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14. ABSTRACT

The fundamental importance of ribosomes to cellular functions and broader biological outcomes is, by now, readily apparent. Recent studies have shown that the central components of the ribosome, including the repertoire of ribosomal proteins (RPs), can be regulated and diversified to control protein translation. This regulation is mediated, in part, by post-translational modifications (PTMs) of ribosomal proteins. PTMs of ribosomal proteins are versatile, may have functional consequences for translational control, and are intimately linked to human disease. In preliminary studies, we have shown that ribosome mono(ADP-ribosyl)ation (MARylation), a PTM of proteins, is dependent on the cytosolic NAD+ synthase, NMNAT-2, and the PARP monoenzyme, PARP-16, an endoplasmic-reticulum-anchored, cytosol-facing protein. In addition, our preliminary results exploring this biology have revealed that MARylation of RPs by NMNAT-2 and PARP-16: (1) attenuates protein translation and reduces protein aggregation; (2) controls loading of mRNAs onto polysomes; and (3) supports the viability of cells by promoting proteostasis. The results suggest a functional link between NAD+ production and consumption in ribosome activity. Our results suggest a model in which ribosome MARylation regulates protein synthesis to prevent protein aggregation and proteotoxic stress. In the context of ovarian cancer cells, this enhances cell growth and likely accounts for the elevated expression of NMNAT-2. Given the important roles of PTMs on RPs in ribosome function, as well as the important role that regulation of protein translation has in maintaining proteostasis, we hypothesize that (1) NAD+-dependent, site-specific MARylation of RPs mediated by NMNAT-2 and PARP-16 affects the assembly and function of ribosomes and (2) alterations in ribosome assembly and function by MARylation of RPs is tied to cytosolic NAD+ metabolism, and affects cellular functions and viability. We will test this hypothesis in two aims: Aim 1: Determine the mechanisms by which ribosome MARylation affects ribosome function and Aim: 2: Determine the effects of RP MARylation on cellular and biological outcomes in ovarian cancer. Our expected outcomes for this aim are definitive connections between site-specific MARylation events and the molecular mechanisms they control (from Aim 1) to well characterized cancer-related outcomes (from Aim 2).

15. SUBJECT TERMS
ADP-ribose, ADP-ribosylation, mono(ADP-ribose), mono(ADP-ribosyl)ation, nicotinamide adenine dinucleotide (NAD+), nicotinamide mononucleotide adenylyl transferase (NMNAT-1), PARP-16, post-translational modification, proteostasis, ribosomal protein, ribosome, translation

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TABLE OF CONTENTS

1. INTRODUCTION:

Ovarian cancer is the deadliest of all gynecologic cancers, with less than 50% of patients surviving 5 years. Despite advances in radical surgery and chemotherapy for the treatment of advanced ovarian cancers, up to 85% eventually relapse and response to subsequent cytotoxic therapies is short-lived. This project investigates the role of mono(ADP-ribosylation) (MARylation) in ovarian cancer. ADP-ribosylation (ADPRylation) is a reversible post-translational modification (PTM) of proteins resulting in the covalent attachment of ADP-ribose (ADPR) units derived from nicotinamide adenine dinucleotide (NAD^+). ADPRylation is catalyzed by the poly(ADP-ribose) polymerase (PARP) family of enzymes, including mono(ADP-ribosyl) transferases (MARTs) that catalyze (MARylation). Recent studies have shown that the central components of the ribosome, including the repertoire of ribosomal proteins (RPs), can be regulated and diversified to control protein translation. This regulation is mediated, at part, by PTMs of RPs. In our work, we have identified sites of MARylation on a number of RPs in mammalian cells. Given the important roles of PTMs on RPs in ribosome function, as well as the important role that regulation of protein translation has in maintaining proteostasis, we hypothesize that (1) NAD^+-dependent, site-specific MARylation of RPs mediated by NMNAT-2 and PARP16 (as well as other cytosolic MARTs) affects the assembly and function of ribosomes and (2) alterations in ribosome assembly and function by MARylation of RPs is tied to cytosolic NAD^+ metabolism, and affects cellular functions and viability. We are using a host of biochemical, cell-based, and proteomic assays to test this hypothesis.

2. KEYWORDS:

- ADP-ribose
- ADP-ribosylation
- mono(ADP-ribose)
- mono(ADP-ribosyl)ation
- nicotinamide adenine dinucleotide (NAD+)
- nicotinamide mononucleotide adenylyl transferase 2 (NMNAT-2)
- PARP14
- PARP16
- post-translational modification
- proteostasis
- ribosomal protein
- ribosome
- TARG1
- translation

3. ACCOMPLISHMENTS:

What were the major goals of the project?

Specific Aim 1: Determine the mechanisms by which ribosome MARylation affects ribosome function

- Major Task 1: Determine which amino acid residues on ribosomal proteins are MARylated in an NMNAT-2 and PARP-16-dependent manner *[Timeline: Months 1-8, Status: 100% Complete]*
- Major Task 2: Determine how site-specific MARylation of ribosomal proteins affect ribosome function *[Timeline: Months 8-18, Status: 75% Complete]*

- Major Task 3: Determine how site-specific MARylation of ribosomal proteins affect cellular proteostasis *[Timeline: Months 12-18, Status: 100% Complete]*
- Major Task 4: Generate site-specific anti-MARylated ribosomal protein antibodies *[Timeline: Months 18-36, Status: 20% Complete]*

Specific Aim 2: Determine the effects of RP MARylation on cellular and biological outcomes in ovarian cancer

- Major Task 5: Determine if ribosome MARylation controls cell-based cancer-related outcomes *[Timeline: Months 18-28, Status: 85% Complete]*
- Major Task 6: Determine if ribosome MARylation controls tumor growth in vivo *[Timeline: Months 18-36, Status: 50% Complete]*
- Major Task 7: Determine how ribosomal protein MARylation affects tumor growth in vivo *[Timeline: Months 18-36, Status: 25% Complete]*.

