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14. ABSTRACT

There are several clinical applications of our Breakthrough studies. First, the proposed studies will validate the CK1d-to-ribosome assembly pathway as an exploitable vulnerability for TNBC, which would support efforts for fast-tracking such CK1d-selective agents into the breast oncology clinic. Second, if our studies prove successful (as we fully expect) they will support new anti-cancer drug campaigns that seek to disable the functions of other assembly factors that are necessary for the proper assembly of ribosomes – with ~200 assembly factors that one could target this is a rich arena for developing a whole new cast of cancer therapeutics, for TNBC, HER2+ and luminal B breast cancers and likely many other tumor types. Third, our studies will define the mechanism by which CK1d inhibitors block TNBC cell growth and survival. This will allow for the identification of biomarkers, which are needed to show that these drugs are indeed on target in the tumors of patients treated with these agents, for example by evaluating effects of these drugs on the quantity of ribosomes in tumor cells (which can be quantified by staining tumor sections with a dye that detects RNA). Finally, our proposed studies may reveal potential mechanisms for the development of resistance to CK1d inhibitors and, using FDA approved drugs, they will test new

15. SUBJECT TERMS
Triple negative breast cancer, ribosome assembly, autophagy, CRISPR/Cas9

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1. INTRODUCTION

Casein kinase 1δ (CK1δ) is a target for cancer drug development, as CK1δ is amplified and/or overexpressed in several human tumor types, in particular TNBC, and treatment of such tumor cells with CK1δ inhibitors blocks their growth, survival and tumorigenic potential [6-9]. Indeed, TNBC are highly sensitive to SR3029, a nanomolar potent and highly selective CK1δ inhibitor [10]. CK1δ is involved in many cellular processes, but in yeast we have shown that its essential cellular role is in the phosphorylation and thus release of the assembly factor Ltv1 from nascent 40S ribosomal subunits [12]. Deletion of Ltv1 in yeast rescues the lethal effects from CK1δ knockdown and, excitingly, the efficacy of SR3029 is impaired by the selective knockdown of the ribosome assembly factor Ltv1 in human TNBC [12]. Nevertheless, while in yeast *Ltv1* was entirely deleted, knockdown of Ltv1 in human cells was incomplete (70-80%), and the deleterious effects from CK1δ inhibition were not completely rescued [12]. Thus, it remains unclear whether other targets of CK1δ contribute to the apoptotic and anti-proliferative effects of SR3029. In this project, we seek to define how CK1δ regulates TNBC cell growth and survival, and how CK1δ inhibitors that are currently in preclinical development provoke tumor regression. We will also test the hypothesis that TNBC can develop resistance to CK1δ inhibitors via phosphomimetic mutations in the Ltv1 phosphorylation site, and we well address the mechanisms by which their activity can be enhanced by co-administration with other drugs that augment the autophagic clearance of ribosome assembly intermediate.

2. KEYWORDS: Triple negative breast cancer, ribosome assembly, autophagy, CRISPR/Cas9.

3. ACCOMPLISHMENTS:

Major goals:

Is ribosome assembly the essential function of CK1d in the maintenance of TNBC?
Is the CK1d-to-Ltv1 circuit necessary for maintenance of TNBC?
Is Ltv1-D sufficient to augment tumorigenic potential of TNBC?

Accomplishments under these goals:

In yeast we have shown that Ltv1 is the main target of the casein kinase Hrr25/CK1δ[12]. Hrr25 phosphorylates Ltv1, thereby releasing it from nascent ribosomes, and allowing its maturation to progress. Thus, deletion of Ltv1 abrogates the requirement for Hrr25/CK1δ for cellular growth [12]; if there is no Ltv1 to be released from nascent 40S ribosomal subunits, there is no need for Hrr25/CK1δ.

