based on
cDNA sequencing in the NCBI database. Most recently, another mutation was described within PACT’s dsRBM3 (S265R) as a compound heterozygote with P222L mutation causing early-onset DYT16\(^6^7\). Interestingly, a spontaneous frameshift mutation was reported in a mouse by the Jackson Laboratory that was described to cause dystonia in the mouse (Fig 1.4)\(^6^7,^6^8\). The identification of 10 DYT16 mutations in PACT, 9 of which are in humans and 1 in a novel mouse model establishes the causative role of PACT in DYT16 disease etiology (Fig 1.4).

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\(^6^9\). They further demonstrated that as compared to wild type (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 DYT16 patient cells show delayed but more prolonged PKR activation and eIF2α phosphorylation in response to ER stress. Studies that were performed on other forms of dystonia after Vaughn et al.’s first report of dysregulated eIF2\(\alpha\) signaling in DYT16, also implicated misregulation of ER stress and eIF2\(\alpha\) signaling in the etiology of both DYT1 and DYT6\(^7^0,^7^1\). 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\(^7^2,^7^3\). This manifests in both the accumulation of misfolded proteins as well as secretory defects. 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)\textsuperscript{60,71}. 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 of ER stress through the de-repression of PKR’s kinase activity. Thus, we propose that the dysregulation of eIF2\(\alpha\) signaling may be a generalized mechanism driving the etiology for some forms of primary dystonia\textsuperscript{74}.

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 kinase activity of the eIF2\(\alpha\) kinases, PERK and PKR in Alzheimer’s disease, ALS, and certain forms of autism\textsuperscript{75-79}. Conversely, both \textit{in vivo} and \textit{in vitro} studies have implicated deficient phosphatase activity of PP1 in severe neurodevelopment disorders\textsuperscript{7,8,80,81}. Patients and disease models deficient in the regulatory subunits of PP1 present with reduced body size, microcephaly, intellectual disability, and in some cases, Alzheimer’s disease\textsuperscript{82,83}. Most recently, mutations in the \(\gamma\) 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, hypogonitalism, microcephaly, and obesity (MEHMO) syndrome\textsuperscript{84}. Samples taken from patients with MEHMO show signs of chronic stress markers as the causative mutations lead to a defect in ternary complex formation\textsuperscript{84}. A noteworthy symptom in a
severe form of MEHMO syndrome is lower limb ataxia. This ultimately results in the expression of stress response transcripts and constitutively stimulates the ISR in these patients. Taken in concert with various DYT subtypes dysregulating protein synthesis through the alpha subunit of eIF2, aberrancies in translation surrounding the eIF2 signaling hub can have dramatic and severe consequences. Because PACT regulates protein synthesis through eIF2α stress response signaling, the studies outlined in this thesis suggest a common mechanism shared between DYT16 and many debilitating neurological disorders.

1.5 STRUCTURE OF DISSERTATION

Chapter 2 of this dissertation describes the biochemical properties of a dominantly inherited DYT16 frameshift (FS) mutation and its effect on mammalian cells. Our results indicate the truncated protein is both incapable of binding dsRNA as well as void of its ability to interact with PKR. Our results further demonstrate that despite this truncation occurring in PACT’s dsRBM1, the mutant polypeptide surprisingly retains its ability to interact with wild type PACT and TRBP. When overexpressing this FS mutation as a fusion protein containing an amino-terminal fluorescent mCherry tag we observe the formation of insoluble cytosolic aggregates. Furthermore, we describe that overexpression of this mutation in mammalian cells induces apoptosis via activation of caspases. Through utilizing PACT−/− or PKR−/− null cells we establish that the apoptosis results from both PACT/PKR dependent and independent mechanisms. Lastly, by utilizing competition assays we demonstrate that the interaction of the FS mutant protein with TRBP can dissociate wild type PACT from the inhibitory
PACT-TRBP complex, possibly causing PKR activation in cells and inducing apoptosis. Our results indicate a mechanism where PKR is activated by the accumulation of insoluble protein aggregates possibly adding to the list of recent studies implicating increased PKR activity in the pathology of neurodegenerative diseases resulting from protein aggregation.

Chapter 3 of this dissertation describes the biochemical properties associated with five previously uncharacterized DYT16 point mutations (C77S, C213F, C213R, N102S, and T34S) and studies the biological consequences of these mutations. Our results demonstrate that these DYT16 mutations have no consequence on PACT’s dsRNA binding, however, are more efficient PKR activators. We further address the impact these DYT16 mutations have on PACT’s network of protein-protein interactions. We further establish that while the dominantly inherited mutations bind PKR with higher affinity as compared to wt PACT, the recessive mutations show no difference in their ability to form PACT-PKR heterodimers. All of the DYT16 mutant proteins form PACT-PACT homodimers with significantly higher affinity relative to that of wt PACT. Finally, using DYT16 patient derived lymphoblast cells we show the dysregulation of eIF2α signaling kinetics in response to ER stress followed by enhanced apoptosis in patient cells as compared to wt cells from an unaffected family member. Surprisingly, in the patient cells we identified heightened PACT-PKR interaction as compared to the unaffected family members. The observed hyper-sensitivity to ER stress was rescued in patient cells by disrupting the high-affinity
PACT-PKR interactions with luteolin treatment, an inhibitor of PACT-PKR interactions.

Chapter 4 of this dissertation outlines our studies with a recessively inherited frameshift mutation, Lear5j, which results in severe developmental and DYT16-like phenotypes in a novel DYT16 mouse model. We identify that this truncated variant of the PACT protein retains its ability to interact with PKR, however its ability to stimulate PKR’s kinase activity is significantly reduced in vitro. Furthermore, our results suggest the Lear5j mRNA is targeted for NMD only partially both in the brains of these mice as well as MEFs, and that we can still detect significant amounts of the mutant transcripts as well as the truncated protein in the brains of these affected mice. Finally, we show the presence of a severe lack of dendritic arborization in the Purkinje neuron layer of the cerebellum and reduced eIF2α phosphorylation in Lear5j mice.

Chapter 5 of this dissertation provides an overall discussion, conclusions, and future directions.
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 eIF2α is dependent on one of two regulatory subunits, CreP or GADD34. The PP1-CreP holophosphatase is critical for maintaining low basal levels of eIF2α phosphorylation in the absence of stress, while the PP1C GADD34 holophosphatase is mainly responsible for downregulating the ISR\textsuperscript{[2]}. 
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 DYT16 mutations in PACT

(A) 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 DYT16. The recessively inherited mutations are indicated in green, and dominantly inherited mutations indicated in red. (B) Schematic representation of the spontaneously arisen mutation in the mouse homolog of PACT. This recessively inherited mutation truncates the protein within PACT’s dsRBM2.
CHAPTER 2:
A TRUNCATED PACT PROTEIN RESULTING FROM A FRAMESHIFT MUTATION REPORTED IN MOVEMENT DISORDER DYT16 TRIGGERS CASPASE ACTIVATION AND APOPTOSIS¹.

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2.1 ABSTRACT

PACT activates the interferon (IFN)-induced double-stranded (ds) RNA-activated protein kinase (PKR) in response to stress signals. Oxidative stress and endoplasmic reticulum (ER) stress causes PACT mediated PKR activation, which leads to phosphorylation of translation initiation factor eIF2α, inhibition of protein synthesis, and apoptosis. A dominantly inherited form of early-onset dystonia 16 (DYT16) has been identified to arise due to a frameshift (FS) mutation in PACT. In order to examine the effect of the resulting truncated mutant PACT protein on PKR pathway we examined the biochemical properties of the mutant protein and its effect on mammalian cells. Our results indicate that the FS mutant protein loses its ability to bind dsRNA as well as its ability to interact with PKR while surprisingly retaining the ability to interact with PACT and PKR-inhibitory protein TRBP. The truncated FS mutant protein, when expressed as a fusion protein with a N-terminal fluorescent mCherry tag aggregates in mammalian cells to induce apoptosis via activation of caspases both in a PKR- and PACT-dependent as well as independent manner. Our results indicate that interaction of FS mutant protein with PKR inhibitor TRBP can dissociate PACT from the TRBP-PACT complex resulting in PKR activation and consequent apoptosis. These findings are relevant to diseases resulting from protein aggregation especially since PKR activation is a characteristic of several neurodegenerative conditions.
2.2 INTRODUCTION

PKR (protein kinase, RNA activated) is activated by binding to its Protein Activator PACT in human cells\textsuperscript{85} and its murine homolog in mouse cells\textsuperscript{34}. PACT induces autophosphorylation and activation of the interferon (IFN)-inducible, serine/threonine protein kinase PKR in response to cellular stress\textsuperscript{34,35,86,87}. Activation of PKR causes phosphorylation of the α subunit of the eukaryotic protein synthesis initiation factor 2 (eIF2α) leading to an inhibition of protein synthesis\textsuperscript{88}.

PKR is expressed in all cell types at low basal levels in the absence of virus infection, mediates IFN's antiviral actions in virally infected cells and also regulates cellular survival and apoptosis in response to stress in uninfected cells\textsuperscript{89}. Activation of PKR's kinase activity requires binding to one of its activators to bring about a conformational change causing enzymatic activation\textsuperscript{90}. PKR's two dsRNA-binding motifs (dsRBMs) bind to the dsRNA produced during viral replication\textsuperscript{26,91} and activate PKR by unmasking the ATP-binding site\textsuperscript{92}. These dsRBMs also mediate dsRNA-independent protein-protein interactions with other proteins that also carry dsRBMs\textsuperscript{93}. Among such proteins, PACT functions to activate PKR in a dsRNA-independent manner in response to cellular stress\textsuperscript{35,85}. There are three copies of dsRBM in PACT (Fig. 2.1 A), of these the two amino-terminal motifs (M1 and M2) are involved in a direct interaction with the dsRBMs of PKR. The third, carboxy-terminal motif 3 (M3) is dispensable for a high-affinity interaction with PKR but is essential for PKR activation as it contacts a specific region in PKR's catalytic domain\textsuperscript{94,95}. PACT-dependent PKR activation in cells
occurs only in response to oxidative stress signals, growth factor withdrawal, endoplasmic reticulum (ER) stress, to cause phosphorylation of the translation initiation factor eIF2α and cellular apoptosis\textsuperscript{34,86,87,96} although the purified, recombinant PACT activates PKR by direct interaction \textit{in vitro}\textsuperscript{85}. Once the stress conditions have resolved, PACT mediated PKR activation is possibly downregulated by the action of phosphatases that may dephosphorylate PACT. Although the identity of such phosphatases remains unknown, our recent work illustrates that a PKR inhibitory protein TRBP that complexes with PACT in the absence of stress signals is phosphorylated later during the stress response and is involved in the negative regulation of PKR activity at late time points after initial stress signal\textsuperscript{39,48,97}.

PACT is encoded by the \textit{Prkra} gene and recently many mutations in this gene have been described to cause a movement disorder dystonia 16 (DYT16). Many different dystonia types exist, which result from several diverse genetic and physiological causes, thereby constituting a heterogeneous group of movement disorders in which affected individuals develop sustained and painful involuntary muscle contractions leading to twisted postures\textsuperscript{98}. Recently a recessively inherited form of early-onset generalized dystonia (DYT16) has been described to arise from a homozygous missense mutation at amino acid position 222 in PACT\textsuperscript{62}. Seven affected members from two unrelated families were originally identified to carry the same P222L mutation\textsuperscript{62}, which lies between the conserved motifs M2 and M3 within PACT. The same mutation was later reported in many more patients with DYT16\textsuperscript{99}. Subsequently, four more recessive mutations
(C77S, C213F, C213R, and S265R) have been identified in DYT16 patients\textsuperscript{64,66,100}. Among the dominant mutations, a frameshift mutation which results in truncation of the protein after 88 amino acids\textsuperscript{63} and three point mutations reported in Polish and German families (T34S, N102S, and c.-14A>G) indicate that PACT mutations lead to DYT16 in a worldwide distribution\textsuperscript{65}.

In spite of the identification of several PACT mutations, the molecular mechanisms involved in the DYT16 onset or progression have not been studied much\textsuperscript{60}. We have previously analyzed the effect of the P222L mutation on PACT’s biochemical properties such as dsRNA binding, PKR interaction, and PKR activation\textsuperscript{69}. The P222L mutation did not affect PACT’s dsRNA-binding, or PKR-interaction properties \textit{in vitro}. However, in DYT16 patient cells the P222L mutant protein caused a delayed but prolonged activation of PKR in response to ER stress. The altered kinetics of eIF2\textalpha phosphorylation brought about by the changes in PKR activation led to defective downstream signaling and a lack of cell recovery and homeostasis. Thus, the DYT16 patient cells underwent enhanced apoptosis in response to the ER stressor tunicamycin in accordance with the altered biochemical properties of P222L protein.

In this report, we analyzed the effect of a dominant acting frameshift mutation in PACT described in a single early onset dystonia case\textsuperscript{63}. This frameshift mutation due to deletion of two nucleotides would produce a truncated PACT protein with 1-88 amino acids of the PACT followed by 21 new amino acids as a result of the frameshift. Our results indicate that when expressed transiently in mammalian cells, a fusion protein with N-terminal mCherry tag on the mutant
frameshift protein (mCherry-FS) forms aggregates to trigger apoptosis. Furthermore, the PKR+/+ as well as PACT+/+ MEFs show increased caspase activation in response to a transient overexpression of mCherry-FS protein and the caspase activation is reduced, but not absent, in PKR-/- and PACT-/- MEFs indicating that apoptosis caused by the FS mutant protein aggregation may result from both PKR-dependent and PKR-independent mechanisms.

2.3 MATERIALS AND METHODS

**Reagent, Cell Lines, and Antibodies:** HeLa (ATCC CCL-2) cells, PKR+/+, PKR-/-, PACT+/+, and PACT-/- mouse embryonic fibroblasts (MEFs) were cultured in Dulbecco’s modified Eagle’s medium (DMEM), containing 10% fetal bovine serum and penicillin/streptomycin. The anti-mCherry antibodies used were from Abcam (ab183628). Transfections were performed with Effectene Transfection Reagent (Qiagen) according to the manufacturer’s protocol.

**Generation of 2 nucleotide deletion frameshift mutation:** The deletion mutation was generated using mutagenic primers for PCR amplification of PACT ORF in two parts to introduce a two-nucleotide deletion as reported in DYT16 patient. The primer sequences were as follows:

- **PACT-UP1:** 5’-GCTCTAGACATATGGAAATGTCCCAGAGCAGGCAC-3’,
- **DYT16-frameshift-DN1:** 5’-GGCAGCCTCTGCAGCTCTGTTTCGCCAGCAGGCAC-3’
- **DYT16-frameshift-UP1:** 5’-GCTGGCGAAACAGAGCTGCAGAGGCTGCC-3’
- **PACT313M3-AS:** 5’-GGGGATCCTTACTTTCTTTCTTCTGCATTATTATC-3’

The two PCR products were sub-cloned into pgEMT-easy vector (Promega). Once the sequence of the frameshift mutation was verified, we generated full-
length FS ORF in pcDNA3.1· by a three-piece ligation of XbaI–Pst1 restriction piece from the 5’ half of FS point mutant/pgEMT-easy, PstI-BamHI piece from 3’ half of FS point mutant/pgEMT-easy, and XbaI-BamHI cut pcDNA3.1·. The full-length FS mutant ORF in pcDNA3.1· has an amino-terminal flag tag that is added from the vector sequence. Full length FS mutant was sub-cloned into pET15b (Novagen) for producing hexahistidine tagged pure recombinant protein and into pmCherryC1 (Clontech) for expression in mammalian cells. The PACT/pmCherryC1 and PACT/pEGFPC1 constructs were generated by sub-cloning the PACT ORF from PACT/pcDNA3.1· and the PKR/pEGFPC1 construct was generated by sub-cloning the PKR ORF with the kinase dead K296R mutation from K296R/pCDNA3.1·. It is well established that the wt PKR overexpression in cells is not feasible as it induces apoptosis very rapidly and thus for all experiments using full length PKR in fluorescence microscopy and co-localization experiments, we used the catalytically dead PKR mutant K296R expression construct.

**dsRNA-binding assay:** The dsRNA-binding assay was performed with the in vitro translated, 35S-labeled PACT and FS proteins synthesized using the expression constructs in pcDNA3.1· and TNT-T7 coupled reticulocyte lysate system from Promega as described before 97.

**Protein–protein interaction assay:** In vitro translated, 35S-labeled PKR, PACT, FS or TRBP proteins were synthesized using the TNT T7 coupled reticulocyte system from Promega as explained before. 5 µl of the in vitro translated 35S-labeled proteins were incubated with either 100 ng of pure
recombinant hexahistidine tagged PKR, PACT or FS mutant as indicated in the individual figures and 20 µl of Ni-agarose (Novagen) in 200 µl of binding buffer [5 mM imidazole, 20 mM Tris–HCl pH 7.9, 200 mM NaCl, 0.2 mM phenylmethylsulfonyl fluoride (PMSF), 0.5% IGEPAL (Sigma)] at 25°C for 30 min on a rotating wheel. The beads were washed four times in 500 µl of binding buffer each time and the washed beads were then boiled in 20 µl Laemmli buffer (150 mM Tris–HCl pH 6.8, 5% SDS, 5% β-mercaptoethanol, 20% glycerol) for 2 min and eluted proteins were analyzed by SDS–PAGE on a 12% gel.

Fluorography was performed at 80°C with intensifying screens. To assay if the FS mutant protein can dissociate TRBP-PACT complex, 5 µl of the in vitro translated 35S-labeled TRBP protein was incubated with 100 ng of pure recombinant hexahistidine tagged PACT and anti-PACT rabbit monoclonal antibody bound to 10 µl of protein A sepharose beads in 200 µl of binding buffer [20 mM Tris–HCl pH 7.9, 200 mM NaCl, 0.2 mM phenylmethylsulfonyl fluoride (PMSF), 0.5% IGEPAL (Sigma)] at 25°C for 30 min on a rotating wheel. Increasing amounts of pure recombinant FS protein was then added as indicated in Fig. 2.6B and the incubation was continued for 30 min longer. The beads were then washed four times in 500 µl of binding buffer each time and the washed beads were then boiled in 20 µl Laemmli buffer (150 mM Tris–HCl pH 6.8, 5% SDS, 5% β-mercaptoethanol, 20% glycerol) for 2 min and eluted proteins were analyzed by SDS–PAGE on a 12% gel. The assays were quantified on Typhoon FLA7000 by analyzing the band intensities in the relevant lanes.

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**Caspase 3/7 assay:** PKR+/+, PKR−/−, PACT+/+ and PACT−/− mouse embryonic fibroblasts (MEFs) were plated at in 6 well dishes and transfected with flag-FS mutant/pCDNA3.1+ or empty vector pCDNA3.1−. Cells were collected at indicated time points and mixed with equal parts of Promega Caspase-Glo 3/7 reagent and incubated for 45 min. Luciferase activity was measured with a negative control of cell culture medium alone used to normalize all readings.

**Western Blot analysis:** HeLa cells were transfected in triplicates in 6 well dishes with 500ng DNA per well of pmCherryC1 empty vector, FS mutant/pmCherryC1, and PACT/pmCherryC1. Transfected cells were collected 12 h after transfection and washed twice with ice cold 1× PBS. Harvested cells were lysed in western lysis buffer (2% Triton X-100, 20 mM Tris–HCl pH 7.5, 100 mM KCl, 200 mM NaCl, 4 mM MgCl2, 40% glycerol, and phosphatase inhibitor cocktail 2 (Sigma) at 1:100 dilution) for 5 min on ice. Lysates were centrifuged at 13,200 rpm for 2 min. Protein concentration in the supernatant was quantified using Bradford reagent. Western blot was performed with the anti-mCherry antibody and western blot images were analyzed using the Typhoon FLA 7000 and ImageQuant LAS 4000 (GE Health).

**Expression and purification of recombinant PKR, wt PACT and FS mutant:** The protein coding regions (PKR, wt PACT or FS mutant) were sub-cloned into pET15b (Novagen) to generate PKR/pET15b, PACT/pET15b, and FS mutant/pET15b resulting in the in-frame fusion of the ORFs to the histidine tag. The recombinant proteins were expressed and purified as described85,103.
Visualization of aggregated FS mutant protein: HeLa cells were grown on coverslips and transfected with 500 ng of either PACT/pmCherryC1, FS mutant/pmCherryC1 empty vector pmCherryC1 using Effectene (Qiagen). 12 h after transfection, the cells were rinsed with ice-cold phosphate buffered saline (PBS) and fixed in 2% paraformaldehyde for 10 min. The cover slips were mounted in Vectashield mounting medium containing DAPI (Vector Laboratories). Cells were then viewed under the inverted fluorescence microscope (EVOS® FL Imaging System).

