AWARD NUMBER: W81XWH-18-1-0088

TITLE: **Dysregulation of the PACT-Mediated Crosstalk Between Protein Kinases PKR and PERK Contributes to Dystonia 16 (DYT16)**

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REPORT DATE: April 2019

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
April 2019	Annual	1 Apr 2018 - 31 Mar 2019

4. TITLE AND SUBTITLE

Dysregulation of the PACT-Mediated Crosstalk Between Protein Kinases PKR and PERK Contributes to Dystonia 16 (DYT16)

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-18-1-0088

5c. PROGRAM ELEMENT NUMBER

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

UNIVERSITY OF SOUTH CAROLINA
1400 WHEAT ST COLUMBIA SC 29210-4112

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

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 serves as a substrate of PERK kinase. 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 remained unknown although a dysregulation of eIF2 alpha signaling has been suggested due to PACT-mediated regulation of PKR. No information was available for PACT's effect on PERK activity. 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.

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

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
Unclassified	Unclassified	Unclassified	Unclassified		19b. TELEPHONE NUMBER *(include area code)*

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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 hypothesize 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 or new target date for completion) is as in the table below.

Research-specific Tasks and Goals

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
Subtask 2: mammalian two hybrid and yeast two hybrid interaction assays to determine a direct interaction between PACT and PERK	07/2018	completed
Subtask 3: *in vitro* PERK kinase activity assays using purified recombinant PERK and PACT proteins:	08/2018	completed
Subtask 4: test the effect of PACT phospho-defective mutants on PERK signaling in response to ER stress:	09/2018	Not yet started
Milestone(s) Achieved: determine PACT's involvement in PERK pathway by direct interaction with PERK and publication of results	09/2018	Completed, Publcn not yet submitted
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	Not yet started
Subtask 2: To determine if PACT 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
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	Not yet started
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	Currently working on
Milestone(s) Achieved: Make all mutants and the plasmid expression constructs using site directed mutagenesis and sub-cloning	05/2019	completed

Subtask 2: determine the effect of PACT mutants on PERK's kinase activity	08/2019	completed
Subtask 3: determine the effect of PACT mutants on PERK signaling pathway in response to ER stress	09/2019	Not yet started
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	Not yet started

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

Specific results 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:

Subtask 1: co-immunoprecipitation (co-IP) assays to determine interaction between PACT and PERK

As seen in Fig. 1, the ^{35}S-methionine labeled *in vitro* synthesized PERK cytoplasmic domain interacts with recombinant PACT protein bound to Ni-agarose beads very efficiently at both 50mM (lane 4) and physiological (150mM) salt concentrations (lane 5). PERK protein shows no binding to Ni-agarose beads in the absence of PACT (lanes 2-3). These results establish that the cytoplasmic domain of PERK interacts with PACT. We also tested this further using purified recombinant hexahistidinetagged PACT protein and GST-tagged PERK cytoplasmic domain. We used Ni-agarose beads to pull down his tagged-PACT and performed western blot analysis with anti-GST antibody to assess if GST-PERK could be pulled down by binding to his-PACT. As seen in Fig. 2. GST-PERK could be pulled down at 50 mM as well as 150 mM salt (lanes 2 and 3) but not when his-PACT was not added to Ni-agarose beads (lane 1). This further confirms a direct interaction between PACT and catalytic cytoplasmic domain of PERK.

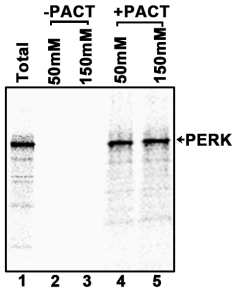

Fig.1. PERK cytoplasmic domain interacts with PACT. 3 µl of ^{35}S-labeled *in vitro* synthesized PERK cytoplasmic domain was bound to either Ni-agarose beads alone (lanes 2-3) or hexahistidine tagged, pure recombinant PACT protein bound to Ni-agarose beads. The beads were washed and the proteins remaining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