Small molecule inhibitors of CK1δ, such as SR3029, show promise as anti-cancer agents particularly in triple-negative breast cancer (TNBC) cell lines and preclinical animal models[9]. Moreover, knockdown of Ltv1 in MDA-MB-231 breast cancer cell lines increased the IC50 (reducing the efficacy) of SR3029[12], suggesting that inhibition of Ltv1 release and thus ribosome assembly contributes to the mechanism of action of SR3029. These findings indicate the importance of the CK1δ-to-Ltv1 circuit in the development and/or maintenance of TNBC.

Nonetheless, our data also indicated that phosphomimetic mutations in Ltv1 could bypass the requirement for Hrr25 in yeast, raising the possibility of a simple mechanism of resistance development[12]. Thus, the study focused on exploring the feasibility of targeting the CK1δ-to-Ltv1 circuit in cancer cells.

While our work did not uncover point mutations in Ltv1 as drivers of evolved resistance, we have nevertheless shown that bypass of the CK1δ-to-Ltv1 circuit occurs "naturally" in cancer cells. Furthermore, the data also provide substantial support for a pro-tumorigenic function of this bypass. Thus, this work has uncovered substantial concerns that in our mind preclude the pursuit of the CK1δ-to-Ltv1 circuit as a target for any cancer, especially TNBC cells. While these findings are disappointing from a therapeutic perspective, they are scientifically important, and have opened an entire new area of study for us, which is already providing new insights into the connection between protein translation and cancer, and how this essential protein homeostatic machinery is subverted by cancer cells.

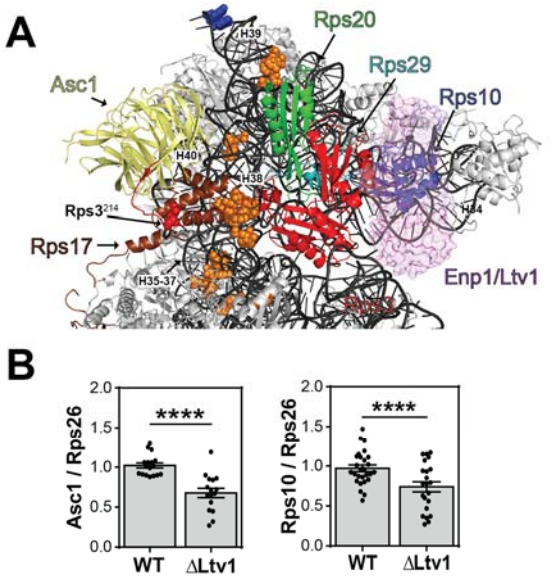

Figure 1: Ltv1 chaperones assembly of the ribosomal head. (A) Structure of the mature ribosomal head (PDBID 4V88). The position of Enp1and Ltv1 was derived from superposition of the head in 4V88 and 6FAI. RNA residues that are more (orange) or less (blue) accessible in mature ribosomes from ΔLtv1 strains are highlighted in space-fill. (B) Levels of Asc1 and Rps10 relative to Rps26 in ribosomes from wild type and ΔLtv1 yeast show reduced Asc1 and Rps10 occupancy.

Ltv1 deficiency leads to the production of ribosomes lacking Rps10 and Asc1, which can promote cancer

Recent analysis of ribosomal protein expression in cancer cells has strongly suggested that cancer is often associated with loss of stoichiometry of ribosomal proteins[13-15], which in healthy cells is typically suppressed by p53[13]. Furthermore, epidemiologic data indicate that loss of ribosomal protein stoichiometry is associated with a poor outcome[14,15]. Intriguingly, haploinsufficiency of many ribosomal proteins leads to cancer in model organisms as well as humans[16-19]. These findings, together with observations from yeast that ribosomes lacking individual ribosomal proteins are stable and accumulate when expression of one protein is downregulated[20,21], suggest that ribosomes lacking individual ribosomal proteins support cancer development and/or progression.