Yeast Two-Hybrid Interaction Assay: To test TRBP-PACT and TRBP-FS interactions, PACT and FS were expressed as GAL4-activation domain fusion proteins from the pgADT7 vector and TRBP was expressed as GAL4 DNA-binding domain fusion proteins from the pgBKT7 vector. We also tested these interactions with TRBP expressed as GAL4-activation domain fusion protein from the pgADT7 vector with PACT and FS proteins expressed as GAL4 DNA-binding domain fusion proteins from the pgBKT7 vector. The appropriate combinations of expression constructs were co-transformed into AH109 yeast cells (Clontech) and the transformed yeast cells were plated on double dropout SD minimal medium lacking tryptophan and leucine. In order to check for the transformants’ ability to grow on quadruple dropout media, transformed yeast cells were grown to an OD$_{600}$ of 2 in YPD media (yeast extract, peptone, and dextrose). 500 µl of each culture was pelleted and resuspended in an appropriate amount of distilled water to yield an OD$_{600}$ of 10. Serial dilutions were then made to yield OD$_{600}$ values of 1, 0.1, and 0.01. 10 µl of each dilution was then spotted onto quadruple
dropout SD minimal media lacking histidine, tryptophan, leucine, and adenine in the presence of 10 mM 3-amino-1,2,4-triazole (3-AT). Plates were incubated at 30°C for 3 days to score the growth.

Quantifications and Statistics: All binding assays and pull-down assays (Typhoon FLA7000) were quantified using GE Life Sciences ImageQuant TL software. To determine statistical significance of results of the dsRNA-binding, protein interaction and caspase assays a two-tailed Student’s T-test was performed, assuming equal variance. Each figure legend indicates p values as denoted by brackets and special characters. Note that our alpha level was p=0.05.

2.4 RESULTS

The FS mutation destroys PACT’s dsRNA binding as well as PKR interaction activity: The early onset dominant mutation causing DYT16 in one German patient is a deletion of two nucleotides (AT) in codon 89 (Fig. 2.1 B) of the PACT open reading frame (ORF). This results in a frameshift that would produce a 109 amino acid long truncated protein with 1-88 amino acids of original PACT protein followed by 21 extraneous amino acids due to the frameshift (Fig. 2.1 A and B). The resulting truncated (mutant FS) protein does not have a complete copy of the evolutionarily conserved dsRNA-binding motif (dsRBM1) and thus is missing the crucial part of the motif that is essential for binding dsRNA as well as protein-protein interactions. Therefore, the FS mutant protein is expected to be devoid of dsRNA-binding ability, as well as interaction with PKR and PACT. In order to determine if the frameshift mutation
affects dsRNA binding activity, an *in vitro* dsRNA-binding assay previously well established for PKR and PACT\(^{85}\) was performed (Fig. 2.2 A and B). As seen in Figure 2 A, the wt PACT protein binds to dsRNA (lane 2) but the frameshift mutant protein shows no binding to dsRNA (lane 4). The binding of wt PACT to dsRNA immobilized on the beads could be competed out by exogenously added dsRNA (lane 6) but not ssRNA (lane 5) confirming specific binding to dsRNA. Firefly luciferase, a protein that does not bind dsRNA used as a negative control showed no binding to the beads further demonstrating the binding specificity. The quantification of percentage binding established that FS mutant protein has no dsRNA-binding activity as compared to wt PACT (Fig. 2.2 B). We next examined if the FS mutant protein can interact with PKR and PACT using protein-protein interaction pull down assay (Fig. 2.3 A-F). \(^{35}\)S-methionine labeled PKR, and PACT were *in vitro* translated using rabbit reticulocyte system and mixed with pure recombinant hexahistidine tagged PKR, PACT, or FS mutant proteins bound to Ni-charged sepharose beads to measure the pull down of \(^{35}\)S-labeled proteins. As seen in Fig. 2.3 A and B, PKR interacts efficiently with PACT (lane 3) and PKR (lane 4) but not with FS mutant protein (lane 5). No \(^{35}\)S-labeled PKR was pulled down by Ni-agarose beads in the absence of any hexahistidine tagged protein (lane 2) indicating absence of any non-specific binding of PKR to the Ni-agarose beads and presence of a specific interaction with PACT and PKR. These results indicate that the mutant FS protein does not interact with PKR *in vitro* under the conditions of this assay. As seen in Fig. 2.3 C and D, PACT interacts efficiently with PACT (lane 3), PKR (lane 4) and surprisingly also with
mutant FS protein (lane 5). No $^{35}$S-labeled PACT was pulled down by Ni-agarose beads in the absence of any hexahistidine tagged protein (lane 2) indicating absence of any non-specific binding of PACT to the Ni-agarose beads and presence of a specific interaction with PACT, PKR, and FS mutant. We further tested the interaction of $^{35}$S-labeled, in vitro translated PKR, PACT and FS proteins with pure recombinant FS protein bound to Ni-charged sepharose beads to measure the pull down of $^{35}$S-labeled PKR, PACT and FS proteins. As seen in Fig. 2.3 E and F, in vitro translated PKR showed no interaction with the recombinant FS protein (lane 4), whereas in vitro translated PACT (lane 5) and FS (lane 6) proteins showed a strong interaction with recombinant FS protein bound to the Ni-agarose beads. There was no non-specific binding of PKR, PACT, or FS proteins to the Ni-agarose beads in the absence of FS protein bound to the beads (lanes 7-9) indicating specific interaction between PACT-FS and FS- FS under the conditions of this assay.

**FS mutant protein forms aggregates in mammalian cells:** In order to test the effect of FS mutant protein expression on mammalian cells, we transfected HeLa cells with mCherry-PACT and mCherry-FS expression constructs. Observing the transfected cells under a fluorescence microscope 12 h after transfection indicated that mCherry-PACT protein shows primarily cytoplasmic localization without aggregates (Fig. 2.4 A, panel a), whereas the mCherry-FS mutant protein forms cytoplasmic aggregates (Fig. 2.4 A, panel b). The mCherry protein itself shows localization both to cytoplasm and nucleus (Fig. 2.4 A, panel c). The mCherry tag is known to be monomeric and any aggregation of mCherry-
FS fusion protein observed in mammalian cells would thus be driven by the FS portion of the fusion protein and not by the mCherry tag. We also analyzed the expression of mCherry fusion proteins by a western blot analysis with anti-mCherry antibody 12 h after transfection. As seen in Fig. 2.4B, the expression of mCherry fusion proteins can be detected on the western blot. The presence of higher molecular weight bands in mCherry-FS lane indicated that the aggregates formed by the mCherry-FS protein persist even in the presence of SDS and during gel electrophoresis (Fig 4 B, lane 2). Such aggregates could be formed by the mCherry-FS protein molecules alone or with endogenous PACT protein and at present we do not know the exact composition of these protein aggregates. At longer time points after transfection, the cells overexpressing mCherry-FS mutant protein showed cell death around 24-36h. Despite multiple efforts, it was not feasible to establish a stable expression of mCherry-FS to achieve 100% cells in transfected population expressing the mutant mCherry-FS. We also attempted to express the FS mutant protein using a smaller tag such as the 8-amino acid long flag tag. Expression of the flag tagged FS mutant protein was not detectable by either immunofluorescence or western blot in the transient transfection system, which may be due to more efficient and quicker induction of cell death or due to nonsense-mediated decay (NMD) of the mRNA. Addition of the mCherry tag on the amino terminus makes the fusion protein size larger and this may allow the mRNA to escape NMD, making detection of the mCherry-FS mutant protein possible. In order to detect any change in PKR and eIF2α phosphorylation in response to FS mutant protein expression, we performed western blot analysis at
shorter time points after transfection. As seen in Fig. 2.5 A, we detected a time-dependent small increase in eIF2α phosphorylation (lanes 2-4) in cells expressing mCherry-FS protein starting at 1-8 h (lanes 2-5) and the cells transfected with empty vector mCherry did not show a similar increase (lanes 7-10). PKR phosphorylation also was detected in mCherry-FS transfected cells (lanes 2-5) but not in cells transfected with empty vector mCherry (lanes 7-10). These results suggest that expression of mCherry-FS protein causes PKR activation leading to eIF2α phosphorylation indicating that PKR activation could be the reason for cell death observed at later time points in response to mCherry-FS expression.

**Transient overexpression of FS mutant protein activates caspase 3/7:**
Activation of PKR in mammalian cells causes apoptosis via activation of caspases. Thus, we tested if transient overexpression of mCherry-FS mutant protein results in activation of caspases. Overexpression of mCherry-FS mutant in mouse embryonic fibroblasts from PKR+/+ and PKR-/- mice resulted in significant induction of caspase activity and apoptosis of transfected cells. As seen in Figure 5 B, there is a significant increase in caspase 3/7 activity in PKR+/+ MEFs transfected with mCherry-FS mutant expression construct at 24 and 30 h (red bars) as compared to the cells transfected with empty vector (blue bars). Compared to the PKR +/- MEFs, the PKR -/- MEFs showed significantly less increase in caspase 3/7 activity at equivalent time points after the transfection. However, the PKR-/- cells overexpressing mCherry-FS mutant also underwent apoptosis similar to PKR +/- cells, but with a slower kinetics. We also
tested if PACT was essential for caspase activation in response to FS mutant protein expression. Overexpression of mCherry-FS mutant in mouse embryonic fibroblasts from PACT+/+ and PACT-/- mice resulted in significant induction of caspase activity. As seen in Figure 5 C, similar to PKR+/+ MEFs, there is a significant increase in caspase 3/7 activity in PACT+/+ MEFs transfected with mCherry-FS mutant expression construct at 24 and 30h (red bars) as compared to the cells transfected with empty vector (blue bars). Compared to the PACT +/- MEFs, the PACT -/- MEFs show significantly less increase in caspase 3/7 activity at equivalent time points after the transfection. These results suggest that the FS mutant protein aggregates depend on PACT and PKR only in part to induce caspase activation and apoptosis.

In order to understand how the mutant FS mutant protein could bring about PKR activation in mammalian cells when it shows no interaction with PKR, we explored the possibility that FS mutant protein may promote PACT-PKR interaction by interacting with TRBP. Our previous work has suggested that in unstressed cells, PACT is complexed with TRBP, a PKR inhibitory protein. TRBP inhibits PKR by directly interacting with PKR as well as by sequestering dsRNA and PACT. In unstressed cells PACT-TRBP heterodimers prevail and PKR remains inactive. In response to stress signals, once PACT is phosphorylated, it dissociates from TRBP to form PACT-PACT homodimers which bind to PKR at higher affinity and activate PKR catalytically. Thus, we investigated the possibility that mutant FS protein could interact with TRBP to prevent its association with PACT and thereby releasing PACT to activate PKR.
In order to investigate this, we first tested if FS mutant protein interacts with TRBP using protein-protein interaction pull down assay (Fig. 2.6 A). $^{35}$S-methionine labeled TRBP was *in vitro* translated using rabbit reticulocyte system and mixed with pure recombinant hexahistidine tagged PKR, PACT, or FS mutant proteins bound to Ni-charged sepharose beads to measure the pull down of $^{35}$S-labeled TRBP. As seen in Fig. 2.6 A and B, TRBP interacts efficiently with PKR (lane 3), PACT (lane 4) as well as with FS mutant protein (lane 5). No $^{35}$S-labeled TRBP was pulled down by Ni-agarose beads in the absence of any hexahistidine tagged protein (lane 2) indicating absence of any non-specific binding of TRBP to the Ni-agarose beads and presence of a specific interaction with PKR, PACT and FS. These results indicate that TRBP interacts with FS mutant protein similar to its interaction with PKR and PACT *in vitro* under the conditions of this assay. We reasoned that if FS mutant protein can displace PACT from PACT-TRBP complex, it may be able to activate PKR in cells by releasing PACT from TRBP. In order to test this, we explored if FS mutant protein can dissociate PACT-TRBP interaction. $^{35}$S-methionine labeled TRBP was *in vitro* translated using rabbit reticulocyte system and mixed with pure recombinant hexahistidine tagged PACT protein bound to anti-PACT monoclonal antibody attached to protein A-sepharose beads in the absence of any FS mutant protein. Increasing amounts of pure recombinant FS mutant protein was then added (lanes 4-7) to measure the pull down of $^{35}$S-labeled TRBP with PACT bound to beads. As seen in Fig. 2.6 C and D, the amount of TRBP pulled down with PACT was inversely correlated to the amount of FS mutant protein added.
(lanes 4-7 compared to lane 3). In order to compare the relative strengths of TRBP-PACT and TRBP-FS interactions, we used the yeast two hybrid protein-protein interaction assay. As seen in Fig. 2.6 E, TRBP-FS interaction was stronger than the TRBP-PACT interaction (compare TRBP-PACT panel with TRBP-FS panel in the TRBP/pgBKT7 set). When TRBP was expressed as the GAL4-activation domain fusion protein, similar results were seen (compare PACT-TRBP panel with FS-TRBP panel in the TRBP/pgADT7 set). These results indicate that FS mutant protein can bind to TRBP to release PACT, which than could bring about PKR activation leading to caspase activation and apoptosis. The FS protein also may bind to PACT to prevent PACT-TRBP association and the FS-PACT heteromeric interactions could bring about PKR activation. Figure 7 depicts these possibilities in a schematic model.

2.5 DISCUSSION

PKR activation in response to cellular stress is regulated by PACT and is involved in modulating cellular survival[34,35,69,86,87]. Although many mutations in PACT have now been identified in a worldwide occurrence of DYT16 cases, the pathomechanisms involved in DYT16 remain poorly understood. Our previous work on the most prevalent P222L mutation in DYT16 has revealed that this mutation leads to a dysregulation of eIF2α phosphorylation in response to cellular stress[69]. Cells homozygous for P222L mutation exhibited enhanced sensitivity to ER stressor tunicamycin due to a delayed but more robust eIF2α phosphorylation. In this study, we aimed to examine the effect of a dominant PACT mutation reported in a single DYT16 patient. We started by characterizing
the biochemical properties of the FS mutant protein \textit{in vitro}. The FS mutant protein exhibited no dsRNA-binding ability as it is truncated at residue 88 and lacks the carboxy-terminal part of M1, the first conserved motif, and also the amino acid residues critical for interaction with dsRNA\textsuperscript{85,91,97}. We have previously characterized the contribution of individual amino acids in this motif to dsRNA-binding and protein-protein interaction and the results obtained with FS mutant are in accordance with our previous work\textsuperscript{97}. The mutant FS protein also showed no interaction with PKR, although it showed interaction with PACT. Based on our previous work on PACT-PKR and PACT-PACT interactions, it is expected that FS mutant protein may not interact with PKR or PACT as it lacks the alanine residues at positions 91 and 92\textsuperscript{52,97}. Thus, it was surprising that the FS mutant protein interacted with PACT \textit{in vitro} and the FS protein aggregates showed co-localization of PKR and PACT in HeLa cells. It is worth noting here is that the pure recombinant hexahistidine tagged FS protein showed aggregate forms on an SDS-PAGE gel indicating that it forms aggregates and such aggregated forms are present in the FS mutant we used to bind to Ni-agarose beads in our pull-down assays. This aggregation may possibly contribute to interaction with PACT in this assay and similarly also in mammalian cells.

In order to study how FS protein affects the PACT-PKR pathway during cellular stress, we wanted to investigate the effect of FS mutant protein expression in mammalian cells. As the mutation is reported to be dominant, we wanted to establish a cell line with a stable expression of the mutant protein in order to analyze its effects on the PKR pathway. Despite multiple attempts with
several different smaller epitope tags (Flag, Myc, HA) on the FS mutant protein, we were unable to detect its expression at early or late time points post-transfection using western blot analyses. Thus, we reasoned that an overexpression of the FS mutant protein may be toxic to cells or the mRNA may be undergoing NMD\textsuperscript{104}. We next studied its expression by tagging the FS protein with a mCherry tag as the expression could be detected in live cells using fluorescence microscopy. We could detect mCherry-FS protein expression as early as 6 h after transfection and the cells expressing mCherry-FS protein showed increasing cell death at later time points. Most strikingly, the mCherry-FS protein showed aggregate formation in cells. The high molecular weight forms of aggregated FS protein observed by us on SDS-PAGE are similar to aggregate forms of other proteins such as mutant Cu/Zn superoxide dismutase that are aggregate prone and cause ALS\textsuperscript{108}. The misfolded \(\alpha\)-synuclein involved in the pathogenesis of Lewy body diseases isolated from the brains of dementia patients also shows similar aggregates that are not disaggregated in presence of SDS\textsuperscript{109}. Since PKR shows no interaction with FS mutant protein but FS mutant protein can interact strongly with TRBP to dissociate PACT from the PACT-TRBP heterodimers, we propose that PKR is activated by PACT released from TRBP when FS mutant protein is present. A schematic model depicting this is presented in Fig. 2.7, which outlines PKR- and PACT-dependent processes leading to apoptosis. In the absence of FS mutant protein when PACT mediated PKR activation takes place in response to stress (Fig. 2.7 A), PACT is phosphorylated and forms homodimers efficiently after its dissociation from
TRBP. When overexpressed, the FS mutant protein can cause dissociation of PACT and the "free" PACT released from TRBP-PACT heterodimers could bring about PKR activation (Fig. 2.7 B). Any possible phosphorylation of PACT due to overexpression of FS mutant protein remains to be explored in future. Under such conditions, PKR activation leads to caspase activation and apoptosis as we observed in cells overexpressing mCherry-FS protein. Our results demonstrated that FS mutant protein expression led to caspase activation in murine fibroblasts which was partly dependent on the presence of PACT and PKR. However, the FS protein aggregates also induced caspase activation in a PKR and PACT independent manner as both PKR as well as PACT null cells showed a slower kinetics of caspase induction. These results are indicative that FS mutant protein aggregates induce apoptosis in both a PACT-PKR-dependent and PACT-PKR-independent manner.

Activation of PKR by protein aggregates observed with the FS mutant could be indicative of a more general process rather than a specific process driven by the FS mutation. In this regard, it could be worth investigating if known aggregation prone proteins involved in neurodegenerative diseases trigger PACT dependent PKR activation. A significant amount of evidence exists to indicate a possible connection between PACT and PKR recruitment to such aggregates. A number of studies have implicated PKR in the pathogenesis of neurodegenerative diseases, such as Alzheimer’s disease (AD)\textsuperscript{110}, Parkinson’s disease (PD)\textsuperscript{75,111}, Huntington’s disease (HD)\textsuperscript{75}, and amyotrophic lateral sclerosis (ALS)\textsuperscript{112}. Of these, PKR activation has been most investigated in Alzheimer’s
disease. The earliest reports of PKR activation in AD demonstrated an accumulation of activated (phosphorylated) PKR in degenerating neurons mainly using histological methods. A direct implication of PKR activation in the pathology of the disease is indicated based on the recent evidence and inhibition of PKR prevents the neuronal apoptosis in AD mouse models as well as in neuronal cultures. Activated PKR aggregates are reported in hippocampal neurons in PD and HD patient brains. More relevantly, there is evidence for PACT being present in potential aggregates in addition to presence of activated PKR in the degenerating neurons. Immunohistochemical studies with the hippocampal neurons showed a co-localization of PACT with phosphorylated PKR in the post-mortem brains of AD patients. A mass spectrometry analysis showed an increased association of PACT with the aggregation prone mutant HTT protein in the affinity-purified complexes from huntingtin mutant (HTT) juvenile mouse brain. Elevated PKR expression or activation is also observed in ALS although co-localization of PACT and PKR has not been investigated so far for ALS. There is no clear evidence for neurodegeneration in monogenic inherited forms dystonia. Although neurodegeneration has been investigated and found to be absent in DYT6, evidence of neurodegeneration has been reported in X-linked dystonia-parkinsonism and in DYT16.