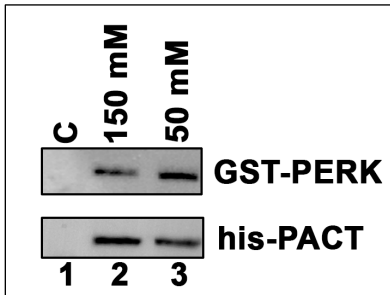

Fig.2. PERK cytoplasmic domain interacts with PACT. 50 ng of pure recombinant GST-PERK cytoplasmic domain was bound to either Ni-agarose beads alone (lane 1) or 50 ng of hexahistidine tagged, pure recombinant PACT protein bound to Ni-agarose beads. The beads were washed at either 50mM salt containing buffer or with 150 mM salt containing buffer and the proteins remaining bound to beads were analyzed by SDS-PAGE followed western blot analysis with anti-GST antibody. Lane 1 shows PERK protein does not bind to the Ni-agarose if PACT is not bound to the beads.

Subtask 2: mammalian two hybrid and yeast two hybrid interaction assays to determine a direct interaction between PACT and PERK.

We tested the interaction between the cytoplasmic domain of PERK and PACT by using a yeast two hybrid and mammalian two hybrid assay. For this purpose, we used the cytoplasmic domain of a catalytically inactive PERK mutant K618A as overexpression of a catalytically active cytoplasmic domain would adversely affect yeast growth. Overexpression of a catalytically active eIF2α kinase drastically slows the yeast growth as seen before and this would prevent us from being able to detect PACT-PERK interaction.

Thus, we tested the interaction between the catalytically inactive PERK mutant K618A catalytic domain and PACT. We detected no interaction in yeast two hybrid assay (data not shown) There may be several reasons for this, a) the catalytically inactive PERK may not interact with PACT especially since our results shown in Figs 4, 6, and 8 demonstrate that PACT acts as a substrate of PERK. Thus, if the catalytically inactive PERK does not assume an active conformation, it may be unable to bind PACT. b) The interaction between PERK and PACT may be transient and thus not detectable in yeast two hybrid assay or c) the interaction between the two proteins takes place in yeast nucleus as dictated by the design of the yeast two hybrid system and PERK and PACT may not interact in the nuclear environment as they are both cytoplasmic proteins. Thus, yeast two hybrid system may not be best suited for detecting and studying PERK-PACT interaction. We further tested the interaction between PERK's catalytic domain and PACT using the mammalian two hybrid system. As seen in Fig. 3, we detected a weak but consistent interaction between PERK catalytic domain and PACT. The weak interaction is expected as the interaction between PERK and PACT is an enzyme-substrate interaction and in many instances, the substrate does not stay bound to the kinase enzyme after phosphorylation. These results conclusively demonstrate interaction between PERK and PACT.

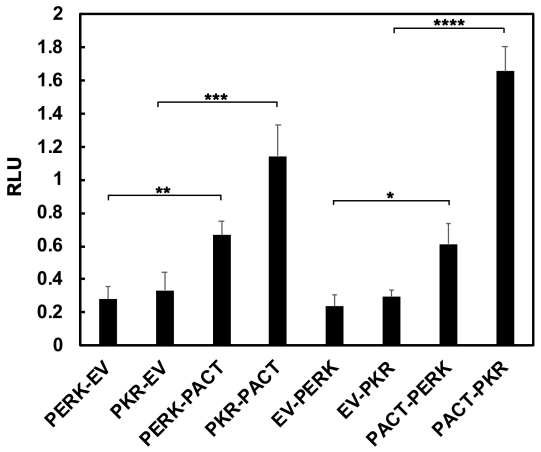

Fig. 3: COS-1 cells were transfected with 200 ng each of the indicated GAL4 DBD and VP16 AD construct pairs, 200 ng of the pG5 Luc reporter construct, as well as 1 ng of the pRL-Null construct to normalize for different transfection efficiencies. Cells were harvested 24 h after transfection, and lysates were assayed for firefly and renilla luciferase activity. Experiments were performed twice in triplicates, and the bars represent the averages of the experiments ± S.D. Student's *t*-tests were performed to determine statistical significance—asterisk **P*-value = 0.0027, double asterisk ***P*-value = 0.0014, triple asterisk ****P*-value = 0.000158, *****P*-value = 0.000115.