Ribosomes are a major player in the protein homeostasis network, not just because they produce all proteins in all cells, but also because they are a major mediator of mRNA quality control via the no-go-decay (NGD) pathway[22,23]. This pathway identifies oxidized or damaged mRNAs, because of the resulting stalled ribosomes[24], and is also responsible for removing ribosomes that read through the stop codon and are then stalled in the polyA tail[25]. Like translation, NGD requires the correct incorporation of all RPs[1,2,26-29]. Thus, misassembled ribosomes can affect protein homeostasis directly via mistranslation, or by affecting mRNA levels.

Our work has uncovered a role for Ltv1 in promoting the proper incorporation of ribosomal proteins into the head structure of the small ribosomal subunit[30]. As a result, ribosomes from cells lacking Ltv1 contain misfolded rRNA (**Figure 1A**), have mispositioned Rps3 and Rps20 (data not shown), and contain substoichiometric amounts of Rps10 and Asc1 **(Figure 1B)**. Importantly, we have also shown that this function of Ltv1 is conserved between yeast and human cells (**Figure 2** and [30]). Furthermore, unpublished structural data also indicate substoichiometric incorporation of Rps12 and unpublished genetic and biochemical data indicate that Rps15 is mispositioned. Thus, Ltv1 is globally required for proper formation of the small subunit head. Furthermore, since Ltv1 functions independent of ribosomal protein concentration[30], these data also demonstrate that haploinsufficiency of Ltv1 allows for the formation of ribosomal protein-deficient ribosomes in the presence of p53, which monitors the stoichiometry of ribosomal protein expression (*not* incorporation). Thus, these data also demonstrate how cancer cells divert the mechanisms used to promote stoichiometric ribosomal protein incorporation to produce ribosomes lacking individual proteins, and demonstrate how these can be formed via bypass of the p53 monitor. These results were published recently[30].

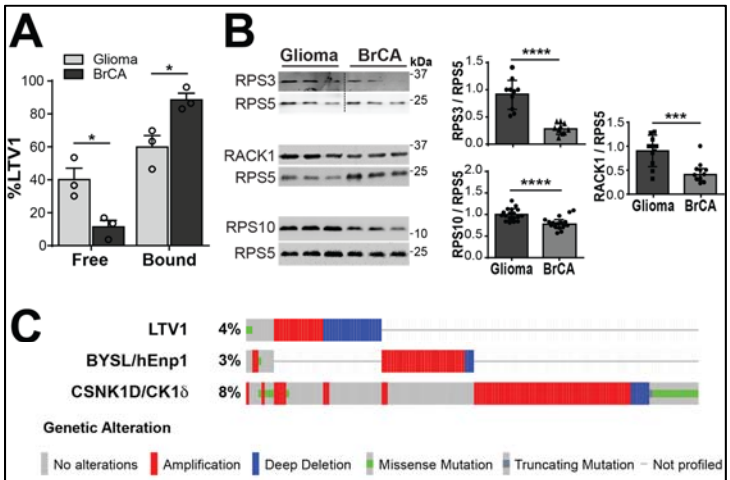

Figure 2: **Cancer cells have insufficient Ltv1.** (A) Levels of free Ltv1 are depleted in breast cancer (BrCA) relative to glioma cells. (B) Ribosomes from breast cancer cells have substoichiometric Rps3, Rps10 and Asc1. (C) Ltv1, but not its binding partners Enp1 (BYSL) or CK1δ, is frequently deleted in cancer cells. From the CBio Portal[4,5].

Ribosomes from Ltv1 deficient cells not only have substoichiometric amounts of Asc1 and Rps10, they also have defects in translational fidelity **(Figure 3A)** and promote resistance to certain stresses **(Figure 3B)**, which can be a result of stochastic stop-codon readthrough[31,32]. Furthermore,

