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CONTRACTING ORGANIZATION: University of South Carolina

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14. ABSTRACT

Currently, the available treatment options for dystonia are merely palliative and the drug development has not progressed significantly due to a lack of understanding about the involved molecular pathomechanisms. We investigated if PACT, the gene mutated in dystonia 16 (DYT16), causes a disruption in the normal regulatory crosstalk between PERK and PKR kinases leading to a loss of cell homeostasis after ER stress. Both PERK and PKR kinases phosphorylate eIF2 alpha and activate a downstream signaling pathway that allows recovery and survival after ER stress. **The most significant finding during the last funding period** was that PACT is a substrate of PERK kinase and PERK phosphorylates PACT *in vivo* after ER stress. This is a paradigm-shifting finding as it was previously unknown that PACT could participate and regulate both PKR and PERK pathways. The molecular etiology of DYT16 has now been conclusively shown to be a dysregulation of eIF2 alpha signaling. Thus, our research has uncovered a PACT-mediated novel regulatory pathway and laid the foundation for more in depth drug development to target PACT-PERK interactions in future. In addition, it has added significant new knowledge about how cells respond to ER stress. In the brief period of 8 months we made significant progress included in this report, before the COVID-19 pandemic slowed down our work significantly.

15. SUBJECT TERMS
Dystonia, kinase, cell survival, stress response, protein interaction, PACT, PERK, PKR

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1. INTRODUCTION:

Subject: Dystonia is a movement disorder in which the affected individuals develop sustained, involuntary and excessive muscle contractions caused by disrupted brain function. Inherited genomic mutations, brain trauma, and use of certain psychiatric drugs are the known factors that can cause dystonia. The military veterans injured in combat often develop dystonia years after the original injury. Thus, understanding the involved pathomechanisms and effective treatments for dystonia is a health issue of particular relevance to the armed forces. Our research aims to characterize the molecular pathways defective in dystonia 16 (DYT16), and will be valuable in developing new therapies not just for DYT16 but also for many forms of dystonia because the ER stress response pathway that is defective in DYT16 is also affected in multiple forms of dystonia including late onset, traumatic brain injury-induced dystonia. Purpose: DYT16 is caused by inherited mutations in PACT protein (encoded by *Prkra* gene) and PACT is a well-established regulator of protein kinase PKR activity. **The overall purpose** of this project is to investigate if in addition to regulating PKR kinase activity, PACT also regulates another kinase, PERK. PERK is one of the central kinases that responds to endoplasmic reticulum (ER) stress to trigger onset of a protective response. So far, it has been believed that PERK and PKR respond to non-overlapping sets of stress signals without any crosstalk between them. We hypothesized that PACT directly participates in PERK signaling pathway to regulate cell survival. Our results will thus demonstrate the cross-regulation between PKR and PERK to identify new druggable targets for dystonia. Scope: Using molecular and biochemical techniques we are investigating (1) if PACT interacts directly with PERK to affect its kinase activity or to function as its substrate, (2) if PACT contributes positively or negatively in the PERK signaling pathway to affect cell survival, and (3) if PACT mutations reported in DYT16 cause a dysregulation of PERK pathway which may lead to the onset of dystonia. While focusing on PACT-mediated regulation of PERK pathway, the scope of our research is broad and far-reaching. PERK activation in response to ER stress regulates cellular fate via eIF2α phosphorylation and as eIF2α phosphorylation is known to regulate various aspects of neuronal development and functioning in the brain, our research offers a new paradigm that will be valuable for other forms of dystonia because disrupted eIF2α signaling has been noted in multiple forms of dystonia. Thus, although being focused on PACT-PERK, our work offers insights to integrate the pathologies operative in various dystonia types and may uncover novel druggable targets for dystonia therapy.

2. KEYWORDS:

Dystonia, DYT16, PACT, Prkra, PERK, PKR, eIF2α, ER stress, kinase, signaling, apoptosis

3. ACCOMPLISHMENTS:

- **What were the major goals of the project?**

The following goals and major tasks were stated in the approved SOW. The expected target date as in SOW and the Status of task (completed, underway, or not started yet) is as in the table below.

Research-specific Tasks and Goals- new progress in the last year in bold font in the "status" column

Specific Aim 1: To test if PACT interacts directly with PERK to affect its kinase activity or to function as its substrate:		
Major Task 1: To determine if PACT interacts directly with PERK and activates it or functions as its substrate	**Target Date**	**Status**
Subtask 1: co-immunoprecipitation (co-IP) assays to determine interaction between PACT and PERK	06/2018	completed in 2019
Subtask 2: mammalian two hybrid and yeast two hybrid interaction assays to determine a direct interaction between PACT and PERK	07/2018	completed in 2019
Subtask 3: *in vitro* PERK kinase activity assays using purified recombinant PERK and PACT proteins:	08/2018	**completed in 2019, more results since then (Fig.2-3**)
Subtask 4: test the effect of PACT phospho-defective mutants on PERK signaling in response to ER stress:	09/2018	**Underway, Fig.1**
Milestone(s) Achieved: determine PACT's involvement in PERK pathway by direct interaction with PERK and publication of results	09/2018	**Completed, Manuscript in preparation**
Specific Aim 2: To investigate the functional contribution of PACT to the PERK signaling pathway		
Major Task 2: to determine PACT's functional role in PERK pathway		
Subtask 1: To determine if PERK phosphorylates PACT in response to ER stress	12/2018	**Completed (Fig. 2 and 3)**
Subtask 2: To determine if PACT is essential for PERK activation in response to ER stress	02/2019	Not yet started
Subtask 3: To investigate if PKR is activated in response to ER stress in PERK null MEFs	04/2019	Completed in 2019
Milestone(s) Achieved: Characterized the functional contribution of PACT to PERK pathway and the crosstalk between PERK and PKR pathways via PACT and publication of results as well as presentation at a national meeting	04/2019	**Underway**
Major Task 3: **To test if PACT mutations reported in DYT16 cause a dysregulation of PERK pathway**		
Subtask 1: Do the DYT16 PACT mutants interact with PERK with similar efficiency as the wt PACT	06/2019	completed

Milestone(s) Achieved: Make all mutants and the plasmid expression constructs using site directed mutagenesis and sub-cloning	05/2019	Completed in 2019
Subtask 2: determine the effect of PACT mutants on PERK's kinase activity	08/2019	Completed in 2019
Subtask 3: determine the effect of PACT mutants on PERK signaling pathway in response to ER stress	09/2019	**completed**
Milestone(s) Achieved: Characterization of effects of DYT16 mutations on PERK signaling pathway and demonstration of a dysregulation of the regulatory crosstalk between PKR and PERK pathways, Publication of results	09/2019	**Manuscript based on part of the results published in Neurobiology of Disease**

- **What was accomplished under these goals?**

Results obtained after last annual report (04/30/2019) are summarized below:

Specific Aim 1: To test if PACT interacts directly with PERK to affect its kinase activity or to function as its substrate:

Subtasks 1-3: were completed in 2019.

Subtask 4: test the effect of PACT phospho-defective mutants on PERK signaling in response to ER stress: We have not yet completed this subtask. We had proposed to test if overexpression of phospho-defective PACT mutants interferes with a normal ER stress response. We plan on testing PERK activation, PKR activation, eIF2α phosphorylation, transcriptional induction of ATF4, CHOP, and GADD34, and apoptotic response of cells overexpressing PACT mutants. We will soon begin this analysis. However, we had also proposed to use a luciferase reporter plasmid (AARE-Renilla Luciferase, SwitchGear Genomics) that measures the amount of transcriptionally active ATF4 in HEK293 cells. We used this reporter system to test if PACT phospho-defective and phospho-mimic mutants show downregulation of ATF4 as compared to wt PACT. We completed this analysis and the results were negative. As seen in Fig. 1 none of the phospho-defective or phosphor-mimic mutants of PACT showed any effect on AARE-Renilla Luciferase activity at the basal or in response to ER stress. The most possible reason for this may be that the serine 246 and serine 287 are not

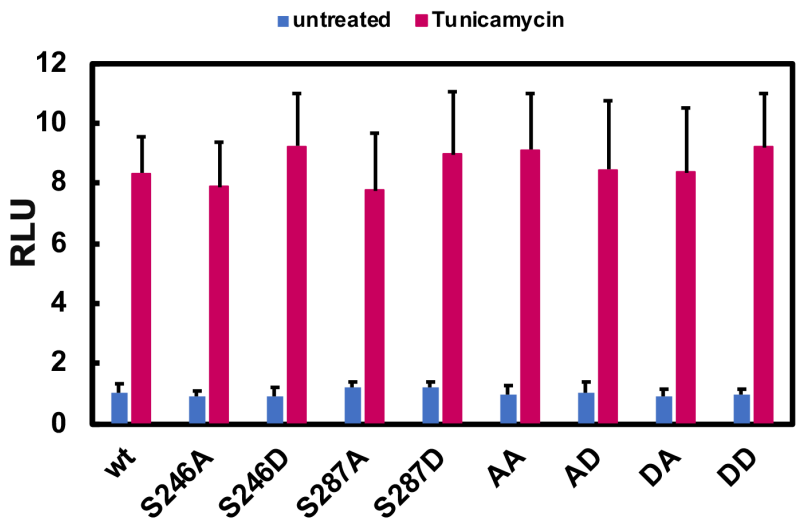

Fig. 1. Effect of phospho-defective or phospho-mimic mutants of PACT on ER stress response. The blue bars represent activity in untreated cells and pink bars indicate activity in cells treated with 10 μg/ml tunicamycin. AARE-Renilla Luciferase was used as the reporter. Firefly luciferase driven by CMV promoter was used to normalize the transfection efficiencies.

the important phosphorylation sites on PACT in response to PERK activation. PERK may phosphorylate PACT at sites different than serine 246 or serine 287. We have previously studied the importance of serines 246 and 287 in response to oxidative stress but the phosphorylation sites in response to ER stress may be different than serines 246 and 287. Since the results of AARE-Renilla Luciferase assays indicated no effect of the phospho-mimic or phospho-defective PACT mutants, we postponed our detailed analysis of their effects on PERK activation, PKR activation, eIF2α phosphorylation, transcriptional induction of ATF4, CHOP, and GADD34, and apoptotic response of cells. We plan on completing this analysis by 09/2021. As our results demonstrated that PACT is a substrate of PERK, we had also tested previously if the phospho-defective (alanine substitutions) or phosphor-mimic (aspartic acid substitutions) PACT mutants are phosphorylated by PERK. In addition, as seen in Fig. 6 of our last progress report, either a mutation of serine 246 or serine 287 did not affect the ability of PERK to phosphorylate PACT. Taken together, these results indicate that PERK phosphorylates sites other than serines 246 and 287 or phosphorylates sites in addition to these two serines.

Major activities during this period for this specific aim: The major activities were to establish reproducible biochemical assays to test PACT's interaction with PERK and to develop and standardize PERK kinase activity assays to determine the effect of PACT on PERK's kinase activity. This was accomplished as shown in our last technical report submitted 04/2019. The remaining activity was subtask 4 and although the initial results are negative for this sub-task, these negative results are very important as they indicate additional phosphorylation sites on PACT that are important for the PERK pathway. We are currently investigating this.

Specific objectives during this period for this specific aim: The major objectives were to (1) investigate if PACT interacts with PERK using biochemical and genetic assays and (2) investigate the effect of PACT on PERK's kinase activity to evaluate if PACT activates or inhibits PERK activity and if PACT is a substrate of PERK. This was accomplished effectively on the last progress report in 04/2019. In addition, since then we have also concluded that the known phosphorylation sites on PACT do not contribute positively or negatively to the regulation of PERK pathway. This conclusion, although negative is extremely valuable as it allowed us to rule out a major possibility and has opened up a new inquiry to characterize the novel sites on PACT that are phosphorylated by PERK.