Subtask 3: *in vitro* PERK kinase activity assays using purified recombinant PERK and PACT proteins: We performed PERK kinase activity assays using the recombinant pure human PERK catalytic domain. We purchased this active enzyme from Sigma-Aldrich (Cat number SRP5024) which is supplied as a pure recombinant protein expressed in *E. coli* and containing residues 563-1115 of human PERK (EIF2AK3) fused to a GST tag. This active enzyme thus contains the cytoplasmic catalytic domain of human PERK fused to a GST tag and is a 115 kd protein. We tested if it is an active kinase and also its ability to phosphorylate PACT by adding purified recombinant PACT protein to the kinase enzymatic reaction mixture. We used PERK's known substrate eIF2α as a positive control. As seen in Fig. 4, PERK shows kinase activity and is efficiently autophosphorylated (lane 1) in absence of any substrate. Addition of increasing amounts of PACT to the reaction showed efficient phosphorylation of PACT by PERK (lanes 2-5) in a dose dependent manner. PERK also

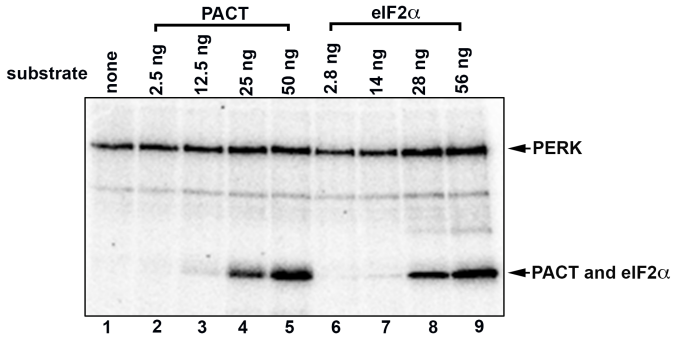

Fig. 4. PERK activity assay: Pure recombinant 20 ng GST-PERK (cytoplasmic catalytic domain) was used to measure phosphorylation of PACT or eIF2α. The amount of substrate proteins added is as indicated above the lanes. Pure recombinant PACT or eIF2α purified from rabbit reticulocytes was added in amounts as indicated.

phosphorylated equivalent amounts of eIF2α very efficiently (lanes 6-9). The level of PACT phosphorylation observed was as efficient as eIF2α phosphorylation (compare lanes 2-5 to lanes 6-9). These results conclusively establish that PACT acts as a substrate of PERK.

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 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. In addition, we 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 mutants show downregulation of ATF4 as compared to wt PACT. We have completed this analysis and the results were negative. As seen in Fig. 5 none of the phospho-defective 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 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 studied the importance of these sites in response to oxidative stress but the phosphorylation sites that play a role 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 phosphor-mimic or phosphor-defective PACT mutants, we have 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 09/2019. As our results demonstrated that PACT is a substrate of PERK, we tested if the phospho-defective (alanine substitutions) or phosphor-mimic (aspartic acid substitutions) PACT mutants are phosphorylated by PERK. As seen in Fig. 6, either a mutation of serine 246 or serine 287 did not affect the ability of PERK to phosphorylate PACT. These results indicate that PERK phosphorylates sites other than serines 246 and 287 or phosphorylates sites in addition to these two serines.