What was accomplished under these goals?

1) Major Activities and Specific Objectives – The goals of this proposal are two-fold: (1) Determine how ADPRylation of ribosomal proteins regulates the functions of the ribosome (i.e., translation, proteostasis and the prevention of proteotoxity/protein aggregation, stress granule formation) and (2) how this regulation drives the biology or ovarian cancers. During Year 2 of this grant, we made substantial progress or completed many aspects of Aim 1 (ADPR site identification, mechanisms) and began working in earnest on Aim 2 (biological outcomes). This work included: (1) Characterizing biochemically PARP16 MART activity, (2) Determining which amino acid residues on RACK1, an integral ribosomal protein in the 40S subunit, are MARylated and the cognate MART that mediates the MARylation, (3) Determining how site-specific MARylation of RACK1 affects ribosome function, especially as it relates to stress granule assembly, and (4) Determining how site-specific MARylation of RACK1 affects the growth of ovarian cancer cells in culture and in vivo. This work has led to a complete manuscript that has been submitted to *bioRxiv* and will soon be submitted to a major journal for publication.

2) Significant Results and Key Findings –

Characterization of PARP16 and its role in the MARylation-dependent regulation of ribosomes.

Following up on our previously published work regarding the control of translation and proteostasis via NMNAT-2/PARP16-mediated MARylation of ribosomes (Challa et al., 2021), we set out to characterize in more detail additional molecular and biochemical mechanisms underlying this regulation. As a starting point, we performed immunoprecipitation (IP)-mass spectrometry analysis of PARP16-interacting proteins (Fig. 1A). From this analysis, we identified a number of high-confidence interactors including proteins involved in nucleocytoplasmic transport (e.g., karyopherins and nucleoporins, such as KPNB1) and translation (e.g., the eIF-2-alpha kinase activator GCN1) (Fig. 1B). We confirmed these interactions in IP-Western blotting assays (Fig. 1C). We also demonstrated that KPNB1 and GCN1 are MARylation substrates of PARP16 using proximity ligation assays (PLAs) with a MAR detection reagent and KPNB1 or GCN1 antibodies in the presence of PARP16 knockdown or treatment with a PARP16 inhibitor (DB008; (Bejan et al., 2022) (Fig. 1D and not shown). In addition, we purified recombinant PARP16 (GST-PARP16 full length) and developed an in vitro transmodification assays with purified ribosomes, demonstrating for the first time the transmodification of a PARP16 substrate in vitro (Fig. 1E). In addition, we developed a target engagement assay to show covalent labeling of PARP16 with DB008 in xenograft tumors in vivo (Fig. 1F). These studies have advanced our work with PARP16 and the PARP16 inhibitor.