Significant results and key outcomes during this period for this specific aim: The significant results and the key outcomes were (1) we demonstrated that the known phosphorylation sited on PACT (serines 246 and 287) are not important for the regulation of PERK pathway. (Fig. 1), and (2) in future investigations, we will need to map the novel sites that are phosphorylated by PERK.

Specific Aim 2: To investigate the functional contribution of PACT to the PERK signaling pathway.

Subtask 1: To determine if PERK phosphorylates PACT in response to ER stress. This subtask was completed in 2019. We conclusively demonstrated in the last progress report that PACT is a substrate of PERK. We have now done some additional work in this direction and we used deletion mutants of PACT to map potential regions for PERK phosphorylation sites within PACT. As seen in Fig. 2 A, wt PACT (lane 2) and FS dletion mutant (lane 7) were the only two proteins that got strongly phosphorylated by PERK. M1 (lane 4), M2 (lane 5), M3 (Lane 6), and M1M2 (lane 3) deletion mutants showed very weak phosphorylation. These

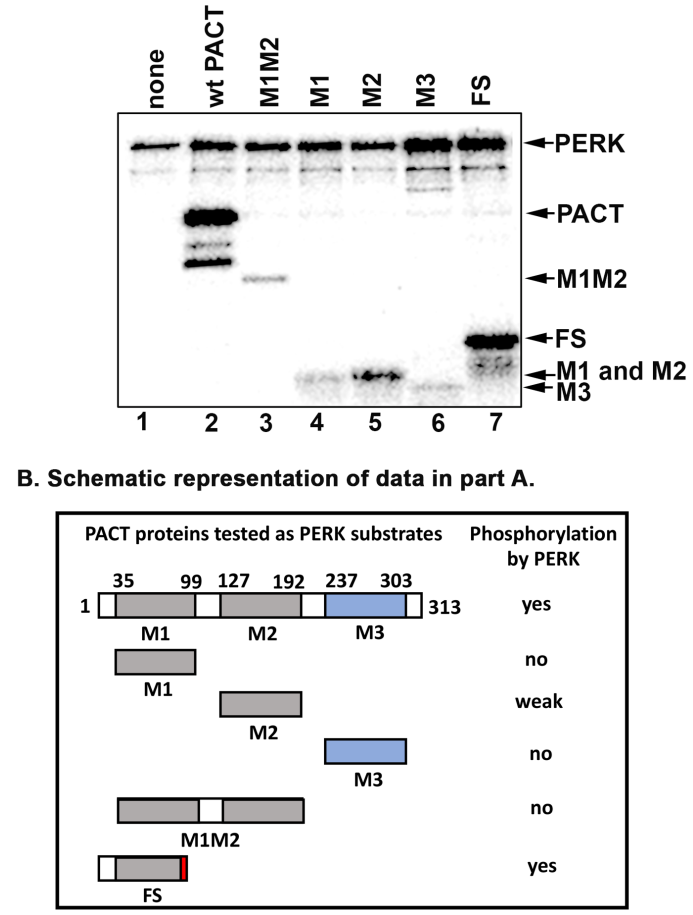

Fig. 2. PERK's ability to phosphorylate deletion mutants of PACT: A. kinase activity assay. Pure recombinant 20 ng GST-PERK (cytoplasmic catalytic domain) was used to measure phosphorylation of various PACT deletion mutants as compared to wt PACT. The PACT proteins added is as indicated above the lanes. Pure recombinant PACT proteins were used as substrates. B. Schematic diagram shows various deletions within PACT and if these deletions showed phosphorylation by PERK. The extraneous amino acids resulting due to the frameshift mutation in FS is shown in red).

results indicate that it is very likely that the PERK phosphorylation sites may reside within the first 35 residues of PACT (Fig. 2 B). Although it is possible that the phosphorylation sites in FS could also potentially reside in the few extraneous amino acids resulting from the frameshift mutation, we think this is highly improbable as the region 1-35 of PACT contains 4 serines and 3 threonines. It is thus, most likely that PERK phosphorylates one or more of these sites. We will explore this further to identify the precise sites of phosphorylation. The results presented in Fig. 2 have offred valuable clues for us to invesigate further and define PACT's functional role in the PERK signaling pathway. This is a major achievement in the last year for us.

In addition to this, we also wanted to demonstrate that PERK phosphorylates PACT in cells after ER stress. Thus, we tested this in PERK+/+ and PERK -/- cells using *in vivo* phosphate labeling during early time points after ER stress. Endogenous PACT was immunoprecipitated and its phosphorylation was analyzed by phosphorimager analysis. As seen in Fig. 3, in PERK +/+ cells, PACT is phosphorylated at 0.5h-2h after ER stress and its phosphorylation decreases at 4h after ER stress. In contrast to this, in PERK-/- cells, PACT is not phosphorylated in response to ER stress. This establishes that in the absence of PERK, PACT is not phosphorylated in response to ER stress. Taken together with our *in vitro* data that purified PERK phosphorylates purified PACT directly, this establishes that PACT is a substrate of PERK after ER stress. **This is a major paradigm-shifting result and opens up a whole new research direction for ER stress response as well as for dystonia.** We anticipate that several forms of dystonia may activate PACT-PKR pathway via ER stress and future research in this direction would unravel novel drug targets that will be beneficial for a variety of dystonia subtypes. Thus, our results are very impactful in the long term and will form a foundation for future research once we have published them, possibly within the next year.

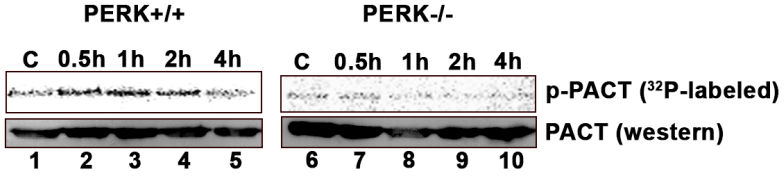

Fig. 3. PERK is essential for PACT phosphorylation in response to ER stress: PERK+/+ and PERK-/- MEFs were treated with 0.5 μg/ml tunicamycin after they were maintained in ^{32}P-labeled orthophosphoric acid in phosphate-free DMEM for 24h. The cell extracts were prepared in buffers containing phosphatase inhibitors at the indicated times and endogenous PACT was immunoprecipitated. Immunoprecipitates were analyzed by SDS-PAGE followed by phosphorimager analysis. 50% of the immunoprecipitates were analyzed by a western blot analysis to ensure that equal amounts of PACT protein is immunoprecipitated in all samples.

Subtask 2: To determine if PACT is essential for PERK activation in response to ER stress. We have not yet started work on this sub task. The lab was shut down due to the COVID-19 pandemic in March 2020 and was closed until end of August 2020. We are now working at 50% capacity as per the CDC guidelines following safe social distancing and thus the work is progressing quite slowly. However, we will be able to complete all the work proposed in this sub task by Sept. 2021.

Subtask 3: To investigate if PKR is activated in response to ER stress in PERK null MEFs. We completed this subtask in 2019 and the results were included on the last progress report. We demonstrated that PERK is essential for PKR activation in response to ER stress.

Major activities during this period for this specific aim: We were able to demonstrate that PACT is phosphorylated by PERK in vivo after ER stress. We were also able to determine that the most likely PERK phosphorylation sites are within the amino terminal 1-35 residues in PACT.

Specific objectives during this period for this specific aim: The specific objectives were to determine if the observed in vitro phosphorylation of PACT by PERK also happens in intact cells after ER stress. This was the main objective we pursued and obtained conclusive evidence that PACT is a legitimate *in vivo* substrate of PERK. The second objective to investigate if PACT is essential for PERK activation was not accomplished due to COVID-19 pandemic imposed shut down of lab in March 2020.

Significant results and key outcomes during this period for this specific aim: The biggest key outcome was the demonstration that PERK phosphorylated PACT in response to ER stress in intact cells. This is expected to form the foundation of further research that will characterize a novel pathway in detail.

Specific aim 3: To test if PACT mutations reported in DYT16 cause a dysregulation of PERK pathway.

Subtask 1: Do the DYT16 PACT mutants interact with PERK with similar efficiency as the wt PACT. We tested the interaction between the cytoplasmic domain of PERK and PACT (wt and the DYT16 PACT mutants) using pure recombinant hexahistidine tagged PACT proteins immobilized on NI agarose and *in vitro* synthesized ^{35}S-methionine labeled cytoplasmic domain of PERK. As seen in Fig. 4, the ^{35}S-methionine labeled *in vitro* synthesized PERK cytoplasmic domain interacts with recombinant wt PACT protein bound to Ni-agarose beads very efficiently at physiological (150mM) salt concentration (lane 3). PERK protein shows no binding to Ni-agarose beads in the absence of PACT (lane 2). As seen in lanes 4-9, various DYT16 PACT mutants interact with PERK equally well as wt PACT. These results establish that the cytoplasmic domain of PERK interacts equally well with wt PACT and all DYT16 point mutants. We are also currently testing this further using purified recombinant hexahistidine tagged PACT protein and GST-tagged PERK cytoplasmic domain. We will use Ni-agarose beads to pull down his tagged-PACT and perform western blot analysis with anti-GST antibody to assess if GST-PERK could be pulled down by binding to his- wt PACT and the his-DYT16 mutant PACT proteins. This will further confirm that the DYT16 mutations do not affect the interaction between PACT and catalytic cytoplasmic domain of PERK.

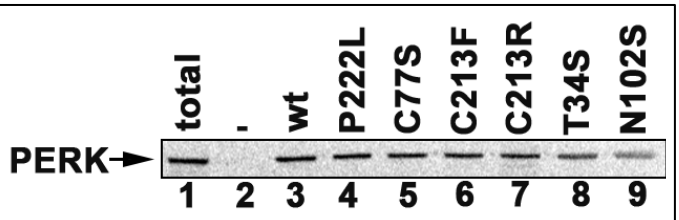

Fig.4. Interaction of DYT16 PACT mutants with PERK cytoplasmic domain. 3 µl of ^{35}S-labeled *in vitro* synthesized PERK cytoplasmic domain was bound to either Ni-agarose beads alone (lane 2) or hexahistidine tagged, pure recombinant PACT protein bound to Ni-agarose beads. The beads were washed and the proteins remaining bound to beads were analyzed by SDS-PAGE followed by phosphorimager analysis. Lane 1 shows PERK protein in the input without binding to beads.

Subtask 2: determine the effect of PACT mutants on PERK's kinase activity: We completed this subtask in 2019. We determined that PERK phosphorylates the recessive DYT16 PACT mutants (C77S, C213F, and C213R) with reduced efficiency and phosphorylates the dominant DYT16 PACT (T34S and N102S) mutants with increased efficiency as compared to wt PACT. At present we do not know the biological significance of these results. The biological significance will become clear after we investigate the functional role of PACT in PERK signaling pathway.

Subtask 3: Determine the effect of PACT mutants on PERK signaling pathway in response to ER stress. Initially we had planned on using expression constructs for DYT16 PACT mutants in HEK293 cells to investigate their effects on PERK signaling pathway. However, when we performed these experiments, it was clear that due to the presence of endogenous wt PACT, we could not see much effect of the recessive DYT16 PACT mutants. Thus, we next used the DYT16 patient lymphoblast cell lines to investigate PERK and eIF2α signaling pathway**.** In both P222L homozygous patient as well as in P222L/C213R compound heterzozygous patient, the downstream events that occur in response to PERK signaling and eIF2α phosphorylation were dysregulated. We have included the P222L/C213R compound heterozygote data in this report. As seen in Fig. 5, the phosphorylation of eIF2α, expression of ATF4, and CHOP (ATF4 and CHOP are downstream effects of PERK activation) were dysregulated. **Some of these results are now published in the following manuscript:** https://doi.org/10.1016/j.nbd.2020.105135.