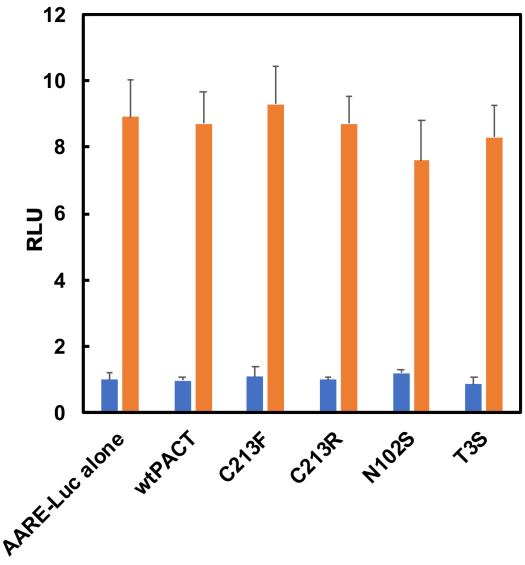

Fig. 5. Effect of dystonia mutants on ability to induce a normal ER stress response. The blue bars represent activity in untreated cells and orange bars indicate activity in cells treated with 10 mg/ml tunicamycin. Firefly luciferase driven by CMV promoter was used to normalize the transfection efficiencies.

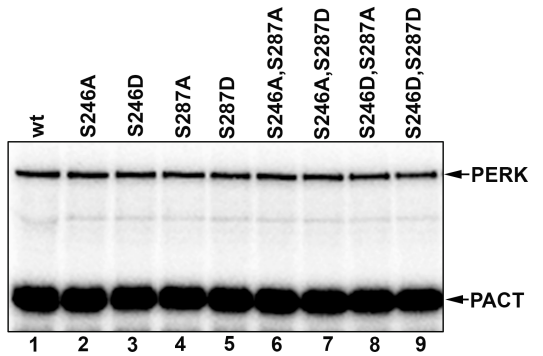

Fig. 6. PERK activity assay: Pure recombinant 20 ng GST-PERK (cytoplasmic catalytic domain) was used to measure phosphorylation of various PACT mutants as compared to wt PACT. The PACT proteins added is as indicated above the lanes. Pure recombinant PACT proteins were used as substrates.

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 Figs. 1,2 and 4-6.

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. As shown in Fig. 1-6 this was accomplished effectively.

Significant results and key outcomes during this period for this specific aim: The significant results and the key outcomes were (1) we conclusively demonstrated the interaction between the cytoplasmic domain of PERK and PACT (Fig. 1-2), and (2) we conclusively demonstrated that PACT is a substrate of PERK in vitro using kinase activity assays (Fig. 5-6).

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

This specific aim was divided in two major tasks. The first one was to determine PACT's functional role in PERK pathway. This was divided into three subtasks. We plan on beginning work on the first two subtasks by 06/2019 and plan on finishing all three subtasks by 09/2019. As seen in Figs. 5-6 we have conclusively demonstrated that PACT acts as a PERK substrate *in vitro* and we feel strongly that these results will be supported by our *in vivo* data as proposed in these subtasks. The third subtask is completed and the results are shown in Fig. 7. As seen in lanes 5-8 PKR is activated in response to tunicamycin in PERK+/+ cells but there is no PKR activation seen in PERK-/- cells (lanes 1-4). This further demonstrates that PERK activation is the upstream signal for PKR activation and PACT is the most likely candidate PKR activator after it is phosphorylated by PERK.

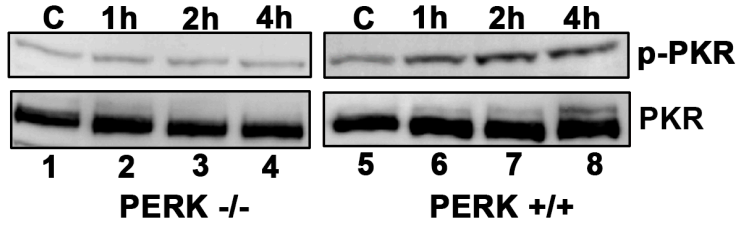

Fig. 7: PERK is essential for PKR activation in response to ER stress: PERK+/+ and PERK-/- MEFs were treated with 0.5 μg/ml tunicamycin. The cell extracts were prepared at the indicated times and analyzed by western blot analysis using a phospho-PKR specific antibody. The same blot was stripped and re-probed with anti-PKR (total) antibody.