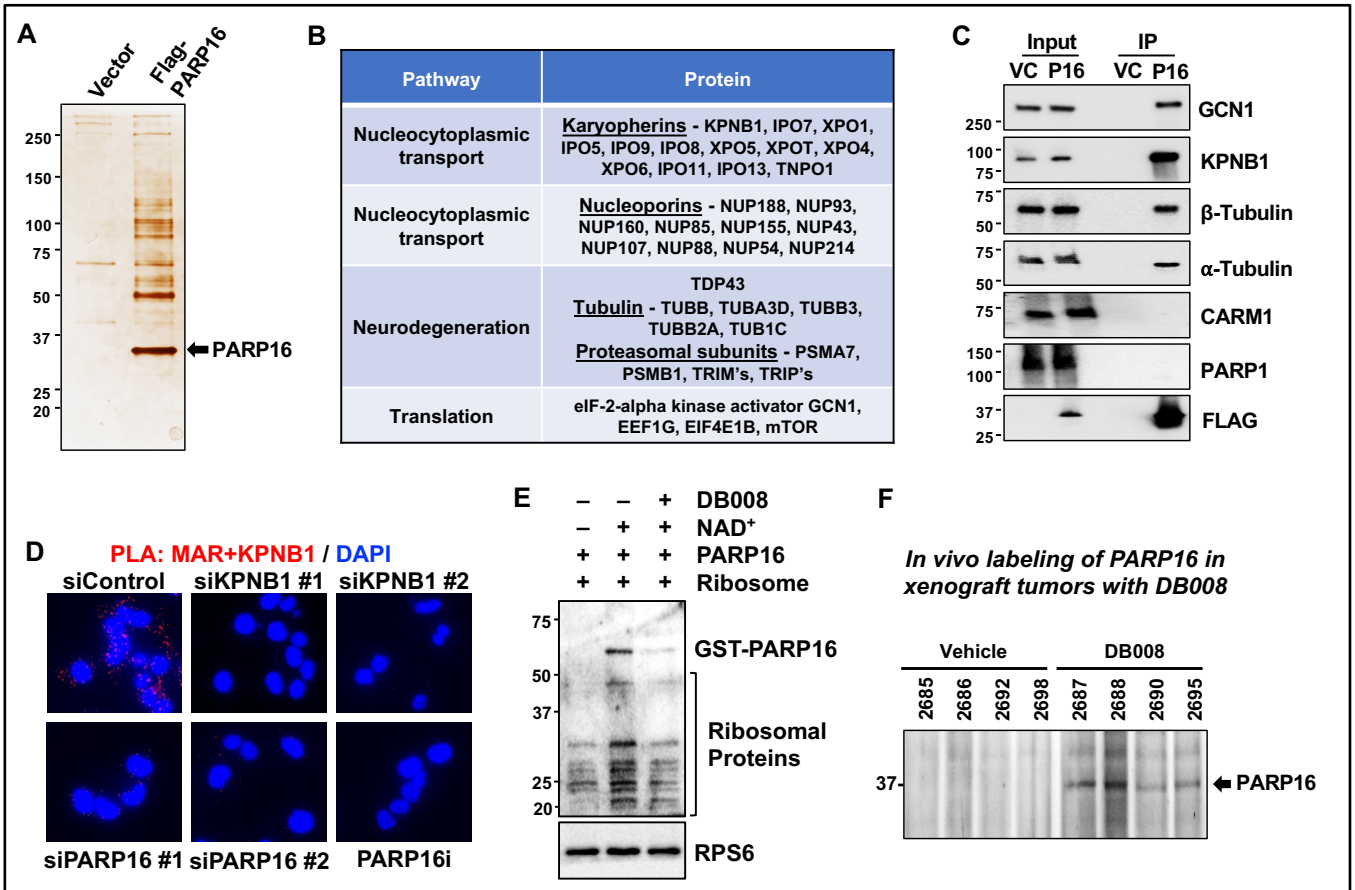

Figure 1. Characterization of PARP16 and its role in the MARylation-dependent regulation of ribosomes.
(A and B) Immunoprecipitation (IP)-mass spectrometry analysis of PARP16-interacting proteins. From this analysis, we identified a number of high-confidence interactors including proteins involved in nucleocytoplasmic transport (e.g., karyopherins and nucleoporins, such as KPNB1) and translation (e.g., the eIF-2-alpha kinase activator GCN1)
(C) Confirmation of these PARP16 interactors from (A) and (B) in IP-Western blotting analyses.
(D) Demonstration that KPNB1 is a MARylation substrate of PARP16 using proximity ligation assays (PLAs) with a MAR detection reagent and KPNB1 or GCN1 antibodies in the presence of PARP16 knockdown or treatment with a PARP16 inhibitor (DB008).
(E) Development of an in vitro transmodification assays with purified full-length recombinant PARP16 (GST-PARP16) and purified ribosomes, demonstrating for the first time the trans-modification of a PARP16 substrate in vitro
(F) Click chemistry-based target engagement assay showing covalent labeling of PARP16 with DB008 in xenograft tumors in vivo.

RACK1, an integral component of the 40S ribosomal subunit, is MARylated in ovarian cancer cells.

RACK1 is an integral component of the ribosome (Rabl et al., 2011) located in a regulatory region of the 40S subunit (Park et al., 2020). It functions as a scaffolding protein, which recruits proteins that are important for quality control during mRNA translation (Arimoto et al., 2008), and serves as an essential component of stress granules (Buchan and Parker, 2009; Zhou et al., 2023). We previously identified sites of MARylation on RACK1 in ovarian cancer cells using mass spectrometry-based proteomics (Challa et al., 2021) (Fig. 2A; Asp 144, Glu 145, and Asp 203). We confirmed that RACK1 is MARylated in OVCAR3 ovarian cancer cells using immunoprecipitation of endogenous RACK1, followed by immunoblotting with an antibody-like MAR detection reagent (Gibson et al., 2017) (Fig. 2B). Next, we generated OVCAR3 cells that, when cultured in doxycycline (Dox), simultaneously knockdown endogenous RACK1 and ectopically express HA-tagged wild-type RACK1 (RACK1-WT) or a MARylation site mutant RACK1 (RACK1-Mut). Immunoprecipitation of the HA tage RACK1 followed by immunoblotting for MAR demonstrated site-specific MARylation of RACK1 at Asp 144, Glu 145,

and Asp 203 (Fig. 2C). We also developed a proximity ligation assay (PLA) for *in situ* detection of site-specific MARylation using MAR and HA (i.e., RACK1) antibodies (Fig. 2D).

RACK1 is required for efficient translation of selected mRNAs

Our previous studies demonstrated that one function of MARylation of ribosomal proteins is to inhibit global protein synthesis by altering polysome formation. Therefore, we measured global protein synthesis levels of RACK1-WT- or RACK1-Mut-expressing cells using puromycin incorporation assays. We did not observe an obvious change in global protein synthesis in cells deficient in RACK1 MARylation (Fig. 2E). We next performed ribosome profiling (Ribo-seq) assays (Chen et al., 2020; McGlincy and Ingolia, 2017) to investigate potential changes in translational efficiency in cells deficient in RACK1 MARylation. We observed changes in the translation levels of 165 transcripts in RACK1-Mut-expressing cells versus RACK1-WT-expressing cells (Fig. 2F). Gene ontology of the affected transcripts showed enrichment in mRNAs encode proteins involved in receptor tyrosine kinase signaling, including AKT1 (Fig. 2G and data not shown). These results implicate RACK1 MARylation in the control of translation of a subset of mRNAs.