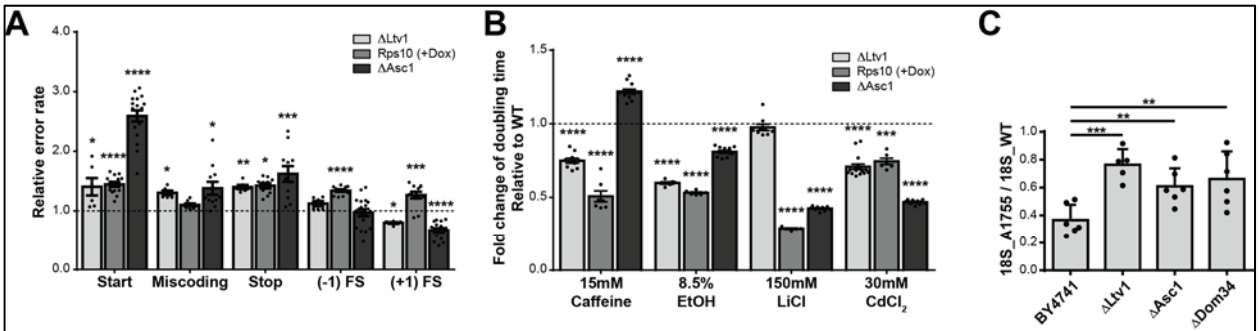

Figure 3: Ribosomes from ΔLtv1 yeast perturb protein homeostasis and ribosome-mediated RNA quality control. (A) Fidelity assays in ΔLtv1, ΔAsc1 and Rps10-insufficient yeast. (B) ΔLtv1, ΔAsc1 and Rps10-insufficient yeast are resistant to some stresses. (C) ΔLtv1 yeast are deficient in NRD. Asc1 and Dom34 are required for NRD and NGD[1-3].

Ltv1-deficient cells are defective for rRNA quality control (NRD, **Figure 3C)**, which requires the same proteins as mRNA quality control (NDG)[33]. Finally, Rps10 haploinsufficiency predisposes to cancer[34]. Thus, the role of Ltv1 in ensuring incorporation of ribosomal proteins into the head, together with the role of fully assembled ribosomes for maintenance of healthy protein homeostasis

strongly suggests that Ltv1 should not be targeted for any form of therapeutic intervention. Because this role for Ltv1 is conserved in human cells (**Figure 2A&B)**, and because of the ability to bypass the requirement for Hrr25/CK1δ for cellular growth in the absence of Ltv1[12], because of the tumorigenic potential of Ltv1-deficient cells described above, and the observation that many tumors already have downregulated or deleted Ltv1 (**Figure 2**), we also suggest that therapeutic targeting of CK1δ might inadvertently select for the development of Ltv1-deficient tumors, which could further *support* instead of deter tumor development.

Rps10/Asc1 deficient ribosomes perturb protein homeostasis

To better understand the role of Rps10/Asc1 deficient cells in tumor development or progression, we have produced yeast cells where Rps10 can be depleted by growth in glucose. These can then be supplemented by plasmids encoding Rps10. Using this system, we have shown that ribosomes lacking Rps10 are stable in yeast (**Figure 4A)**. Furthermore, we have used a luciferase reporter assay to test if Rps10 affects mRNA selectivity. These data indicate that Rps10 senses the identity of the nucleotide at the +13 position relative to the P-site (**Figure 4B**). Thus, Rps10 might affect mRNA selectivity, akin to what we have recently shown for Rps26[21]. Better characterization of these effects from Rps10 (and Asc1) on translation could expose vulnerabilities of Ltv1-deficient cancers.

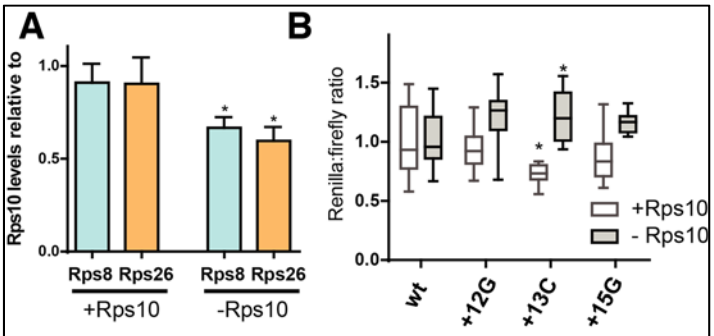

Figure 4: Rps10-depleted ribosomes can accumulate and affect translation. (A) Reducing Rps10 expression decreases Rps10 occupancy in purified ribosomes relative to Rps8 and Rps26. (B) Point mutation of the +13 position, but not the +12 or 15 position near the start site affect translation. This effect is abolished in Rps10-deficient yeast, demonstrating sensitivity of mRNA binding to Rps10 concentration.