The second major task under this aim was to test if PACT mutations reported in DYT16 cause a dysregulation of PERK pathway. Subtask 1 was to determine if DYT16 PACT mutants interact with PERK with similar efficiency as the wt PACT and subtask 2 was to determine the effect of DYT16 PACT mutants on PERK's kinase activity. We have completed task 2 and the results are shown below. As demonstrated in Fig. 5-6 PERK phosphorylates PACT, thus we investigated if the DYT16 mutants of PACT are phosphorylated with similar efficiency as that of wt PACT. As seen in Fig. 8 (next page) 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 once we investigate the functional role of PACT in PERK signaling pathway. Once phosphorylated by PERK, PACT

could either activate PKR or it could have a PKR-independent role in PERK signaling pathway. We will soon begin work in this direction and anticipate completion of this subtask by 09/2019.

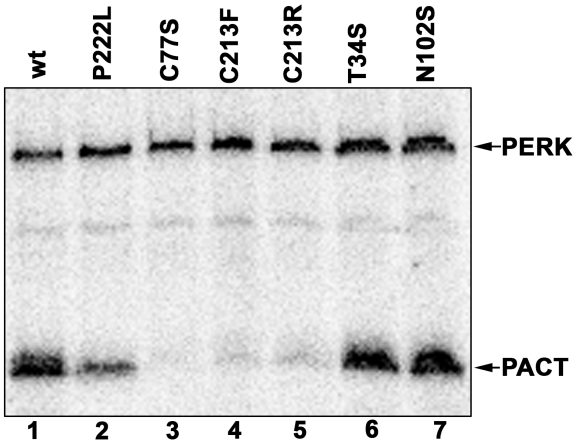

Fig. 8. Effect of DYT16 PACT mutations on PACT's ability to serve as PERK substrate: Pure recombinant 20 ng GST-PERK (cytoplasmic catalytic domain) was used to measure phosphorylation of various PACT mutants as compared to wt PACT. The PACT proteins added is as indicated above the lanes. Pure recombinant PACT proteins were used as substrates.

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

The project has provided great opportunity to train one graduate student who is working towards his PhD and he has worked on this project with me. I train the students one-on one as I work in the lab. The project allows to train the graduate student to perform biochemical and molecular work. In particular the graduate student has learned 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 student has now become very proficient in performing these experiments and will defend his thesis by Fall 2020. In addition to training the student at bench, the project has allowed me to train the student in interpreting the data, and preparing the results for presentation and publication. I have routine weekly lab meetings when students present their data effectively and answer questions pertaining to their results. This prepares the student to attend national/international meetings to present their data. This project has allowed ample scope to train the graduate student and he is planning on presenting his results at the EMBO workshop "Proteostasis: From organelles to organisms" in November 2019 in Ericeira, Portugal. The project has allowed me to train the graduate student to become successful in biochemical research pertaining to dystonia as well as protein homeostasis. In addition, the student has given multiple seminar presentations locally at our university and department in weekly seminar series. He 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.

- **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.

- **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. This resulted in a new target date for some of the specific tasks. The experiments that we postponed to the next reporting period involve cell culture experiments and *in vivo* demonstration of PERK-PACT interactions and the effect of altered PERK-PACT interactions or activities of the cell survival. 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:	09/2019
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	09/2019
Subtask 2: To determine if PACT essential for PERK activation in response to ER stress	09/2019
Subtask 3 is completed	
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
Subtask 2 is completed	
Subtask 3: determine the effect of PACT mutants on PERK signaling pathway in response to ER stress	09/2019

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. Once our results are published, it will have a major influence on the field. It is unknown that PERK has any substrate other than eIF2a 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-eIF2a 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:

Nothing to report yet. Publications, presentations, and conference paper expected in next 6 months.

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	
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	
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

NONE