It also remains an open question if dysregulation of eIF2α pathway observed in a few primary dystonias is the primary cause of the pathology or a secondary effect related to activation of stress signaling pathways. Our research identified the dysregulation of PKR-eIF2α signaling pathway as a consequence of P222L
mutation in DYT16\textsuperscript{69}. Subsequently DYT1, DYT6 as well as the sporadic cervical dystonia also were reported to show an impairment of the eIF2α signaling pathway\textsuperscript{74,120-122}. Although we could not analyze eIF2α dysregulation with expression of FS mutant protein in the transient expression system, it is worth a mention that a spontaneously arisen, recessive insertion mutation in \textit{Prkra} identified at the Jackson Laboratory results in a progressive dystonia\textsuperscript{123}, kinked tails, and mortality in mice. Some neurons in the dorsal root ganglia and the trigeminal ganglion were apoptotic in the homozygous mutant mice, consistent with the observed neurodegenerative phenotype. The described mutation could result in the production of a truncated PACT protein with seven extraneous amino acids after first 178 AA before a premature stop codon. It remains to be determined if such a truncated protein is present in these mice and may cause the observed dystonia phenotype. The \textit{Prkra} null mice show no dystonia symptoms\textsuperscript{51}, thereby raising the possibility that the dystonia phenotype may be directly correlated to presence of mutant PACT proteins. On the contrary, it is also possible that the mutant truncated PACT protein is not produced in patient cells or in mice due to NMD of the mRNA due to the presence of an early termination codon. As no patient cells are available for the reported FS mutation, this spontaneously created mouse model can be utilized to detect if the truncated protein is present in brain or any other cell types and may help understand the pathomechanisms involved in DYT16.

In summary, our results presented here report the biochemical properties of a truncated PACT protein resulting from a frameshift mutation in a DYT16 patient.
Interestingly, these results indicate that although the FS mutant protein shows no direct interaction with PKR, the aggregates of the FS mutant protein formed in mammalian cells result in PKR activation to cause caspase activation leading to apoptosis. In the context of neurodegenerative diseases caused by protein aggregates in neurons, these findings beg for more in depth investigations for the possible role of PKR activation in triggering neuronal loss.
Figure 2.1: Schematic representation of the frameshift mutation in PACT. A. Domain structure of PACT and frameshift (FS) mutant. Orange boxes: conserved dsRNA-binding motifs dsRBM1 and dsRBM2 that mediate dsRNA-independent high-affinity interaction with PKR, Green box: M3 motif that does not bind dsRNA and mediates low-affinity interaction with the PACT-binding motif (PBM) within PKR’s catalytic domain. The residues added as a result of frameshift mutation in FS are indicated as a red box. B. FS mutation in PACT ORF. The first 330 nucleotides of the PACT ORF are shown. The two-nucleotide deletion (266-267) is indicated in bold and underlined font. The shifted reading frame is indicated in red font. Thus, the frameshift mutation produces a protein that has amino acids 1-88 and 21 new amino acids (PACT88-21).
Figure 2.2: Effect of FS mutation on dsRNA-binding. dsRNA-binding activity of wt PACT and FS mutant was measured by poly(I)-poly(C)-agarose binding assay with in vitro translated $^{35}$S-labeled proteins. The positions of PACT and FS bands are indicated by arrows and the molecular weights are as indicated. T, total input; B, proteins bound to poly(I)-poly(C)-agarose. Competition lanes 5 and 6: competition with 100-fold molar excess of ssRNA (ss) or dsRNA (ds). The fainter bands below the parent PACT bands represent products of in vitro translation from internal methionine codons. The firefly luciferase, which does not bind dsRNA, was used as a negative control. **B. Quantification of dsRNA-binding assay.** Bands were quantified by phosphorimager analysis and % bound was calculated. Error bars: standard error of mean from 4 independent experiments. wt PACT: blue bar and FS mutant: red bar.
Figure 2.3: Effect of FS mutation on interaction with PKR and PACT. (A, C, and E) Pull down assay of *in vitro* translated proteins with pure recombinant PKR, PACT, and FS proteins. 5 µl of *in vitro* translated, 35S-labeled flag-tagged PKR (A) or PACT (C) or FS (E) protein was mixed with 100 ng of purified, hexahistidine tagged recombinant PKR, PACT, and FS proteins immobilized in Ni-charged Sepharose beads. Pull down of 35S-labelled PKR, PACT and FS was analyzed by SDS-PAGE after washing the beads five times with wash buffer. Total lane: total input PKR, PACT, and FS as indicated (50% of the bound samples); Bound lanes: the pulled down PKR (A) or PACT (C) or PKR, PACT and FS (E).

B, D, and F. Quantification of data in 3 A, 3 C, and 3E. The radioactivity present in the bands was measured by phosphorimager analysis and the % pull down was calculated as (radioactivity present in the pull down (bound) PKR, PACT, FS bands/the radioactivity present in the PKR, PACT, FS bands in the total lane) X 100. Error bars: standard error of mean from 4 independent experiments. Student T-tests were performed, and p values are as follows (B) * = 0.00018, ** = 0.00013, (D) * = 0.00019, and ** = 0.00009, (F) * = 0.00002 and ** = 0.00001, n=4.
A. The mCherry-FS mutant protein aggregates in mammalian cells. HeLa cells were transfected with mCherry-PACT/pmCherryC1, Cherry-FS/pmCherryC1, and pmCherryC1 empty vector (EV). Expression of proteins was examined 12 h after transfection using a fluorescence microscope. a: overlay of mCherry-PACT (red) and DAPI nuclear stain (blue), b: overlay of mCherry-FS fusion protein (red) DAPI nuclear stain (blue), c: overlay of mCherry (red) and DAPI nuclear stain (blue).

B. Western blot analysis of mCherry tagged proteins. HeLa cells were transfected with mCherry-PACT/pmCherryC1, Cherry-FS/pmCherryC1, and pmCherryC1 empty vector (EV). 12 h post transfection, the cells were harvested, cell extracts were made and analyzed by western blot analysis with anti-mCherry antibody. Lane 1: mCherry-PACT, lane2: mCherry-FS, lane 3: mCherry EV. The positions of mCherry-PACT, mCherry-FS and mCherry are as indicated by arrows. The multimers of mCherry-FS seen in lane 2 are indicated by arrowheads.
Figure 2.5: A. PKR activation and eIF2α phosphorylation in response to transient overexpression of FS mutant protein.
Figure 2.5 (Continued): A. HeLa cells were transfected with Cherry-FS/pmCherryC1, and pmCherryC1 empty vector. At 1h-8h post transfection the cells were harvested, cell extracts were made and analyzed by western blot analysis with the indicated antibodies. Lanes 1-5: mCherry-PACT/pmCherryC1 transfected cell extracts, lanes 6-10: pmCherryC1 empty vector transfected cell extracts. B. mCherry-FS mutant induces caspase3/7 activation in a PKR-dependent as well as PKR-independent manner. The PKR+/+ and PKR-/- mouse embryonic fibroblasts were transfected with either the mCherry-FS/pmCherryC1 expression construct or empty vector pmCherryC1. Caspase 3 and 7 activities were measured, at indicated time points. blue bars: EV (pmCherryC1) transfected cells and the red bars: mCherry-FS/pmCherryC1 transfected cells. Student T-tests were performed, and p values are as follows * = 0.0073 (significant), ** = 0.0065 (significant), *** = 0.0027 (significant), **** = 0.00051, and ***** = 0.00023 (significant), n=4. C. FS mutant induces caspase 3/7 activation in a PACT-dependent as well as PACT-independent manner. The PACT+/+ and PACT-/- mouse embryonic fibroblasts were transfected with either the mCherry-FS/pmCherryC1 expression construct or empty vector pmCherryC1. Caspase 3 and 7 activities were measured, at indicated time points. blue bars: EV (pmCherryC1) transfected cells and the red bars: mCherry-FS/pmCherryC1 transfected cells. Student T-tests were performed, and p values are as follows * = 0.0067 (significant), ** = 0.0051 (significant), *** = 0.0043 (significant), **** = 0.00065, and ***** = 0.00027 (significant), n=4.
Figure 2.6: Effect of FS mutation on TRBP-PACT interaction. (A) Pull down assay of *in vitro* translated TRBP protein with pure recombinant PKR, PACT, and FS proteins. 5 µl of *in vitro* translated, $^{35}$S-labeled flag-tagged TRBP protein was mixed with 100 ng of purified, hexahistidine tagged recombinant PKR, PACT, and FS proteins immobilized in Ni-charged Sepharose beads. Pull down of $^{35}$S-labelled TRBP was analyzed by SDS-PAGE after washing the beads five times with wash buffer. Total lane: total input TRBP (50% of the bound samples); Bound lanes: pulled down TRBP. The "-" lane shows TRBP pull down with no recombinant protein on Ni-charged Sepharose beads as a negative control.
Figure 2.6 (Continued): (C) FS mutant protein can dissociate TRBP from PACT. 5 µl of the *in vitro* translated 35S-labeled TRBP protein was incubated with 100 ng of pure recombinant hexahistidine tagged PACT and anti-PACT rabbit monoclonal antibody bound to 10 µl of protein A sepharose beads. Increasing amounts of pure recombinant FS protein was added as indicated in lanes 4-7. Pull down of 35S-labelled TRBP was analyzed by SDS-PAGE after washing the beads five times with wash buffer. Total lane: total input TRBP (50% of the bound samples); Bound lanes: pulled down TRBP. The "-" lane shows TRBP pull down with no recombinant protein on Ni-charged Sepharose beads as a negative control. Lanes 4-7: pure recombinant FS mutant protein added in increasing amounts (100 pg, 1 ng, 10 ng, 20 ng). (B and D) Quantification of data in 6 A, and 6 C. The radioactivity present in the bands was measured by phosphorimager analysis and the % pull down was calculated as (radioactivity present in the pull down (bound) TRBP bands/the radioactivity present in the TRBP band in the total lane) X 100. Error bars: standard error of mean from 4 independent experiments. Student T-tests were performed, and p values are as follows (B) * = 0.00011, (D) * = 0.000012, n=4. **E. TRBP-FS interaction is stronger than TRBP-PACT interaction in yeast two-hybrid assay.** PACT/pGADT7, FS/pGADT7 or empty vector (EV) pGADT7 were co-transformed with TRBP/pGBK7 into AH109 yeast cells and selected on SD double dropout media (-tryptophan, -leucine). Ten microliters of transformed yeast cells (OD600 = 10, 1, 0.1, 0.01) were spotted on SD quadruple dropout media (-tryptophan, -leucine, -histidine, -adenine) containing 10 mM 3-amino-1,2,4-triazole (3-AT). Plates were incubated for 3 days at 30°C. The assay was also performed with either PACT/pGBK7 or FS/pGBK7 and TRBP/pGADT7 in a similar manner (TRBP/pGADT7 panels). Co-transformation of PACT or FS in pGBK7 and empty vector pGADT7 served as negative controls.
Figure 2.7: A schematic model of PKR activation in cells expressing FS mutant. (A) wt cells. As previously established, in the absence of stress, PACT heterodimerizes with TRBP, PKR is catalytically inactive and eIF2α is not phosphorylated. In response to a stress signal, PACT dissociates from TRBP due to its phosphorylation, forms homodimers that bind to PKR with high affinity, activate its kinase activity leading to eIF2α phosphorylation. (B) Cells expressing FS mutant protein. In the absence of stress, FS mutant protein heterodimerizes with TRBP with high affinity (and possibly also with PACT) to displace PACT from TRBP-PACT heterodimers. Consequently, PACT homodimers form due to high affinity interactions between FS mutant protein molecules. Such homodimers bind to PKR with high affinity and activate its kinase activity leading to eIF2α phosphorylation leading to apoptosis via caspase activation.
CHAPTER 3:

DYSTONIA 16 (DYT16) MUTATIONS IN PACT CAUSE ENHANCED PKR ACTIVATION LEADING TO A DYSREGULATION OF EIF2α SIGNALING AND A COMPROMISED STRESS RESPONSE

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3.1 ABSTRACT

Dystonia 16 (DYT16) is one of many types of dystonias, which are neuromuscular movement disorders characterized by involuntary movements and twisting or tensing of one or more muscles leading to abnormal and often painful postures. Although DYT16 can appear at any age, it most often is early-onset and begins in childhood. DYT16 is caused by mutations in PACT, the protein activator of interferon-induced double-stranded RNA-activated protein kinase (PKR). PKR is one of the four mammalian protein kinases that regulates the integrated stress response (ISR) via phosphorylation of the translation initiation factor eIF2α. This post-translational modification attenuates general protein synthesis while concomitantly triggering the eIF2α-independent enhanced translation of a few specific transcripts leading either to recovery and homeostasis or cellular apoptosis depending on the intensity and duration of stress signals. PKR plays a regulatory role in determining the cellular response to viral infections, oxidative stress, endoplasmic reticulum (ER) stress, and growth factor deprivation. In the absence of stress, both PACT and PKR are bound by their inhibitor transactivation RNA-binding protein (TRBP) thereby keeping PKR inactive. Under conditions of cellular stress these inhibitory interactions dissociate thus facilitating PACT-PACT interactions that are critical for PKR interaction and activation. While both PACT-TRBP and PKR-TRBP interactions are pro-survival, both PACT-PACT and PACT-PKR interactions are pro-apoptotic. We previously reported that the most prevalent DYT16 substitution mutation P222L activates PKR more robustly and for longer duration resulting in
enhanced apoptosis of patient cells in response to ER stress. To evaluate if the additional DYT16 mutations reported more recently also alter PKR activation and ISR, we examined the ability of five DYT16 mutants to activate PKR via these protein-protein interactions. Our results indicate that the mutant DYT16 proteins show stronger PACT-PACT interactions as well as enhanced PKR activation. Using DYT16 patient derived lymphoblasts we demonstrate that the enhanced PACT-PKR binding affinity and heightened PKR activation leads to a dysregulation of ISR and increased apoptosis in response to ER stress. More importantly, this enhanced sensitivity to ER stress can be partially rescued by luteolin, which disrupts PACT-PKR interactions. Our results not only demonstrate the impact of DYT16 mutations on regulation of ISR and a plausible disease etiology, but also indicate that therapeutic interventions for DYT16 could be possible in future after further careful research and evaluation of such strategies.

3.2 INTRODUCTION

Integrated stress response (ISR) is an evolutionarily conserved pathway activated in eukaryotic cells by many different types of stress stimuli in order to restore cellular homeostasis. The central event in this pathway is the phosphorylation of eukaryotic translation initiation factor 2 (eIF2α) on serine 51 by one of the four members of a family of serine/threonine kinases\(^1\). This post-translational modification prevents the formation of the ternary complex essential for translation initiation, thus leading to a decrease in general protein synthesis while allowing induction of selected specific genes that promote cellular recovery\(^1\). While transient eIF2α phosphorylation is favorable for cellular survival,
prolonged eIF2α phosphorylation is pro-apoptotic due to the upregulation as well as preferential translation of pro-apoptotic transcripts \(^1\). Thus, although ISR is primarily a pro-survival response to restore cellular homeostasis, exposure to severe stress drives signaling towards cellular death due to induction of genes that promote apoptosis. The gene expression program regulated by ISR is thus central to tailoring the cellular response to stress in a manner that is dependent on the cellular context, as well as the nature and severity of the stress signal.

The interferon (IFN) inducible double-stranded RNA (dsRNA)-activated protein kinase (PKR) is an eIF2α kinase that is ubiquitously expressed at low levels in all cell types \(^1\). While PKR’s kinase activity was initially characterized to be stimulated under conditions of viral stress, it has also been well documented to be active under conditions of oxidative stress, endoplasmic reticulum (ER) stress, and serum deprivation \(^1,13,17,18,35,36\). In virally infected cells, PKR is activated by direct interactions with dsRNA, a viral replication intermediate used by many viruses. However, in the absence of viral infections and in presence of other stress signals, PKR is activated by its protein activator (PACT) \(^13,14,25\) in a dsRNA-independent manner. Two evolutionarily conserved amino terminal dsRNA binding motifs (dsRBMs) of PKR \(^1,124,125\) mediate its interactions with dsRNA as well as with PACT and other regulatory proteins. Upon binding dsRNA or PACT, PKR undergoes a conformational change unveiling the ATP binding site within its kinase domain (KD) which results in the autophosphorylation and activation of PKR \(^1,15,31\). In the absence of stress, however, PKR is inhibited through direct interactions with the transactivation response element (TAR) RNA
binding protein (TRBP) which are also facilitated by the dsRBMs on the amino termini of each protein\textsuperscript{46,126}.

TRBP is a dsRNA binding protein that was initially discovered due to its strong binding affinity to the TAR RNA element of HIV\textsuperscript{126}. Since then, TRBP has been well characterized for its ability to inhibit PKR both by sequestration of dsRNA under conditions of viral infection, and by direct interactions with PACT as well as PKR mainly in the absence of cellular stress\textsuperscript{47,127,128}. These interactions are facilitated by the conserved dsRBM motifs of TRBP such that the two amino terminal motifs facilitate dsRNA and protein-protein interactions, while the third motif that does not bind dsRNA mediates interactions with dicer, merlin, and PACT\textsuperscript{42,43,46}. In the absence of stress, TRBP positively regulates translation by inhibiting PKR through the formation of both TRBP-PACT and TRBP-PKR heterodimers, consequently preventing PKR from being activated\textsuperscript{47,49,52}.

PACT is best characterized as a stress-modulated protein activator of PKR bringing about PKR activation via a direct, dsRNA-independent interaction in response to ER stress, oxidative stress, and serum deprivation\textsuperscript{25,35}. Similar to TRBP, PACT contains three copies of the conserved dsRBMs such that the two amino terminal motifs, dsRBM1 and 2, are critical for dsRNA binding and protein-protein interactions and a carboxy terminal dsRBM3 motif that does not bind dsRNA being required for PKR activation\textsuperscript{25,37,38}. Within dsRBM3, two serine residues, S246 and S287, serve as phosphorylation sites to induce PACT-PACT homomeric and PACT-PKR heteromeric interactions\textsuperscript{52,53}. In the absence of stress, PACT is constitutively phosphorylated on S246 and is bound to TRBP.
and thus is unable to activate PKR \(^{53}\). In response to cellular stress, PACT is phosphorylated on S287 by a yet uncharacterized kinase which promotes its dissociation from TRBP to trigger PACT-PACT homomeric interactions\(^{43,49,52,53}\). Such PACT-PACT homodimers are required for PKR activation and their formation is triggered by the stress-induced phosphorylation of PACT's dsRBM3\(^{49,52,53}\). The PACT-PACT homodimers with the S246 and S287 phosphorylations bind PKR with higher affinity thereby bringing about PKR activation\(^{49}\). The dsRBM3 of PACT is thus essential for PKR activation and interacts directly with the PACT-binding motif (PBM) within the KD of PKR \(^{15,31,49}\). Once activated, PKR phosphorylates the alpha subunit of eIF2 on serine 51 resulting in the attenuation of general protein synthesis\(^1\). This triggers all the downstream ISR events such as but not limiting to ATF4 and CHOP induction which in turn regulate cellular fate either restoring homeostasis or inducing apoptosis.

Recently, several PACT mutations have been identified in patients (OMIM: DYT16 (612067)), with a neuromuscular movement disorder dystonia 16 (DYT16) \(^{62,67,129}\). PACT is encoded by the gene \textit{Prkra} and currently the list of \textit{Prkra} mutations leading to DYT16 has grown to eight in a worldwide occurrence \(^{60,63,65}\). The dystonias are a heterogeneous group of movement disorders in which the affected individuals exhibit repetitive and painful movements of the affected limbs, as well as compromised posture and gait patterns\(^{59,99}\). DYT16 is a rare early-onset dystonia parkinsonism disorder characterized by progressive limb dystonia, laryngeal and oromandibular dystonia and parkinsonism. Although
DYT16 was originally described to have an autosomal recessive inheritance pattern, four dominantly inherited variants of DYT16 have been reported in patients after the initial report. Previously, our lab has reported on how a recessively inherited P222L mutation increases cell susceptibility to ER stress through the dysregulation of eIF2α stress response signaling in DYT16 patient derived lymphoblasts. Furthermore, using an *in-vitro* approach we have demonstrated that a dominantly inherited frameshift mutation expresses a truncated PACT protein that disrupts PACT-TRBP heterodimers resulting in an increase in PACT mediated PKR activation leading to an enhanced sensitivity to ER stress via dysregulation of the eIF2α stress response signaling pathway. In accordance with our findings, subsequent reports also identified the dysregulation of eIF2α signaling in both DTY1 and DYT6. Collectively, these findings indicate a potential common link among several forms of dystonia.

In the present study we characterize the effects of three recessively inherited DYT16 point mutations (C77S, C213F, C213R) and two dominantly inherited DYT16 point mutations (N102S and T34S) on their ability to regulate PKR activation and ISR. Our data demonstrates that although these DYT16 point mutations have no effect on PACT’s dsRNA binding ability and PACT-TRBP interactions, the dominant mutations show enhanced ability to interact with PKR. Most significantly, all the DYT16 mutations under study demonstrated a heightened capacity to form PACT-PACT homodimers in the absence of stress. Furthermore, using lymphoblasts derived from a compound heterozygous DYT16 patient containing both C213R and P222L mutations as independent alleles, we
identified stronger binding affinity between PACT and PKR in the DYT16 patient cells and a dysregulation of the eIF2α stress response signaling and downstream ISR genes. The DYT16 patient lymphoblasts also demonstrated an increase in cell susceptibility to ER stress that could be rescued in the presence of luteolin, a potent inhibitor of PACT-PKR interactions. Our work further strengthens the case for involvement of dysregulated eIF2α signaling as a mechanism in the disease etiology and lays the groundwork for exploring possible therapeutic options for DYT16.