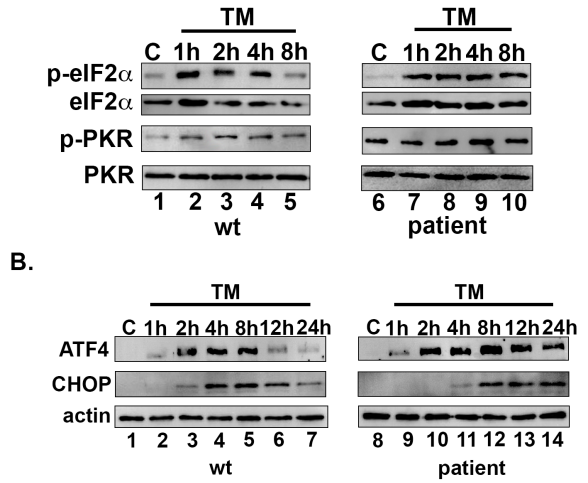

Fig. 5. eIF2α signaling pathway dysregulation in normal and DYT16 patient lymphoblasts. (A) Western blot analysis for p-PKR and p-eIF2α. Whole cell extracts from normal (wt) and DYT16 patient derived lymphoblasts treated with 5 μg/ml of tunicamycin (TM) were analyzed at indicated time points. Blots were 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**. Whole cell extracts from normal (wt) and DYT16 patient derived lymphoblasts treated same as in 3A were analyzed at indicated time points. Blots were probed for ATF4, and CHOP. Best of four representative blots are shown. β-actin was used as a loading control to ensure equal amounts of protein was loaded in each lane.

A PDF of this manuscript is also attached to the report.

Major activities during this period for this specific aim: We could conclusively establish that the DYT16 PACT mutants interact equally well as wt PACT with PERK's cytoplasmic domain. In addition, we also established that PERK signaling is dysregulated in DYT16 patient cells in response to ER stress. We published our research in Neurobiology of Disease (link to publication above).

Specific objectives during this period for this specific aim: The specific objectives during this period for this aim was to investigate the effect of DYT16 PACT mutations on PERK signaling pathway and to study any perturbations in the PERK signaling due to DYT16 mutations in PACT. Both were achieved conclusively.

Significant results and key outcomes during this period for this specific aim: Two significant results were achieved. (1) DYT16 PACT mutants interact with PERK at same efficiency as wt PACT. (2) PERK signaling is dysregulated in DYT16 patient cells. We also published a major manuscript that includes some of these results.

- **What opportunities for training and professional development has the project provided?**

The project has provided great opportunity to train two graduate students. One has graduated with PhD in May 2020 and one is currently working towards his PhD in my lab. The student who graduated with a PhD is the first author of our recent publication in Neurobiology of Disease (link above). He has since moved on to his postdoctoral job. I train the students one-on one as I also work in the lab. The project allows me to train the graduate students for cutting edge biochemical and molecular work. In particular, the graduate students learn cell culture, protein-protein interaction assays, kinase activity assays, yeast and mammalian two hybrid protein interaction methodology and assays, reporter assays using luciferase reporter plasmids, transfection of cells in culture, western blot analysis, and co-immunoprecipitation assays. The current PhD student has become very proficient in performing these experiments and will defend his thesis by May 2021. In addition to training the students at bench, the project has allowed me to train the student in interpreting the data to prepare the results for presentation and publication. I have weekly lab meetings when students present their data and answer questions pertaining to their results. This prepares the students to attend national/international meetings. This project has allowed ample scope to train the current graduate student and he is planning on presenting his results at the next ASBMB meeting in April 2021. The project has allowed me to train the graduate students to become successful in biochemical research pertaining to dystonia as well as protein homeostasis. The students have given seminar presentations locally at our university and department in weekly seminar series. The current graduate student is also training one undergraduate under him so he is also able to pass on his knowledge and skills to students engaged in undergraduate research. The student who graduated with PhD in May 2020 trained an undergraduate researcher under him and this undergraduate student is a co-author on the recent manuscript published in Neurobiology of Disease.

- **How were the results disseminated to communities of interest?**

I serve as a mentor for local high school students who would like to engage themselves in learning about how basic research impacts all major medical breakthroughs for treating human diseases. I visit local high schools to give talks to the honors and Advanced Placement Biology class. This has been very successful strategy to generate interest in having a career in science. Since this project focuses on molecular pathways that are defective in a movement disorder DYT16, my audience in high schools has found it very interesting to learn about the disorder and see how research done in my lab may be helping to find novel cures for the dystonia patients. Currently this activity is on hold since March 2020 due to the COVID19 pandemic.

- **What do you plan to do during the next reporting period to accomplish the goals?**

We have made great progress in accomplishing the goals outlined in the original proposal and SOW. However, we had to change the order in which we complete the designated tasks. Some of the specific tasks that were to be completed in the next reporting period we have already completed as of now and some other tasks that were to be completed during the last reporting period will be completed in the next reporting period. We had to make this change as the pure recombinant PERK enzyme that we use to perform the enzyme assays has a shelf life and we wanted to get as many assays finished as possible using the single batch of enzyme to minimize variations. Thus, we completed the tasks that needed the purified PERK enzyme first

during this reporting period. The COVID19 pandemic related shutdown of lab also caused significant disruption in completing the work as planned. Nevertheless, we are very successful in completing the work as proposed, which will result in one more major manuscript in 2021 or 2022. We also plan on submitting major federal grant proposals to extend this work and continue working on dystonia. This has resulted in a new target date for some of the specific tasks. We plan on completing this as outlined below:

Specific Aim 1: To test if PACT interacts directly with PERK to affect its kinase activity or to function as its substrate:	
Major Task 1: To determine if PACT interacts directly with PERK and activates it or functions as its substrate	target date
Subtasks 1-3 are completed	
Subtask 4: test the effect of PACT phospho-defective mutants on PERK signaling in response to ER stress:	09/2020
Specific Aim 2: To investigate the functional contribution of PACT to the PERK signaling pathway	
Major Task 2: to determine PACT's functional role in PERK pathway	
Subtask 1 is complete	
Subtask 2: To determine if PACT essential for PERK activation in response to ER stress	09/2020
Subtask 3 is completed	
Major Task 3: To test if PACT mutations reported in DYT16 cause a dysregulation of PERK pathway	
Subtask 1 is completed	
Subtask 2 is completed	
Subtask 3: is completed	

4. IMPACT:

- **What was the impact on the development of the principal discipline of the project?**

The major finding from our research so far is that PACT is phosphorylated by PERK. In addition, the DYT16 mutants affect the eIF2α signaling and this is an emerging common theme in many different types of dystonia, possibly including injury induced late-onset dystonias in veterans. Once all of our results are published, it will have a major influence on the field. It is unknown that PERK has any substrate other than eIF2α and NRF2. PERK initiates a cascade of signaling events in response to ER stress such as protein misfolding that enable cell adaptation and ER stress resolution. (In case of dystonia (DYT16) this would be the stress caused by misfolded PACT proteins) The signaling pathways initiated by PERK activation are not only essential for the survival of normal cells undergoing ER stress, but are also co-opted by tumor cells in order to survive the oxygen and nutrient-restricted conditions of the tumor microenvironment and PERK signaling is known to

influence a variety of pro-tumorigenic processes. Therefore, from a purely biological standpoint as well as from a clinical perspective, it is important to understand this critical cell adaptive pathway in greater detail through identifying its interacting partners and thereby elucidating additional downstream signaling branches. Our research aims at identifying and characterizing such novel consequences of PERK activation and is thus very important for many branches of cell molecular biology and biochemistry disciplines. It is expected to have a major impact and offer paradigm shifting views of this central stress response pathway.

- **What was the impact on other disciplines?**

Our research explores basic biochemistry pertaining to stress response and cell survival. However, as PERK-PACT-eIF2α pathway is an integral part of protein and cell homeostasis, our work is relevant to drug development for many neurodegenerative diseases as well as diabetes, and cancer. Thus, upon completion of our research novel druggable targets are expected to emerge which can be developed further by pharmacologists and researchers involved in translational research.

- **What was the impact of technology transfer?**

  Nothing to report.

- **What was the impact on society beyond science and technology?**

  Nothing significant to report during this period.

5. CHANGES/PROBLEMS:

Nothing to report. Work is progressing well as planned.

6. PRODUCTS:

Burnett SB, Vaughn LS, Sharma N, Kulkarni R, Patel RC. Dystonia 16 (DYT16) mutations in PACT cause dysregulated PKR activation and eIF2α signaling leading to a compromised stress response. **Neurobiol Dis.** 2020 Oct 10;146:105135. doi: 10.1016/j.nbd.2020.105135. Epub ahead of print. PMID: 33049316.

Additional Publications, presentations, and conference papers expected in next year.

7. PARTICIPANTS AND OTHER COLLABORATING ORGANIZATIONS

- **What individuals have worked on the project?**

Name	**Rekha Patel (no change from the proposed)**
Project Role	**PI**
Researcher Identifier (ORCID ID)	
Nearest Person Month worked	**1 summer month**
Contribution to the project	**Planning, Supervision**
Funding Support	**No change**
Name	**Sumuel Burnett (no change from the proposed)**
Project Role	**Graduate Student**
Researcher Identifier (ORCID ID)	
Nearest Person Month worked	**12 months, full academic year**
Contribution to the project	**Experiments, Manuscript writing**

Funding Support	**No change**
Name	**Indhira Handy (no change from the proposed)**
Project Role	**Technician-Research assistant**
Researcher Identifier (ORCID ID)	
Nearest Person Month worked	**12 months, full academic year**
Contribution to the project	
Funding Support	**No change**

- **Has there been a change in the active other support of the PD/PI or senior/key personnel since the last reporting period?**

  Nothing to report

- **What other organizations were involved as partners?**

  Nothing to report.

8. SPECIAL REPORTING REQUIREMENTS

NOT APPLICABLE

9. APPENDICES

PDF copy of published manuscript - pages 14-28

Burnett SB, Vaughn LS, Sharma N, Kulkarni R, Patel RC. Dystonia 16 (DYT16) mutations in PACT cause dysregulated PKR activation and eIF2α signaling leading to a compromised stress response. **Neurobiol Dis.** 2020 Oct 10;146:105135. doi: 10.1016/j.nbd.2020.105135. Epub ahead of print. PMID: 33049316.

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Dystonia 16 (DYT16) mutations in PACT cause dysregulated PKR activation and eIF2α signaling leading to a compromised stress response

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ABSTRACT

Dystonia 16 (DYT16) is caused by mutations in PACT, the **p**rotein **act**ivator of interferon-induced double-stranded **R**NA-activated **p**rotein **k**inase (PKR). PKR 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 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 **t**ransactivation **R**NA-**b**inding **p**rotein (TRBP) thereby keeping PKR inactive. Under conditions of cellular stress these inhibitory interactions dissociate facilitating PACT-PACT interactions critical for PKR activation. While both PACT-TRBP and PKR-TRBP interactions are pro-survival, PACT-PACT and PACT-PKR interactions are pro-apoptotic. In this study we evaluate if five DYT16 substitution mutations alter PKR activation and ISR. Our results indicate that the mutant DYT16 proteins show stronger PACT-PACT interactions and enhanced PKR activation. In DYT16 patient derived lymphoblasts the enhanced PACT-PKR interactions and heightened PKR activation leads to a dysregulation of ISR and increased apoptosis. More importantly, this enhanced sensitivity to ER stress can be rescued by luteolin, which disrupts PACT-PKR interactions. Our results not only demonstrate the impact of DYT16 mutations on regulation of ISR and DYT16 etiology but indicate that therapeutic interventions could be possible after a further evaluation of such strategies.