However, in addition to mRNA translation and selectivity, Rps10 and Asc1 are both required for NGD and NRD[1-3,26-29]. Previous genome-wide analyses with Asc1-deleted cells were interpreted as indicating a role for Asc1 in translating mRNAs with short open reading frames[11]. Mechanistically, it was suggested that Asc1 plays a role in coupling mRNAs to the cap-dependent translation-factors eIF4G and eIF4E[11]. Nonetheless, the analysis did not take into consideration the now much better characterized role of Asc1 in NGD. Thus, when we reanalyze the data by splitting them into mRNAs likely affected by blocked NGD (for these we expect increased reads from the mRNA and the ribosome footprints), and by Asc1 directly (for these we expect unchanged or decreased mRNAs and decreased ribosome footprints), a different picture emerges. We find 744 candidate genes whose abundance may be regulated by NGD, and who are therefore expected to be sensitive to Asc1 and Rps10 incorporation into ribosomes, mediated by

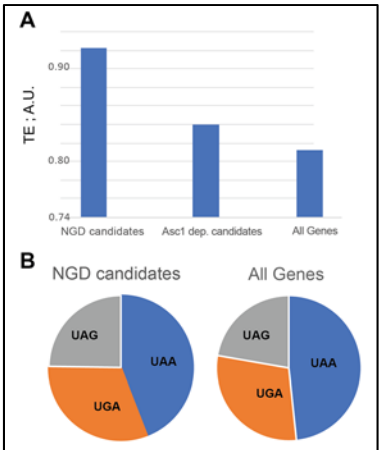

Figure 5: Characteristics of candidate genes for regulation via no-go-decay (NGD). (A) NGD candidates have high TE values/high ribosome density. (B) NGD candidates are more likely to have weak stop codons such as UAG and UGA. Data from [11].

Ltv1. Importantly, the physical characteristics of these genes are consistent with what we expect from NGD-regulated genes. Because NGD involves collision of two ribosomes[27,35] it is dependent on ribosome density on the mRNA[35]. Consistently, candidate NGD-regulated genes have high translational efficiency (TE, **Figure 5A**), a measure of ribosome density on an mRNA. Furthermore, a major substrate of NGD are ribosomes stalled on the polyA tail after read-through of the stop codon[25]. Accordingly, NGD candidate genes are enriched in UGA and UAG stop codons, which have a 2-4 fold higher readthrough propensity than UAA codons[36] (**Figure 5B)**. Together these data indicate that Ltv1 deficiency might affect homeostasis of over 10% of the yeast genome by perturbation of endogenous NGD genes. Again, a more thorough assessment of these genes, not just in yeast, but also human cells would potentially reveal vulnerabilities of Ltv1-deficient cancer.

NGD also resolves ribosomes stalled at internal polyA sequences of 11 and more length[25], and the dataset includes several of these candidates, including Rqc2, a downstream component of the NGD pathway, and Ltv1 (data not shown). These findings are especially interesting, as they suggest that Ltv1 levels are feedback regulated, such that low Ltv1 concentrations will produce ribosomes lacking Asc1 and Rps10, which will stabilize the Ltv1 mRNA, which is otherwise subject to NGD due to its internal polyA stretch. Furthermore, they also suggest that NGD might be autoregulated. We are currently testing these ideas experimentally.

Training opportunities and professional development

Dr. Ghalei has recently moved on to a position as an Assistant Professor at Emory University.

How were the results disseminated?