3.3 MATERIALS AND METHODS

**Cell Lines and Antibodies**—Both HeLaM and COS-1 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% Fetal Bovine Serum and penicillin/streptomycin. wt and DYT16 Patient B-Lymphoblasts were cultured in RPMI 1640 medium containing 10% FBS and penicillin/streptomycin. Both wt and DYT16 patient lymphoblast cell lines were Epstein-Barr Virus-transformed to create stable cell lines as previously described. 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: anti-Cleaved-PARP monoclonal (CST, #32563), FLAG-HRP: anti-FLAG
monoclonal M2-HRP (Sigma A8592), MYC-HRP: anti-MYC monoclonal (Santa Cruz, 9E10), β-Actin: Anti-β-Actin-Peroxidase monoclonal (Sigma-Aldrich, A3854).

**Generation of DYT16 Point Mutations**—We generated each DYT16 mutant construct using site specific mutagenesis through PCR amplification changing the codon within the PRKRA gene to be consistent with DYT16 patients and code for the appropriate amino acid substitution. The following site-specific mutagenic primer pairs were used:

**C77S Sense:** 5'-'GCT CTA GAC ATA TGG AAA TGT CCC AGA GCA GGC AC-3'

**C77S Antisense:** 5'-'GCC TCT GCA GCT CTA TGT TTC GCC AGC TTC TTA CTT GTA CCT TCA CCT GTG GAG GTT ATG TCA CCA ACG G-3'

**C213F Sense:** 5'-'GCT CTA GAC ATA TGG AAA TGT CCC AGA GCA GGC AC-3'

**C213F Antisense:** 5'-'GGA GAA TTC CTC AAG GAA TGC CAA GTA AAT CCT AAA GAA TGT CC-3'

**C213R Sense:** 5'-'GCT CTA GAC ATA TGG AAA TGT CCC AGA GCA GGC AC-3'

**C213R Antisense:** 5'-'GGA GAA TTC CTC AAG GAA TGC CAA GTA C GT CCT AAA GAA TGT CC-3'

**N102S Sense:** 5'-'GCT GCA GAG GCT GCC ATA AAC ATT TTG AAA GCC AGT GCA AGT ATT TGC TTT GC -3'

**N102S Antisense:** 5'-'GGG GAT CCT TAC TTT CTT TCT GCT ATT ATC-3'
**T34S Sense:** 5′-GCT CTA GAC ATA TGG AAA TGT CCC AGA GCA GGC AC-3′

**T34S Antisense:** 5′-CGT GTA ATA CCT GAA TCG GTG ATT TCC CTG GCT TAG C-3′

To generate each construct, we performed PCR amplification in order to change the corresponding codon sequence to code for the amino acid residue consistent with the DYT16 patients. Each PCR product was then subcloned into pgEMT-easy vector (Promega) and sequences were validated through DNA sequencing from Eton Biosciences. After sequence validation, we generated full length DYT16 ORFs through cutting: (i) partial DYT16 ORF in pgEMT-easy with construct specific restriction enzymes, (ii) Amino terminal FLAG or Myc-tagged wt PACT in BSIIKS+ with compatible restriction sites. Cloning scheme was as follows: C77S in pgEMT-easy cut with NdeI-PstI ligated into FLAG/Myc-PACT-BSIIKS+ cut with PstI-BamHI. C213F and C213R in pgEMT-easy cut with NdeI-EcoRI ligated into FLAG/Myc-PACT-BSIIKS+ cut with EcoRI-BamHI. N102S in pgEMT-easy cut with NdeI-PstII and ligated into FLAG/Myc-PACT-BSIIKS+ cut with PstI-BamH1. T34S in pgEMT-easy cut with NdeI-TfiI ligated into FLAG/Myc-PACT-BSIIKS+ cut with TfiI-BamHI. Once full length DYT16 ORFs were generated with amino terminal FLAG or myc tags we then subcloned each ORF into pCDNA3.1- using XbaI-BamHI restriction sites. All DYT16 constructs were also cloned into mammalian two-hybrid system vectors and pET15b (Novagen) using NdeI-BamH1 restriction sites. TRBP and Flag-K296R (PKR) constructs were generated as previously described49.
Expression and purification of PACT from E. coli – The ORFs of both wt PACT and all DYT16 point mutations were subcloned into pET15b (Novagen) to generate an in-frame fusion protein with a hexahistidine (his) tag. Recombinant proteins were then expressed and purified as previously described.25

dsRNA Binding Assay – Both wt PACT and DYT16 PACT constructs in pCDNA3.1- were in-vitro translated using the TNT-T7-coupled rabbit reticulocyte system from Promega while incorporating an 35S-Methionine radiolabel and the dsRNA binding ability was measured using poly(I:C) conjugated agarose beads. We diluted 4 ul of in-vitro translation in 25 ul of binding buffer (20 mM Tris–HCl, pH 7.5, 0.3 M NaCl, 5 mM MgCl₂, 1 mM DTT, 0.1 mM PMSF, 0.5% NP-40, 10% glycerol) and incubated in 25 ul of poly(I:C)-agarose beads and incubated at 30°C for 30-minutes. We then washed the beads 4 times with 500 ul of binding buffer and bound proteins were analyzed via SDS-PAGE gel electrophoresis and autoradiography. The competition assay was performed incubating either soluble single-stranded RNA, poly(C), or dsRNA, poly(I:C), with the in-vitro translated proteins before the adding the poly(I:C)-agarose beads. To ensure the presence of PACT was due to the dsRNA binding capacity we assayed in-vitro translated 35S-Methionine labeled firefly luciferase which has no dsRNA binding ability. T-lanes represent total in-vitro translated radiolabeled proteins, whereas, B-lanes represent fraction of labeled protein that remained bound to poly(I:C)-agarose beads after washing. Bound fraction was quantified using Typhoon FLA7000 by analyzing relative band intensities of both T and B-lanes. Percentage of PACT
was bound to beads was then determined by dividing values obtained from B-lanes by values obtained by T-lanes and then plotted as bar graphs.

**PKR Activity Assay** – HeLa M cells were treated with IFN-β for 24-hours and harvested at 70% confluency. Cells were washed using ice-cold PBS and centrifuged at 600 g for 5-minutes. Cells were then 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 then centrifuged at 10,000 g for an additional 5-minutes. PKR was then immunoprecipitated from 100 ug of this lysate using anti-PKR monoclonal antibody (R&D Systems Technology: 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°C on a rotating wheel for 30-minutes. We then added 10 ul of Protein A-Sepharose beads to each immunoprecipitate followed by an additional 1-hour incubation under the same conditions. Protein A-Sepharose beads were then 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, 2 mM MnCl₂, 100 U/ml aprotinin, 0.1 mM PMSF, 5% glycerol). PKR activity assay using PKR bound to protein A-Sepharose beads was then conducted by incorporating: 500 ng of purified eIF-2 as the PKR substrate, 0.1 mM ATP, 10 uCi of [γ-³²P] ATP, and increasing amounts of either recombinant wt PACT or recombinant DYT16 PACT (400 pg – 4 ng) as the PKR activator. Reaction was
then incubated at 30°C for 10 min and resolved on a 12% SDS-PAGE gel and analyzed via autoradiography.

**Western Blot Analysis** – Lymphoblasts derived from a compound heterozygous DYT16 patient containing both P222L and C213R mutations on independent alleles were cultured alongside lymphoblasts derived from a family member containing no mutations in PACT as our control cells. Cells were cultured 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 (Sigma) and phosphatase inhibitor (Sigma). Concentration of total protein extract was then determined through BCA assay and appropriate amounts resolved on SDS-PAGE denaturing gels to detect the proteins of interest. Proteins were detected via chemiluminescence and antibodies indicated above.

**Co-Immunoprecipitation Assays with Endogenous Proteins** – For co-immunoprecipitation (co-IP) of endogenous proteins DYT16 and wt lymphoblasts were seeded at a concentration of 300,000 cells/mL in RPMI complete media and treated with 50 μM of luteolin (Santa Cruz) over a 24 hour time course. Cells were then harvested and whole cell extract was immunoprecipitated using anti-PKR antibody (71/10, R&D Systems) conjugated to protein A sepharose beads (GE Healthcare) overnight at 4°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).

Immunoprecipitation was carried out using 100 ng of anti-PKR antibody and 10 ul of protein A sepharose beads slurry per immunoprecipitation.

Immunoprecipitates were then washed 3 times in 500 ul of IP buffer followed by resuspension and boiling for 5 minutes in 2x Laemmli buffer (150 mM Tris–HCl pH 6.8, 5% SDS, 5% β-mercaptoethanol, 20% glycerol). Samples were then 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. Input blots of whole cell extract without immunoprecipitation are shown to indicate equal amounts of protein in each sample.

**Co-Immunoprecipitation Assays in HeLa Cells** – In all cases HeLa M cells were seeded at 20% confluency in 6-well dishes 24-hours prior to co-transfecting 250 ng of each flag or myc tagged construct using Effectene transfection reagent (Qiagen). Cells were harvested 24-hours post transfection in IP buffer. Whole cell extract was then immunoprecipitated overnight at 4°C on a rotating wheel in either pre-conjugated anti-flag agarose beads (Sigma) or pre-conjugated anti-myc agarose beads (Thermo Scientific). Immunoprecipitates were then washed 3-5 times in IP buffer followed by resuspension and boiling for 5 minutes in 2x Laemmli buffer. Samples were then resolved on 10% SDS-PAGE denaturing gels and transferred onto PVDF membranes. To evaluate PACT-PACT homodimerization and PACT-TRBP heterodimerization, flag-tagged constructs were immunoprecipitated using 15 ul of pre-conjugated flag agarose
beads and blots were initially probed with anti c-myc antibody to detect co-IP (PACT), followed by re-probing with anti-flag antibody to detect efficiency of IP (PACT or TRBP). PACT-PACT homodimerization co-IPs were incubated at 50°C for 30 minutes in stripping buffer (62.5 mM Tris-HCl pH 6.8, 10% SDS, 0.75% β-mercaptoethanol) prior to re-probing with anti-flag antibody. To evaluate PACT-PKR interactions, we co-transfected myc-tagged PACT constructs in pCDNA3.1 with a flag tagged dominant negative PKR mutant, K296R, also in the pCDNA3.1- backbone. We then immunoprecipitated whole cell lysates in 15 ul of pre-conjugated myc-agarose beads and resolved on 10% SDS-PAGE denaturing gels as described above. Blots were initially probed with anti-flag antibody to detect co-IP (PKR) followed by re-probing with anti-myc antibody to determine equal amount of IP (PACT) per sample. Input blots of whole cell lysate exempt from immunoprecipitation are shown to demonstrate equal expression of each construct prior to immunoprecipitation.

**Mammalian-2-Hybrid** – In all cases, wt PACT, DYT16, TRBP, or PKR ORFs were subcloned 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). COS-1 cells were then transfected with: (i) 200 ng each of the GAL4-DBD and the VP16-AD constructs, (ii) 200 ng of pg5Luc (Firefly luciferase) reporter construct, (iii) 1 ng of pRL Null vector (Renilla luciferase) (Promega) to normalize for transfection efficiencies. Cells were then harvested 24-hours post transfection.
and assayed for both firefly and renilla luciferase activity 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 media and treated with a concentration of 5 ug /mL of tunicamycin over a 24-hour time course. Samples were collected at each indicated time point and mixed with equal parts Promega Caspase-Glo 3/7 reagent (Promega (G8090)) and incubated for 45 minutes. Luciferase activity was then measured and compared to cell culture medium alone as our negative control. All values were normalized to luciferase activity detected in negative control. 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 ug /mL of tunicamycin in luteolin free media over the same time course.

### 3.4 RESULTS

**DYT16 mutations do not affect PACT’s dsRNA-binding activity.** The majority of DYT16 mutations characterized in the present study occur outside of PACT’s highly conserved dsRBMs (Fig. 3.1 A). Four of the mutations associated with the recessively inherited DYT16 (C77S, C213F, C213R, and P222L) result in the loss of a cysteine or proline residues which could have dramatic consequences on the 3-dimensional conformation of the protein (Fig. 3.1 A). Furthermore, the two dominantly inherited mutations (N102S and T34S) occur on
the flanking ends of PACT’s first dsRBM that is most critical for dsRNA binding and protein-protein interactions (Fig. 3.1 A). As seen in Fig. 3.1 B and C, the DYT16 point mutants show no change in their dsRNA binding capabilities in comparison to the wt PACT (lanes 1-14). In order to ascertain the specificity of the dsRNA-binding assay, we used in vitro translated firefly luciferase, which has no dsRNA-binding activity as a negative control (lanes 19-20). Additionally, we demonstrate the specificity of the interaction for dsRNA by adding excess dsRNA or ssRNA as competitors. As seen in lanes 15-18, the binding to dsRNA immobilized on beads can be effectively competed by exogenously added dsRNA but not single-stranded (ss) RNA (lanes 15-18).

**DYT16 mutants activate PKR more efficiently.** PACT is best characterized for its ability to activate PKR under conditions of cellular stress. Therefore, we next evaluated the consequence of each of the DYT16 mutations for PACT’s ability to activate PKR using an in vitro PKR activity assay. His tagged wt PACT and DYT16 mutant proteins were expressed and purified from bacterial cells using nickel affinity chromatography. The purified recombinant proteins were used as activators in an in vitro PKR activity assay by adding increasing amounts to PKR immunoprecipitated from HeLa cells. We are then able to determine efficiency of PKR activation by comparing PKR autophosphorylation in the presence of wt PACT and the various PACT mutants (Fig. 3.2 A). Basal levels of activated PKR are observed in lanes 1 and 10 (upper panel) and lanes 1 and 8 (lower panel) in the absence of any added activator. When the purified recombinant PACT proteins are added, a dose dependent
increase (left: 400 pg, right: 4.0 ng) in activated autophosphorylated PKR is observed (lanes 2-9, 11-16 for upper panel and lanes 2-7, 9-12 for lower panel). The amount of radioactivity present in PKR bands was quantified using a phosphorimager analysis and is shown in Fig. 3.2 B. In all cases, recessive mutations demonstrated a slightly increased capacity to activate PKR (Fig. 3.2 A, lanes 4-12 and Fig. 3.2 B) as compared to wt PACT (Fig. 3.2 A, lanes 2-3). Interestingly, when tested in combinations as reported in DYT16 patients, the recessive mutants showed significantly enhanced ability to activate PKR (Fig. 3.2 A upper panel lanes 13-16, and Fig. 3.2 B) The dominant mutants (lower panel) showed enhanced ability to activate PKR only at 4 ng but not at 400 pg (Fig. 3.2 A, lower panel lanes 4-7). Interestingly, when tested in combination with wt PACT, both the dominant mutants demonstrated significantly higher PKR activation (Fig. 3.2A lower panel: lanes 9-12, and Fig. 3.2 B). These results indicate that the DYT16 point mutants have enhanced ability to activate PKR as compared to wt PACT.

**DYT16 patient derived lymphoblasts are more susceptible to ER stress.** As the DYT16 mutant proteins exhibited an increased ability to activate PKR, we next utilized the lymphoblast lines derived from a DYT16 patient and his normal, wt parent to determine the effect of one particular DYT16 mutation combination on cell viability in response to stress. It is important to note that DYT16 is a rare, early-onset movement disorder and patient cells are not available from most of the DYT16 patients. Here we characterize the effect of ER stress on DYT16 patient derived lymphoblast cells expressing both P222L and
C213R mutations on independent alleles. We compared these cells to wt lymphoblast cell lines derived from an unaffected family member. Consequently, we utilized the ER stress inducing agent, tunicamycin (TM), which results in the accumulation of misfolded proteins in the ER due to inhibition of protein glycosylation\textsuperscript{17}. In case of wt lymphoblasts, over a 24-hour time course in response to TM treatment we observed a marginal increase in expression of cleaved PARP1, a marker of cellular apoptosis (Fig. 3.2 C, lanes 6-7) indicating efficient recovery and homeostasis. In contrast to this, in the DYT16 patient derived lymphoblasts, there was a dramatically significant increase in cleaved PARP1 in response to tunicamycin (Fig. 3.2 C, lanes 13-14). To further validate these results, we performed caspase 3/7 activity assays under the same conditions to measure cell apoptosis. In wt lymphoblasts we detect caspase activity at 24 h but not at 6 h post-treatment (Fig. 3.2 D, blue bars). In contrast, the DYT16 patient lymphoblasts demonstrate significantly elevated caspase activity at 6h which further increases at 24 h post-treatment (Fig. 3.2 D, red bars). Thus, further supports that the DYT16 patient lymphoblasts are significantly more susceptible to ER stress and exhibit increased apoptosis as compared to wt cells possibly due to a failure to restore homeostasis.

**eIF2α phosphorylation and ISR is dysregulated in DYT16 patient lymphoblasts.** In order to elucidate the underlying mechanism driving heightened sensitivity to ER stress in DYT16 lymphoblasts, we performed western blot analysis on cells treated under the same conditions as figure 2C-D probing for markers of cellular stress response (Fig. 3.3). We compared the
kinetics of both eIF2α phosphorylation and PKR activation in the DYT16 lymphoblasts to the wt lymphoblasts from the unaffected family member. In wt lymphoblasts (left) we observe a low basal level of eIF2α phosphorylation in the untreated cells (Fig. 3.3 A, lane 1) followed by increased eIF2α phosphorylation at 1-4 hours post treatment (lanes 2-4) and then restoration to basal levels by 8 hours (lane 5). In contrast to this, in the DYT16 lymphoblasts (right), we observe a similar increase in eIF2α phosphorylation 1 hour after treatment (lane 7), however, the eIF2α phosphorylation is sustained even at 8-hours post treatment (lanes 8-10). We also studied the time course of PKR activation in DYT16 patient lymphoblasts under the same conditions. In wt lymphoblasts (left) we observe PKR activation at 1 hour after TM treatment that is sustained until 4 hours (lanes 1-4) and shows a slight decrease by 8 hours (Fig. 3.3 A). In contrast to this, the DYT16 lymphoblasts (right) exhibit a dramatically elevated level of activated PKR even in untreated cells (lane 6) that does not show any stress-dependent increase after treatment with TM (lanes 7-10). As we noted the differences in eIF2α and PKR phosphorylation responses between wt and DYT16 lymphoblasts, we examined if the downstream effects of eIF2α phosphorylation also show similar differences. In wt lymphoblasts (left), ATF4 is undetectable in untreated cells (Fig. 3.3 B, lane 1) and its expression increases in a time dependent manner from 1-8 hours post treatment (lanes 2-5) and declines at 12 and 24 hours after treatment (lanes 6-7). In contrast, in the DYT16 patient lymphoblasts (right) although we observe increased expression of ATF4 from 1-8 hours post treatment (lanes 9-11), it persists at high levels even at 12 hours post
treatment and shows only a small decline at 24 hours after treatment. Finally, we compared levels of CHOP, an ATF4-induced pro-apoptotic protein, in response to TM treatment in wt and DYT16 lymphoblasts. Collectively these results demonstrate a dysregulation of ISR pathway due to the prolonged phosphorylation of eIF2α, elevated levels of activated PKR, the prolonged elevated levels of ATF4 translation, and delayed but sustained induction of CHOP.