1. 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 (Pakos-Zebrucka et al., 2016). The central event in this pathway is the phosphorylation of eukaryotic translation initiation factor 2 (eIF2α) on serine 51 by one of the four serine/threonine kinases (Donnelly et al., 2013; Taniuchi et al., 2016). This post-translational modification prevents the formation of the ternary complex during translation initiation, leading to a decrease in general protein synthesis while allowing induction of selected genes that promote cellular recovery (Wek, 2018). 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 (Donnelly et al., 2013). Thus, although ISR is primarily a pro-survival response to restore cellular homeostasis, exposure to severe stress drives signaling towards cellular death. Thus, ISR tailors the cellular stress response in a specific manner to 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 a ubiquitous eIF2α kinase (Garcia et al., 2007; Meurs et al., 1990) active under cellular stress conditions such as viral infection, oxidative and endoplasmic reticulum (ER) stress, and serum deprivation (Ito et al., 1999; Patel et al., 2000). In virally infected cells, PKR is activated by direct interactions with dsRNA, a viral replication intermediate for many viruses (Barber, 2001). However, in the absence of viral infections other stress signals activate PKR *via* its

Abbreviations: PKR, Protein kinase RNA-activated; IFN, interferon; ds, double-stranded; eIF2, eukaryotic initiation factor; ER, endoplasmic reticulum; dsRBM, dsRNA-binding motif; TRBP, TAR RNA-binding protein; PACT, PKR activator protein; Prkra, Protein Activator of interferon-induced protein kinase; DYT, dystonia; ISR, integrated stress response; HIV, human immunodeficiency virus

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protein activator (PACT) (Patel and Sen, 1998) in a dsRNA-independent manner. Two evolutionarily conserved amino terminal dsRNA binding motifs (dsRBMs) of PKR mediate its interactions with dsRNA (Feng et al., 1992; Green and Mathews, 1992; Patel and Sen, 1992) as well as with PACT (Huang et al., 2002; Peters et al., 2001) and other regulatory proteins (Chang and Ramos, 2005). Upon binding dsRNA or PACT, PKR undergoes a conformational change which results in the autophosphorylation and activation of PKR (Cole, 2007; Nanduri et al., 1998). In the absence of stress, however, PKR is inhibited through direct interactions with the transactivation response element (TAR) RNA binding protein (TRBP) *via* the dsRBMs of each protein (Benkirane et al., 1997; Laraki et al., 2008). TRBP was initially discovered due to its strong affinity to the TAR RNA element of HIV (Benkirane et al., 1997) inhibits PKR both by sequestration of dsRNA and by direct interaction during viral infections (Daniels and Gatignol, 2012). In the absence of stress, TRBP inhibits PKR *via* the formation of both TRBP-PACT and TRBP-PKR heterodimers (Daher et al., 2009; Singh et al., 2011; Singh and Patel, 2012).

PACT is a stress-modulated activator of PKR that works *via* a direct, dsRNA-independent interaction in response to ER stress, oxidative stress, and serum deprivation (Bennett et al., 2004; Bennett et al., 2012; Ito et al., 1999; Patel et al., 2000). Similar to TRBP, PACT contains three copies of the conserved dsRBMs and 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 essential for PKR activation (Huang et al., 2002; Patel and Sen, 1998; Peters et al., 2001). Within dsRBM3, serines 246 and 287 serve as phosphorylation sites to promote PACT-PACT homomeric and PACT-PKR heteromeric interactions (Peters et al., 2006; Singh and Patel, 2012). In the absence of stress, PACT is constitutively phosphorylated on S246 (Peters et al., 2006), bound to TRBP (Daher et al., 2009) and is unable to activate PKR. In response to cellular stress, PACT is phosphorylated on S287 which promotes its dissociation from TRBP to trigger PACT-PACT homomeric interactions (Daher et al., 2009; Peters et al., 2006; Singh et al., 2011; Singh and Patel, 2012) which are required for PKR activation (Peters et al., 2006; Singh et al., 2011; Singh and Patel, 2012). Once activated, PKR phosphorylates eIF2α on serine 51 resulting in the attenuation of general protein synthesis (Garcia et al., 2006) and triggering downstream ISR events including ATF4 and CHOP induction that in turn regulate cellular fate either by restoring homeostasis or inducing apoptosis.

Recently, eight different mutations have been identified in Prkra gene (encoding PACT, OMIM: DYT16, 612067) in patients with a neuromuscular movement disorder dystonia 16 (DYT16) (Camargos et al., 2012; Camargos et al., 2008; de Carvalho Aguiar et al., 2015; Dos Santos et al., 2018; Lemmon et al., 2013; Quadri et al., 2016; Seibler et al., 2008; Zech et al., 2014). 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 (Bragg et al., 2011; Geyer and Bressman, 2006). 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 (Camargos et al., 2008), four dominantly inherited variants of DYT16 have also been reported (Seibler et al., 2008; Zech et al., 2014). Previously, our lab reported that a recessively inherited P222L mutation increases cell susceptibility to ER stress through the dysregulation of eIF2α stress response signaling in DYT16 patient derived lymphoblasts (Vaughn et al., 2015). 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 increasing PACT mediated PKR activation causing an enhanced sensitivity to ER stress *via* dysregulation of the eIF2α signaling pathway (Burnett et al., 2019). In accordance with our findings, subsequent reports identified dysregulation of eIF2α signaling in both DTY1 and DYT6 (Beauvais et al., 2016; Beauvais et al., 2018; Rittiner et al., 2016; Zakirova et al., 2018). 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 (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 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 and ISR. 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.

2. Results

2.1. 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. 1A). 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 dramatical consequences on the 3-dimensional conformation of the protein (Fig. 1A). Furthermore, the two dominantly inherited mutations (N102S and T34S) occur on flanking ends of PACT's first dsRBM that is most critical for dsRNA binding and protein-protein interactions (Fig. 1A) (Chukwurah et al., 2018). As seen in Fig. 1B 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).

2.2. DYT16 mutants activate PKR more efficiently

PACT is best characterized for its ability to activate PKR under conditions of cellular stress (Patel et al., 2000; Singh et al., 2011; Singh et al., 2009; Singh and Patel, 2012). 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. Hexahistidine 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 in 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. 2A). Some amount of 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

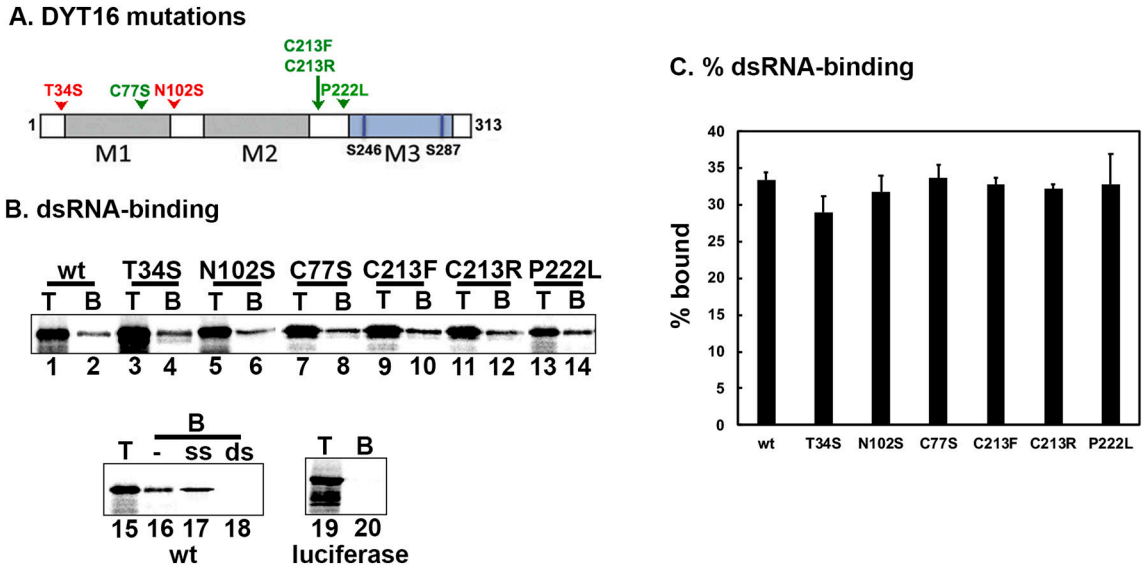

Fig. 1. Effect of DYT16 mutations on dsRNA-binding. (A) Schematic representation of DYT16 mutations: Of the three conserved dsRBMs, M1 and M2 are shown in grey and the third motif lacking dsRNA-binding (M3) is shaded blue with the two phosphorylation sites represented as dark blue lines. Dominant DYT16 mutations are indicated in red while recessive mutations are indicated in green. (B) dsRNA-binding assay: dsRNA binding activity of wt PACT and DYT16 point mutants was measured by a poly(I)·poly(C)-agarose binding assay with *in vitro* translated ^{35}S-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 full-length 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. Lanes 19 and 20 represent binding of firefly luciferase protein to poly(I)·poly(C)-agarose, used as a negative control to demonstrate specificity. (C) Quantification of the dsRNA binding assay. Bands were quantified by phosphorimaging analyses, 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. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

PKR bands was quantified using a phosphorimager analysis and is shown in Fig. 2 B. In all cases, recessive mutations demonstrated a slightly increased capacity to activate PKR (Fig. 2A, lanes 4–12 and Fig. 2B) as compared to wt PACT (Fig. 2A, lanes 2–3). Interestingly, when tested in combinations as reported in DYT16 patients, the recessive mutants showed significantly enhanced ability to activate PKR (Fig. 2 A upper panel lanes 13–16, and Fig. 2B) The dominant mutants (lower panel) also showed enhanced ability to activate PKR at 400 pg (Fig. 2A, lower panel lanes 4–7). Interestingly, when tested in combination with wt PACT, both the dominant mutants demonstrated significantly higher PKR activation (Fig. 2A lower panel: lanes 9–12, and Fig. 2B). These results indicate that the DYT16 point mutants have enhanced ability to activate PKR as compared to wt PACT.

2.3. 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 for most of the DYT16 patients. Here we characterize the effect of ER stress on DYT16 compound heterozygote patient derived lymphoblast cells expressing both P222L and C213R mutations as 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. In the case of wt lymphoblasts, over a 24-h time course in response to TM treatment we observed a marginal increase in expression of cleaved PARP1, a marker of cellular apoptosis (Fig. 2C lanes 2–7) (Oslowski and Urano, 2011). In contrast to this, in the DYT16 patient derived lymphoblasts, there was a dramatically significant increase in cleaved PARP1 in response to tunicamycin (Fig. 2C, lanes 13–14). To further validate these results, we performed caspase 3/7 activity assays under the same conditions to measure apoptosis. In wt lymphoblasts we detect caspase activity at 24 h but not at 6 h post-treatment (Fig. 2D, blue bars). In contrast, the DYT16 patient lymphoblasts demonstrate significantly elevated caspase activity at 6 h which further increases at 24 h post-treatment (Fig. 2D, red bars). This 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.