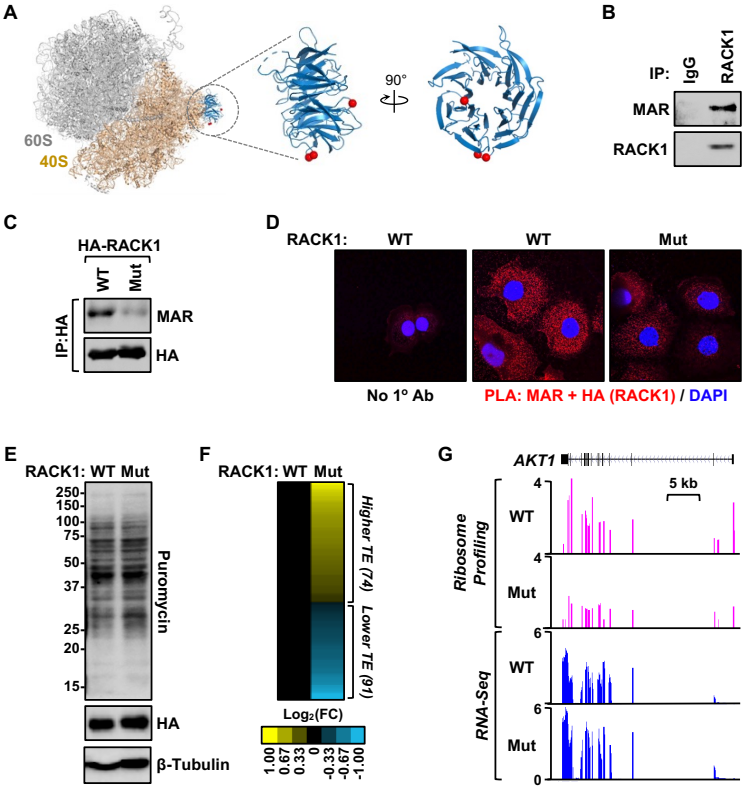

Figure 2. Site-specific MARylation of RACK1 in ovarian cancer cells.

(A) *(Left)* Spatial distribution of the proteins modified by MARylation in the 80S ribosome (PDB ID: 4V6X). *(Middle and right)* Sites of MARylation within RACK1 (Asp 144, Glu 145, and Asp 203; blue ribbon) are indicated in two expanded views, with the structure in the right rotated by 90°.

(B) RACK1 is MARylated. Endogenous RACK1 was immunoprecipitated from OVCAR3 cells and subjected to immunoblotting for MAR and RACK1.

(C) RACK1 is MARylated at Asp 144, Glu 145, and Asp 203. HA-tagged RACK1 was immunoprecipitated from OVCAR3 cells ectopically expressing wild-type (WT) or MARylation site mutant (Mut) RACK1 and subjected to Immunoblotting for MAR and HA.

(D) In situ detection of RACK1 MARylation. Proximity ligation assay (PLA) of HA-tagged RACK1 and MAR in OVCAR3 cells subjected to Dox-induced knockdown of endogenous and re-expression of RACK1. DNA was stained with DAPI. Scale bar is 10 μm.

(E) RACK1-Mut expression does not alter global protein synthesis in OVCAR3 cells. Immunoblot analysis of puromycin incorporation assays from OVCAR3 cells subjected to Dox-induced knockdown of endogenous and re-expression of RACK1. β-tubulin serves as a loading control.

(F) Regulation of mRNA translation by RACK1 MARylation. Ribosome profiling of OVCAR3 cells subjected to Dox-induced knockdown of endogenous RACK1 followed by re-expression of exogenous RACK1 (WT or Mut). Heatmap representation of mRNAs that exhibit altered translation efficiency when RACK1-Mut was expressed.

(G) RACK1 MARylation regulates translation of *AKT1*. Example ribosome profiling and RNA-seq traces of AKT1 in OVCAR3 cells subjected to Dox-induced knockdown of endogenous RACK1 and re-expression of exogenous RACK1 (WT or Mut). A schematic of the *AKT1* gene with a scale bar is shown.

Site-specific MARylation of RACK1 by PARP14 is required for stress granule assembly.

Since RACK1 is a key player in stress granule assembly (Buchan and Parker, 2009; Zhou et al., 2023), we tested whether MARylation alters the localization of RACK1 to stress granules. In a variety of assays, we showed that a loss of RACK1 MARylation reduces the interaction of G3BP1 with ribosomes. G3BP1 is a marker of stress granule formation (Asadi et al., 2021; Zhou et al., 2023) (data not shown).

Collectively, our data demonstrate that sites-specific MARylation of RACK1 drives protein-protein interactions that are required for stress gran ule assembly.

In our previous work, we identified PARP16 as a MART that MARylates selected ribosomal proteins to regulate the loading of mRNAs onto ribosomes and their translation (Challa et al., 2021). To determine which MART MARylates RACK1 in OVCAR3 cells, we used the RACK1+MAR PLA coupled with an siRNA screen of MART enzymes, focusing on those that are expressed in OVCAR3 cells and are primarily cytosolic. We observed that knockdown of *PARP14* mRNA caused the most consisten and dramatic reduction in RACK1 MARylation (data not shown). To confirm this observation, we used a chemical inhibitor of PARP14 (PARP14i), RBN012579 (Schenkel et al., 2021), which inhibits PARP14 activity, as shown by a reduction in autoMARylation (Fig. 3A). The PARP14i also inhibited RACK1 MARylation in a PLA (Fig. 3B). We also observed that chemical inhibition of PARP14 activity leads to phenotypes that mimic expression of RACK1-Mut: (1) reduced association of G3BP1 with ribosomes (Fig. 3, C and D), (2) reduced interactions between G3BP1 and SG factors (Fig. 3E), and (3) reduced SG assembly (Fig. 3F). We observed similar regulation of PARP14-mediated RACK1-MARylation and stress granule assembly in additional ovarian cancer cell lines, SKOV3 and HCC5044 (data not shown). Together, these data show that PARP14-mediated, site-specific MARylation of RACK1 drives stress granule assembly in ovarian cancer cells.