**Effect of DYT16 mutations on PACT-PKR interactions.** In light of the heightened basal levels of PKR activation observed in the DYT16 patient cells (Fig. 3.3 A), we next wanted to investigate the effect of these DYT16 mutations on PACT-PKR interactions. To address this, we performed co-immunoprecipitation (co-IP) assays using cells expressing a combination of myc-epitope tagged wt or DYT16 mutant PACT and flag-epitope tagged PKR. PKR is expressed at low basal levels in cells and both increased PKR activation and increased PKR expression levels are toxic to cells due to increased apoptosis. Thus, in order to evaluate PACT-PKR heterodimer formation we utilized an expression vector encoding Flag-tagged K296R, a catalytically inactive, PKR mutant, which has been established to affect PKR’s kinase activity without affecting PACT-PKR interactions. Previously our lab has reported that the recessively inherited DYT16 mutation, P222L, shows an increased ability to form PACT-PKR heterodimers relative to wt PACT\(^69\). Here our results show that the other recessively inherited mutations (C77S, C213F, and C213R) show no difference in their ability to interact with PKR relative to wt PACT (Fig. 3.4 A,
lanes 7-10). In the absence of myc-PACT, no flag-PKR is immunoprecipitated confirming that there is no non-specific binding of flag-PKR to the beads in the absence of myc-PACT (co-IP panel, lane 6). Lanes 1-5 demonstrate equal amounts of Myc-PACT proteins were immunoprecipitated in each lane (top panel) while input gels (lower panel) demonstrate equal expression from each myc-PACT expression construct (lanes 2-5) and Flag-PKR (lanes 6-10). In contrast, we do observe an increase in the PACT-PKR heterodimer formation in case of dominantly inherited mutations (N102S and T34S) under the same conditions (Fig. 3.4 B). As compared to wt PACT (lane 2), co-IP of the dominant mutants N102S and T34S (lanes 3-4) is significantly increased. No co-IP of myc-PACT is seen in the absence of flag-PKR (lane 1), thus demonstrating that there is no non-specific interaction of PACT proteins with the beads in the absence of flag-PKR. Lanes 6-8 (upper IP panel) demonstrate equal amounts of flag-PKR was immunoprecipitated in each lane, while input panels demonstrate equal expression of all constructs (lower panel, lanes 1-4, and 6-8).

In order to validate the co-immunoprecipitation results, we tested the PACT-PKR interactions using the mammalian-two-hybrid (M2H) assay. In agreement with co-IP data, our results demonstrate that the recessively inherited mutations C77S, C213F and C213R have no difference in their ability to interact with PKR (Fig. 3.4 C). Consistent with our previously reported data, the P222L mutant demonstrates a stronger binding to PKR as indicated by greater induction of the luciferase reporter gene compared to wt PACT (Fig. 3.4 C). In the case of the P222L mutation, we observed about 2.5-fold increase in the PKR interaction
as compared to wt PACT, whereas, the other recessive mutants have similar PKR interaction as the wt PACT. Similarly, our results from the co-IP data were confirmed in case of the dominant mutations (Fig. 3.4 D). The T34S mutant showed about 2.25-fold increase and the N102S mutant showed about 4.25-fold increase in PKR interaction relative to wt PACT (Fig. 3.4 D).

*Effect of DYT16 mutations on PACT-PACT interactions.* PACT-PACT interactions are critical for the interaction with and activation of PKR. Consequently, using the same protein-protein interaction studies outlined in Fig. 3.4 we addressed whether PACT-PACT interactions were affected by the DYT16 mutations (Fig. 3.5). We co-expressed myc- or flag-epitope tagged PACT proteins by transient transfection of the respective expression constructs in combinations as reported in patients. Our data shown in Fig. 3.5 A-C demonstrates that all DYT16 mutants show a dramatic increase in their ability to form PACT-PACT homodimers in the absence of stress as compared to wt PACT (Fig. 3.5). In the case of the recessively inherited mutations, we observe minimal wt PACT homodimerization (Fig. 3.5 A, lane 2) with this being variable and no interaction being detected in few experimental repeats as it is established that in the absence of stress, PACT-PACT dimerization is usually absent. The recessively inherited DYT16 mutations show enhanced C77S-C213F and P222L-C213R interactions as compared to wt PACT-wt PACT interactions (compare lanes 3-4 to lane 2). In case of the dominantly inherited mutations we tested their ability to form wt PACT-mutant dimers (Fig. 3.5 B), as well as mutant-mutant dimers (Fig. 3.5 C). We did not observe any wt PACT homodimerization in the
absence of stress (Fig. 3.5 B and C, lane 2), however, both the dominant DYT16 mutants N102S and T34S showed enhanced interaction with wt PACT (Fig. 3.5 B, lanes 3-4) with N102S showing the strongest interaction with wt PACT. When evaluating these dominant mutations for their ability to interact with themselves, we observe very strong interaction between N102S-N102S and T34S-T34S (Fig. 3.5 C, lanes 3-4) as compared to wt PACT-wt PACT with the strongest interaction being T34S-T34S. We do not observe any co-IP of myc tagged wt PACT in the absence of flag tagged wt PACT (lane 1) demonstrating the absence of any non-specific binding to the beads (Fig. 3.5 A-C). The IP panels show that equal amounts of flag-tagged PACT protein is immunoprecipitated in each lane (Fig. 3.5 A-C, upper panel, lanes 5-8), and input blots indicate equal expression of each construct (Fig. 3.5 A-C, lower panel, lanes 1-8).

To further confirm our co-IP data we tested the interaction between DYT16 PACT mutants utilizing the M2H (Fig. 3.5 D-E). As seen in Fig. 3.5 D and E, in the patient specific combinations all the recessive mutants show enhanced interactions relative to wt PACT-wt PACT interaction (Fig. 3.5 D). The P222L-C213R and C213F-C77S interactions are ~5-fold and ~9-fold higher than wt PACT-wt PACT interaction respectively (Fig. 3.5 D). Furthermore, the dominant mutants T34S and N102S also show enhanced interactions (Fig. 3.5 E). The wt PACT-T34S and N102S-wt PACT interactions are ~10-fold and ~30-fold higher than wt PACT-wt PACT interactions. Finally, the T34S-T34S and N102-N102S interactions are enhanced ~10-fold and ~20-fold respectively compared to wt PACT-wt PACT interactions.
PACT’s ability to interact with TRBP is not affected by the DYT16 mutations. In the absence of stress TRBP binds PACT thereby preventing the formation of PACT-PACT homodimers that result in PKR interaction and activation. Thus, changes in PACT’s interaction with TRBP can consequentially affect PKR activation. Previously, our lab has reported that the recessively inherited DYT16 P222L mutation increases PACT’s binding affinity to TRBP ultimately resulting in delayed PKR activation. We determined the consequence of the DYT16 mutations under study on PACT-TRBP heterodimer formation and our results indicate that the recessively inherited mutations, C77S, C213F, and C213R (Fig. 3.6 A, lanes 2-5) as well as the dominantly inherited N102S and T34S mutations (Fig. 3.6 B, lanes 2-4) have equal binding affinity to TRBP relative to wt PACT. As we do not detect the presence of myc-wt PACT in the absence of flag-TRBP expression (lane 1, Fig. 3.5 A and B) we can rule out any nonspecific binding of PACT to the beads. Finally, immunoprecipitation blots indicating that equal amount of myc-TRBP protein was immunoprecipitated (Fig. 3.5 A and B, IP panels, lanes 7-10 and lanes 6-8) and input blots demonstrating equal protein expression are shown (Fig. 3.5 A and B, input panels, lanes 1-10 and lanes 1-8).

We also validated these results using the M2H to determine the relative strengths of PACT-TRBP interactions. Consistent with our previously reported data, the P222L mutation shows ~2-fold increase in interaction with TRBP relative to wt PACT (Fig. 3.6 C). The other recessively inherited mutations, however, show no difference in their ability to interact with TRBP relative to wt
PACT (Fig. 3.6 C). Consistent with the co-IP data, we also do not observe any difference in the PACT-TRBP interaction for the dominantly inherited mutations relative to wt PACT (Fig. 3.6 D).

**DYT16 Patient lymphoblasts show a stronger PACT-PKR interactions and its disruption rescues their higher sensitivity to ER stress.** In light of the increased PACT-PACT interaction independent of cellular stress observed in fig. 5 and the elevated basal levels of activated PKR observed in fig. 3, we next wanted to investigate if the PACT-PKR interaction in patient derived lymphoblasts is also stronger as compared to wt lymphoblasts derived from the unaffected family member. In order to address this question, we treated these cells with luteolin, a flavonoid that has been established to efficiently inhibit or disrupt PACT-PKR interactions\textsuperscript{134}. As seen in Fig. 3.7 A, in the wt lymphoblasts we can detect some PACT-PKR interaction (upper panel) prior to luteolin treatment (lane 2), and at 1-hour after luteolin treatment PACT-PKR interactions are barely detectable (lane 3). We observe a time dependent decrease in the PACT-PKR interaction from 1-8 hours (lanes 3-5), and at 24h after luteolin treatment the interaction can no longer be detected (lane 6). In the DYT16 patient lymphoblasts, we observe much higher PACT-PKR interaction prior to luteolin treatment (lane 8) and the interaction persists until 2 hours (lanes 9-10) and then decreases slowly at 4 and 8 hours after luteolin treatment (lanes 11-12). We do see the loss of PACT-PKR interactions at 24 hours after treatment in the DYT16 patient lymphoblasts (lane 12). Immunoprecipitation blots (lower panel) demonstrate equal amounts of PKR were immunoprecipitated in all lanes (lanes 1-12), and the input blots
demonstrate that equal amount of protein was present in all immunoprecipitations. We do not detect the presence of PACT or PKR in samples incubated overnight in the absence of PKR antibody thus demonstrating that there is no nonspecific binding of PACT to the beads in the absence of PKR immunoprecipitation (lanes 1 and 7). These results confirm that PACT-PKR interaction is stronger in DYT16 patient cells as compared to the wt cells and that a 24 h treatment with luteolin disrupts the interaction in wt as well as DYT16 cells.

Prevalence of PACT-TRBP heteromeric interactions promote cell survival while both PACT-PACT homomeric and PACT-PKR heteromeric interactions promote apoptosis. Our data indicates that there is an increase in both PACT-PACT (Fig. 3.5) and PACT-PKR interactions (Fig. 3.4 and Fig. 3.7 A), and the DYT16 patient lymphoblasts are more susceptible to ER stress induced apoptosis (Fig. 3.2 D). Therefore, we next wanted to determine if disrupting the PACT-PKR interaction in the DYT16 patient lymphoblasts would lead to an increase in cell viability in response to ER stress. As a 24 h treatment with luteolin of both wt control and DYT16 patient lymphoblasts could completely disrupt PACT-PKR interactions (Fig. 3.7 A, lanes 6 and 12), we tested if prior luteolin treatment would be protective for DYT16 patient lymphoblasts after ER stress. As seen in Fig. 3.7 B, the wt control lymphoblasts we do not detect any caspase 3/7 activity in our untreated samples or at 6-hours after TM treatment but there is a significant increase in caspase activity is detected at 24 h post treatment (Fig. 3.7 B, blue bars). The cells treated for 24 hours in luteolin prior to
TM treatment show a marked reduction in caspase 3/7 activity (Fig. 3.7 B, red bars). In contrast, the DYT16 patient cells show higher basal levels of caspase 3/7 activity prior to TM treatment that is further enhanced at 6 hours post-treatment. We see the most abundant caspase 3/7 activity in DYT16 cells treated for 24-hours in TM (Fig. 3.7 B, blue bars) that is dramatically reduced when cells are treated with luteolin 24-hours prior to treatment (Fig. 3.7 B, red bars). These results demonstrate that disrupting PACT-PKR interactions with luteolin in DYT16 cells can protect the cells from ER stress-induced apoptosis.

3.5 DISCUSSION

DYT16 is an early-onset, generalized dystonia caused by mutations in the Prkra gene, which encodes for PACT\textsuperscript{85}, a stress-modulated activator of PKR\textsuperscript{35}. In response to cellular stress, PACT activates PKR leading to eIF2\textalpha phosphorylation and inhibition of general protein synthesis\textsuperscript{35}. Although primarily a protective response to restore homeostasis, if PKR remains active for prolonged periods it triggers cell death via apoptosis\textsuperscript{17,105}. Our previous work has established that the regulation of PKR activation in response to stress depends on shifting the PKR inhibitory (PACT-TRBP and TRBP-PKR) interactions to PKR-activating (PACT-PKR and PACT-PACT) interactions soon after the cell encounters the initial stress signal\textsuperscript{135}. This is regulated by stress-induced PACT phosphorylation at serine 287, which dissociates PACT from TRBP and allows for its interaction with PKR\textsuperscript{52,136}. Previously we investigated the effects of a recessively inherited DYT16 missense mutation P222L on PACT-induced PKR activation in response to ER stress\textsuperscript{69}. Our results indicated that P222L activates...
PKR more robustly and for longer duration but with initial lag and slower kinetics as compared to wt PACT. In addition, the affinity of PACT-TRBP, PACT-PACT as well as PACT-PKR interactions was also enhanced in DYT16 patient lymphoblasts homozygous for P222L mutation. The initial lag in PKR activation and eIF2α phosphorylation was due to stronger TRBP-PACT interaction ultimately leading to a delayed and prolonged intense PKR activation due to stronger PACT-PACT and PACT-PKR interactions causing enhanced cellular death. In addition, our previous work on a dominant frameshift DYT16 mutation that results in truncation of the PACT protein after 88 amino acids also demonstrated a dysregulation of PACT-PKR-eIF2α pathway. The truncated mutant PACT protein formed aggregates in cells and caused PKR activation by displacing TRBP from PACT-TRBP complexes to promote PACT-PKR interaction, eIF2α phosphorylation, caspase activation and apoptosis.

In the present study we evaluated the effects three recessive (C77S, C213R, and C213F) and two dominant (T34S and N102S) DYT16 mutations on PKR activation. Our results establish that similar to two previously examined DYT16 mutants, dysregulation of ISR is also a common feature of all five DYT16 mutations. However, there are some important differences in the mechanism by which the dysregulation of ISR occurs. Similar to the P222L mutant, all five mutants show stronger PACT-PACT interactions as well as enhanced PKR activation. But unlike the previously studied P222L and the frameshift DYT16 mutants, none of these five mutants exhibited any changes in PACT-TRBP interactions. The recessive mutants tested in combinations as
found in DYT16 patients, as well as the two dominant mutants exhibited marked enhancement of PACT-PACT interactions in both co-IP and mammalian two hybrid assays (Fig. 3.5). Using DYT16 lymphoblasts from a compound heterozygote patient, we observe that the enhanced PACT-PKR interactions (Fig. 3.7) and elevated PKR kinase activity (Fig. 3.2 A and B) leads to a dysregulation of ISR and increased apoptosis in response to ER stress (Fig. 3.2, C and D).

These results further strengthen the case for a maladaptive ISR as disease etiology for DYT16. Our previous study on the DYT16 patient cells carrying a homozygous P222L mutation was the first report on dysregulated eIF2α signaling in any type of dystonia69. Subsequently DYT174,121, DYT6121 as well as DYT11138 studies also suggested the maladaptive ISR pathway as a point of convergence for neuronal dysfunctions observed in dystonia. Two independent studies support the involvement of aberrant eIF2α signaling in brain to DYT1 synaptic defects. Using an unbiased proteomics approach abnormal eIF2α pathway activation in DYT1 mouse and rat brain was identified, which also correlated with human brain samples121. Rittiner et al. used an RNAi-based functional genomic screening in HEK293T cells that also indicated dysregulated eIF2α pathway in DYT1. Moreover, in this study, pharmacological restoration of eIF2α signaling was reported to restore the cortico-striatal long term synaptic depression (LTD) in DYT1 knock-in mice74. In addition, this report also examined patients with focal cervical dystonia and reported sequence variants in ATF4, which is a direct target of eIF2α signaling74. RNA-Seq analysis to identify the
effect of heterozygous DYT6 Thap1 mutations on the gene transcription signatures in neonatal mouse striatum and cerebellum identified eIF2α signaling as one of the top dysregulated pathways. The neuronal plasticity defects in DYT6 could also partially be corrected by salubrinal, a selective inhibitor of the eIF2α phosphatase which downregulates the ISR in a timely manner\textsuperscript{121}. A gene-expression analysis in adult cerebellar tissue from a mouse model of DYT11 also has identified genes associated with protein translation among the top down-regulated mRNAs\textsuperscript{138}.

Stress-induced eIF2α phosphorylation by any of the four ISR kinases results in a suppression of general translation, but at the same time selectively stimulates the translation of some specific mRNAs\textsuperscript{139}. Typically, these mRNAs have long 5’-UTR with complicated secondary structure and one or more short upstream open reading frames (uORFs). Such mRNAs are preferentially translated when eIF2α is phosphorylated and initiation from other mRNAs is suppressed. Thus, eIF2α phosphorylation during cell stress not only achieves conservation of energy by a reduction of total translation but also allows new synthesis of a few proteins such as transcription factors ATF4 and CHOP whose translation is upregulated by eIF2α phosphorylation\textsuperscript{140,141}. These in turn induce the transcription of several genes either coding for ER enzymes and chaperones to cope with the accumulated unfolded proteins in the ER, or trigger apoptosis when homeostasis cannot be achieved due to intense or prolonged stress\textsuperscript{142}. The dysregulation of ISR observed in DYT16 patient lymphoblasts although present in all cell types of the patients, it is likely to be especially detrimental to
neuronal function. There is large amount of evidence indicating that in neurons, eIF2α phosphorylation driven translational changes are an essential feature of normal neuronal functions in the absence of stress and all four eIF2α kinases participate either individually, synergistically or even interchangeably in regulating neuronal activity\textsuperscript{143}. The eIF2α phosphorylation dependent translation regulation allows the neurons to quickly change protein compositions at the synapse in a stimulus-dependent manner, and such regulation is known to be important for maintaining healthy neuronal functions. For example, ATF4, which presumably is the most important protein known to be regulated at translational level by eIF2α phosphorylation, is known to be associated with regulation of neuronal activity in the absence of stress\textsuperscript{144}. When PKR-mediated eIF2α phosphorylation was specifically increased in hippocampal CA1 pyramidal cells by a chemical inducer, ATF4 expression increased significantly\textsuperscript{145}. Increased levels of ATF4 led to impairment of hippocampal long-term potentiation (L-LTP) and memory consolidation. Despite the well-established role of ATF4 as a suppressor of synaptic plasticity, it has to be understood that the changes in ATF4 concentrations are complicated and sometimes can be bidirectional. For example, the GCN2\textsuperscript{-/-} mice have decreased eIF2α phosphorylation, and thus have decreased ATF4 in hippocampal neurons\textsuperscript{146}. These mice showed strong and sustained L-LTP and their spatial memory was improved compared to control wt mice. Thus, it may seem that low levels of ATF4 make neurons more sensitive to stimulation and their potentiation occurs too easily. It is certainly possible that neuronal activity-dependent shifts in ATF4 levels are important for
LTP to take place normally. Any perturbation in such shifts, in either direction due to lower or higher ATF4 may be detrimental for normal neuronal functions. This becomes relevant to dystonia as both higher and lower ATF4 levels seem to be detrimental in different forms of dystonia. Rittiner et al observed reduced ATF4 induction in DYT1 cells and also identified the presence of inactivating mutations in ATF4 in sporadic cervical dystonia patients. In our DYT16 lymphoblasts, we observe a sustained and higher level of ATF4 expression in response to ER stress (Fig 3.3). This was true both in P222L homozygous as well as P222L (data not shown) and C213R compound heterozygous DYT16 patients. Our studies are thus strongly indicative that a dysregulation of ATF4 expression occurs in DYT16 and this could derail normal healthy neuronal function.

PKR has emerged as a major player in several neurodegenerative diseases in recent years as aberrant elevated PKR activation has been observed in human patients in post-mortem studies as well as in several mouse models. Increased levels of PKR phosphorylation have been detected in the brains of patients with neurodegenerative diseases such as Alzheimer’s disease (AD), Parkinson’s disease, Huntington’s disease, dementia, and prion disease. Activated PKR was recently shown to be responsible for the behavioral and neurophysiological abnormalities in a mouse model of Down syndrome and PKR inhibitory drugs partially rescued the synaptic plasticity and long-term memory deficits in mice. Drugs that target the eIF2α signaling pathway have shown benefits in many mouse models for neurodegenerative diseases and in particular, inhibiting PKR has proven to be effective, showing
rescue of synaptic and learning deficits in two different AD mouse models\textsuperscript{153}. In case of DYT16 we wanted to take a more specific approach as C16, a widely used chemical inhibitor of PKR has been documented to have off target effects\textsuperscript{144} thus questioning its suitability in treating DYT16. We have previously reported that luteolin disrupts the PACT-PKR interaction efficiently and can inhibit stress-induced ISR and inflammation\textsuperscript{154}. We tested if luteolin is able to rescue the DYT16 cells from stress induced apoptosis. Luteolin was able to dissociate PACT-PKR interactions efficiently in both normal as well as DYT16 lymphoblasts (Fig. 3.7). The observation that it takes significantly longer to disrupt PACT-PKR interactions in DYT16 patient cells as compared to normal wt cells, further supports that PKR interacts much stronger with P222L and C213R mutant PACT molecules. Luteolin treatment rescues the higher apoptosis phenotype in DYT16 cells and offers a promising lead into possible future therapies aimed at disrupting PACT-PKR interactions. In addition to DYT16, such therapies may also show promise in AD as PACT mediated PKR activation has been implicated in AD\textsuperscript{116}.