2.4. 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 with TM under the same conditions probing for markers of cellular stress response (Fig. 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. 3A, lane 1) followed by increased eIF2α phosphorylation at 1–4 h post treatment (lanes 2–4) and then restoration to basal levels by 8 h (lane 5). In contrast to this, in the DYT16 lymphoblasts (right), we observe a similar increase in eIF2α phosphorylation 1 h after treatment (lane 7), however, the eIF2α phosphorylation is sustained even at 8-h 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 h after TM treatment that is sustained until 4 h (lanes 1–4) and shows a slight decrease by 8 h (Fig. 3A). 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

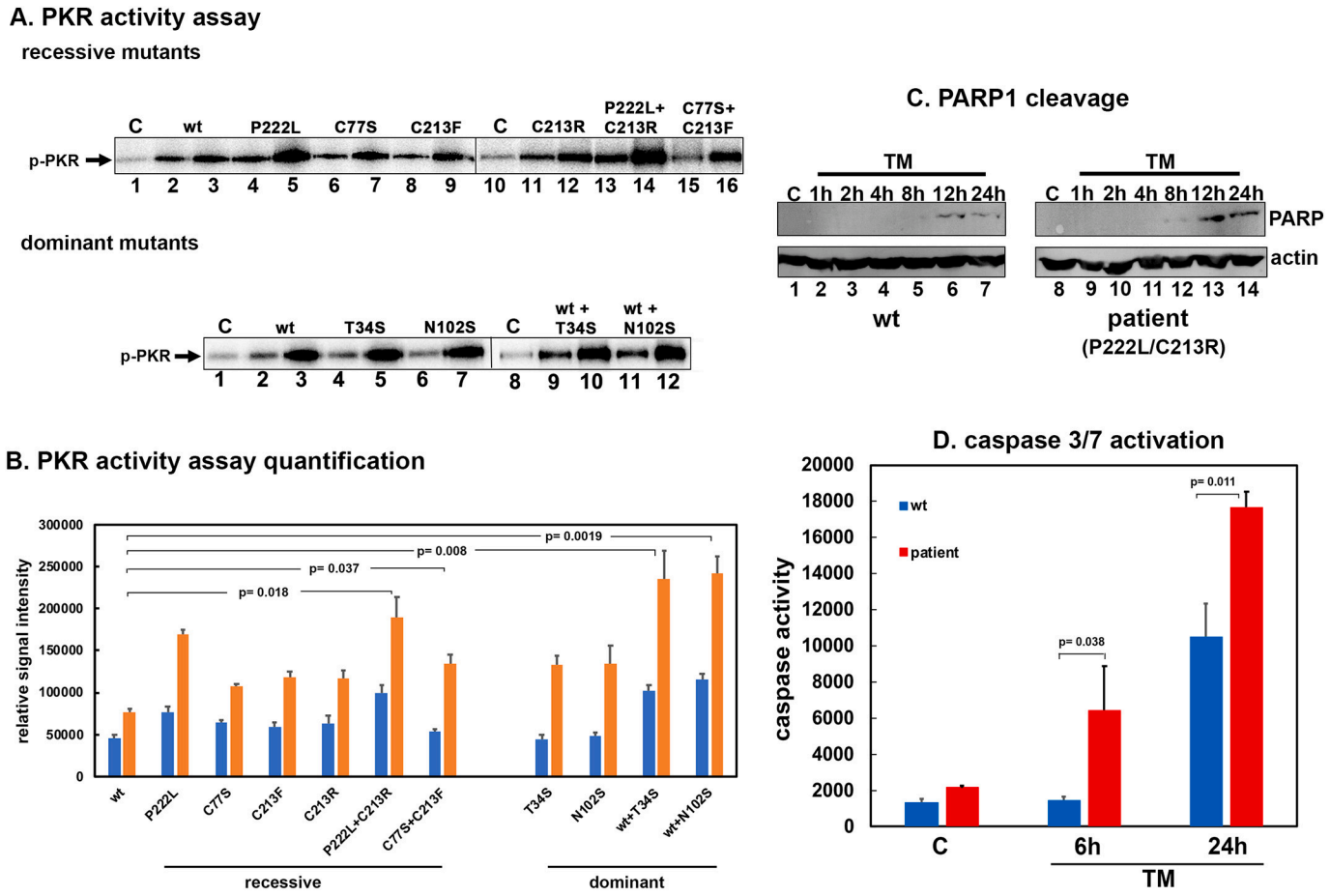

Fig. 2. Effect of DYT16 mutations on PKR activation and cell fate: (A) PKR kinase activity assay. Kinase activity assay was performed using PKR immunoprecipitated from HeLa cell extracts using a monoclonal PKR antibody (R&D Systems) and protein A-sepharose beads. Either 400 pg (lanes 2,4,6,8 top panel, and lanes 2,4, 6 bottom panel) or 4 ng (lanes 3,5,7,9 top panel, and lanes 3,5,7 bottom panel) of recombinant wt PACT or DYT16 mutant proteins were used as PKR activators. 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 (lanes 11,13,15 top panel and lanes 9,11 bottom panel) or 2 ng (lanes 12,14,16 top panel and lanes 10,12 bottom panel) of each mutant protein. (B) Quantification of kinase activity assay. Radioactivity in each band was quantified using phosphoimaging analysis and the relative signal intensities were plotted. Blue bars: PKR activity seen with 400 pg and orange bars: PKR activity seen with 4 ng of the corresponding pure recombinant PACT protein. The *p* values 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 using anti-cleaved PARP1 and anti-β-actin antibodies. (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 bars: DYT16 cells. The data is an average of three independent experiments and the p values are as indicated. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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. 3B, lane 1) and its expression increases in a time dependent manner from 1 to 8 h post treatment (lanes 2–5) and declines at 12 and 24 h after treatment (lanes 6–7). In contrast, in the DYT16 patient lymphoblasts (right) although we observe increased expression of ATF4 from 1 to 8 h post treatment (lanes 9–11), it persists at high levels even at 12 h post treatment and shows only a small decline at 24 h after treatment. Finally, we compared levels of CHOP, an ATF4-induced pro-apoptotic protein, in response to TM treatment in wt and DYT16 lymphoblasts. CHOP is undetectable in untreated cells (Fig. 3B, lane 1) and its expression increases in a time dependent manner from 2 to 8 h post treatment (lanes 3–5) and declines at 12 and 24 h after treatment (lanes 6–7). In contrast, in the DYT16 patient lymphoblasts (right) we observe a delay in expression of CHOP and it is not detected until 4 h post treatment (lane), and it persists at high levels at 8–24 h post treatment (lanes 12–14). Collectively these results demonstrate a dysregulation of ISR pathway, prolonged phosphorylation of eIF2α, elevated levels of activated PKR, prolonged elevated levels of ATF4 translation, and delayed but sustained induction of CHOP.

2.5. 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. 3A), 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 as it induces 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 inactivates PKR's catalytic activity without affecting PACT-PKR or any other interactions (Cosentino et al., 1995). 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 (Vaughn et al., 2015). Here our results show that the other

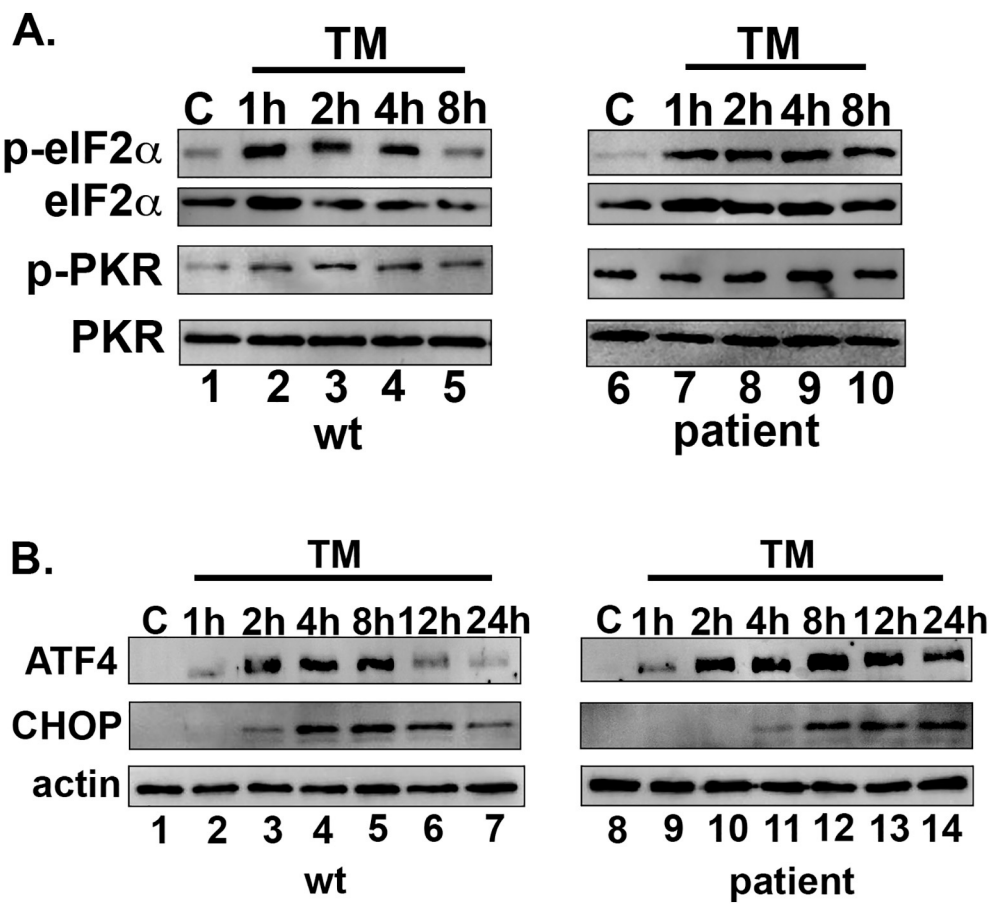

Fig. 3. PKR activation and ISR in response to tunicamycin in normal and DYT16 patient lymphoblasts. (A) Western blot analysis for p-PKR and p-eIF2α. Whole cell extracts from normal (wt) and DYT16 patient derived lymphoblasts treated with 5 μg/ml of tunicamycin (TM) were analyzed at indicated time points. 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. Whole cell extracts from normal (wt) and DYT16 patient derived lymphoblasts treated same as in 3A were analyzed at indicated time points. Blots were probed for ATF4, and CHOP. Best of four representative blots are shown. β-actin was used as a loading control to ensure equal amounts of protein was loaded in each lane.

recessively inherited mutations (C77S, C213F, and C213R) show no difference in their ability to interact with PKR relative to wt PACT (Fig. 4A, lanes 2–5). 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 1). Lanes 7–10 demonstrate equal amounts of myc-PACT proteins were immunoprecipitated in each lane (top panel) while input gels (lower panel) demonstrate equal expression of each myc-PACT expression construct (lanes 7–10) and flag-PKR (lanes 1–5). 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. 4B). 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-IP 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. 4C). 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. 4C). 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 showed similar PKR interaction as the wt PACT. Similarly, our results from the co-IP data were confirmed in case of the dominant mutations (Fig. 4D). 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. 4D).