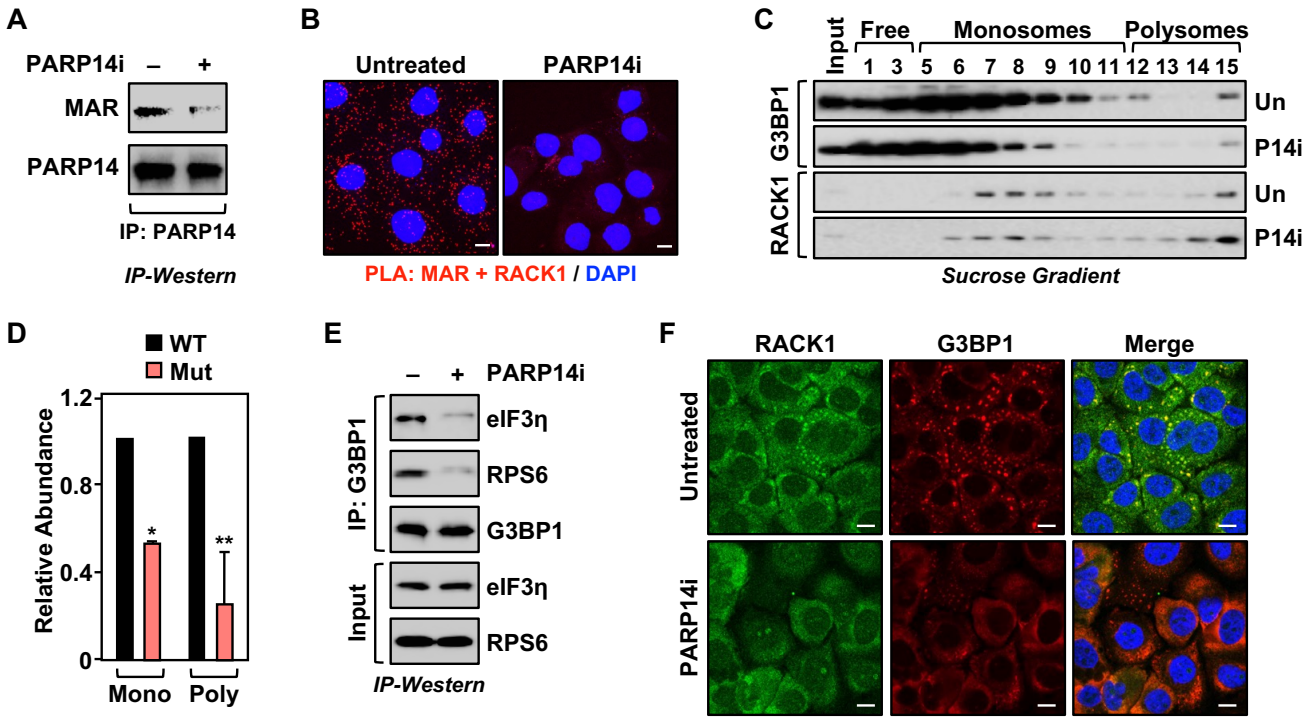

Figure 3. PARP14 inhibition reduces stress granule assembly.
(A) PARP14 inhibitor blocks PARP14 autoMARylation. OVCAR3 cells were treated with 10 μM PARP14 inhibitor (RBN012759) for 24 hours. PARP14 was immunoprecipitated and subjected to immunoblotting for PARP14 and MAR.
(B) Inhibition of PARP14 catalytic activity blocks RACK1 MARylation. PLA using MAR and RACK1 antibodies in OVCAR3 cells treated with 10 μM PARP14 inhibitor (RBN012759) for 24 hours. DNA was stained with DAPI. Scale bar is 10 μm.
(C and D) PARP14 inhibition reduces the recruitment of G3BP1 to ribosomes. (C) Immunoblot analysis of RACK1 and G3BP1 in sucrose density gradient fractions of ribosomes prepared from OVCAR3 cells treated with 10 μM PARP14 inhibitor for 24 hours. Each bar in the graph in (D) represents the mean + SEM of the relative abundance of G3BP1 in monosomes or polysomes (n = 3, two-way ANOVA, * $p < 0.05$ and ** $p<0.01$).
(E and F) PARP14 inhibition reduces G3BP1 localization to stress granules and its interaction with translation factors that are key components of stress granules. (E) G3BP1 was immunoprecipitated from OVCAR3 cells treated with 10 μM PARP14 inhibitor for 24 hours and subjected to immunoblotting for eIF3η, RPS6, and G3BP1 as indicated. (F) Immunofluorescent staining assays of OVCAR3 cells treated with 10 μM PARP14 inhibitor for 24 hours and subjected to 15 minutes of treatment with 250 μM sodium arsenite ($NaAsO_2$). Staining for RACK1 and G3BP1. DNA was stained with DAPI. Scale bar is 10 μm.