The results presented here not only strengthen our previous research on DYT16, they also demonstrate the merit in developing drugs to disrupt PACT-PKR interactions for possible clinical application in the future. Further efforts to discover compounds similar to luteolin that disrupt PACT-PKR interactions at lower concentrations or to develop specific peptides for this purpose may be fruitful. In this regard, it is worth mentioning that an interaction between dsRBM3 of PACT with PKR's catalytic domain is essential for PKR activation and a
disruption of such an interaction may also prove beneficial as it would block PKR's catalytic activity even after PACT-PKR interactions have taken place. In combination with luteolin such peptides could offer valuable therapeutic options by lowering the effective luteolin dose significantly.
Figure 3.1: Effect of DYT16 mutations on dsRNA-binding. (A) Schematic representation of DYT16 mutations: Three conserved dsRBMs that bind dsRNA (M1 and M2) are shown in grey and the third domain lacking dsRNA-binding (M3) is shaded blue with the two phosphorylation sites represented as dark blue lines. Dominant mutations are indicated in red while recessive mutations are indicated in green.
Figure 3.1: Effect of DYT16 mutations on dsRNA-binding. (Continued) (B) dsRNA-binding assay: The 35S-methionine labeled wild type PACT and DYT16 mutant proteins were in-vitro translated from 100 nG of appropriate plasmid DNAs, using rabbit reticulocyte lysate. dsRNA binding activity of wt PACT and point mutants was measured by a poly(I)·poly(C)-agarose binding assay with in vitro translated 35S-labeled proteins. T, total input; B, proteins bound to poly(I)·poly(C)-agarose. Competition lanes (15-18): - - no competitor, competition with 100-fold molar excess of single-stranded RNA (ss) or dsRNA (ds). The minor bands below the parent PACT bands represent products of in vitro translation from internal methionine codons and thus are not produced in similar quantities in all translation reactions and thus are of variable intensity in lanes 1-3. Lanes 19 and 20 represent binding of firefly luciferase protein to dsRNA, used as a negative control to demonstrate specificity. C, quantification of the dsRNA binding assay. Bands were quantified by phosphorimaging analysis, and % bound was calculated. Error bars: S.D. from three independent experiments. The p values were calculated using statistical analyses indicated no significant difference between % dsRNA-binding of wt and point mutants.
Figure 3.2: Effect of DYT16 mutations on PKR activation and cell fate. (A) PKR kinase activity assay was performed using PKR immunoprecipitated from HeLa cells with a monoclonal PKR antibody (R&D Systems) and protein-A-sepharose beads. Recombinant wild type PACT or DYT16 mutant protein either 400 pg or 4 ng was used as PKR activator. Lanes 13-16 (upper panel) and Lanes 9-12 (lower panel): PACT mutants in combinations reported in DYT16 patients were used as PKR activator with 200 pg or 2 ng of each mutant protein.
Figure 3.2: Effect of DYT16 mutations on PKR activation and cell fate. (Continued) (B) Quantification of kinase activity assay. Radioactivity in each band was quantified using phosphoimaging analysis and the relative signal intensities are plotted. The p values calculated using Student’s t-tests are as indicated. (C) Western blot analysis for cleaved PARP1. Whole cell extracts from normal (wt) and DYT16 patient derived lymphoblasts treated with 5 μg/mL of tunicamycin (TM) were analyzed at indicated time points. (D) Caspase-Glo 3/7 activity. Lymphoblast lines established from wt and DYT16 patient were treated with 5 μg/mL tunicamycin and the caspase 3/7 activities were measured at indicated time points. Blue bars: wt cells, and red bar: DYT16 cells. Student’s t-tests were performed, and the p values are as indicated.
Figure 3.3: PKR activation, eIF2α phosphorylation, ATF4 and CHOP induction in response to tunicamycin in normal and dystonia patient lymphoblasts. (A) Western blot analysis for p-PKR and p-eIF2α. Whole cell extracts from normal (wt) and DYT16 patient derived lymphoblasts treated with 5 μg/mL of tunicamycin (TM) 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) Western blot analysis for ATF4 and CHOP under the same conditions as 3.3A. Beta-actin was used as a loading control to ensure equal amounts of protein was loaded in each lane.
Figure 3.4: Effect of DYT16 mutations on PACT-PKR interaction. (A & B) Co-immunoprecipitation assays: HeLa cells were co-transfected with the trans dominant negative Flag-PKR (K296R) and Myc-PACT expression plasmids in pCDNA3.1+. Twenty-four hours post-transfection, cells were harvested and myc-PACT
Figure 3.4: Effect of DYT16 mutations on PACT-PKR interaction. (Continued) (A) or flag-PKR(B) was immunoprecipitated with myc-agarose or flag agarose beads. The immunoprecipitates were then analyzed by western blot analysis with anti-flag or anti-myc antibodies (co-IP panel). Input gels show expression levels of proteins without immunoprecipitation. (A) Recessive DYT16 mutants and (B) Dominant DYT16 mutants. (C and D) Mammalian two-hybrid assays. 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. Cells were harvested 24 h after transfection, 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. The experiment was repeated twice with each sample in triplicate, and the averages with standard error bars are presented. Student’s $t$-tests were performed to calculate $p$ values and they are as indicated. *RLU*, relative light units.
Figure 3.5: Effect of DYT16 mutations on PACT-PACT interactions. (A-C) Co-Immunoprecipitation assays to measure PACT-PACT interaction with mutant protein combinations seen in DYT16 patients. HeLa cells were co-transfected with Flag-PACT and Myc-PACT expression plasmids in pCDNA3.1-. Twenty-four hours post-transfection, cells were harvested, and flag-PACT was immunoprecipitated with flag agarose beads. The immunoprecipitates were then analyzed by western blot analysis with anti-myc antibodies (co-IP panel). Input gels show expression levels of proteins without immunoprecipitation.
Figure 3.5: Effect of DYT16 mutations on PACT-PACT interactions. (Continued)
(A) Recessive DYT16 mutants, (B) Dominant DYT16 mutant interactions with wt PACT and (C) Dominant DYT16 mutant interactions with dominant mutants (homomeric interactions). Input gels show expression levels of proteins without immunoprecipitation. (D and E) Mammalian two-hybrid assays. 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. Cells were harvested 24 h after transfection, and cell extracts were assayed for luciferase activity. The plasmid combinations are as indicated, various PACT proteins were expressed as a GAL4 DNA-binding domain fusion proteins (bait) and also as VP16-activation domain fusion proteins. The experiment was repeated twice with each sample in triplicate, and the averages with standard error bars are presented. Student's t-tests were performed to calculate $p$ values and they are as indicated. RLU, relative light units.
Figure 3.6: Effect of DYT16 mutations on PACT-TRBP interactions. (A, B) Co-Immunoprecipitation assays. HeLa cells were co-transfected with Flag-TRBP and Myc-PACT expression plasmids in pCDNA3.1- . Twenty-four hours post-transfection, cells were harvested and flag-TRBP was immunoprecipitated with flag-agarose beads. The immunoprecipitates were then analyzed by western blot analysis with anti-myc antibodies (co-IP panel) and anti-flag antibody (IP panel). Input gels show expression levels of proteins without immunoprecipitation. (A) Recessive DYT16 mutants, (B) Dominant DYT16 mutants. (C and D) Mammalian two-hybrid assays. 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. Cells were harvested 24 h after transfection, and cell extracts were assayed for luciferase activity. The plasmid combinations are as indicated, TRBP protein was expressed as a GAL4 DNA-binding domain fusion protein (bait) and various PACT proteins as VP16-activation domain fusion proteins. The experiment was repeated twice with each sample in triplicate, and the averages with standard error bars are presented. RLU, relative light units.
Figure 3.7: Effect of luteolin on PACT-PKR interactions and caspase activation in response to tunicamycin. (A) co-immunoprecipitation of endogenous PKR and PACT proteins. Lymphoblasts from unaffected family member (wt) or dystonia patient (patient) were treated with 50 μM luteolin. The cell extracts were prepared at the indicated times, 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 and anti-PKR mAbs showing equal amount of PACT and PKR in all samples.
Figure 3.7: Effect of luteolin on PACT-PKR interactions and caspase activation in response to tunicamycin. (Continued) (B) Effect of luteolin on Caspase 3/7 activity in lymphoblasts. Lymphoblasts from unaffected family member (wt) and DYT16 patient (patient) were treated for 24-hours in 50 μM Luteolin (red) or left untreated (blue) followed by treatment with 5 μG/ml tunicamycin. Caspase 3/7 activity was measured at the indicated time points after tunicamycin treatment. The p values calculated using Student's t-tests are as indicated.
CHAPTER 4:
CHARACTERIZATION OF A RECESSIVELY INHERITED FRAMESHIFT MUTATION IN PACT WHICH LEADS TO EARLY ONSET DYSTONIA 16 (DYT16) IN MICE

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4.1 ABSTRACT

In response to stimuli from a diverse range of cellular stressors, protein synthesis is negatively regulated through the phosphorylation of the α subunit of the eukaryotic translation initiation factor 2 (eIF2α). The interferon induced double stranded RNA (dsRNA) binding protein kinase (PKR) is an eIF2α kinase that has been shown to be activated in response to viral stress, endoplasmic reticulum stress, oxidative stress, and serum starvation. Under non-viral stress conditions, PKR is activated through direct high affinity interactions with its protein activator, PACT. Mutations in PACT have been identified as the causative factor in the development of early onset primary dystonia subtype 16 (DYT16) in human patient populations. Recently, a recessively inherited de novo frameshift mutation, Prkra^lear-5J (hereafter termed lear-5J), was identified in the mouse PACT. Mice homozygous for this mutation exhibited craniofacial developmental abnormalities, reduced body size, kinked tails, and DYT16-like symptoms. In the present study we investigate the biochemical and developmental consequences of this lear-5J mutation within this novel DYT16 model system. We identify that this truncated variant of the PACT protein retains its ability to interact with PKR, however, its ability to stimulate PKR’s kinase activity is dramatically reduced in vitro. Furthermore, our results suggest the lear-5J mRNA is partially targeted for NMD both in the brains of these mice as well as MEFs. Surprisingly, we only detect the truncated protein the brains of these affected mice which could indicate proteostatic mechanisms are differentially regulated in the brain relative to other cell types. When examining the cerebellum lear-5J mice, our results
demonstrated abnormalities in the development of the folia. Finally, upon further examination of the cerebellum our results reveal a severe lack of dendritic arborization in the Purkinje neuron layer and reduced eIF2α phosphorylation in the brains of the lear-5J mice relative to unaffected controls.

4.2 INTRODUCTION

Translational regulation is a fundamental modulator of cellular homeostasis in response to environmental stimuli. One of the primary nodes of translational regulation is the prevention of the formation of the ternary complex which is required for the synthesis of cap dependent mRNAs. In response to amino acid deprivation, viral infection, accumulation of unfolded proteins or reactive oxygen species, the alpha subunit of the eukaryotic translation initiation factor 2 (eIF2α) is phosphorylated resulting in the attenuation of general protein synthesis. This post-translational modification is facilitated by a family four eIF2α kinases, such that the activity of each kinase is stimulated in a stress signal-specific manner in order to trigger the integrated stress response (ISR). Under such conditions, specialized stress response mRNAs are induced and preferentially translated in a cap- and eIF2α-independent manner in order to recover from stress. While transient eIF2α phosphorylation is favorable for recovery and cell survival, chronic or prolonged eIF2α phosphorylation is detrimental by inducing apoptosis.

The interferon induced double-stranded RNA (dsRNA) activated protein kinase (PKR) is an eIF2α kinase that has been extensively studied for its role in attenuating protein synthesis in response to viral infection, endoplasmic reticulum
(ER) stress, oxidative stress, and amino acid deprivation\(^1,13,17,18,34,35\). PKR’s kinase activity is stimulated through direct interactions with dsRNA, a replication intermediate used by many viruses, or its protein activator (PACT)\(^{13,14,25}\). Both of these interactions are facilitated by two highly conserved dsRNA binding motifs (dsRBMs) on the amino terminus of PKR\(^{13,14,25,124}\). Interactions with dsRNA and PACT induce a conformational change in PKR revealing a conserved carboxy terminal kinase domain (KD) which is then activated through a trans-autophosphorylation event\(^1,15,31\). Conversely, in the absence of stress PKR is negatively regulated through direct interactions the HIV transactivation response element (TAR) RNA binding protein (TRBP)\(^{46,126}\). While interactions with dsRNA and PACT that activate PKR are pro-apoptotic, PKR-TRBP interactions that inhibit PKR are favorable to cell viability.

PACT is a dsRNA binding protein that activates PKR through direct protein-protein interactions under conditions of oxidative stress, ER stress, and amino acid deprivation\(^{25,35}\). Similar to PKR, PACT contains two highly conserved amino terminal dsRBMs (dsRBM1 and 2) that facilitate dsRNA and protein-protein interactions; however, PACT also contains a third carboxy-terminal dsRBM (dsRBM3) that does not bind dsRNA but is required for PKR activation\(^{25,37,38}\). Within PACT’s dsRBM3 are two serine residues that serve as sites for post translational modifications, S246 and S287\(^{53}\). While S246 is constitutively phosphorylated, S287 is only phosphorylated under conditions of cellular stress\(^{49,53}\). Under homeostatic conditions, PACT is in a heterodimeric complex with TRBP and thus low basal levels of active PKR are maintained \(^{53}\).
Under the conditions of cellular stress outlined above, PACT is phosphorylated by an unknown kinase on S287 which results in the dissociation of PACT-TRBP heterodimers and promotes the formation of PACT-PACT homodimers which are essential for PKR activation.31,49,52,53

PACT is the product of the PRKRA gene which is ubiquitously expressed in all cell types. Mutations in PACT have been shown to be directly causative of the neuromuscular disorder, early onset dystonia and parkinsonism subtype 16 (DYT16).62,67,129 The dystonias are a group of heterogeneous movement disorders consisting of 18 categories that are linked to mutations in 25 known genes.60 The patient presentation of dystonia consists of repetitive movements, involuntary muscle contractions, as well as compromised posture and gate59. Previous studies in DYT1, DYT6, and DYT16 have all identified the dysregulation of eIF2α signaling in the etiology of the disorders.74,131,132 Interestingly, recent studies have identified mutations in eIF2γ to be the causative factor in the intellectual disability disorder, MEHMO84,155. These patients also demonstrated dysregulation of eIF2 signaling surrounding the formation of the ternary complex resulting in the induction of ISR proteins84,155. Furthermore, these patients present with craniofacial development defects, reduced body size, ataxic gate, and lower limb spasticity155. Collectively, these studies suggest that the dysregulation of eIF2 signaling results in severe neuromuscular and intellectual disability disorders.

Recently, Palmer et al. described a spontaneous frameshift mutation (Lear5j) in the mouse Prkra gene which codes for the mouse homolog of PACT34,68. This frameshift mutation results in a truncation within the dsRBM2
functional domain of the PACT protein\textsuperscript{68}. PACT is highly conserved among human and mouse differing only in 6 amino acids 4 of which have identical chemical properties\textsuperscript{34,36}. These mice present with craniofacial developmental abnormalities, small ear size (giving the name "Lear" for "little ears") drastically reduced body size, kinked tails, and ascending dystonia that progresses until fatal at 3-6 weeks of age\textsuperscript{68}. This mouse model provides an animal model to examine the molecular pathways involved in DYT16 and also test some possible therapeutic options. Although it remains unknown if an equivalent mutation in humans would also produce a dystonia phenotype and if such a mutation could be viable in human population. In the present study we undertook a very preliminary characterization of both the \textit{in vitro} and \textit{in vivo} consequences of this mutation. Ultimately, our findings demonstrate that the truncated protein is detectable in mouse brain but not in MEFs, there is more detectable mRNA in mouse brain relative to MEFs, and that the mutant PACT protein retains its ability to interact with and activate PKR albeit with much reduced efficiency \textit{in vitro}. Our \textit{in vivo} data evaluating the brains of these mice demonstrates somewhat defective foliation of the cerebellum. Upon further evaluation of this phenotype, we observed that the cerebellar Purkinje neuron layer has a dramatic reduction in the dendritic arborization. Finally, we identified a reduction in total phosphorylated eIF2\textalpha{}, elevated eIF2\textalpha{} phosphatase levels, and no detectable difference in ATF4 levels in Lear5j mice compared to wt control. Consistent with previously published data, our results demonstrate that dysregulating eIF2 signaling pathway in mouse has severe consequences on development and may
contribute to the etiology of the observed neuromuscular phenotype similar to DYT16.

4.3 MATERIALS AND METHODS

**Cell Lines and Antibodies** – Both HeLaM and Mouse Embryonic Fibroblasts (MEFs) cell lines were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% Fetal Bovine Serum and penicillin/streptomycin. All transfections were carried out using Effectene transfection reagent (Qiagen) per manufacturer protocol. Antibodies used were as follows: PKR: anti-PKR (human) monoclonal (71/10, R&D Systems), p-eIF2α: anti-phospho-eIF2α (Ser-51) polyclonal (CST, #9721), PACT: anti-PACT polyclonal (ProteinTech, 10771-1-AP), ATF4: Anti-ATF4 monoclonal (CST, #11815), FLAG-HRP: anti-FLAG monoclonal M2-HRP (Sigma A8592), β-Actin: Anti- β-Actin-Peroxidase monoclonal (Sigma-Aldrich, A3854), CreP: PPP1R15B rabbit polyclonal (ProteinTech, 14634-1-AP).

**Generation of lear-5J Frameshift Mutation** – We generated the lear-5J frameshift mutation using site specific mutagenesis through PCR amplification such that antisense primer contained a single adenine insertion relative to the *Prkra* template DNA. The following site-specific mutagenic primers were used: lear-5J Sense: GCCTCGAGCACATATGTCCCATAGCAGGCATCG, lear-5J Antisense:

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GCGGATCCGAAATATTACTAAACTTTGGCGAGAAATTTCAGCGAGCATCG
CTTGAGCTTGTGTGTTTTGATGCCCCCTTTC
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The subsequent PCR product was then ligated into pgEMT-Easy vector (Promega) and the presence of the
mutation was validated through DNA sequencing services provided by Eton Biosciences. After sequence validation we generated amino terminal flag-tagged lear-5J constructs in pBSIIKS+ using Ndel and BamHI restriction sites. We next subcloned the Flag-lear-5J ORF into the mammalian expression construct, pCDNA3.1-, using XbaI-BamHI restriction sites.

**Expression and purification of PACT/lear-5J from E. coli** – The ORFs of both wt PACT and lear-5J frameshift mutation were subcloned into pET15b (Novagen) to generate an in-frame fusion protein with a histidine tag. Recombinant proteins were then expressed and purified as previously described.

**PKR Activity Assay** – HeLa M cells treated with IFN-β for 24-hours and harvested at 70% confluency. Cells were washed using ice-cold PBS and centrifuged at 600 g for 5-minutes. Cells then 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. Whole cell lysates were then centrifuged at 10,000 g for an additional 5-minutes. PKR was then immunoprecipitated from 100 ug of this lysate using anti-PKR monoclonal antibody (R&D Systems Technology: 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°C on a rotating wheel for 30-minutes. We then added 10 ul of Protein A-Sepharose beads to each immunoprecipitate followed by an additional 1-hour incubation under the same conditions. Protein A-Sepharose beads were then
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, 2 mM MnCl₂, 100 U/ml aprotinin, 0.1 mM PMSF, 5% glycerol). The PKR activity assay using the PKR bound to protein A-Sepharose beads was then conducted by incorporating: 500 ng of purified eIF-2 as the PKR substrate, 0.1 mM ATP, 10 uCi of [γ³²P] ATP, and increasing amounts of either recombinant wt PACT or recombinant lear-5J (400 pg – 4 ng) as the PKR activator. Reaction was then incubated at 30°C for 10 min and resolved on a 12% SDS-PAGE gel and analyzed via autoradiography.

**Western Blot Analysis** – Protein extracts were prepared from a fraction of the brains of wt mice, mice heterozygous for the lear-5J mutation, and mice homozygous for the lear-5J mutation using western 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) containing a 1:100 dilution of protease inhibitor (Sigma) and phosphatase inhibitor (Sigma). Tissue was initially homogenized in the lysis buffer cocktail described above and incubated on ice for 5 minutes followed by centrifugation at 13,000 g for 10 minutes. Concentration of total protein extract was then determined through Bradford assays and appropriate amounts were resolved on SDS-PAGE denaturing gels to detect the proteins of interest. Protein extract derived from MEFs were prepared from wt and lear-5J MEFs cultured as described above. Cells were initially seeded at 40% confluence and harvested 24-hours later using the lysis buffer cocktail described above. Concentration of whole cell extracts were determined by
Bradford assays. Proteins were detected via chemiluminescence and antibodies indicated above.