2.6. Effect of DYT16 mutations on PACT-PACT interactions

As PACT-PACT interactions are critical for activation of PKR, it is most relevant to assess if the DYT16 mutations affect PACTs ability to form homomeric interactions. Consequently, using the same protein-protein interaction studies outlined in Fig. 4 we addressed whether PACT-PACT interactions were affected by the DYT16 mutations (Fig. 5). We co-expressed myc- or flag-epitope tagged PACT proteins transiently by co-transfection of the respective expression constructs in combinations seen in patients. Our data shown in Fig. 5A–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. We observe minimal wt PACT homodimerization (Fig. 5A, 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. 5B), as well as mutant-mutant dimers (Fig. 5C). We did not observe any wt PACT homodimerization in the absence of stress (Fig. 5B and C, lane 2), however, both the dominant DYT16 mutants N102S and T34S showed enhanced interaction with wt PACT (Fig. 5B, 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. 5C, 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)

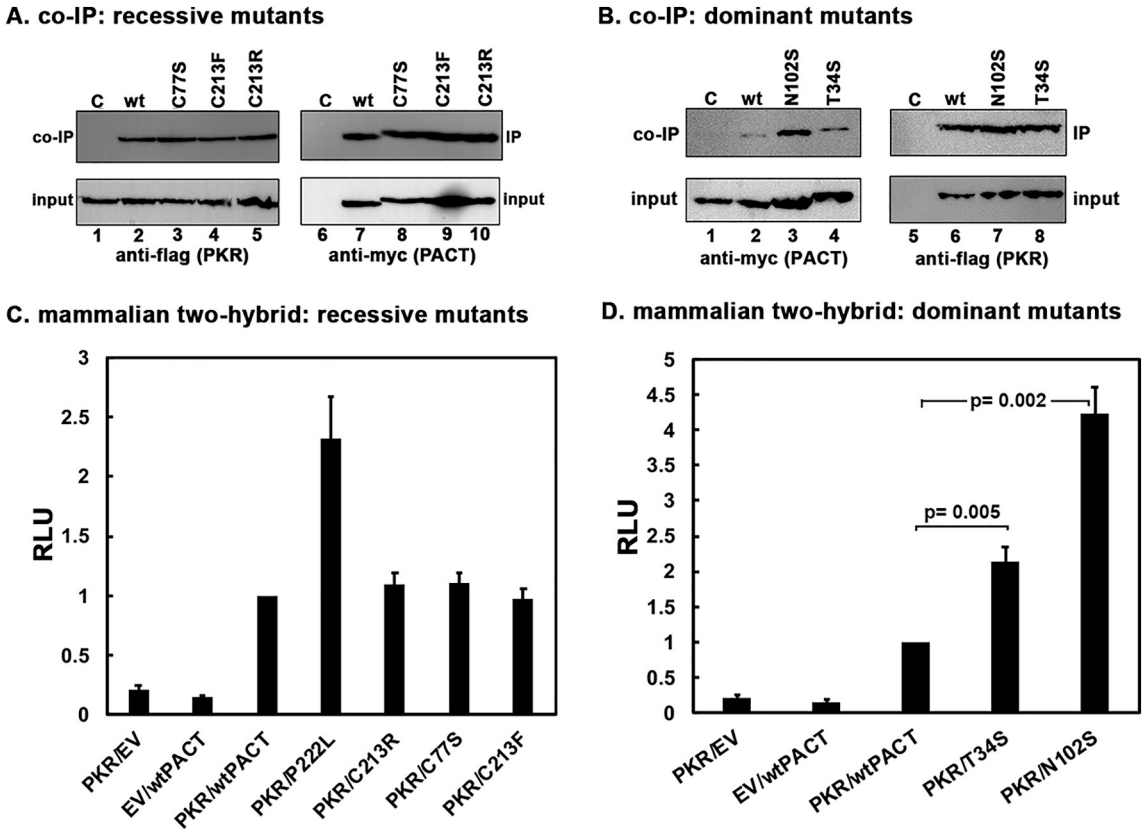

Fig. 4. Effect of DYT16 mutations on PACT-PKR interaction. (A & B) Co-IP assays: HeLa cells were co-transfected with flag-PKR and myc-PACT expression plasmids in pCDNA3.1-. 24 h post-transfection, cells were harvested and myc-PACT (A) or flag-PKR (B) was immunoprecipitated using myc-agarose or flag agarose beads. The immunoprecipitates were analyzed by western blot analysis with anti-flag (A) or anti-myc (B) antibodies (co-IP panel) and with anti-myc (A) or anti-flag (B) for IP panels. 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 (preys). The experiment was repeated twice with each sample in triplicate, and the averages with standard error bars are presented. The *p* values are as indicated for samples with significant differences in interaction. *RLU*, relative luciferase units.

demonstrating the absence of any non-specific binding to the beads (Fig. 5A–C). The IP panels show that equal amounts of flag-tagged PACT protein was immunoprecipitated in each lane (Fig. 5A–C, upper panel, lanes 5–8), and input blots indicate equal expression of each construct (Fig. 5A–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. 5D–E). As seen in Fig. 5D and E, in the patient specific combinations all the recessive mutants show enhanced interactions relative to wt PACT-wt PACT interaction (Fig. 5D). The P222L-C213R and C213F-C77S interactions are ~5-fold and ~9-fold higher than wt PACT-wt PACT interaction respectively (Fig. 5D). Furthermore, the dominant mutants T34S and N102S also show enhanced interactions (Fig. 5E). 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. These results further strengthen our co-IP data that the DYT16 mutations enhance PACT's ability for forming PKR activating homomeric interactions.

2.7. PACT's ability to interact with TRBP is not affected by the DYT16 mutations

In the absence of stress, TRBP binds PACT and prevents the formation of PACT-PACT homodimers that could result in PKR activation. Thus, changes in PACT's interaction with TRBP can consequentially affect PKR activation and 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 (Vaughn et al., 2015). We thus 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. 6A, lanes 2–5) as well as the dominantly inherited N102S and T34S mutations (Fig. 6B, lanes 2–4) have similar 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. 5A and B) we can rule out any nonspecific binding of myc-PACT to the beads. Finally, IP blots indicating that equal amount of myc-TRBP protein was immunoprecipitated (Fig. 5A and B, IP panels, lanes 7–10 and lanes 6–8) and input blots demonstrating equal protein expression are shown (Fig. 5A 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. 6C). The other recessively inherited mutations, however, show no difference in their ability to interact with TRBP relative to wt PACT (Fig. 6C). 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. 6D). These results confirm that the new DYT16 mutations examined here do not change PACT's interactions with TRBP and validate our earlier report that the P222L DYT16 mutation enhances PACT's

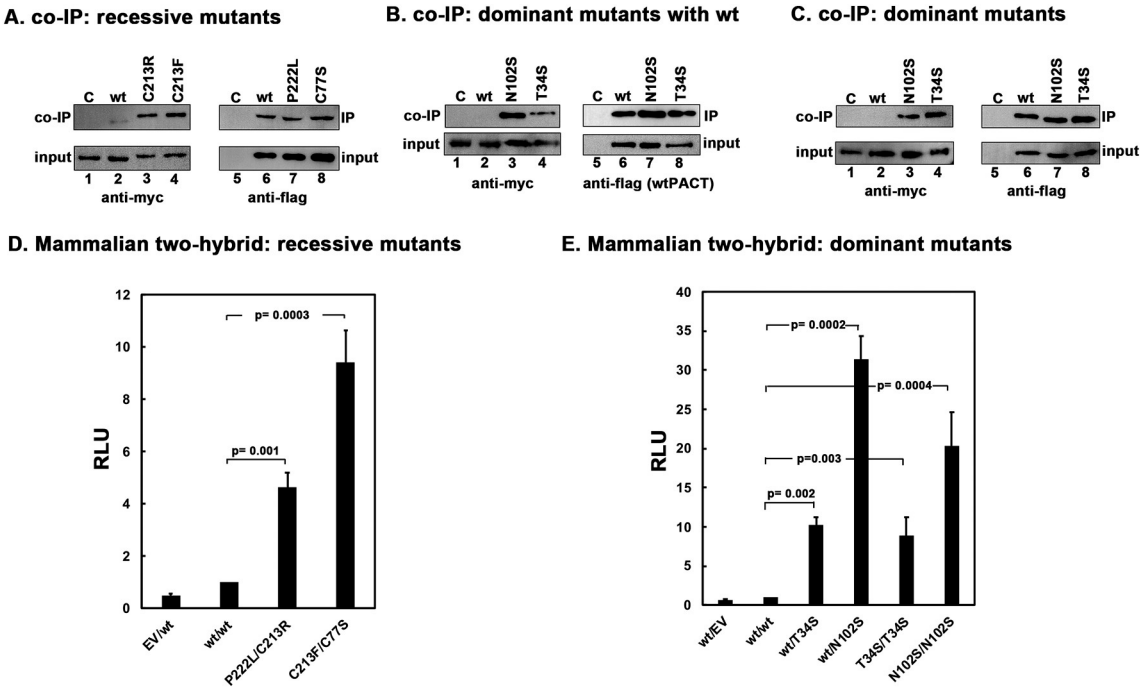

Fig. 5. Effect of DYT16 mutations on PACT-PACT interactions. (A-C) Co-IP assays to measure PACT-PACT interaction with mutant protein combinations as present in DYT16 patients. HeLa cells were co-transfected with flag-PACT and myc-PACT expression plasmids in pCDNA3.1-. 24 h post-transfection, cells were harvested and flag-PACT was immunoprecipitated with flag-agarose beads. The immunoprecipitates were analyzed by western blot analysis with anti-myc antibodies (co-IP panel) or anti-flag antibodies (IP panel). Input gels show expression levels of proteins without immunoprecipitation. (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 (preys). The experiment was repeated twice with each sample in triplicate, and the averages with standard error bars are presented. The *p* values are as indicated. *RLU*, relative luciferase units.

interaction with TRBP (Vaughn et al., 2015).

2.8. DYT16 patient lymphoblasts show 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 previously been established to efficiently inhibit or disrupt PACT-PKR interactions (Dabo et al., 2017). As seen in Fig. 7A, in the wt lymphoblasts we can detect some PACT-PKR interaction (upper panel) prior to luteolin treatment (lane 2), and at 1 h after luteolin treatment PACT-PKR interactions are barely detectable (lane 3) and a further time dependent decrease in the PACT-PKR interaction is seen from 1 to 8 h (lanes 3–5), with the interaction no longer be detected at 24 h post-treatment (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 h (lanes 9–10) then decreasing slowly at 4 h and 8 h after luteolin treatment (lanes 11–12). We do see a complete loss of PACT-PKR interactions at 24 h after treatment in the DYT16 patient lymphoblasts (lane 12). IP blots (lower panel) demonstrate that 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 IP samples. 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 PKR or PACT to the beads in the absence of PKR antibody (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. 5) and PACT-PKR interactions (Fig. 4 and Fig. 7A), and the DYT16 patient lymphoblasts are more susceptible to ER stress induced apoptosis (Fig. 2D). 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 significantly disrupt PACT-PKR interactions (Fig. 7A, lanes 6 and 12), we tested if prior luteolin treatment would be protective for DYT16 patient lymphoblasts after ER stress. As seen in Fig. 7B, the wt control lymphoblasts we do not detect any caspase 3/7 activity in our untreated samples or at 6 h after TM treatment but there is a significant increase in caspase activity at 24 h post treatment (Fig. 7B, blue bars). The cells treated for 24 h with luteolin prior to TM treatment show a marked reduction in caspase 3/7 activity (Fig. 7B, red bars). In contrast, the DYT16 patient cells show higher basal levels of caspase 3/7 activity prior to TM treatment that is enhanced at 6 h post-treatment and a further increase is seen at 24 h (Fig. 7B, blue bars). This increase is dramatically reduced, especially at 24 h post treatment, when cells are treated with luteolin 24 h prior to TM treatment (Fig. 7B, red bars). These results demonstrate that disrupting PACT-PKR interactions with luteolin in DYT16 cells can protect the cells from ER stress-induced apoptosis.