Loss of RACK1 MARylation sensitizes ovarian cancer cells to stress.

Since loss of RACK1-MARylation suppresses stress granule assembly, which is crucial for overcoming stress (Arimoto et al., 2008; Park et al., 2020), we surmised that the loss of PARP14-mediated site-specific MARylation of RACK1 will sensitize the cells to external stressors. To test this, we performed cell growth assays using ovarian cancer cells cultured in the presence of thapsigargin, which induces endoplasmic reticulum (ER) stress (Sagara and Inesi, 1991). Ovarian cancer cells expressing RACK1-Mut or treated with PARP14i exhibited slower growth than cells expressing RACK1-WT or treated with vehicle (Fig. 4, A and B). Similar effects were observed in OVCAR3 xenograft tumors grown in immunodeficient mice (Fig. 4, C and D). Further analysis demonstrated that the expression of RACK1-Mut or PARP14i treatment increased ER stress as indicated by increased phosphorylation of eIF2a is, causing apoptosis as indicated by increased cleaved caspase-3 (data not shown). These results connect the site-specific MARylation of RACK1 to cellular and biological outcomes.

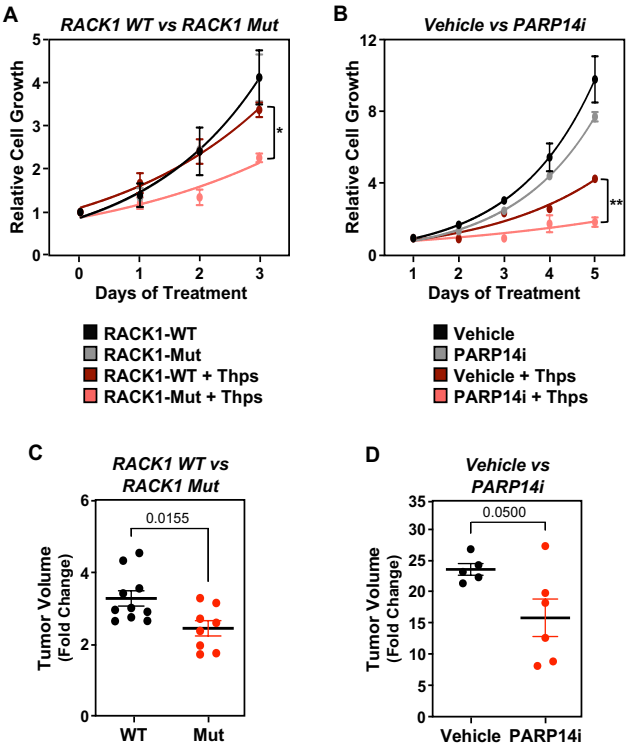

Figure 4. Loss of RACK1 MARylation sensitizes ovarian cancer cells to stress and inhibits their growth.
(A) RACK1-Mut expressing cells are sensitive to ER stress and inhibits their growth. Growth curves of OVCAR3 cells with Dox-induced knockdown of endogenous RACK1 and re-expression of exogenous RACK1 (WT or Mut) in the presence or absence of 3 nM thapsigargin (Thps) for the indicated times. Each point represents the mean ± SEM of the growth of the cells relative to Day 0 of treatment (n = 3, two-way ANOVA, * $p < 0.01$).
(B) PARP14 inhibition sensitizes ovarian cancer cells to ER stress and inhibits their growth. Growth curves of OVCAR3 cells in the presence or absence of 10 μM PARP14 inhibitor (PARP14i) and 3 nM thapsigargin (Thps) for the indicated times. Each point represents the mean ± SEM of the growth of the cells relative to Day 0 of treatments (n = 3, two-way ANOVA, ** $p < 0.001$).
(C and D) Expression of RACK1-Mut or treatment with PARP14 inhibitor (PARP14i) inhibits the growth of OVCAR xenograft tumors derived from cells like those described in (A) and (B). The xenograft tumors were established in immunocompromised NSG mice subjected to the treatments indicated. (C) Tumor growth at Day 69. Each cluster in the graph shows the mean and the individual data points for n = 10 or 8 mice (WT or Mut, respectively), Student's t-test, $p = 0.0155$. (D) Tumor growth at Day 19 post-treatment. Each cluster in the graph shows the mean and the individual data points for n = 5 or 6 mice (vehicle or PARP14i, respectively), Student's t-test, $p = 0.05$.

3) <u>Major Conclusions</u> - Our new data generated during year two have advanced the aims of the proposed research, tested our hypotheses, strengthened our conclusions, and opened up new areas of investigation. Specifically, they allowed us to characterize in more detail additional molecular and biochemical mechanisms underlying translation by PARP16. In addition, they allowed us to identify additional MARylated proteins that regulate translation (e.g., RACK1) and the MARTs that regulated them (e.g, PARP14). These results strengthen our conclusion that cytosolic MARylation is a key regulator of translational regulation in ovarian cancers.