**Reverse Transcriptase PCR** – RNA was isolated from either: (i) a fraction of mouse brain derived from wt mice, lear-5J heterozygous mice, lear-5J homozygous mice or (ii) wt and lear-5J homozygous MEFs. In all cases, tissue or cells were incubated on ice for 5-minutes in RNA-Bee (Tel-Test, Inc) and in the presence of CIA (chloroform:isoamylacetate (24:1)). Lysates were then centrifuged at 12,000 g for 15-minutes at 4°C. The fraction of lysate containing RNA was then carefully collected and precipitated overnight in an equal volume of isopropanol at -20°C. Samples were then centrifuged at 12,000 g for 15 minutes to pellet the RNA. Supernatant was removed and RNA pellet was washed 2x in 70% ETOH and centrifuged at 12,000g for 8 minutes at 4°C followed by a 1-hour incubation at room temperature to ensure RNA was devoid of alcohol contamination. Purified RNA pellets were then resuspended in nuclease free water. In order to compare relative levels of mRNA, cDNA was generated using 1 ug of total RNA, 75 nG of random hexamer, and reverse transcriptase (Thermo Scientific) per manufacturer protocol. Finally, relative mRNA levels of *Prkra* were then compared using *Prkra* specific primers and either S15 or GAPDH housekeeping primers were used to amplify these housekeeping genes to determine equivalent amounts of cDNA were loaded into each reaction. The following primer sequences were utilized: *Prkra* Sense: ATGTCCCATAGCAGGCATCGTGCCG, *Prkra* Antisense: CCTTCCTGGGAAAGGTTATATCAGG, S15 Sense:
Co-Immunoprecipitation Assays with Endogenous Proteins – HeLa M cells were seeded at 20% confluency in 6-well dishes 24-hours prior to transfecting 500 ng of either flag-wt PACT or flag-lear-5J constructs using Effectene transfection reagent (Qiagen). Cells were harvested 24-hours post transfection and harvested in IP buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 20% Glycerol). Whole cell extract was then immunoprecipitated overnight at 4°C on a rotating wheel using anti-PKR antibody (71/10, R&D Systems) conjugated to protein A sepharose beads (GE Healthcare) in IP buffer. Immunoprecipitates were then washed 3 times in IP buffer followed by resuspension and boiling for 5 minutes in 2x Laemmli buffer (150 mM Tris–HCl pH 6.8, 5% SDS, 5% β-mercaptoethanol, 20% glycerol). Samples were then resolved on 12% SDS-PAGE denaturing gels and transferred onto PVDF membranes. Blots were initially probed for flag-PACT/lear-5J constructs (coIP) using anti-flag antibody (described above) followed by incubation in anti-PKR antibody (described above) to determine equal amounts of PKR were immunoprecipitated. Input blots of whole cell extract without immunoprecipitation are shown to indicate equal amounts of protein in each sample.

Histology and Immunostaining – Brains derived from day 28 wt or lear-5J homozygous mice were fixed at 4°C overnight in 4% paraformaldehyde in PBS. The fixative was then removed and tissues were then washed in PBS
followed by dehydration in absolute ethanol. Following the dehydration step, tissues were then permeabilized with methyl salicylate and embedded in paraffin. We then generated 6 μM sagittal sections from each tissue and evaluated through hematoxylin and eosin (H&E) staining.

Immunostaining was carried out as previously described and antibodies, dilutions, and detection methods are indicated in Table 1. In brief, experiments utilizing mouse primary antibodies utilized blocking reagents from the M.O.M. kit (Vector Laboratories) and experiments utilizing primary antibodies from other species used the blocking reagent from tyramide signal amplification (TSA) kit no. 22 dissolved in PBS (Thermo Fisher Scientific) each per manufacturer protocol. In each case, primary antibodies were incubated overnight at 4°C in the appropriate blocking reagent. Samples were then incubated in species specific secondary antibodies in TSA block for 30 minutes at room temperature. Biotin-conjugated secondary antibodies were incubated in streptavidin conjugates (Alexa Fluor 488, Alexa Fluor 594, or HRP) at a 1:100 dilution in TSA block at room temperature for 30 minutes (Thermo Fisher Scientific). Following this incubation, the streptavidin-HRP conjugates were either subjected to SigmaFast 3,3’-diaminobenzidine (DAB) staining (Sigma-Aldrich) or TSA with Alexa Fluor 488 (TSA kit no.22, Thermo Fisher Scientific) for 3 minutes in each case. Fluorescent detection methods were counterstained with 300 nM 4’,6-diamidino-2-phenylindole (DAPI) for 5 minutes prior to mounting in fluorescence mounting media. DAB detection was counterstained with 0.5% methyl green in 0.1 M sodium acetate buffer (pH 4.2) for 5 minutes at room temperature.
4.4 RESULTS

A single nucleotide insertion truncates PACT in dsRBM2 – The lear-5J mice arise from a spontaneously arisen, recessively inherited frameshift mutation in the Prkra gene encoding for the mouse homolog of PACT\(^68\). This mutation is caused by a single adenine insertion in codon 178 which leads to a premature stop codon within one of PACT’s functional motifs (Fig. 4.1A)\(^68\). Consequently, the frameshift introduces 7 extraneous amino acids before truncating the protein within dsRBM2 (Fig. 4.1A-B). The resultant protein contains a full copy of PACT’s dsRBM1 and only a partial copy of PACT’s dsRBM2 eliminating some of the residues within this domain considered to be important for protein-protein interactions (Fig. 4.1A-B). However, the truncated protein still retains the entire dsRBM1, which is most crucial for dsRNA-binding and protein-protein interactions.

Lear-5J has both cytoplasmic and nuclear localization – The wt PACT protein is ubiquitously expressed with a predominant cytosolic distribution. Recently, our lab demonstrated that a human DYT16 frameshift mutant protein forms protein aggregates when overexpressed in human cells\(^130\). Therefore, we next tested the effect of overexpressing the truncated lear-5J protein in cells (Fig. 4.2A). To address this question, we generated amino terminal GFP tagged wt PACT and lear-5J constructs. These expression constructs were then transiently transfected in HeLa cells and the cells were examined by fluorescence microscopy 24-hours post transfection (Fig. 4.2A). Interestingly, while majority of
wt PACT is localized to the cytoplasm (left), the lear-5J mutant protein can be detected in both the cytoplasm and nucleus (right).

**Lear-5J interacts with and activates PKR in vitro** – Previously, our lab has reported that mutating specific hydrophobic residues within PACT’s dsRBM1 significantly disrupts PACT-PKR interactions, while disrupting hydrophobic residues in dsRBM2 has minimal consequences for PACT-PKR interaction. In light of the lear-5J mutation truncating the protein within PACT’s dsRBM2, we wanted to address whether this truncated protein retains its ability to interact with PKR (Fig. 4.3A). To address this question, we performed co-immunoprecipitation assays (co-IP) from cells overexpressing either flag-tagged wt PACT or flag-tagged lear-5J constructs. Our results indicate that despite the truncation, the lear-5J protein interacts with PKR with equal binding affinity to that of wt PACT under the conditions of this assay (upper panel, lanes 1-2) (Fig. 4.3A). In the absence of PKR, we do not detect flag-wt PACT or flag- lear-5J thus indicating that there is no binding to the beads nonspecifically in the absence of PKR protein (upper panel, lanes 3-4) (Fig. 4.3A). Lanes 1-2 (lower panel) indicate that equal amount of endogenous PKR was immunoprecipitated in both samples while lanes 3-4 (lower panel) indicate the presence of PKR only in the presence of PKR antibody (Fig. 4.3A). Input blot (left) demonstrates equal expression of Flag-wt PACT and Flag- lear-5J prior to immunoprecipitation (Fig. 4.3A).

PACT activates PKR under conditions of cellular stress. Therefore, we next tested the effect the lear-5J frameshift mutation has on its ability to activate PKR in vitro. While dsRBM1 and 2 are responsible for dsRNA...
and protein-protein interactions, dsRBM3 is critical PACT’s ability to activate PKR\textsuperscript{38}. To address this question, we performed \textit{in vitro} PKR activity assays using either purified recombinant hexahistidine-tagged wt PACT or lear-5J to stimulate PKR activation. Both hexahistidine-tagged wt PACT and lear-5J proteins were expressed in bacteria and purified using affinity chromatography on Ni-agarose. Two different amounts of the purified recombinant proteins were tested for activation of PKR immunoprecipitated from HeLa cells (Fig. 4.3B). Our results indicate that at lower concentration (400 pg), lear-5J does not activate PKR above background (lanes 1-2), whereas, wt PACT results in a clear activation of PKR (lane 4). Interestingly, lear-5J activates PKR slightly at the higher concentration (4 ng) (lane 3). Although lear-5J is a less potent activator of PKR as compared to wt PACT (lanes 4-5), these results indicate that the truncated lear-5J protein can activate PKR with a reduced efficiency, which is a surprising result and we will need to validate and explore this further to understand the biochemical and biological significance of this if any.

\textit{Lear-5J protein is detectable in mouse brain but not MEFs –}

Frameshift mutations are often silenced through the quality control mechanism nonsense mediated decay (NMD) of mRNAs with an early stop codon\textsuperscript{159}. We next wanted to address whether the lear-5J mutant protein and mRNA is detectable in mice, or if the gene is silenced through NMD. To address this question, we isolated protein and total RNA from the brains of wt, heterozygous lear-5J, and homozygous lear-5J mice. We performed western blot analysis utilizing a polyclonal antibody for PACT to recognize the truncated lear-5J protein
wt PACT has a molecular weight of 36 kDa (upper band), whereas, the lear-5J truncated protein has a predicted molecular weight of 22 kDa (lower band) (Fig. 4.4A). Lane 1 shows whole cell protein extract derived from mice heterozygous for the lear-5J mutation where both wt PACT (upper band) and lear-5J (lower band) are detectable (Fig. 4.4A) as expected. Comparably, whole cell protein extract derived from two independent lear-5J homozygous mouse brains have no detectable wt PACT band at the 36 kDa marker, however, both have a detectable band at the predicted molecular weight for the truncated lear-5J protein at 22 kDa (lanes 2-3) (Fig. 4.4A). Finally, we detect the presence of wt PACT but not the lear-5J truncated protein in whole cell protein extract derived from wt mice (lane 4). Blots were then probed for β-actin to ensure equal protein loading (lower panel, lanes 1-4). To further validate that the bands observed in Fig. 4.4A are specific to lear-5J and the mRNA corresponding to the Prkra gene is present in the brain (and not degraded via NMD), we performed reverse transcriptase PCR (RT-PCR) on total RNA isolated from the brains of the mice described above in comparison to wt MEF. Our results indicate that compared to the wt mice (lane 2) the Prkra transcripts are present at reduced levels in lear-5J mice (lanes 3-4) (Fig. 4.4B). We observe the greatest reduction in mRNA levels in lear-5J homozygous mice (lane 4) (Fig. 4.4B). These results indicate that although NMD may be operative on lear-5J mRNA, it does not cause complete absence of the transcripts from lear-5J brains. This also offers support to the idea that the protein bands observed at the expected position in lear-5J brain extracts (Fig. 4.4A) are indeed the truncated mutant lear-5J protein. RT-PCR for the
ribosomal protein S15 was used as a positive control to ensure equal amount of
cDNA was added to each PCR reaction (lower panel) (Fig. 4.4B).

We next wanted to evaluate whether the truncated lear-5J protein was
also present in MEFs derived from lear-5J mice. Therefore, we performed
western blot and RT-PCR analysis on MEFs derived from mice of the same
genotypes as Fig. 4A-B. HeLa cells over-expressing flag- lear-5J were used as a
positive control (Lane 4) (Fig. 4.4C). Our results indicate that wt PACT is
abundant in these cells (upper panel, lane 1), however, no lear-5J mutant protein
is detectable in MEFs (lanes 2-3). To ensure equal protein loading we then
probed for β-actin as our loading control (lower panel, lanes 1-4). Finally, we
wanted to determine if the observed absence of lear-5J protein in MEFs is due to
the absence of Prkra mRNA, as it could be degraded completely via NMD. We
assessed this using RT-PCR as described before. Our results (Fig. 4.4D) show a
dramatic reduction in detectable Prkra mRNA in MEFs homozygous for the lear-
5J mutation (lane 2) compared to the wt control (lane 1). GADPH was used as
the positive control to ensure equal quantities of cDNA were used for each
reaction. These results indicate that MEFs could be more efficient at NMD of the
aberrant mutant lear-5J transcript than neuronal cells. In addition, the neuronal
cells may also be less efficient in degrading the truncated mutant protein as the
mutant protein is clearly detected in brain extracts of lear-5J mice whereas no
lear-5J protein is detected in lear-5J MEFs corresponding to the reduced but
detectable levels of Prkra mRNA.
**PACT is expressed in the mouse cerebellum:** Studies have demonstrated that eIF2α plays an important role in regulating translation during the development of an organism, however, the functional contribution of PACT during development remains an open question\(^8,82\). The cerebellum is the best characterized region of the brain for coordinating motor control and proprioception. As dystonia is mainly thought to result from defects in neuronal circuits, we next wanted to evaluate the presence of PACT in the cerebellum of developing mouse brain. In order to address this question, we performed two forms of IHC: (i) 3,3’-diaminobenzidine (DAB) such labeling the protein brown (Fig. 4.5A), (ii) immunostaining for wt PACT (green) and DAPI (4’,6-diamidino-2-phenylindole) (blue) (Fig. 4.5B). In both cases, our results show that wt PACT is abundantly expressed in the cerebellum (Fig. 4.5A-B). The cerebellum is subdivided into three layers: the granular layer, the Purkinje cell layer, and a sparse molecular layer that contains axons from neurons within the internal granular layer\(^160\). Notably, we observe the highest concentration of wt PACT in the Purkinje cell layer (brown) (Fig. 4.5A-B). In light of this observation, we further evaluated the expression of wt PACT within the Purkinje neurons by performing double immunostaining for the Purkinje neuron specific marker, calbindin (red), and PACT (green) counterstained with DAPI (blue) (Fig. 4.5C). In agreement with the data described above, we are able to accurately detect abundant levels of wt PACT (green) in the Purkinje neurons (red) (Fig. 4.5C).

**Lear-5J mice show defects in cerebellum development and deficiencies in Purkinje cell arborization** – The cerebellum of all mammals
share a common developmental pattern in that it is divided into 10 distinct folia\textsuperscript{160}. After establishing that wt PACT is abundantly expressed in mouse cerebellum, we next wanted to assess whether the lear-5J frameshift mutation has an effect on cerebellar development. To address this question, we compared mid-sagittal sections of wt and lear-5J cerebellum derived from 28-day old mice using Hematoxylin and Eosin (H&E) staining using brains of 2 lear-5J mice (Fig. 4.6A-B). In both wt and lear-5J mice we observe the characteristic 10 folia within each of the cerebellum (Fig. 4.6A-B). Interestingly, while the total number of folia was unaffected in lear-5J mice compared to wt, the complexity of the folia was significantly reduced (Fig. 4.6A-B). The folia within the cerebellum in the lear-5J mice demonstrate an elongated and less branched pattern relative to that of wt mice (Fig.6A-B). The most distinguishing factor when comparing these foliations is within folium IX. In wt mice, this folium is subdivided into three lobules (IXa, IXb, IXc)\textsuperscript{160}. While we can observe these distinct lobules in cerebellar tissue derived from wt mice, we do not detect such lobules in the lear-5J cerebellum (Fig. 4.6A-B). Although these initial observations provide valuable insight into the development of lear-5J mice, it is worth noting that although both lear-5J mice brains that were evaluated were drastically different than the wt control, there was also substantial differences between the two lear-5J mice. Consequently, these more mice need to be evaluated to support these initial observations. Due to the abundance of wt PACT in the Purkinje cell layer identified in Fig. 4.5, we next wanted to determine if there were any aberrant phenotypes within this cerebellar layer between these two mice (Fig. 4.6C-H). To this end, we
performed immunostaining on sagittal sections of wt and lear-5J cerebellar tissue using calbindin (green) to specifically mark Purkinje cells and DAPI (blue) as the nuclear stain (Fig. 4.6C-F). In wt mice, we are able to accurately detect Purkinje cells lining the folia of the cerebellum (Fig. 4.6C). Upon magnification, we further observe that the wt Purkinje neurons demonstrate the characteristic arborizations branching from the cell body (Fig. 4.6C-D). By comparison, we do not observe any aberrant phenotype in the overall spatial organization of Purkinje neurons within the lear-5J mice (Fig. 4.6C, 6E). Interestingly, upon magnification we identified a dramatic reduction in the dendritic branching of each Purkinje neuron in the lear-5J mice compared to that of wt (Fig. 4.6D, 4.6F).

**Lear-5J cerebellum shows dysregulated eIF2α signaling:** A perturbation of the basal eIF2α phosphorylation levels as well as dysregulation of stress-induced phosphorylation has been previously linked to the dystonia subtypes DYT1, DYT6, and DYT16,120,131,132. It is well established that neurons maintain higher levels of phosphorylated eIF2α compared to most cell types in order to ensure that translation is tightly regulated in a stimulus-specific manner to regulate various neuronal functions possibly including muscle movement77. The mechanisms that regulate this phosphorylation state of eIF2α, however, remain largely uncharacterized. Consequently, we next determined if the lear-5J mutation resulted in any changes in eIF2α phosphorylation in the mouse cerebellum. To answer this question, we performed immunostaining on mid-sagittal sections of mouse cerebellum probing for phosphorylated eIF2α, the constitutively expressed regulator of eIF2α phosphorylation (CreP), and ATF4
(Fig. 4.7A-D). Our results indicate that mice homozygous for the lear-5J mutation show a dramatic reduction in levels of phosphorylated eIF2α as compared to wt controls (Fig. 4.7A). In light of this observation, we next evaluated levels of a negative regulator of eIF2α phosphorylation, CreP. CreP is a regulatory subunit of the protein phosphatase 1 (PP1) which works in a complex to provide substrate specificity to its catalytic subunit PP1C towards eIF2α. Interestingly, our results indicate that the lear-5J mice have elevated levels of CreP as compared to wt controls (Fig. 4.7B). To further evaluate this question, we examined events downstream of eIF2α phosphorylation. The ATF4 transcript contains upstream short ORFs that allow its preferential translation under conditions of eIF2α phosphorylation in response to ER stress. Therefore, we compared levels of ATF4 protein in lear-5J mice to wt controls (Fig. 4.7C-D). Under these conditions, we do not observe any detectable difference in relative ATF4 levels in lear-5J mice compared to that of wt (Fig. 4.7C-D).

4.5 DISCUSSION

PACT serves as a negative regulator of general protein synthesis under conditions of cellular stress as it triggers eIF2α phosphorylation via PKR activation. Previous work from our lab have established how DYT16 mutations in PACT lead to the dysregulation of eIF2α stress response signaling. In these studies, we identified that the recessively inherited P222L mutation leads to a delayed but heightened eIF2α phosphorylation to increase cell susceptibility to ER stress. We further described that a dominantly inherited frameshift (FS) mutation in PACT results in insoluble protein aggregates, PKR
activation, and significant apoptosis in the absence of stress\textsuperscript{130}. Interestingly, this truncated PACT protein lost its ability to directly interact with PKR and dsRNA, however, retained its ability to interact with wt PACT and TRBP \textit{in vitro}. With respect to both the P222L mutation and the FS mutation, we observe the dysregulation in eIF2α stress response signaling\textsuperscript{69,130}. In light of the aggregates previously observed from the DYT16 FS mutation, we wanted to address whether the lear-5J mutation would also form these insoluble protein aggregates. To this end, we expressed an amino terminal GFP tagged lear-5J in HeLa cells and compared the localization of the protein to GFP-wt PACT (Fig. 4.2A). While we do not observe any protein aggregates in the lear-5J expressing cells, we do observe the unusual nuclear localization of the lear-5J polypeptide in addition to the usual cytoplasmic localization of wt protein (Fig. 4.2A).