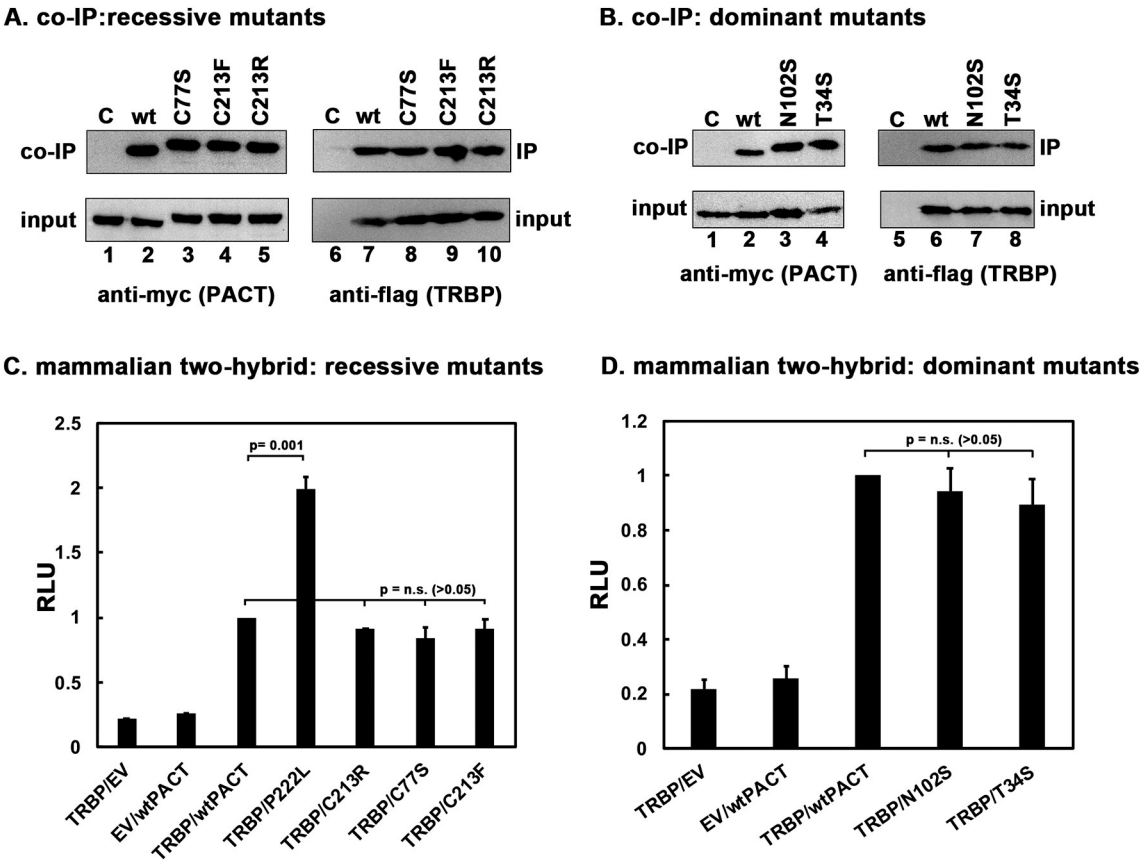

Fig. 6. Effect of DYT16 mutations on PACT-TRBP interactions. (A, B) Co-IP assays. HeLa cells were co-transfected with flag-TRBP and myc-PACT expression plasmids in pCDNA3.1-. 24 h post-transfection, cells were harvested and flag-TRBP was immunoprecipitated with flag-agarose beads. The immunoprecipitates were 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 (preys). The experiment was repeated twice with each sample in triplicate, and the averages with standard error bars are presented. *RLU*, relative light units. The *p* values are as indicated, n.s. indicates not significant.

3. Discussion

DYT16 is an early-onset, generalized dystonia caused by mutations in the *Prkra* gene, which encodes for PACT (Patel and Sen, 1998), a stress-modulated activator of PKR (Patel et al., 2000). In response to cellular stress, PACT activates PKR leading to eIF2α phosphorylation and inhibition of general protein synthesis (Patel et al., 2000). Although primarily a protective response to restore homeostasis, if PKR remains active for prolonged periods it triggers cell death *via* apoptosis (Gil and Esteban, 2000; Singh et al., 2009). 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 (Daher et al., 2009). This is regulated by stress-induced PACT phosphorylation at serine 287, which dissociates PACT from TRBP and allows for its interaction with PKR (Singh et al., 2011; Singh and Patel, 2012). Previously we investigated the effects of a recessively inherited DYT16 missense mutation P222L on PACT-induced PKR activation in response to ER stress (Vaughn et al., 2015). 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 but prolonged and 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 (Seibler et al., 2008) also demonstrated a dysregulation of PACT-PKR-eIF2α pathway (Burnett et al., 2019). 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) (de Carvalho Aguiar et al., 2015) and two dominant DYT16 mutations (T34S and N102S) (Zech et al., 2014) on PKR activation. Our results establish that similar to two previously examined DYT16 mutants (Burnett et al., 2019; Vaughn et al., 2015), dysregulation of ISR is a common feature of all five DYT16 mutations. However, there are some important differences in the mechanism by which the dysregulation of ISR is brought about by these five mutants. Similar to the P222L mutant, all five mutants show stronger PACT-PACT interactions as well as enhanced PKR activation but unlike the P222L and the frameshift DYT16 mutants, none of these five mutants exhibited 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. 5). Using DYT16

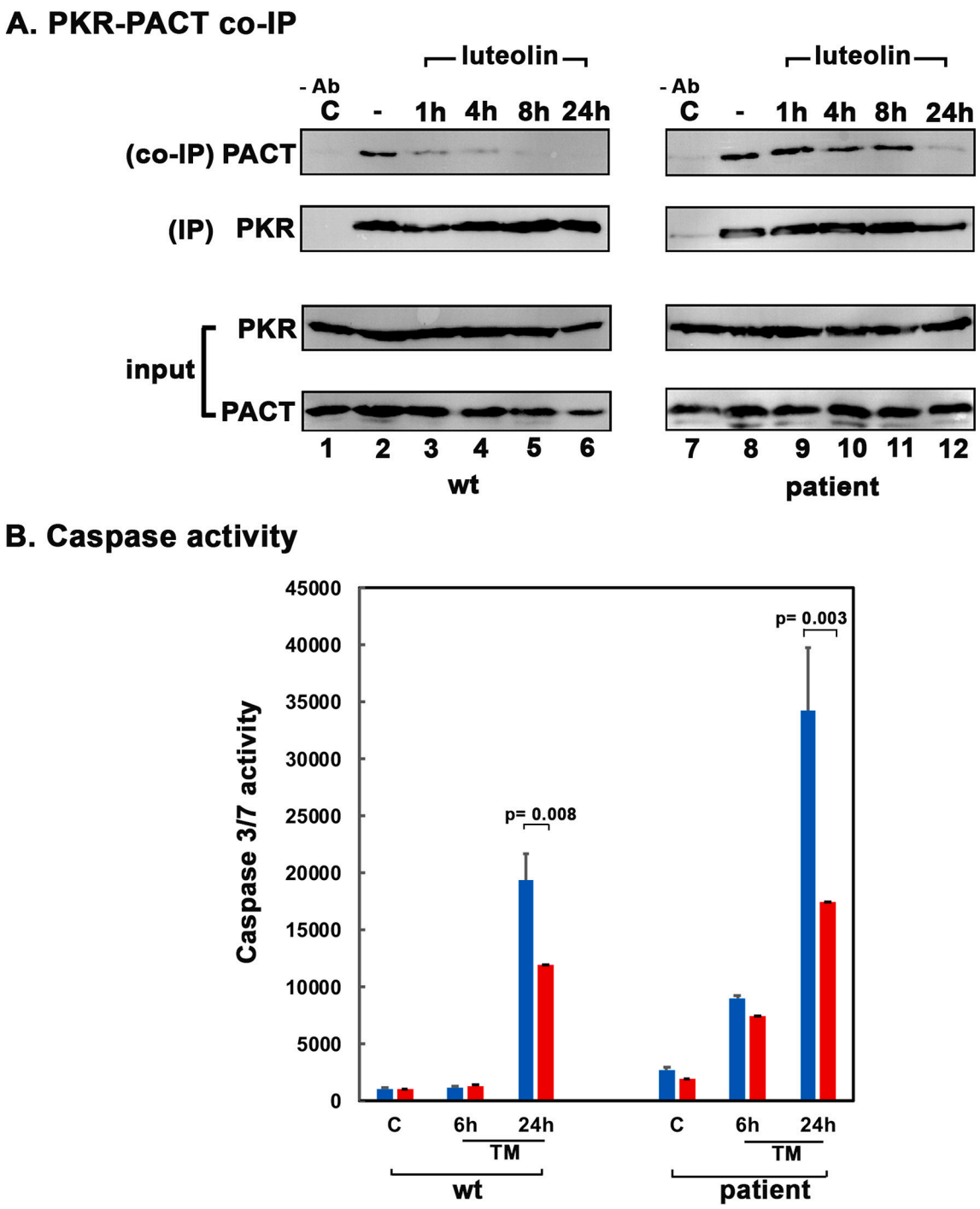

Fig. 7. Effect of luteolin on PACT-PKR interaction and caspase activation in response to tunicamycin in DYT16 patient lymphoblasts. (A) Co-IP of endogenous PKR and PACT proteins. Lymphoblasts from unaffected family member (wt) or DYT16 patient (patient) were treated with 50 μM luteolin. The cell extracts 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. (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 h with 50 μM Luteolin (red) or left untreated (blue) followed by treatment with 5 μg/ml tunicamycin (TM). Caspase 3/7 activity was measured at indicated time points after tunicamycin treatment. The p values are as indicated. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

lymphoblasts from a compound heterozygote patient, we observe that the enhanced PACT-PKR interactions (Fig. 7) and elevated PKR kinase activity (Fig. 2A and B) leads to a dysregulation of ISR and increased apoptosis in response to ER stress (Fig. 2C and D).

Based on our results, we predict that the neuronal cells carrying mutant PACT proteins will be unable to cope well with cellular stress and restore homeostasis. Although the observed enhanced apoptosis in lymphoblast cells is significant and indicative of possible increased neuronal apoptosis, it is of limited scope and must be taken with caution and more meaningful molecular studies on neurons derived from

DYT16 patient cells should be undertaken in the future. There is significant expression of PACT as well as PKR in neurons based on our studies on murine brain as well as human cultured neurons (data not shown). The PACT-PKR stress response pathway functions ubiquitously in all cell types including neurons (Chen et al., 2006; Paquet et al., 2012; Vaughn et al., 2014) and PACT mediated PKR activation and its involvement in neurodegeneration has been noted in Alzheimer's patients and mouse models (Paquet et al., 2012). As currently there are no derived DYT16 neurons available, our studies on patient lymphoblasts indicate that considerable efforts involved in undertaking in-depth studies using DYT16 derived neurons would be worthwhile in future.

Although neurodegeneration is the expected long-term outcome of enhanced neuronal apoptosis, neither apoptosis not neurodegeneration has so far been systematically investigated in blood or brain of dystonia patients, and this lack of information is usually interpreted as neurodegeneration generally being absent in dystonia patients. On the other hand, there is some evidence of increased neuronal apoptosis in the context of DYT16. Most DYT16 patients develop symptoms in early childhood and in the case of the compound heterozygous patient carrying P222L and C213R mutant alleles included in this study, imaging studies had revealed progressive MRI abnormalities with significant bilateral volume loss in the basal ganglia (Brashear, 2013; Lemmon et al., 2013), which agrees with the enhanced apoptosis observed in our experiments. This patient developed dystonia after a febrile illness, which could be a possible cellular stress event that may have triggered progressive cellular dysfunction or loss. In accordance with our earlier *in vitro* studies on three Brazilian P222L homozygous patients that showed enhanced apoptosis (Vaughn et al., 2015), the imaging studies performed on one Portuguese P222L homozygous patient showed marked bilateral loss of striatal presynaptic dopamine transporters, suggesting nigrostriatal neurodegeneration as a possible feature of the disease (Pinto et al., 2020). In addition to these imaging studies on DYT16 patients, there is evidence of neuronal apoptosis in dystonic mice with Prkra mutations. A spontaneously arisen, recessive insertion mutation in *Prkra* was identified at the Jackson Laboratory (JAX) that results in a progressive dystonia, kinked tails, and mortality (Palmer et al., 2015). Some neurons in the dorsal root ganglia and the trigeminal ganglion were noted to be apoptotic in the homozygous mutant mice, consistent with the observed neurodegenerative phenotype. In agreement with this, our *in vitro* studies on a similar frameshift mutation reported in a German patient indicated increased apoptosis as an outcome, although patient cells were not included in our study (Burnett et al., 2019). Thus, it would be very informative if neurodegeneration is investigated in DY16 patients in the future in order to shed light on DYT16 pathogenesis.

This study further strengthens the case for a maladaptive ISR as possible disease etiology for DYT16 as our previous report with homozygous P222L patients was the first on dysregulated eIF2α signaling in any type of dystonia (Vaughn et al., 2015). Subsequently DYT1 (Beauvais et al., 2018; Rittiner et al., 2016), DYT6 (Zakirova et al., 2018) as well as DYT11 (Xiao et al., 2017) 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 samples (Beauvais et al., 2018). 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 LTD in DYT1 knock-in mice (Rittiner et al., 2016). In addition, this report also examined patients with focal cervical dystonia and reported sequence variants in ATF4, which is a direct target of eIF2α signaling (Rittiner et al., 2016). 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 (Zakirova et al., 2018). A gene-expression analysis in adult cerebellar tissue from a mouse model of DYT11 also have identified genes associated with protein translation among the top down-regulated mRNAs (Xiao et al., 2017).