4) <u>Progress Toward Goals</u> - We are making excellent progress toward our goals, which are twofold: (1) Determine how ADPRylation of ribosomal proteins regulates the functions of the ribosome (i.e., translation, proteostasis and the prevention of proteotoxity/protein aggregation, stress granule formation) and (2) how this regulation drives the biology or ovarian cancers.. Since the submission of this proposal in July 2020, significant fractions of Aim 1 and Aim 2 have been completed or are nearing completion. We have identified a host of MARylation sites on ribosomal proteins and are systematically analyzing their roles in ribosome function, protein translation, proteostasis, and broader cancer-related biological

outcomes. Our initial focus was on specific MARylation sites on RPL24 and RPS6 mediated by PARP16. In each case, we have verified the sites and performed a plethora of biochemical, cellular, and cancer-related assays to assess the function of MARylation at these sites. We are continuing by investigating other MARylation sites on other ribosomal proteins (e.g., RACK1) mediated by other MARTs (e.g., PARP14).

During Year 2 of this grant, we made substantial progress or completed many aspects of Aim 1 (ADPR site identification, mechanisms) and began working in earnest on Aim 2 (biological outcomes). This work included: (1) Characterizing biochemically PARP16 MART activity, (2) Determining which amino acid residues on RACK1, an integral ribosomal protein in the 40S subunit, are MARylated and the cognate MART that mediates the MARylation, (3) Determining how site-specific MARylation of RACK1 affects ribosome function, especially as it relates to stress granule assembly, and (4) Determining how site-specific MARylation of RACK1 affects the growth of ovarian cancer cells in culture and in vivo. This work has led to a complete manuscript that has been submitted to *bioRxiv* and will soon be submitted to a major journal for publication.

5) <u>Other Accomplishments</u> – Some of our initial work on this project was presented in an abstract submitted to a major conference (as indicated elsewhere in this report) and a preprint was submitted to *bioRxiv* as follows: Challa S., Nandu T., Kim H.B., Gong X., Renshaw C.W., Li W-C., Tan X., Camacho C.V., Chen J., Kraus W.L. (2023) A PARP14/TARG1-regulated RACK1 MARylation cycle drives stress granule dynamics in ovarian cancer cells. *bioRxiv* 10.1101/2023.10.13.562273. This will soon be submitted to a journal for publication.

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

This grant is a basic science research grant that supports various types of personnel, which may include postdocs, graduate students, and research staff. Although the research is not specifically intended to provide training and professional development opportunities, the personnel do get those opportunities through their work supported by the grant. Of particular note is that Dr. Sridevi Challa, a former postdoc in the Kraus lab who has been a key contributor to the work on this grant and is the first author of the paper noted above, was able to launch her independent professional career due, in part, to her efforts on this grant. She started a tenure-track Assistant Professor position at the University of Chicago, Department of Obstetrics & Gynecology and the Cancer Center in January 2023.

Training activities for postdoc and graduate students include one-on-one work with the PI (mentor), lab journal clubs and data clubs, works in progress, manuscript reviewing, and other opportunities to present and write about aspects of their work. Professional development activities for postdocs, graduate students, and research staff include participation in conferences, workshops, and seminars on science-related topics, as well as career development-related topics. In addition, the PI (mentor) has regular one-on-one career development and planning discussions with the PI (mentor).

All postdoctoral and graduate student trainees at UT Southwestern Medical Center are required by their respective training programs (the Graduate School and Postdoctoral Office) to have Individual Development Plans (IDPs). These plans are formulated in conjunction with their advisory committees and may include professional development coursework. The IDPs are used to help the trainees track progress, set goals, and plan for the future. They are also used as a mechanism to come to a course of action mutually agreed upon by the trainee and mentor.

Trainees in the Green Center for Reproductive Biology Sciences at UT Southwestern Medical Center participate in a weekly 'Works in Progress' meeting with all of the other trainees and faculty in the Center, where they present their work at least once a year. In addition, they participate in a yearly career advising workshops sponsored by the Director. Finally, they also participate in a computationally-oriented hands-on workshop to learn how to conduct genomic data analyses sponsored by the Center's Computational Core Facility.

Trainees and research staff in the Kraus lab also participate in a weekly lab journal club, a weekly lab research meeting, a weekly small group data analysis meeting related to the topic of their project, and a regular individual meetings with the mentor/PI. They also attend at least one scientific conference per year related to their area of research, where they present their work and get feedback on their science.

How were the results disseminated to communities of interest? If there is nothing significant to report during this reporting period, state "Nothing to Report."

Nothing to report.

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

Over the past year, we (1) generated data mapping the sites of MARylation on ribosomal proteins in ovarian cancers, including RPL24 and RPS6, and (2) exploring how MARylation at those sites is regulated by NMNAT-2 and PARP-16. In the next reporting period, we will continue with the work outlined in the aims including the effects of a PARP-16 inhibitor using assays similar to those described herein as an alternative to depletion of NMNAT-2 and PARP-16. In addition, we will explore additional sites in other ribosomal proteins, such as RACK1 (Receptor for Activated C Kinase 1). RACK1 is a member of the tryptophan-aspartate repeat (WD-repeat) family of proteins that functions in the shuttling and chaperoning of proteins in the cell, anchoring proteins at particular cellular locations, and stabilizing protein activity. Importantly, RACK1 interacts with the ribosome and is even considered to be an integral ribosomal protein. As with we have done with RPL24 and RPS6, we have identified sites of MARylation on RACK1. We will verify the sites, determine the PARP family member responsible for MARylation those sites, and perform a plethora of biochemical, cellular, and cancer-related assays to assess the function of MARylation at these sites.

6) Literature Cited –

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4. IMPACT:

What was the impact on the development of the principal discipline(s) of the project?