In the present study, our objective was to determine if the recessively inherited lear-5J mutation results in the dysregulation of eIF2α signaling thus possibly contributing to underlying craniofacial abnormalities and DYT16-like symptoms. The accumulation of misfolded proteins and protein aggregates has been directly linked to many neurodegenerative and cognitive disorders. To combat this, two fundamental quality control systems exist within the cell in order to maintain homeostasis. Firstly, NMD serves as an RNA surveillance pathway that protects the organism from the deleterious effects of frameshift mutations by the selective degradation of transcripts containing premature termination codons (PTC). By selectively degrading such transcripts, the NMD pathway prevents the accumulation of truncated proteins commonly associated with proteopathies as
the PTC containing transcript is not translated into the truncated protein. The second protective mechanism within cells is the protein quality control (PQC) system. This homeostatic mechanism serves to prevent the accumulation of misfolded or truncated proteins through selectively degrading the defective proteins themselves. As the lear-5J mutation is a frameshift mutation encoding for a truncated protein, we wanted to investigate whether we could detect the PTC containing mRNA as well as the truncated protein. We identified the lear-5J truncated protein in the brains of lear-5J mice at equivalent levels to that of wt PACT in control mice (Fig. 4.4A). Furthermore, while we were also able to detect the mRNA in the brains of the mutant mice the relative abundance of the lear-5J PTC containing transcript was drastically reduced relative to wt Prkra transcript levels found in wt controls (Fig. 4.4B). To validate these findings, we conducted the same series of experiments in mouse embryonic fibroblasts (MEFs). Interestingly, while we saw the same decreased levels of the Prkra transcript between lear-5J MEFs compared to wt controls, we were unable to detect the truncated protein in these cells (Fig. 4.4C-D). As we are able to detect the lear-5J mRNA in both brain and MEFs, this indicates the NMD quality control system is not sufficient on its own to prevent accumulation of Prkra mRNA (Fig. 4.4B, 4.4D). Because we are able to detect the truncated lear-5J protein in the brains of these mice but not MEFs, we postulate that the PQC system is less efficient in the brains of lear-5J mice compared to lear-5J MEFs (Fig. 4.4A, 4.4C).

While it is well established that eIF2α phosphorylation plays an integral role in maintaining homeostasis within neurons, the underlying details of specific
mechanisms remain unknown. PACT is best characterized for its role in activating PKR under conditions of ER stress, oxidative stress, and serum starvation. After identifying that the truncated lear-5J protein is present in the brains of lear-5J, we next determined the consequences of this truncation on the biochemical properties of PACT. PACT’s ability to interact with and activate PKR is facilitated by PACT’s dsRBM that serve as functional domains. PACT facilitates high affinity interactions with PKR via its two amino terminal dsRBMs (Fig. 4.1A). As the lear-5J frameshift mutation truncates the protein within dsRBM2, we initially wanted to evaluate the effect of this mutation on PACT’s ability to interact with PKR (Fig. 1B). Previous studies from our lab established the contribution of individual amino acid residues within dsRBM1 and dsRBM2 on PACT’s protein-protein interactions. Consistent with these studies, our results demonstrate that despite the severe truncation in dsRBM2, lear-5J retains its ability to interact with PKR (Fig. 4.3A). While dsRBM1 is the most critical functional domain in PACT for protein-protein and dsRNA interactions, dsRBM3 has been shown to be required for PACT’s ability to activate PKR. We next wanted to determine if the lear-5J mutation could activate PKR as it lacks the critical carboxy terminal dsRBM3. Much to our surprise the lear-5J mutation is able to weakly activate PKR independent of dsRBM3 under the conditions of this assay (Fig. 4.3B). It is worth noting that when resolving recombinant hexahistidine tagged lear-5J on SDS-PAGE gel we consistently observe multimers of this truncated protein. We cannot exclude the possibility that these aggregates are helping stabilize the conformational change in PKR required for
its activation in response to interaction with PACT or lear-5J. It is plausible that the PKR’s kinase activity is stimulated by PACT in a stress independent manner in order to contribute the basal state of eIF2α phosphorylation in neurons and the truncated lear-5J protein could disrupt such regulation.

Rowe et. al previously described a Prkra−/− mouse model that does not demonstrate DYT16-like symptoms, however, it shares many overlapping developmental phenotypes with the lear-5J mice102. Both PACT −/− mice and lear-5J +/+ mice exhibit (i) reduced body size, (ii) craniofacial abnormalities, (iii) underdeveloped ears, (iv) and hearing loss68,102. The PACT−/− mice do not exhibit any dystonia phenotype. A noteworthy difference between the PACT−/− mice and lear-5J mice lies within their genetic backgrounds. The PACT−/− mice are described on both a mixed and pure C57BL/6 background, whereas, the lear-5J +/+ mice are on the BTBR T+ Itpr3tf/J background68,102. This BTBR genetic background contains a mutation in the Itpr3 gene which codes for an inositol 1,4,5-triphosphate receptor type 3. This receptor plays a critical role in mediating intracellular calcium levels. As calcium signaling is directly linked to ER homeostasis, it is reasonable that the combination of calcium signaling dysregulation and lear-5J results in the additional DYT16-like phenotypes, especially since we have previously linked PACT mediated PKR regulation to play a central role in cellular fate in response to ER stress.

The cerebellum is a region of the brain that is indispensable for motor control161. We focused our analysis of lear-5J brains to cerebellum as it is also recently shown to be important for dystonia phenotypes in other forms of
dystonia. When comparing the overall developmental pattern of the cerebellum between the lean-5J mice to wt mice we see an overall reduction in the complexity of the foliation (Fig. 4.6A-B). The fully developed cerebellum contains 10 distinct folia some of which are subdivided into distinct lobules. This degree of complexity has evolved in order to increase the surface area of this region, thus increasing both total cell number and circuit formation. Although the lean-5J mice have all 10 foliations, some of the folial subdivisions are less defined (Fig. 4.6B). Interestingly, when evaluating the Purkinje neuron layer within the cerebellum we observed a severe deficiency in the arborization of the Purkinje neurons in lean-5J +/- mice as compared to wt mice (Fig. 4.6C-F). The Purkinje neurons function as inhibitory neurons and are responsible for the output of all motor coordination signals to the cerebellar cortex. They facilitate this control through their extensive arborization of dendrites from the cell body. Under normal conditions, this network of dendrites branching from the Purkinje cells facilitates communication between the deep nuclei and the spinal cord. Interestingly, previous studies have specifically identified that the dysregulation of either sodium or potassium pumps in Purkinje neurons leads to the rapid onset of dystonia and parkinsonism or ataxia. Other studies have also shown a direct correlation between abnormal Purkinje neuron development in patients with autism and ataxias. Our results suggest that the Purkinje neurons in lean-5J +/- mice could be dysregulated as a consequence of the severe deficit of dendritic arborizations, thus could be contributing to the underlying DYT16-like phenotype (Fig. 4.6E-F). It is worth noting that the mutation in the Itpr3 receptor...
mutation in the BTBR genetic background itself could be working in tandem with the lear-5J mutation to show the resultant dystonia phenotype.

Our cerebellar immunohistochemistry results indicate that not only do the lear-5J mice have a reduction in their basal state of eIF2α phosphorylation, they also have elevated levels of CreP (Fig. 4.7A-B). These elevated phosphatase levels in conjunction with the weakened ability for lear-5J to activate PKR could be contributing to the underlying mechanism of the reduced levels of phosphorylated eIF2α in these neurons. Finally, as eIF2α is the primary node of the ISR, we wanted to address whether the cerebellar tissue in these mice were eliciting a stress response. To this end, we performed immunostaining identifying activating transcription factor 4 (ATF4), which is preferentially translated in a cap-independent manner under conditions of stress (Fig. 4.7C-D). While we do not observe any noticeable difference in ATF4 level between wt and lear-5J (Fig. 4.7C-D).

The dysregulation of protein synthesis is an emerging theme in the field of neurodevelopment, intellectual disability disorders, and neurodegenerative disorders. Neurodegeneration is a consequence of proteopathies associated with the accumulation of insoluble protein aggregates both intra- and extracellularly. PKR has been linked to the pathology of Alzheimer’s disease (AD), Parkinson’s disease (PD), and Huntington’s disease (HD). The pathology of AD is largely understood to be attributed to the accumulation of Aβ peptides and hyperphosphorylated tau proteins. Previous studies have identified a correlation between the neurotoxic Amyloid beta (Aβ) peptide burden
and p-PKR levels in patient samples as well as the colocalization of hyperphosphorylated tau protein and p-PKR in AD patients. The development of immunotherapies targeting the Aβ plaques in AD has shown promising insight in reducing the Aβ burden of immunized patients. Interestingly, the reduction of Aβ plaques also correlated with a reduction of p-PKR in the immunized cohort.

HD results from expanded numbers of polyglutamine repeats in the huntingtin protein which leads to the accumulation of cytotoxic insoluble protein aggregates. Although clinical studies have identified elevated activated PKR in HD tissue, the underlying mechanism describing how PKR is activated in HD remains unknown. It is worth noting that Peel et al. have shown PKR is capable of binding to CAG repeats within exon 1 of the mutant huntingtin transcript such that the interaction increased with the number of repeats. Although the authors of this study indicate this interaction is capable of activating PKR, they also indicate that it is not the causative mechanism driving HD pathology, but rather a contributing factor. The underlying pathology of PD lies within the loss of dopaminergic neurons and the accumulation of cytotoxic α-synuclein protein aggregates. Three specific point mutations in α-synuclein (A30P, A53T, E46K) have been identified to cause the aggregation of α-synuclein and thus the causative factor in familial PD. Several studies have demonstrated that the phosphorylation of serine 129 in α-synuclein induces the protein aggregation. A recent study identified that PKR facilitates the α-synuclein S129 phosphorylation. Most recently, MEHMO (Mental retardation, Epileptic seizures, Hypogenitalism, Microcephaly and Obesity) syndrome identified the
dysregulation of the ternary complex through mutation in the gamma subunit of eIF2 (eIF2γ), resulting in a constitutive active ISR\textsuperscript{84,155}. In some cases, these patients present with lower limb ataxia consistent with the observed phenotype in DYT16\textsuperscript{84,155}. Collectively, these studies point to a common mechanism of translational dysregulation and the ISR in neuronal and neurodegenerative disease pathologies\textsuperscript{84,155}.

In the context of the dystonias, three DYT subtypes, DYT1, DYT6, and DYT16 have all implicated the dysregulation of translation through eIF2α signaling in the pathology of the disease\textsuperscript{74,120,129,132}. In the present study, we characterized how a recessively inherited frameshift mutation, lear-5J, results in developmental abnormalities and could possibly contribute to DYT16-like phenotypes. We identified that this truncated variant of PACT possesses the ability to interact with and weakly activate PKR \textit{in vitro}. Furthermore, we demonstrate that the lear-5J mRNA is targeted for NMD, however, we are able to detect both reduced levels of the mRNA and the truncated protein in the brains of these mice. Finally, we show the severe lack of dendritic arborization in the Purkinje neuron layer of the cerebellum with reduced eIF2α phosphorylation in lear-5J mice. In future studies, it would be highly beneficial to create this lear-5J mutation, as well as other causative DYT16 mutations, in a C57BL/6 genetic background. In the context of the lear-5J mice, this would clarify if the resulting phenotypes are due to the truncated PACT protein alone or a combination of lear-5J mutation and the BTBR T\textsup+ Itrp3\textsup/sub/J genetic background. In addition, the
lear-5J mice may offer a great mouse model for screening potential therapeutic options to alleviate the dystonia symptoms.
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Abbreviations: **DAB**: 3,3’-diaminobenzidine, **HRP**: horseradish peroxidase
**Figure 4.1:** Schematic representations of the lear-5J frameshift mutation in the *Prkra* gene. (A) Functional domains of PACT and lear-5J frameshift mutation. Orange boxes: highly conserved dsRBM1 and dsRBM2 that facilitate high affinity dsRNA interaction and protein-protein interactions. Green: dsRBM3 that does not bind dsRNA but has weak binding affinity to the PACT binding motif (PBM) within the catalytic (kinase) domain (KD) of PKR. The frameshift mutation resulting from a single nucleotide insertion results in the addition of 7 novel amino acid represented in red before the stop codon. (B) Frameshift mutation in *Prkra* ORF. Single adenine insertion at nucleotide position 534 results in a truncated protein with the original 178 AA PACT AA followed by 7 novel AA before a premature stop codon truncating the protein. This truncation occurs within the dsRBM2 functional domain.
Figure 4.2: Lear-5J protein accumulates both in cytoplasm and nucleus. (A) GFP-Tagged wt PACT (left) and GFP-Tagged lear-5J (right) constructs were transiently transfected in HeLa cells at 40% confluency and imaged 24-Hours post transfection.
Figure 4.3: Lear-5J protein interacts with and activates PKR. (A) Co-Immunoprecipitation of endogenous PKR and Flag-lear-5J overexpressed in HeLa cells. HeLa cells were transfected with Flag wt PACT or Flag-lear-5J in pCDNA3.1- expression constructs at 40% confluency and harvested 24-hour post transfection. Whole cell extracts were immunoprecipitated at 4°C overnight using 100 ng of anti-PKR antibody per IP. Samples were then analyzed via SDS-PAGE gel electrophoresis and western blot analysis probing for Flag tagged wt PACT or lear-5J (co-IP panel) using monoclonal anti-Flag-M2 (Sigma) antibody. To ascertain that an equal amount of protein was immunoprecipitated, blots were re-probed using an anti-PKR antibody (IP panel). Input blots without immunoprecipitation demonstrate equal amounts of each protein were present prior to IP.
Figure 4.3: Lear-5J protein interacts with and activates PKR. (Continued)
(B) PKR kinase activity assay was performed using PKR immunoprecipitated from HeLa cells, recombinant lear-5J and wt PACT proteins, and 1 μCi of [γ-32P] ATP per reaction. Either pure recombinant lear-5J (left) or wt PACT (right) protein was added as activator in amounts indicated above each lane. Phosphorylated PKR was visualized after SDS-PAGE and phosphorimager analysis.
Figure 4.4: Lear-5J truncated mutant protein can be detected in mouse brains but not MEFs. (Continued) (B) Reverse transcriptase PCR (RT-PCR) using total RNA from the brains of the indicated lear-5J genotypes compared with the mouse embryonic fibroblasts (MEFs) using S15 as the positive control to ascertain presence of equal amount of total RNA in all samples. (C) Western blot analysis of cell extracts derived from MEFs of the indicatedlear-5J genotypes and HeLa cells overexpressing Flag-Lear-5J protein. Blots were probed for PACT using a polyclonal antibody and the best of 3 representative blots is shown. The position of the full-length PACT and the truncated lear-5J protein is indicated by arrows. (D) RT-PCR using total RNA isolated from wt and homozygous lear-5J MEFs. GAPDH was used as a positive control to ascertain equal amount of total RNA was analyzed in each sample.
Figure 4.5: PACT protein is abundantly expressed in mouse cerebellum and especially in Purkinje neurons. (A) Immunohistochemistry on day 28 sagittal sections of wt C57BL/6 cerebellum using anti-PACT antibody. Brown staining indicates presence of PACT protein.
Figure 4.5: PACT protein is abundantly expressed in mouse cerebellum and especially in Purkinje neurons. (B) Immunohistochemistry on Day 28 sagittal sections of cerebellum from wt C57BL/6 mice showing wild type PACT (green) counterstained with DAPI (blue) as the nuclear marker. (C) Immunohistochemistry of tissue described in B showing co-localization of PACT staining with a Purkinje neuron-specific marker calbindin. PACT (red), DAPI (blue), and calbindin (green).
Figure 4.6: Lear-5J mutation affects cerebellar development and reduces arborization in Purkinje neurons.

(A-B) Hematoxylin and eosin staining on day 28 sagittal sections of wt and lear-5J cerebellum. Fully developed mouse cerebellum has ten lobules denoted here as I-X. (C-F) Immunohistochemistry of Day 28 sagittal sections of mouse cerebellar tissue stained with purkinje neuron marker, calbindin (green), and nuclear stain DAPI (blue). Dashed boxes (left panel) indicate areas of magnification (right panel).
Figure 4.7: Lear-5J cerebellum exhibits dysregulation of eIF2α signaling. (A-D) Immunohistochemistry of Day 28 sagittal sections of BTBR (wt) (left) and lear-5J (right) mouse cerebellar tissue stained with the nuclear marker DAPI (blue) and protein of interest (green). (A) DAPI (blue), phosphorylated eIF2α (green), (B) DAPI (blue), CreP (green), (C-D) DAPI (blue), ATF4 (green).
CHAPTER 5:
GENERAL DISCUSSION
This dissertation focuses on how mutations in PACT contribute to the underlying pathomechanisms associated with early onset dystonia, DYT16. This work demonstrates how the interactions between a family of dsRNA binding proteins, PACT, TRBP, and PKR regulate cell fate decisions in response to cellular stress through their ability to control translation and induce the ISR. Taken in concert, our results highlight the critical role PACT plays in the maintenance of cellular homeostasis and development, as well as how the dysregulation of this protein family contributes to the etiology of DYT16.

In chapter 2 of this dissertation we describe a mechanism whereby a dominantly inherited FS mutation in PACT identified in a DYT16 patient dysregulates eIF2α stress response signaling. Interestingly, our results show this FS mutation does not interact with PKR, however, when overexpressed in mammalian cells forms protein aggregates that lead to PKR’s activation, the phosphorylation of eIF2α, and caspase mediated apoptosis. We report that this FS mutation ablates PKR interactions, it results in higher affinity interactions with both wt PACT and TRBP in vitro. We go on to show that the FS protein is capable of disrupting PACT-TRBP heterodimers in vitro. Therefore, we proposed a mechanism where overexpressing this FS protein disrupts the inhibitory PACT-TRBP heterodimers while forming FS-TRBP and possibly FS-wt PACT heterodimers in the absence of stress. As a consequence, this promotes the formation of PACT-PACT homodimers required for PKR activation and subsequent eIF2α phosphorylation ultimately leading to caspase mediated apoptosis. Many neurodegenerative diseases are caused by protein
aggregates in neurons, our findings demonstrate a need for further investigation in
the possible role of PKR activation in neuronal loss within this context.

In chapter 3 of this dissertation we outline the biochemical consequences five
causative DYT16 point mutations have on PACT’s ability to activate PKR, interact
with dsRNA, and form high affinity homomeric and heterodimeric protein-protein
interactions. We then further describe maladaptive ISR kinetics in DYT16 derived
patient cells as compared to the unaffected control cells. In all cases, our results
indicate that the DYT16 mutations show a significant increase in their homomeric
interactions in the absence of stress and enhanced PKR activation in vitro. Although
none of these DYT16 mutations demonstrated a change in their ability to bind
dsRNA or heterodimerize with TRBP, it is notable that only the dominantly inherited
N102S and T34S mutations resulted in enhanced heterodimer formation with PKR.
Then using DYT16 patient derived lymphoblasts we demonstrate in the absence of
stress, PACT-PKR interactions are enhanced and observe an increase in basal
levels of PKR activation. Consequently, in response to ER stress the DYT16
lymphoblasts demonstrated a prolonged and more robust ISR that ultimately
resulted in an increase in apoptosis in patient cells relative to unaffected controls.
Finally, our study demonstrates for the first time that this hypersensitivity to ER
stress observed in patient cells is partially rescuable through disrupting the high
affinity PACT-PKR interactions with the flavonoid, luteolin. Our results here support
the hypothesis that the dysregulation of the ISR could be a common modality in
many forms of primary dystonia. We also provide the first insights into potential
therapeutic targets for DYT16.
In chapter 4 of this dissertation we characterize a novel DYT16 mouse model resulting from a de novo mutation in the Prkra gene which codes for the mouse homolog of PACT. This recessively inherited frameshift mutation was identified by the Jackson Laboratories and termed “lear-5J” as mice homozygous for this mutation presented with small ears. Furthermore, these mice presented with severe craniofacial developmental abnormalities, underdeveloped ears, reduced body size, kinked tails, and DYT16-like symptoms. We investigated how this lear-5J truncated variant of PACT alters the protein’s ability to interact with and activate PKR, as well as identify any developmental abnormalities within the cerebellum. We demonstrate that the lear-5J truncated protein retains its ability to interact with PKR, however, its ability to stimulate PKR’s kinase activity is dramatically reduced in vitro.

Furthermore, we show that the lear-5J mRNA is partially targeted for NMD both in the brains of these mice as well as MEFs, however, we only detect the truncated protein in the brains of affected mice. Our histological analysis on the cerebellum of lear-5J mice revealed abnormalities in the development of the folia as compared to unaffected wt mice. Finally, upon further examination of these cerebellum we identified a severe lack of dendritic arborization in the Purkinje neuron layer of the cerebellum and reduced eIF2α phosphorylation in the brains of the lear-5J mice relative unaffected controls. Although significantly more work needs to be pursued in order to further elucidate the underlying mechanisms contributing to the etiology of DYT16-like symptoms in these mice, this provides insights into the first model system for this disease that could prove to be a valuable tool for testing potential therapeutic interventions for DYT16 dystonia.
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