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 (Pakos-Zebrucka et al., 2016). 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 (Sano and Reed, 2013; Wek, 1994). 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 (Tabas and Ron, 2011). 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 (Chesnokova et al., 2017). 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 (Chen et al., 2003). When PKR- mediated eIF2α phosphorylation was specifically increased in hippocampal CA1 pyramidal cells by a chemical inducer, ATF4 expression increased significantly (Jiang et al., 2010). Increased levels of ATF4 led to impairment of hippocampal long-term potentiation (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−/− mice have decreased eIF2α phosphorylation, and thus have decreased ATF4 in hippocampal neurons (Costa-Mattioli et al., 2005). 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 presence of inactivating mutations in ATF4 in sporadic cervical dystonia patients (Rittiner et al., 2016). In our DYT16 lymphoblasts, we observe a sustained and higher level of ATF4 expression in response to ER stress (Fig. 3). This was true both in P222L homozygous (data not shown) as well as P222L 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 (Gal-Ben-Ari et al., 2018; Hugon et al., 2017; Marchal et al., 2014). Increased levels of PKR phosphorylation have been detected in the brains of patients with neurodegenerative diseases such as Alzheimer's disease (AD) (Chang et al., 2002), Parkinson's disease, Huntington's disease (Peel and Bredesen, 2003; Peel et al., 2001), dementia (Taga et al., 2017), prion disease (Paquet et al., 2009). It is important to note that DYT16 patient lymphoblasts show higher levels of apoptosis in the absence of a stress signal in our analyses and it is unclear at present if this results from chronic low levels of PKR activation we noted in our studies. 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 (Zhu et al., 2019). 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 (Hwang et al., 2017). 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 (Chen et al., 2008) thereby 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 (Dabo et al., 2017). 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. 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 (Paquet et al., 2012).

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 future. Further research is essential on exploring the efficacy of luteolin on dystonic symptoms and the recently described *Prkra* mutant mouse model with dystonia symptoms would prove valuable and needs to be evaluated carefully (Palmer et al., 2015). Further efforts to discover compounds similar to luteolin that disrupt PACT-PKR interactions at lower concentrations or to develop specific peptides that disrupt PACT-PKR interaction may also be fruitful. In this regard, it is worth a mention that an interaction between dsRBM3 of PACT with PKR's catalytic domain is essential for PKR activation (Li et al., 2006) and a disruption of such an interaction can be 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.

4. Methods and materials

4.1. Cell lines and antibodies

Both HeLaM and COS-1 cells were cultured Dulbecco's Modified Eagle's Medium (DMEM) containing 10% Fetal Bovine Serum and penicillin/streptomycin. wt and 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 (Anderson and Gusella, 1984; Vaughn et al., 2015). 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).

4.2. 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 ***G***AG 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 ***A***AT 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***G***T CCT AAA GAA TGT CC-3′

N102S Sense: 5′-GCT GCA GAG GCT GCC ATA AAC ATT TTG AAA GCC A***G***T 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 ***A***TT TCC CTG GCT TAG C-3′.

To generate each construct, we performed PCR amplification in order to mutate the corresponding wild type sequence to code for the amino acid residue consistent with the DYT16 patients. Each PCR product was then sub-cloned into pGEMT-easy vector (Promega) and sequences were validated through DNA sequencing. 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 *Nde*I-*Pst*I ligated into FLAG/Myc-PACT-BSIIKS + cut with PstI-BamHI. C213F and C213R in pGEMT-easy cut with NdeI-*Eco*RI ligated into FLAG/Myc-PACT-BSIIKS + cut with EcoRI-BamHI. N102S in pGEMT-easy cut with NdeI-PstI and ligated into FLAG/Myc-PACT-BSIIKS + cut with PstI-BamH1. T34S in pGEMT-easy cut with NdeI-*Tfi*I 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 sub-cloned each ORF into pCDNA3.1- using *Xba*I-BamHI restriction sites. All DYT16 constructs were also cloned into Mammalian two-hybrid system vectors and pET15b (Novagen) using NdeI-BamHI restriction sites. TRBP and Flag-PKR constructs were as previously described (Singh et al., 2011).

4.3. Expression and purification of PACT from E. coli

The ORFs of both wt PACT and all DYT16 point mutations were sub-

cloned into pET15b (Novagen) to generate an in-frame fusion protein with a histidine tag. Recombinant proteins were then expressed and purified as previously described (Patel and Sen, 1998).

4.4. dsRNA binding assays

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 ^{35}S-Methionine radiolabel and the dsRNA binding ability was measured using poly(I:C) conjugated agarose beads. We diluted 4 μl of *in vitro* translation in 25 μl of binding buffer (20 mM Tris–HCl, pH 7.5, 0.3 M NaCl, 5 mM $MgCl_2$, 1 mM DTT, 0.1 mM PMSF, 0.5% NP-40, 10% glycerol) and incubated in 25 μl of poly(I:C)-agarose beads and incubated at 30 °C for 30-min. We then washed the beads 4 times with 500 μl 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 poly(I:C)-agarose beads before the adding the *in vitro* translated proteins. To ensure the presence of PACT was due to the dsRNA binding capacity we assayed *in vitro* translated ^{35}S-Methionine labeled firefly luciferase which has no dsRNA binding ability. Bands in bound and total lanes were quantified using Typhoon FLA7000 by analyzing relative band intensities of both T and B lanes. Percentage of PACT bound to beads was calculated and plotted as bar graphs.

4.5. PKR activity assays

HeLa M cells treated with IFN-β for 24-h and harvested at 70% confluency, washed using ice-cold PBS and centrifuged at 600 *g* for 5-min. Cell were resuspended in lysis buffer (20 mM Tris–HCl pH 7.5, 5 mM MgCl2, 50 mM KCl, 400 mM NaCl, 2 mM DTT, 1% Triton X-100, 100 U/ml aprotinin, 0.2 mM PMSF, 20% glycerol) and incubated on ice for 5 min. Lysates were centrifuged at 10,000 *g* for an additional 5-min. PKR was immunoprecipitated from 100 μg of this protein extract using anti-PKR monoclonal antibody (R&D Systems: MAB1980) in a high salt buffer (20 mM Tris–HCl pH 7.5, 50 mM KCl, 400 mM NaCl, 1 mM EDTA, 1 mM DTT, 100 U/ml aprotinin, 0.2 mM PMSF, 20% glycerol, 1% Triton X-100) at 4 °C on a rotating wheel for 30-min. We then added 10 μL of protein A-Sepharose beads to each immunoprecipitate followed by an additional 1 h incubation under the same conditions. Protein A-Sepharose beads were washed 4 times in high salt buffer followed by an additional two washes in activity buffer (20 mM Tris–HCl pH 7.5, 50 mM KCl, 2 mM MgCl2, 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 conducted by incorporating: 0.1 mM ATP, 10 μCi of [γ-^{32}P] ATP, and increasing amounts of either pure recombinant wt PACT or DYT16 PACT (400 pG – 4 ng) as the PKR activator. Reaction was incubated at 30 °C for 10 min and resolved on a 12% SDS-PAGE gel followed by phosphorimager analysis on Typhoon FLA7000.

4.6. Western blot analysis

Lymphoblasts derived from a compound heterozygous DYT16 patient containing both P222L and C213R mutations as independent alleles were cultured alongside lymphoblasts derived from a family member containing no mutations in PACT as our control cells. Cells were plated at a concentration of 300,000 cells/ml of RPMI media containing 10% fetal bovine serum and penicillin/streptomycin. To analyze cellular response to ER stress, we treated cells with 5 μg/ml of tunicamycin (Santa Cruz) over a 24-h time course and harvested cells in RIPA (150 mM NaCl, 1.0% IGEPAL® CA-630, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0) buffer containing a 1:100 dilution of protease inhibitor cocktail (Sigma) and phosphatase inhibitor (Sigma). Concentration of total protein extract was then determined using BCA assay and appropriate amounts of extracts were analyzed by western blot analyses using appropriate antibodies as indicated.

4.7. 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 of RPMI complete media and treated with 50 μM of luteolin (Santa Cruz) over a 24 h time course. Cells were harvested at indicated time points and whole cell extract was immunoprecipitated 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) using anti-PKR antibody (71/10, R&D Systems) and protein A sepharose beads (GE Healthcare). Immunoprecipitation was carried out using 100 ng of anti-PKR antibody and 10 μl of protein A sepharose beads slurry per immunoprecipitation. Immunoprecipitates were washed 3 times in 500 μl of IP buffer followed by resuspension and boiling for 5 min in 1× Laemmli buffer (150 mM Tris–HCl pH 6.8, 5% SDS, 5% β-mercaptoethanol, 20% glycerol). Samples were resolved on 10% SDS-PAGE denaturing gel and probed with anti-PACT antibody to determine co-IP efficiency and anti-PKR antibody to determine equal amounts of PKR were immunoprecipitated in each sample. Input blots of whole cell extract without immunoprecipitation are shown to indicate equal amounts of protein in each sample.

4.8. Co-immunoprecipitation Assays in HeLa Cells

In all cases HeLa M cells were seeded at 20% confluency in 6-well dishes 24-h prior to co-transfecting 250 ng of each flag- and/or myc-tagged constructs using Effectene reagent (Qiagen). Cells were harvested 24-h post transfection and harvested in IP buffer. Whole cell extract was then immunoprecipitated overnight at 4 °C on a rotating wheel with either flag-agarose (Sigma) or myc-agarose beads (Thermo Scientific). Immunoprecipitates were then washed 3–5 times in IP buffer followed by resuspension and boiling for 5 min in 1× Laemmli buffer. Samples were then resolved on 10% SDS-PAGE denaturing gels and transferred to PVDF membranes. To evaluate PACT-PACT homodimerization and PACT-TRBP heterodimerization, flag-tagged constructs were immunoprecipitated using 15 μl of flag-agarose beads and blots were initially probed with anti-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-IP blots were incubated at 50 °C for 30 min 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-. We immunoprecipitated the cell lysates in 15 μl of myc-agarose beads and resolved on 10% SDS-PAGE denaturing gels and transferred to PVDF membranes. Blots were initially probed 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.

4.9. Mammalian 2-hybrid interaction assays

In all cases, wt PACT, DYT16, TRBP, or PKR ORFs were sub-cloned into both pSG424 expression vector such that it created an in-frame fusion to a GAL4 DNA binding domain (GAL4-DBD), and pVP16AASV19N expression vector such that it maintains an in-frame fusion to the activation domain of the herpes simplex virus protein VP16 (VP16-AD). COS-1 cells were then transfected with: (i) 250 ng each of the GAL4-DBD and the VP16-AD constructs, (ii) 50 ng of pG5Luc a firefly luciferase reporter construct, and (iii) 1 ng of pRLNull

plasmid (Promega), to normalize for transfection efficiencies. Cells were then harvested 24-h post transfection and assayed for both firefly and renilla luciferase activities using Dual Luciferase® Reporter Assay System (Promega). Fusion proteins were assayed for interaction in all combinations.

4.10. Caspase 3/7 activity assays

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

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Author contributions

S.B. and L. V.: formal analysis, investigation, methodology, validation, writing original draft, review and editing. R. P.: funding acquisition, conceptualization, project administration, supervision, writing original draft, review and editing, N.S.: methodology, resources, review and editing, R.K.: formal analysis, investigation, methodology..

Declaration of Competing Interest

The authors declare that they have no conflicts of interest with the contents of this article.

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