We have recently published a paper describing the importance of Ltv1 for proper head assembly, as well as in initial description of the defects on translational fidelity and ribosome-mediated RNA quality control[30]. We are currently starting the preparation of a follow-up manuscript describing the role of Hrr25-mediated Ltv1 release in quality control of ribosomal head assembly. Furthermore, we are working on a dissection of the roles of Rps10 and Asc1 in protein homeostasis via both translation and NGD as described above.

Dr. Collins has presented this work in a talk at the 2018 RNA Society Meeting, Dr. Ghalei has presented this work at seminars she has given at Emory University, and at the 2017 Zing Conference in Tampa. Dr. Doherty has presented this work at the FSU Brain Cancer Meeting. I have presented it in multiple seminar visits.

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4. IMPACT

What was the impact on the development of the principal discipline(s) of the project? This work is part of a developing body of work, first documented by our paper on Rps26[21] that demonstrates that ribosomes lacking specific ribosomal proteins are formed under specific physiological conditions. In addition to building this body of literature, the work under this grant goes beyond, as it describes a *mechanism* by which ribosomes lacking specific ribosomal proteins can be formed. Thus, this work is at the forefront of not just the ribosome field, but also at a frontier of the cancer field.
Our work also cautions against therapeutic targeting of the CK1δ-to-Ltv1 circuit in cancer.

What was the impact on other disciplines? Our analyses suggest that other cancers are likely prone to Ltv1-deficiency, leading to substoichiometric ribosomal protein incorporation. Furthermore, the data also suggest mechanisms whereby stoichiometry of ribosomal proteins can be lost without affecting their expression. This strongly suggests the need for a global survey of ribosome proteomics in cancer cells.

What was the impact on technology transfer? To date there has been no technology transfer. However, CK1δ inhibitors are being further developed by our collaborators Drs. Roush and Dr. Bannister (Scripps), who continue to seek the generation of clinically suitable safety assessment candidates.

What was the impact on society beyond science and technology? Our data demonstrate that Ltv1-deficiency is also associated with unique vulnerabilities, including a resistance to oxidative stress, which should be explored more widely and can be exploited therapeutically.

5. CHANGES/PRODUCTS

Changes in approach and reasons for change. We were unable to generate a true knockout of Ltv1 in TNBC cells. Nonetheless, the analysis of the heterozygous Ltv1 mutants showed that TNBC cells responded differently to Ltv1 deletion than our glioma control cells, indicating that they were already experiencing low Ltv1 levels[30]. Because we had learned about the role of Ltv1 from experiments in yeast, it seemed natural to confirm this conclusion by investigating ribosomal protein incorporation. This coincided with a growing literature that suggests a role for p53 in ensuring equal cellular concentrations of ribosomal proteins[13], and for the accumulation of ribosomes lacking such stoichiometry in cancer cells[13-15] (typically containing p53 mutations). These results suggested both that the CK1δ-to-Ltv1 circuit might be a less valuable target than originally thought, but also indicated that Ltv1 had an important underappreciated tumor-suppressive role tied to its role in ribosome assembly. We chose to focus on this role, as it is likely of conceptual importance beyond Rps10 and Ltv1, and because it is an important emerging area of ribosome cancer biology.

Changes that had a significant impact on expenditures. Nothing to report.

Significant changes in use or care of human subjects. Not applicable.

Significant changes in use or care of vertebrate animals. Nothing to report.

Significant changes in use of biohazards and/or select agents. Nothing to report.