Our studies are some of the first to explore site-specific MARylation mediated by a cytosolic MART and connect it to cellular NAD^+ metabolism. We developed a variety of tools and approaches for these studies. As such, we believe that our work has the potential to change the way that MARylation is studied and understood, especially in the context of human disease. We have applied these approaches to characterize the regulatory activity of two poorly characterized MARTs, PARP16 and PARP14, and identify their ribosomal substrates.

What was the impact on other disciplines?

Nothing to report.

What was the impact on technology transfer?

Nothing to report.

What was the impact on society beyond science and technology?

Nothing to report.

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change

None. The work is going very well and according to plan.

Actual or anticipated problems or delays and actions or plans to resolve them

Overall, the work is going very well and according to plan. However, Aim 1 - Major Task 4: Generation of site-specific anti-MARylated ribosomal protein antibodies, has been more challenging than expected. The chemistry and enzymology to generate site-specific ADPRylated peptide is difficult and these peptides are needed as antigens to produce the antibodies. Moreover, an initial attempt at making a site-specific ADPR antibody against a site-specific ADPRylated peptide has not yet yielded a usable antibody. We are troubleshooting and have a variety of steps in the process that we can adjust and improve. We will keep at it. Note that this is not an essential aspect of the work, but it would be helpful if we can make an antibody against specific ADPRylated ribosomal proteins.

Changes that had a significant impact on expenditures

None.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

None.

Significant changes in use or care of human subjects. Significant changes in use or care of vertebrate animals. Significant changes in use of biohazards and/or select agents.

None.

6. PRODUCTS:

Publications, conference papers, and presentations.

The following abstract was submitted and presented at the conference listed: W. L. Kraus, The Role of PARPs, NAD^+, and Site-Specific ADP-ribosylation in the Control of Complex Biological Processes. Presented at the FEBS Advanced Lecture Course PARP 2023: Research on the family of poly(ADP-ribose) polymerases. Hvar, Croatia, 4-8 June 2023.

Journal publications.

The following preprint was submitted to *bioRxiv*: Challa S., Nandu T., Kim H.B., Gong X., Renshaw C.W., Li W-C., Tan X., Camacho C.V., Chen J., Kraus W.L. (2023) A PARP14/TARG1-regulated RACK1 MARylation cycle drives stress granule dynamics in ovarian cancer cells. *bioRxiv* 10.1101/2023.10.13.562273. This will soon be submitted to a journal for publication.

Books or other non-periodical, one-time publications.

None.

Other publications, conference papers, and presentations.

None.

Website(s) or other Internet site(s)

None.

Technologies or techniques

None.

Inventions, patent applications, and/or licenses

None.

Other Products

This work led to a postdoctoral fellowship from the Ovarian Cancer Research Alliance (Grant Number: 813060) awarded to Dr. Sridevi Challa. This award helped her obtain a faculty position at the University of Chicago, which she started in January 2023.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	**W. Lee Kraus, Ph.D.**
Project Role:	PI
Researcher Identifier (e.g., ORCID ID):	0000-0002-8786-2986
Nearest person month worked:	1.5
Contribution to Project:	Dr. Kraus was responsible for the overall administration and scientific direction of the project. He assisted with the design, implementation, and analysis of all experiments, coordinated the efforts of laboratory personnel, and met weekly with personnel for project direction.
Funding Support:	This award plus additional funding as described below.
Name:	**Sridevi Challa, Ph.D.**
Project Role:	Postdoctoral Researcher
Researcher Identifier (e.g., ORCID ID):	0000-0002-5308-9243
Nearest person month worked:	0.6 (20% effort for 3 months Oct 22 - Dec 2022 before leaving to start a faculty position and who own lab at the University of Chicago).
Contribution to Project:	Dr. Challa planned and executed experiments associated with both aims of this project, including data collection and interpretation.
Funding Support:	This award plus a postdoctoral fellowship from the Ovarian Cancer Research Alliance.
Name:	**Komal Pekhale, Ph.D.**
Project Role:	Postdoctoral Researcher
Researcher Identifier (e.g., ORCID ID):	0000-0002-6372-3690
Nearest person month worked:	12.0
Contribution to Project:	Dr. Pekhale planned and executed experiments associated with both aims of this project, including data collection and interpretation.
Funding Support:	This award plus the PI's grant funds.
Name:	**Tulip Nandu, M.S.**
Project Role:	Computational Biologist
Researcher Identifier (e.g., ORCID ID):	0000-0001-9786-614X
Nearest person month worked:	1.8

Contribution to Project:	Mr. Nandu was responsible for all of the computational analyses on this project.
Funding Support:	This award plus the PI's grant funds.
Name:	**Luka Zrnic, B.S.**
Project Role:	Research Technician II
Researcher Identifier (e.g., ORCID ID):	0000-0003-1947-7901
Nearest person month worked:	6.0
Contribution to Project:	Mr. Zrnic provided technical support for all aspects of the project.
Funding Support:	This award plus the PI's grant funds.

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

Since the last reporting period, there have been no changes in the active other support for W. L. Kraus (PI), as indicated below. In addition, Dr. Challa left UT Southwestern to start a faculty position at the University of Chicago and, as such, she terminated her postdoctoral fellowship from the Ovarian Cancer Research Alliance.

What other organizations were involved as partners?

Nothing to report.

8. SPECIAL REPORTING REQUIREMENTS

Not Applicable.

9. APPENDICES:

No items included this year.