6. PRODUCTS

Website(s) or other Internet site(s): *Nothing to report.*

Technologies or techniques: *Nothing to report.*

Inventions, patent applications, and/or licenses: *Nothing to report.*

Other Products:

Publications:

- Ferretti MB, **Karbstein, K** (2019) Does functional specialization of ribosomes really exist? RNA. 2019 May;25(5):521-538. PMID 30733326 PMID
- Collins, JC*, Ghalei, H*, Doherty, JR, Huang, H, Culver, RN & **Karbstein, K** (2018) Quality Control During Small Ribosomal Subunit Maturation: Ltv1 Chaperones Assembly of the Head. J Cell Biol. 217(12): 4141-4154. PMID: 30348748
- Johnson MC*, Ghalei H*, Doxtader KA, **Karbstein K**, Stroupe, ME (2017) Structural Heterogeneity in Pre-40S Ribosomes. Structure. 2017 Feb 7;25(2):329-340. PMID: 28111018

Conference Presentations:

5/2020	Machines on Genes Meeting, Alicante, Spain
7/2019	FASEB Helicase Meeting, Steamboat Springs, CO
4/2019	ASBMB Meeting, Symposium on RNA Dynamics, Orlando, FL
1/2019	Ribosome Meeting, Merida, Mexico
12/2018	ACS/Beijing University Symposium: Innovation at the Frontiers of Chemistry and Life Sciences
7/2017	Helicase Meeting, Kloster Banz, Germany
8/2016	Machines on Genes Meeting, Chesire, UK
3/2016	ASBMB meeting, San Diego, CA
2/2016	ACS meeting, Carol Fierke symposium, San Diego, CA

Invited Lectures:

12/2018	Heidelberg University, Germany
8/2018	University of Vermont, Burlington, VT
1/2018	Florida Southern College, Spencer Symposium, Lakeland, FL
12/2017	University of Colorado, Boulder, CO
12/2017	University of Colorado, Denver, CO

2/2017 Purdue University, IN
10/2016 Florida State University, FL
3/2016 University of Georgia, Student Symposium

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	*Katrin Karbstein*
Project Role:	*PDPI*
Researcher Identifier (Era commons ID)	*kkarbst*
Nearest person month worked:	*2*
Contribution to Project:	*Dr. Karbstein oversees the effort in the Karbstein lab.*
Funding Support:	5R01 GM086451-11 (NIH); 5R01 GM117093-04 (NIH); HHMI 55108536

Name:	*Joanne Doherty*
Project Role:	*Staff Scientist*
Researcher Identifier (e.g. ORCID ID):	*jrdoherty*
Nearest person month worked:	*12*
Contribution to Project:	*Dr. Doherty has carried out all the experiments with mammalian cells described herein.*
Funding Support:	Florida Brain Cancer Initiative (to Dr. Doherty), and HHMI 55108536 (to Dr. Karbstein)

Name:	*Homa Ghalei*
Project Role:	*Research Associate (Postdoc)*
Researcher Identifier (e.g. ORCID ID):	*hghalei*
Nearest person month worked:	*12*
Contribution to Project:	*Dr. Ghalei has been working on the analysis of yeast cells and ribosomes lacking Ltv1.*
Funding Support:	HHMI 55108536 (to Dr. Karbstein)

Name:	*Max Ferretti*
Project Role:	*Graduate student*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*1*
Contribution to Project:	*Mr Ferretti has prepared yeast strains allowing for the purification and analysis of Rps10-deficient ribosomes*
Funding Support:	F31 NIH predoctoral fellowship (to Mr. Ferretti)

Name:	*Roberto Salatino*
Project Role:	*Graduate student*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*1*
Contribution to Project:	*Mr Salatino has prepared worked on a computational analysis of translation in Asc1-deficient yeast*
Funding Support:	5R01 GM086451-11 (NIH); 5R01 GM117093-04 (NIH)

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

Katrin Karbstein, PDPI:

5R01 GM086451-11 (Karbstein) 07/01/2018 – 06/30/2022 4.08 calendar
NIH/NIGMS $287,614 ***(NEW)***
Quality Control in Ribosome Assembly – the Function of Regulatory Proteins
The goal of this project is to dissect the interplay between translation factors and ribosome assembly factors in quality control of late steps of 40S ribosome assembly.

What other organizations were involved as partners?

H. Lee Moffitt Cancer Center & Research Institute, Inc.
12902 Magnolia Drive, MBC-OSR
Tampa, FL 33612-9497.

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS:
QUAD CHARTS:

9. APPENDICES: