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UNIVERSITY OF CALIFORNIA, SAN DIEGO

Using Proximity-based Proteomics to Identify Interacting Partners and Substrates for the Listerin Ubiquitin Ligase

A dissertation submitted in partial satisfaction of the
requirements for the degree Doctor of Philosophy in Biology

in

Biology

by

Nathaniel David Zuzow

Committee in charge:

Professor Eric Bennett, Chair
Professor Gourisankar Ghosh
Professor Randy Hampton
Professor Jens Lykke-Andersen
Professor Gentry Patrick

2016

The Dissertation of Nathaniel David Zuzow is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

Chair

University of California, San Diego

2016

DEDICATION

Since 2012, I have watched the Bennett Lab grow, and I have grown along with it. The wonderful people who have donned pipettes in soldiering effort to understand their place in a vast network of ideas almost as complicated as the signaling pathways we strive to understand are almost too many to name individually.

In particular, I would like to thank Lisa Rising for her support and kind guidance during my early growing pains in the lab. In its most recent iteration, Marilyn Leonard and Nambi Sundaramoorthy have formed the strongest core of knowledge and support this lab has ever seen, and I wish that I didn't have to leave such a great team. And a stoic thank you to my advisor, Dr. Eric Bennett, for always keeping the bar high, and allowing me to constantly test his patience as I pushed past my personal struggles. He has grown as a mentor just as I have as a graduate student.

I would like to thank my cousin Rick Zuzow—more of a brother, actually. He inspired me to double down on my interest in science, and pursue this oft-unforgiving path in earnest. He was my best friend during childhood, and has been my greatest advocate through fleeting adult years. I have a deep, abiding admiration for both his temperamental brilliance and sense of humor. His mind is the type that does not bow fast to authority, pursues knowledge for its own sake, and eventually builds empires.

My sister Monica and her boisterous family were instrumental in my upbringing, and are steady constants in my life. I love them dearly. Family friend Jackie Bettencourt was my partner in crime growing up, and is one of my most loyal cheerleaders moving forward with my academic pursuits. I won't ever forget those embarrassing stories of my youth, because you all won't let me!

My uncle Gary was nothing short of a second father to me growing up. Some of my fondest memories of childhood involve visits to Griffith Observatory, or the L.A. Arboretum—the back seat of the car filled with children mockingly singing, "Camphor tree! Camphor tree!" after Uncle Gary gave us an unsolicited lesson in botany. And today, I can sit with him and proudly teach him a bit about the science I am doing. I imagine it takes all of his willpower to stop himself from mockingly chanting, "Ubiquitin! Ubiquitin!" back at me.

This is where I speak to the true core of my inspiration, and my muse before all others. I would like to offer my deepest gratitude to my mother, Sonia Sara Zuzow, whose quiet grace and understated demeanor belies unbelievable strength and tenacity. Our family has overcome so much in the past sixteen years, and yet those days still burn bright in memory.

And, most importantly, to my father, David Ralph Zuzow: I never would have made it as far as I have without his sacrifices for his family. One can never know anything with complete certainty, but there is one thing of which I am truly certain: being at my thesis defense would have been one of the proudest moments of his life. We all miss you dearly, and this is for you.

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Chapters 2 and 3 are currently being prepared for submission for publication of the material. Zuzow, Nathan; Leonard, Marilyn; Liao, Jeffrey; Bennett, Eric J. The dissertation author was the primary investigator and author of this material.

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ABSTRACT OF THE DISSERTATION

Using Proximity-based Proteomics to Identify Interacting Partners and Substrates for the Listerin Ubiquitin Ligase

by

Nathaniel David Zuzow

Doctor of Philosophy in Biology

University of California, San Diego, 2016

Professor Eric Bennett, Chair

The ribosomal quality control complex (RQC) is a multiprotein complex which senses, modifies, and extracts defective protein products at the 60S ribosomal subunit for ubiquitin-mediated degradation. Central to the function of the RQC is the RING finger E3 ubiquitin ligase Ltn1, which catalyzes the ubiquitylation and subsequent degradation of defective translation products. Listerin, the mammalian ortholog of Ltn1, is crucial for early development and neuronal function, most likely preventing the formation of toxic protein aggregates in neurons.

Little is known about how Listerin functions in mammalian cells. In Chapter 1, we show that Listerin and other mammalian RQC (mRQC) components interact with each other, as well as with the ribosome. Contrary to our expectations, mRQC interactions are not induced by widespread translational stalling. We also show that translational stress causes mRQC proteins to shift into biochemical fractions known to contain the 60S ribosome.

Because detection of endogenous E3 ubiquitin ligase substrates presents a formidable challenge, traditional affinity purification and mass spectrometry (AP-MS) approaches are of limited use for this purpose. Chapter 2 details our use of tandem mass spectrometry to analyze potential Listerin substrates captured via three separate protein interaction mapping platforms: AP-MS, BioID, and APEX. Here, we describe two new classes of Listerin substrates: signaling kinases of the RSK family, and members of the tubulin glutamylation complex (TGC).

In Chapter 3, we study the functional interactions between Listerin and the putative substrates identified in Chapter 2. We show that RSK1, RSK2 and a subset of TGC proteins are ubiquitylated by Listerin in a RING-dependent manner, and that this modification is consistent with regulatory polyubiquitylation. Additionally, we observe that disruption of Listerin positively regulates mTOR via RSK-mediated inhibitory phosphorylation of TSC2, and this novel function of Listerin is possibly independent of the mRQC.

Taken together, our work demonstrates that mRQC proteins interact with each other and the ribosome, and these interactions are altered by translational stress. Our application of orthologous proteomic approaches led to the discovery of diverse

Listerin substrates, opening the door to more detailed studies of this ligase with respect to human disease.

Introduction

Protein Homeostasis and Human Disease

At the two extremes of a protein's lifespan lie synthesis and degradation—two processes which require exquisite regulatory coordination and specificity to adapt to a dynamic cellular environment. The cell must coordinately regulate protein synthesis, nascent chain folding, post-translational modifications, binding interactions and cellular localization, disaggregation, and protein degradation to maintain protein homeostasis (*1-3*). Protein homeostasis is accomplished through an elaborate network of signaling pathways which integrate a wide range of inputs to ultimately control protein expression levels. Competing and redundant regulatory systems endow the proteome with the ability to rapidly adapt to changing conditions (*4*).

Whereas errors in DNA replication and transcription are fairly rare, protein biosynthesis is an error-prone process. It is estimated that amino acid misincorporations occur once in every 1,000 to 10,000 codons translated (*5*). This leads to at least 15% of average-length proteins (~480aa) (*6*) containing at least one missense substitution. While not all errors arising from erroneous translation lead to disease, and some may in fact be advantageous to the organism (*7*), mistranslated proteins are prone to accumulation, and interfere with normal biological processes. The cell possesses quality control systems to mitigate the potentially toxic effects of these proteins.

A number of human maladies, classified as protein misfolding diseases arise from the failure of specific proteins to achieve the proper conformational state (*8*). Misfolded polypeptides are prone to aggregation, and can accumulate as toxic

aggregates (*8, 9*). As protein homeostasis declines with age, the prevention and clearance of protein aggregates becomes an exceedingly important cellular task (*3*). Aging-related pathologies are tightly linked to a progressive decline in the function of both chaperone and ubiquitin proteasome systems—both of which are considered gatekeepers of protein quality control (*10*).

Protein homeostasis diseases can arise when misfolded or otherwise normal but aberrantly-expressed proteins exceed the clearance capacity of the protein degradation systems of the cell, or when protein degradation systems themselves are impaired (*3*). Using *C. elegans* as a model system for aging, it has been shown that protein aggregation increases exponentially with the age of the organism. This phenomenon is likely a result of accumulated mutations affecting protein synthesis and degradation machinery (*11*).

Neurodegenerative disorders form a large subset of known proteinopathies in aged individuals. A number of these disorders are suspected to arise from, or are at least contributed to in part by, impaired clearance of toxic protein aggregates (*12*). Protein misfolding and aggregation into amyloid fibrils are implicated in the cellular pathologies of numerous human disorders, including Alzheimer's Disease, Parkinson's Disease, as well as the prion and polyglutamine diseases (*8*). For example, one significant and well-studied contributor to the pathology of Alzheimer's Disease involves the aberrant processing of amyloid precursor protein APP, leading to an overproduction of short Aβ peptides which aggregate to form plaques and neurofibrillary tangles (*13, 14*).

Defects in translation have also been implicated in the development of neurodegenerative diseases. Mutations affecting translation and co-translational protein folding have a direct impact on translational fidelity, leading to protein misfolding. For instance, mischarged aminoacyl tRNAs result in miscoding during protein synthesis, causing the accumulation of protein aggregates which manifest pathologies of the central nervous system (*15*).

Impairment of ribosome-associated quality control factors can also directly impinge upon neuronal survival. This includes a loss of function in GTPBP2, a factor required to split stalled ribosomes (*16*), and loss of VCP/Cdc48, an ATPase which plays an essential role in chaperoning substrates for degradation via the ubiquitin proteasome system and autophagy (*17, 18*). The link between translational fidelity and neurodegeneration underscores the importance of co-translational protein quality control systems in neurons.

Ribosomal and co-translational protein quality control

When a newly-synthesized polypeptide is misfolded at the ribosome, co-translational protein quality control systems engage to either refold or degrade the protein (*10*). During normal translation, as a nascent polypeptide emerges from the ribosome it immediately encounters a host of protein folding and localization factors which are required for the successful maturation of newly-synthesized proteins (*19*). Most nascent polypeptides require folding assistance through the action of chaperones present in the cytosol and the ER (*20, 21*).

Errors in protein biogenesis can arise from faulty mRNA or intrinsic features of the nascent chain polypeptide (*22*). Stressors such as heat shock and oxidative stress

can lead to widespread protein misfolding and aggregation (*23*, *24*). Newly-synthesized proteins are particularly vulnerable to misfolding, especially when the protein homeostasis network is compromised (*25*, *26*). Ribosome-associated molecular chaperones such as heat shock proteins are essential for the repair and adaptation of newly-synthesized proteins to stress (*27*). The ubiquitin proteasome system (UPS) can degrade co-translationally-misfolded proteins which cannot be refolded by chaperones (*28*). Chaperone and protein degradation systems are tightly coupled, and function together to prevent toxic protein aggregation (*29*).

While extracellular cues such as heat shock can activate co-translational degradation pathways, a considerable fraction of newly-synthesized polypeptides are co-translationally degraded even in the absence of stress. Early studies in mammalian cells demonstrated that as much as 30% of newly-synthesized polypeptides are marked for degradation upon pharmacological proteasome inhibition (*28*, *30*). More recent studies demonstrate that 12-15% of nascent chains are ubiquitylated at steady state (*31*, *32*).

The energy expenditure of protein synthesis is the largest of all ATP-consuming pathways in the cell (*33*). In light of this, it should be surprising that such a large fraction of newly-synthesized peptides are destroyed. However, it has been shown that the products of co-translational degradation can serve an important function as self biomarkers presented via MHC class I molecules for detection by the immune system (*34-36*).

The antigenic peptides presented by MHC class I molecules originate from defective translation products (*36*). At steady state, defects in translation generally

arise from misprocessing of mRNA, or intrinsic features encoded into the transcript (*22, 37, 38*). Translation of defective mRNAs results in the production of aberrant nascent polypeptides, which are addressed by the ribosomal quality control complex (RQC) (*39, 40*). This complex senses, modifies, and extracts aberrant nascent chains from the ribosome for proteasomal degradation. In light of the collective impact of aberrant translation products on the physiology of the cell, the RQC is extraordinarily important to cell survival.

Ribosome-associated quality control and the RQC

Ribosome-associated quality control involves the convergence of protein and mRNA quality control systems at the ribosome to target defective proteins (*41*). Ribosomal quality control events resulting from aberrant characteristics of the mRNA transcript are divided into three major categories: nonsense-mediated decay (NMD), no-go decay (NGD), and non-stop decay (NGD) (*22, 39*). Factors associated with these pathways are conserved from *S. cerevisiae* to mammals (*42*).

The NMD response is activated when a ribosome encounters a premature termination codon, which is recognized through a mechanism which is still poorly understood, leading to the engagement of mRNA decay systems and release of the nascent polypeptide (*43*). NGD recognizes ribosome stalls as a result of difficult-to-decode sequences or secondary structures, and targets both the mRNA and nascent peptide for degradation (*44*). NSD is mechanistically related to NGD, and is activated in response to ribosome stalls on transcripts which lack an in-frame stop codon (*45*).

The RQC has been linked to the degradation of NSD and NGD substrates in yeast, and has been shown to target both cytosolic and ER-bound nascent chains (*46*-

50). In both NSD and NGD pathways, ribosome stalling triggers the recruitment of eukaryotic release factor-like proteins Hbs1L and Pelota which assist in the exonucleolytic degradation of mRNA, and coordinate with ABCE1 to catalyze the splitting of the ribosomal subunits (*51-53*). After ribosome splitting, the 60S ribosome-bound nascent chain (60S-RNC) can be targeted for degradation by the RQC.

The RQC targets 60S-RNCs through a series of sequential steps. Tae2/Rqc2 engages the 60S subunit to prevent reassociation, and interacts with E3 ubiquitin ligase Ltn1, which catalyzes the polyubiquitylation of the nascent chain (*54*). Cdc48 extracts the ubiquitylated polypeptide from the 60S subunit for degradation by the proteasome (*49*). Rqc2 has also been shown to modify the C-terminal tails of nascent polypeptides through the addition of alanine- and threonine-rich extensions, termed CAT tails (*55*). CAT tail sequences specifically mark these nascent proteins for aggregation (*56*). Rqc1 recruits Cdc48 to 60S-RNCs, and is important for the prevention of protein aggregates arising from CAT tail addition (*47, 57*).

Ltn1 and Rqc2

Ltn1 is a large (~200kDa) E3 ubiquitin ligase which catalyzes the ubiquitylation of nascent polypeptides through its C-terminal catalytic RING domain. When Ltn1 is engaged on the 60S ribosome, the RING domain is positioned near the nascent chain exit tunnel (*54*). Connecting the N- and C- termini of this E3 is a long, flexible HEAT repeat domain which allows this protein to span the 60S ribosomal subunit. The N-terminal domain of Ltn1 is positioned near the canonical translational GTPase binding site, where it makes contact with RQC co-factor Rqc2 (*54*).

The interaction between Rqc2 and Ltn1 at the 60S ribosome creates a steric hindrance to the reassociation of ribosomal subunits, and facilitates binding of the N-terminal helix of Ltn1 to the 60S subunit (*54*). This interaction changes the conformation of Ltn1, placing the RING domain in closer proximity to the peptide exit tunnel, allowing greater access to its nascent chain substrates (*54*).

Importantly, Rqc2 also mediates CAT tail addition to nascent chain polypeptides (*55*). When the RQC is impaired in its ability to ubiquitylate its substrates, either through deletion of Ltn1 or Rqc1, cytosolic CAT tail aggregates form (*57*). Amyloids containing CATylated proteins engage the chaperone system. These aggregates activate the heat shock response through Hsf1 (*55*), and have been shown to contain Hsp40/J protein Sis1 (*56*). Sis1 may function in the recognition of these amyloids by heat shock proteins, leading to their fragmentation and clearance (*58*).

Cdc48 and Rqc1

Cdc48 is a highly-conserved protein which plays an essential protein in cellular homeostasis (*59*). This protein has diverse biological functions including ubiquitin-mediated protein degradation, ER-associated degradation, transcription activation, and cell cycle control (*59*). Cdc48 acts as both a molecular motor and a ubiquitin-dependent chaperone, catalyzing the extraction of its substrates from protein complexes, aggregates, or membrane surfaces (*60*). As a component of the RQC, Cdc48 extracts ubiquitylated nascent chains from the 60S ribosome for proteasomal degradation (*49*).

The factor Rqc1 is important for efficient substrate degradation through enhancing recruitment of Cdc48 to 60S-RNCs (*46, 47*). While Rqc1 is not essential for ubiquitylation of nascent chain substrates, it is important for the proteasomal axis of RQC function, and is necessary, along with Ltn1 and Cdc48, to prevent the formation of CATylated protein aggregates (*57*). Rqc1 contains a highly-conserved N-terminal polybasic region, suggesting a possible negative feedback mechanism in which the RQC complex may regulate its own activity through the degradation of this low-abundance protein (*46, 61*).

Loss of RQC function

While the RQC performs a vital function in protein quality control under normal growth conditions, translational stress reveals the severity of RQC loss-of-function phenotypes. Growth of *ltn1Δ*, *rqc1Δ*, and *rqc2Δ* strains is indistinguishable from wild-type at steady state, but is severely impaired with translational stress (*47, 62*). These growth defects are enhanced in double deletion strains for loss of function in RNA surveillance proteins, such as *Ski2*, illustrating the cooperation between these two quality control pathways.

Proteotoxic stress attenuates global translation during the elongation phase (*63*). The protein products arising from widespread ribosome stalling sequester molecular chaperones to mediate refolding (*63*). While *de novo* folding of misfolded newly synthesized proteins is favored over protein degradation (*31, 64*), a significant fraction of these proteins are targeted for co-translational degradation (*65*). The RQC plays a large role in the cellular response to protein misfolding during translation (*65*). However, with one molecule of Ltn1 for every 100 ribosomes (*50*), this complex is

limited in abundance and saturated during widespread ribosome stalling. Use of CHX at high concentrations (>10μg/mL) results in a depletion of Ltn1 from its free cytoplasmic pool in favor of its ribosome-bound state (*46*).

Saturation of the RQC may negatively impact cellular physiology. As an organism ages, progressive decline in protein homeostasis mechanisms places an increasing burden on protein quality control systems (*66*). Newly synthesized proteins contribute significantly to proteasomal load (*67*), making ribosome-associated protein quality control important to overall cellular homeostasis.

Impairment of RQC function has a direct impact on cell survival. Loss of Rqc2 impairs the ability of the cell to form detoxifying aggregates of aberrant proteins, including NSD and NGD reporters, as well as a toxic variant of Huntingtin protein (*68, 69*). Clearance of these proteins is particularly important in the context of Ltn1 deletion, which imparts an increased sensitivity to proteotoxic stress (*68*).

The RQC in mammals

In *S. cerevisiae*, the RQC is comprised of Ltn1, Rqc1, Rqc2, and Cdc48. The orthologous RQC proteins in mammals are Listerin, TCF25, NEMF, and VCP/p97, respectively (Table 1.0). The mammalian RQC (mRQC) has been studied using cryo-EM and *in vitro* reconstitution assays. While structural studies have confirmed the binding and association of Listerin and NEMF to the 60S ribosomal subunit, there is currently no evidence that VCP or TCF25 associate directly with the mRQC (*40, 70*).

Structural studies reveal that Listerin and NEMF bind the ribosome in a manner similarly to their counterparts in *S. cerevisiae*. Similar to Rqc1 in yeast, NEMF binds to the 60S subunit, creating a steric block to ribosomal re-association

(*70*). Additionally, Listerin binds to the 60S subunit with its C-terminal RING domain poised at the polypeptide exit tunnel in a manner that is enhanced through N-terminal interaction with NEMF (*70*).

Like its counterpart in yeast, the mRQC is capable of targeting nascent polypeptides stalled in translation. In an *in vitro* reconstituted system, ubiquitylation of a model substrate by Listerin is dependent on the ribosome-splitting activity of Pelota, Hbs1, and ABCE1 (*71*). Though a comprehensive study of mRQC substrate targeting has yet to be published, Listerin has been shown to act downstream of ribosome splitting by Hbs1L and Pelota (orthologs of yeast Hbs1 and Dom34, respectively) in the degradation of NSD and NGD substrates (*71, 72*).

The importance of the Listerin and the mRQC

Deletion of Listerin has a significant impact on the central nervous system. Mice homozygous for a dominant-negative variant of Listerin die in early embryonic development (*73*). Those expressing a hypomorphic variant of Listerin exhibit a severe neurodegenerative phenotype characterized by progressive ataxia, which is attributed to the dying back of motor and sensory neurons. This condition mimics well-characterized neuropathologies such as ALS (*73*).

The mRQC protein NEMF is also essential. Deletion of NEMF results in early embryonic lethality (*74*). Though NEMF has been shown to cooperate with Listerin in the degradation of NGD and NSD proteins, no studies have directly linked the lethality of NEMF deletion to its mRQC function. However, this protein is well conserved from *D. melanogaster* to humans, and has been shown to have a tumor suppressive function in lung cancer cells (*75*).

Few studies have been done on the function of the mRQC protein TCF25. However, human carriers of a mutated allele of TCF25 containing a large deletion are healthy and show no signs of clinical abnormality (*76*). Consistent with its role in numerous cellular processes, deletion of VCP is lethal in early development (*77*). Autosomal dominant mutations in *VCP* lead to the development of a rare multisystem degenerative disorder, while loss-of-function mutations manifest as progressive developmental disorders (*78*).

Both Hbs1L and Pelota function in ribosome splitting prior to mRQC engagement. There is limited information on the deletion and loss-of-function phenotypes of Hbs1L. While cell lines harboring Hbs1L deletions are viable, polymorphisms at *HBS1L* locus are associated with β-thalassemia and sickle cell anemia (*79, 80*). Deletion of Pelota results in early embryonic lethality, and this protein appears to be critical for progression of the mitotic cell cycle (*81*).

It is clear that disruption of mRQC proteins and their associated factors can have a severe negative impact on cellular physiology. However, it is important to dissect the roles of these proteins with respect to the mRQC. Listerin is a particularly interesting candidate for study because of its direct connection to protein homeostasis, and the known importance of E3 ubiquitin ligases in the prevention of neurodegenerative diseases (*82*).

While no studies have directly connected Listerin to human pathology, this ligase likely plays an important role in the clearance of co-translational quality control substrates. In humans, polybasic proteins have enhanced potential to form toxic aggregates, as evidenced by the arginine-containing dipeptide repeats encoded by

C9orf72 mutant genes, which cause amyotrophic lateral sclerosis and frontotemporal dementia (*83, 84*). Dysregulation of this protein and others which are sensitive to co-translational quality control mechanisms, could provide a link between RQC function and human disease. However, no studies to date have identified endogenous RQC targets.

Capturing the mRQC *in vivo*

While Listerin is an important ligase in mammals, and the RQC has been shown to be important for protein homeostasis in *S. cerevisiae*, little is known about how the mRQC assembles *in vivo*, or the substrates it targets. This dissertation details our approach towards the functional characterization of the mRQC, and outlines the discovery and validation of new Listerin interactors uncovered using proximity-based proteomics in addition to traditional affinity purification methods.

In Chapter 1, we identify and characterize the core components of the mammalian ribosomal quality control complex. Using *in vitro* cell-based assays, we were able to detect co-interaction between the putative components of the mammalian RQC, and observed increased association between these components with translational stress. We then used a series of reporter-based assays to stimulate specific quality control events at the ribosome, and assayed the function of the mRQC and other co-translational quality control factors in modulating the expression of these proteins. However, these results were inconclusive due to technical issues with our reporter systems.

We then expanded our proteomics approach to include proximity-based biotinylation to detect substrates of Listerin. In Chapter 2, we compare proteomic

strategies using the well-studied molecular chaperone VCP as a test case, before applying this approach to study Listerin interactors and substrates. Although our methods capture overlapping-but-distinct groups of interacting proteins, we are able to show that the proximity-based BioID method is uniquely able to capture both known and unknown Ltn1 interactors and substrates.

BioID revealed two new classes of Listerin substrates: signaling proteins in the ribosomal S6 kinase family, as well as members of the tubulin glutamylation complex. In Chapter 3, we validate these interactions biochemically. Through functional studies, we were able to confirm that Listerin affects downstream MAPK signaling through a regulatory interaction with RSK2. To a lesser extent, we were also able to observe a regulatory interaction between Listerin and members of the tubulin glutamylation complex. Together, our findings cast a new role for Listerin in the regulation of cellular growth and survival, as well as a potential new function in microtubule stability.

Table 1.0: Co-translational quality control proteins associated with NGD and NSD in *S. cerevisiae*, and their mammalian orthologs. Proteins indicated with bold lettering are members of the core RQC complex in yeast. Proteins indicated with dark blue lettering are members of the mammalian RQC.

H. sapiens	*S. cerevisiae*	Description
Listerin	**Ltn1/Rkr1**	**RING E3 ubiquitin ligase**
TCF25	**Rqc1**	**bHLH transcription factor**
NEMF	**Rqc2/Tae2**	**Nuclear export mediator factor**
p97/VCP	**Cdc48**	**AAA ATPase, ubiquitin-dependent chaperone**
RACK1	Asc1	40S ribosomal protein, mRNA binding/translation
ZNF598	Hel2	RING E3 ubiquitin ligase
Hbs1L	Hbs1	Translational GTPase, eRF3 homologue
Pelota	Dom34	eRF1 homologue
ABCE1	Rli1	Ribosome splitting factor

Chapter 1: Functional Characterization of the E3 ubiquitin ligase Listerin and the mRQC

Abstract

The RQC is a multiprotein complex involved in the degradation and sequestration of defective translation products in *S. cerevisiae*. Although the protein orthologs of the RQC are expressed in mammalian cells, studies of the function and composition of this complex have been limited to *in vitro* biochemical assays and structural analysis through cryo-EM. Comprising the known mRQC are the E3 ubiquitin ligase Listerin and the tRNA binding protein NEMF.

In yeast, the function of the RQC is dependent on the interplay between its protein constituents. Canonically, these proteins facilitate substrate ubiquitylation, extraction, and degradation. However, RQC can also promote substrate aggregation. Whether the conserved orthologs of these proteins perform the same function in mammalian cells has yet to be demonstrated. To address this, we used an approach combining molecular biology and proteomics to detect the formation of the mRQC in human cells.

Consistent with our knowledge of the RQC in *S. cerevisiae*, we were able to observe interactions among mRQC components at steady state. In response to translational stalling, we observed altered mRQC protein associations which may indicate a change in how this complex addresses its substrates. Additionally, we were able to observe a shift in the higher-order complex assembly of these proteins into ribosome-containing cellular fractions following translation elongation inhibition, which is consistent with the RQC in yeast.

Listerin, the E3 ligase central to the function of the mRQC, is crucial for viability during development. Partial loss-of-function mutants exhibit a terminal neurodegenerative phenotype in mice. Because protein homeostasis defects manifest strongly in post-mitotic cells such as neurons, a likely explanation for the *Listerin* phenotype is that this ligase normally prevents toxic accumulation of defective translation products. However, little is known about the endogenous substrates of Listerin.

To study the classes of substrates targeted by Listerin, we used model reporter systems engineered to induce translational stalling, in conjunction with genetic overexpression or knockdown of mRQC pathway associated proteins. While our results were inconclusive, here we describe the rationale and implementation of our reporter systems, as well as the pitfalls we encountered while using these reporters in practice.

Introduction and Results

The RQC in yeast and mammals

Although the RQC has been studied extensively in *S. cerevisiae*, very little is known about the orthologous complex, the mRQC, in mammals. The mRQC shares structural and functional aspects with its counterpart in yeast (*40, 54, 70*). However, most of the current literature on the mRQC is derived from *in vitro* studies, and the assembly of the mRQC has yet to be studied in a biological context. Additionally, while the RQC has been shown to target the protein products of aberrant translation in yeast and humans, a single endogenous substrate of this complex has yet to be identified in either organism.

The importance of studying the mRQC is underscored by the embryonic lethality observed in mice harboring individual deletions of mRQC factors Listerin and NEMF (*73*, *74*). Despite the individual importance of these proteins to survival, very little literature exists on their collective contribution to ribosome-associated protein quality control as members of the mRQC. As evidenced by the neurodegenerative phenotype of the *Listerin* mouse (*73*), mRQC function is likely critical in long-lived cells like neurons, where defective protein clearance leads to a pronounced susceptibility to the formation of protein inclusions.

To understand the contribution of the mRQC to protein homeostasis, it is important to study the composition of the complex in a biological context. Studies in *S. cerevisiae* have revealed the genetic and physical interplay between RQC proteins in the cellular response to stalled protein translation (*46*, *56*, *85*). However, the mammalian complex is far less studied. While biochemical reconstitution assays show that Listerin and NEMF cooperate in the ubiquitylation of stalled nascent chains (*40*, *70*), there have not been any published studies on the formation of the mRQC in cells.

The mRQC has proven to be difficult to detect outside of *in vitro* biochemical assays. The known interaction between Listerin and NEMF has evaded detection in solution, and NEMF has a very weak affinity for tRNA despite its function as a tRNA-binding protein (*70*). Inclusion of TCF25 in cryo-EM and biochemical studies of the mRQC show that this protein does not associate with 60S-RNCs or stimulate ubiquitylation *in vitro* (*70*). Additionally, VCP acts downstream of ubiquitylation and there is no evidence that it interacts directly with Listerin or NEMF at the 60S.

The composition and function of the RQC are intimately connected. The constituent proteins of the RQC must coordinate to determine the fate of stalled nascent polypeptides, marking them as targets for proteasomal degradation or aggregation into detoxifying inclusions. Therefore, a functional understanding of the mRQC begins with a study of its constituency in mammalian cells, as well as how these proteins respond to proteotoxic stress.

In this chapter, we detail our approach for the study of mRQC composition and assembly. We generated molecular reagents for the expression of tagged RQC orthologs proteins in mammalian cells (Table 1.0), allowing us to perform co-immunoprecipitation (co-IP) studies of the interactions between putative mRQC proteins. We used immunoblot assays in conjunction with affinity-purification mass spectrometry (AP-MS) to detect interactions amongst mRQC proteins, as well as with the 60S ribosome.

Because the RQC is recruited to ribosomes stalled during the elongation phase of translation, we used elongation inhibitor CHX in conjunction with immunoblot and AP-MS assays to observe whether widespread ribosome stalling induces mRQC formation. Because Listerin is an inherently unstable protein which can catalyze its own ubiquitylation, we included the catalytically-inactive ΔRING variant of this protein in our analysis. This form of Listerin is able to bind to its substrates, but is unable to transfer ubiquitin. It is also more stable than wild type Listerin, making it better suited for cryo-EM studies (*40*).

The RQC appears to be important to protein homeostasis in both yeast and mammals, however it is not known what substrates are targeted by this complex.

Functional studies of the RQC in *S. cerevisiae* show that this complex is required for the degradation of NSD and NGD model substrates (*56*). Although a comprehensive study of the mRQC substrate targeting is lacking in current literature, Listerin has been shown to degrade model NGD and NSD substrates *in vitro* (*71*), and siRNA-mediated knockdown of this E3 stabilizes a NSD reporter in HeLa cells (*72*).

While the proteins of the RQC are well conserved in humans, it is unknown how they function individually, or as part of the mRQC, to degrade aberrant nascent chains arising from ribosome stalls. An overarching goal of the studies set forth in this dissertation is the identification of the endogenous substrates of the mRQC. From a biological perspective, an understanding of how the mRQC assembles to target its substrates at the ribosome, as well as the general classes of ribosomal stalls which trigger substrate targeting, will offer insight into the importance of this complex to mammalian physiology.

To trigger ribosomal quality control events in an inducible manner, we engineered model reporter substrates with stall-inducing sequence elements for expression in mammalian cells. We studied the mRQC in a biological context, using the expression of model reporters in conjunction with genetic knockdown or overexpression of mRQC components. Though this approach yielded mixed results, here we briefly describe the approach we employed, as well as the obstacles we encountered while developing and testing these assays.

Detection of mRQC interactions

We generated a series of HEK293T cell lines for the inducible expression of HA-FLAG-tagged mRQC proteins Listerin, TCF25, NEMF, and VCP, as well as also

the ΔRING variant of Listerin. We also included FLAG-GFP as a negative control for co-IP. These cell lines were treated with and without CHX, and lysates were subjected to IP, and the eluate was divided for protein detection via immunoblot and mass spectrometry.

Immunoblot for Listerin reveals that this E3 co-immunoprecipitates with overexpressed mRQC components TCF25 and NEMF (Figure 1.1). However, there was no observable interaction between Listerin and VCP above GFP control. GFP does appear to bind a low level of background protein, which includes Listerin. GFP background binding poses some difficulty in our detection of *bonafide* interactions between mRQC proteins in both our immunoblot and AP-MS experiments.

In addition to steady state conditions, we assayed for induction of the mRQC upon treatment of cells with the translation elongation inhibitor CHX. Treatment with CHX at 100μg/mL completely abolishes translation. After CHX treatment, there was an observable increase in the association between Listerin and NEMF, which was expected based on previous studies of this interaction. The mild interaction between Listerin and TCF25 was also induced (Figure 1.1).

Because the scope of detection of protein-protein interactions via immunoblot is limited by time and reagent availability, we analyzed FLAG-IP samples for each of the mRQC proteins using AP-MS. We used a label-free proteomics approach for detection of interacting proteins with Listerin, TCF25, NEMF, and VCP, with GFP included as the negative control (Figure 1.2). These samples were harvested in parallel with the samples from Figure 1.1.

To control for differences in bait protein abundance, we normalized for bait abundance across each untreated/treated pair (Figure 1.2A). The only observable interaction in the negative control IP was with VCP (Figure 1.2B). This differs from the immunoblot results, which indicate some level of binding with Listerin. These results confirm that this is indeed background binding which did not sustain through the more stringent wash steps required for AP-MS sample preparation.

Of the possible interactions amongst mRQC proteins, the interaction between Listerin and NEMF is the only one which has been extensively validated. This interaction was only detectable in our AP-MS experiment following CHX treatment (Figure 1.2C). The ΔRING variant of Listerin did not prove to be superior to wild type at detecting this interaction (Figure 1.2D). This is in conflict with the immunoblot results, in which this interaction is detected well above GFP background binding levels (Figure 1.1).

Rqc1 is a core component of the RQC pathway in *S. cerevisiae*, interacting directly with Rqc2 and Cdc48. Based on previous literature, we did not expect to observe any appreciable interaction between the Rqc1 ortholog TCF25 and other mRQC proteins. Using wild type and ΔRING Listerin as bait in an AP-MS experiment, the interaction with TCF25 was undetectable (Figure 1.2C,D). However, when TCF25 was used as bait, we were able to observe a steady-state interaction with Listerin, which was also inducible with CHX (Figure 1.2E). This interaction was confirmed in our previous immunoblot (Figure 1.1).

There is evidence of a physical interaction between Rqc1 and Rqc2 in yeast (*46*), which may be essential for the interplay between the ubiquitylation and

aggregate-forming modalities of the RQC response. The interaction between TCF25 and NEMF was likewise confirmed in our AP-MS experiment, using either protein as bait (Figure 1.2E,F). Using NEMF as bait, it appears as though this interaction may be induced by CHX (Figure 1.2F). However, no significant induction was observed using TCF25 as bait (Figure 1.2E).

Rqc1 is responsible for the effective recruitment of Cdc48 to the ribosome in yeast (*47*). In our AP-MS experiments using either TCF25 and VCP, we were able to detect an interaction between these two proteins above background (Figure 1.2E,G). This interaction was significantly diminished following treatment with CHX (Figure 1.2E,G). In fact, analysis of the AP-MS results for VCP reveals that all steady state interactions with the mRQC are abolished following CHX treatment (Figure 1.2G).

mRQC association with ribosomes

In yeast, the RQC exclusively assembles at 60S-RNCs resulting from stalled translation (*46*). Similarly *in vitro* studies show that mRQC components Listerin and NEMF bind uniquely to 60S-RNC complexes (*70*). The association between the mRQC and the 60S subunit underpins the structure and function of this complex. However, no studies, outside of biochemical assays *in vitro,* show that mRQC proteins nucleate a complex at the 60S ribosome. To confirm these interactions in mammalian cells, we used both sucrose density gradient analysis and AP-MS.

Sucrose density centrifugation is a biochemical technique which allows for the density-based separation of ribosomal subunits from 80S ribosomes and polysomes. To test whether translational stress shifts mRQC proteins to ribosome-containing fractions, we performed sucrose density gradient analysis using HEK293T cells

expressing mRQC proteins in the presence or absence of CHX (Figure 1.3). Using a doxycycline-inducible system, we expressed FLAG-tagged Listerin, TCF25, or NEMF, respectively (Figure 1.3A-C).

After density centrifugation, 12 fractions were collected per gradient, and protein was isolated from the individual fractions. To confirm the separation of ribosomes across these fractions, we performed immunoblot for RPS3 and RPL7, which are markers of the 40S and 60S ribosomal subunits, respectively. Fraction 1 is the ribosome-devoid cytosolic fraction.

Free 60S subunits are observed to occupy both fractions 3 and 4. RPS3 modification, as indicated in Western blots by a higher molecular weight band in addition to the endogenous protein band, is an indicator that CHX treatment was effective. In our previous work, we discovered that this band corresponds with regulatory ubiquitylation of RPS3 (*86*).

Using cells expressing FLAG-tagged Listerin, we observed a shift of Listerin into heavier, 60S-containing fractions 3 and 4 following CHX treatment (Figure 1.3A). However, the shift of Listerin was more evident in the TCF25 and NEMF sucrose gradients (Figure 1.3B,C). Consistent with data that show that Listerin specifically binds to 60S ribosomal subunits, we did not observe a shift of Listerin into heavier 80S or polysome-containing fractions in any of our gradients. These results are quantified in Supplementary Figure 1.1.

TCF25 sucrose gradients show a detectable shift in this protein into higher molecular weight fractions (Figure 1.3B). To contrast, we observed a large shift of NEMF into the 60S fraction, as well as denser 80S and polysome-containing fractions,

following CHX treatment (Figure 1.3C). As expected, the majority of VCP remains in lighter gradient fractions following CHX treatment, though we were able to observe a moderate recruitment to the 60S fraction (Figure 1.3A-C).

While the shift of mRQC proteins to ribosome-containing fractions indicates that these proteins may in fact be induced to associate with the ribosome, this is not definitive proof of association. We analyzed the mass spectrometry results from the experiment presented in Figures 1.2 for the abundance of ribosomal proteins detected in each IP.

Using Listerin as bait, proteins of the 60S ribosomal subunit were detected in greater abundance than in the GFP control experiment at steady state (Supplementary Figure 1.2A-C). However, treatment with CHX had the effect of either decreasing or leaving most interactions with the 60S ribosome unchanged. We observed a similar trend using TCF25, NEMF, and VCP as bait (Supplementary Figure 1.3A-D).

Functional Characterization of the mRQC

Functional data in *S. cerevisiae* show that the RQC is involved in nascent protein degradation as a result of ribosome stalling, particularly in the NSD and NGD pathways. During normal translation, ribosome stalls can occur as a result of readthrough, misprocessing, or regulatory processing of an mRNA transcript. For instance, cryptic polyadenylation sites or read-through of a normal stop codon leads to the coordinate recruitment of co-translational quality control factors for both mRNA decay and protein degradation (*87, 88*). These quality control events generally occur at a low frequency, and the RQC is tuned to respond accordingly.

Little is known about the targets of the RQC in yeast, or the orthologous complex in mammals. Functional data on Listerin has shown that it is involved in degradation of NSD proteins (*72*). However, it is unclear whether clearance of NSD proteins by Listerin is dependent on the mRQC. It is unknown how the mRQC modulates the quality control response to this and other types of ribosome stalls. To study the effects of mRQC overexpression or knockdown on the expression of stalled proteins, we used a β-globin-based reporter system designed to trigger specific co-translational quality control events (Figure 1.4A).

Using the β-globin system to assay the products of defective translation, we discovered that the wild type β-globin reporter is unstable and therefore stabilized with proteasome inhibition. (Figure 1.4B,C). The instability of β-globin, which is inherently unstable in the absence of α-globin expression, created difficulty deconvoluting ribosomal stall-specific degradation from post-translational degradation. To overcome this issue, we engineered a series of GFP-based reporters with the same sequence elements as the β-globin reporter set. However, this new GFP-based system did not offer any advantages over the β-globin system in detection of ribosomal quality control events (data not shown).

In an attempt to align our experimental approach with the prevailing literature, we used a suite of reporters which have been described in a number of publications in the study of co-translational RNA and protein quality *S. cerevisiae* (*89-91*). However, we determined that these reporters are stable, and do not trigger ribosomal quality control in mammalian cells. Therefore, these reporters were not useful for application in studying the mRQC (Data not shown).

Discussion

The mRQC *in vivo*

The results of our two AP-MS experiments indicate that a complex orthologous to the RQC in *S. cerevisiae* forms in mammals *in vivo*. However, it is unclear whether the assembly of this complex is stimulated under conditions of translational stress. Previous studies of the mRQC have shown that interactions between its individual protein components are weak, and have evaded detection in solution (*70*).

Our AP-MS results reflect the labile nature of these interactions, the overcoming of which is largely attributed to avidity effects. Analysis of individual mRQC protein interactions illustrates the relationship between core mRQC components Listerin and NEMF, and generally confirms previously-published biochemical interaction data. While sucrose gradient analysis reveals that these proteins do indeed shift to 60S-containing fractions upon translational stress, AP-MS failed to confirm the induction of interactions amongst mRQC proteins, or with the 60S ribosome.

The interaction between Listerin and NEMF is well documented. While we were able to capture this interaction at steady state using NEMF as bait, it was only apparent in our Listerin AP-MS experiment following CHX treatment. This is consistent with the literature suggesting that these two proteins interact weakly. From both the AP-MS and immunoblot data, we have evidence that this interaction does indeed occur in mammalian cells. Our data on whether this interaction is inducible by CHX is inconclusive.

There is no evidence, in both the prevailing literature and our own data, to support the claim that Listerin and VCP interact. If VCP plays the same role in the mRQC as Cdc48 does in the yeast RQC, this protein interacts directly with ubiquitylated nascent chain substrates which have been polyubiquitylated by Listerin. It may be the case that this is a low-affinity interaction that we cannot detect above background using the GFP negative control. However, it may also be the case that Listerin and VCP simply do not interact, either due to lack of affinity, or a temporal separation between ubiquitylation and extraction steps.

In general, it appears that mRQC interactions are particularly difficult to detect in the Listerin overexpression setting. Overexpression of Listerin may reduce our ability to pull down full complexes, due to the limiting nature of the low-abundance mRQC factor TCF25. TCF25 also encodes a polybasic sequence, which is a known characteristic of Listerin substrates. It is possible that upregulation of Listerin suppresses TCF25 which, in turn, reduces recruitment of VCP to stalled 60S ribosomes. This is another potential explanation for the observed lack of interaction between Listerin and VCP, and is consistent with the Ltn1Δ aggregation phenotype in yeast (*56*).

In *S. cerevisiae*¸ the orthologs of TCF25, NEMF, and VCP have been shown to and functionally interact with Ltn1 at the 60S ribosome (*46*). Our analysis of mRQC localization in response to CHX treatment shows that Listerin, TCF25, NEMF, and VCP are shifted to 60S-subunit containing fractions in sucrose density gradients. As expected due to the high abundance of VCP in the cell, of which only a small amount is likely to be designated to operate with the mRQC, only a small amount of VCP was

shifted to ribosomal fractions, while the rest remained cytosolic. It is uncertain whether our AP-MS interaction data are consistent with this result, since VCP may not only be recruited to ribosomes as part of the mRQC, but as part of a general response to translational stalling.

Interestingly, contrary to the shift we observed in our sucrose gradient experiments, our AP-MS data do not reflect a CHX-inducible association between mRQC proteins and the ribosome. It may be the case that the mRQC proteins are recruited to ribosome-containing fractions in a manner not dependent on the ribosome itself. However, it is more plausible to suggest that the full 60S subunit does not survive the sample preparation steps for mass spectrometry, and what our data represent is background binding, with only a small number of ribosomal proteins representing *bonafide* interactions.

Formation of the mRQC in response to cycloheximide

The response of the mRQC to CHX treatment does not appear to be a straightforward induction of assembly. Instead, what emerges from our data is a picture of a ribosome-associated complex with factors that respond variably to translational stress. The interplay between ubiquitin-proteasome and aggregate-forming modalities may allow the mRQC to dynamically adapt to proteotoxic stress.

The decreased interaction between TCF25 and VCP following CHX treatment is particularly interesting. TCF25 ortholog Rqc1 is important for the recruitment of Cdc48 to the ribosome for peptide extraction. If this functional link is conserved in mammals, then widespread ribosome stalling appears to disfavor the extraction of the

stalled polypeptide. Instead, it is possible that the RQC switches nascent protein fate from proteasomal degradation to aggregation through activation of Rqc2.

TCF25 may act as a molecular switch determining the fate of the nascent polypeptide substrate. Increased association with NEMF may favor protein aggregation, while increased association with VCP favors proteasomal degradation. Further studies of this mechanism, including assays for protein aggregate formation in cells expressing TCF25 which is incompetent for VCP binding, will illuminate the perhaps understated importance of TCF25 to the mRQC.

In yeast impaired for RQC function, growth defects are not apparent unless proteotoxic stress is applied. In mammals, impaired Listerin function presents as a progressive neurodegenerative disorder as protein homeostasis mechanisms decline with age and fail to clear toxic proteins. Given these observations, and in light of the low abundance of Listerin, it stands to reason that the mRQC is fairly specialized, and performs its function infrequently at steady state.

This is evidenced by the low occupancy of mRQC proteins in ribosome-containing fractions in the absence of CHX treatment. The induction of mRQC association with the ribosome suggests that the complex is indeed recruited to ribosomes following widespread translational repression. However, our AP-MS results with VCP suggest that the ubiquitin-proteasome modality of mRQC function is not the favored response to widespread stalling. Immunofluorescence assays targeting either endogenous or fluorophore-tagged model substrates in the presence or absence of CHX will reveal if this is indeed the case.

Reporter-based assays for functional characterization of the mRQC

While these reporter systems have been used to successfully study protein turnover in *S. cerevisiae*, our adaptation of these assays provided us with mixed results. The β-globin based reporter system used for most of our functional assays was inherently flawed due to the unstable nature of β-globin when it is unable to bind α-globin.

We were able to observe that protein expression level of the NMD substrate, β-globin containing a premature termination codon, was much lower than that of the other constructs, and was insensitive to proteasome inhibition. This is consistent with the notion that NMD is primarily triaged at the mRNA level.

The NGD and NSD reporters are capable of generating a protein product that is stabilized by proteasome inhibition. However, the inherent instability of maturely-folded β-globin obfuscates the instability of any protein products arising from stalled translation. Our assays probing the effect of mRQC protein overexpression or depletion on the expression of the products of stalled translation were inconsistent, and therefore inconclusive.

We engineered and tested a series of GFP-based reporter systems to overcome the stability issues of the β-globin reporters, but these reporters did not provide any advantage in the specific detection of defective translation products. We also made use of a reporter system which has successfully been used to probe protein quality control and RNA surveillance mechanisms (*90*). However, this reporter system was entirely stable in our application of it, despite the presence of encoded features to induce translational stalls. These reporters were originally optimized for use in yeast, and it

may be possible that the sequence and structural elements encoded by these reporters are ignored by mammalian quality control systems.

The biology of Listerin further confounds functional characterization of the mRQC. While co-translational degradation rates have been estimated at 12-15% in mammalian cells (*32*), the low abundance of Listerin suggests that the mRQC can only address stalls at 1% of ribosomes (*61*). This highlights the fact that other ribosomal quality control mechanisms such as the CCR4-Not complex may possess some functional overlap with the mRQC in clearance of stalled nascent polypeptides. Future work should focus on a combinatorial approach in studying ligase function, perhaps assaying the stability of stalled nascent chains using CRISPR/Cas9 single and double deletion lines for Listerin, CNOT4, and other ligases.

Conclusions

Together, our data support the hypothesis that the mRQC assembles at the ribosome at steady state. Protein-protein interactions between mRQC orthologs mirror the structural and biochemical data in yeast. However, in response to CHX-mediated ribosome stalling, mRQC components are not uniformly induced to interact. Instead, it seems likely that CHX stalling favors an mRQC modality which does not recruit VCP to the complex. Future studies should take these results into consideration, and perform AP-MS experiments probing mRQC function in response to targeted quality control events.

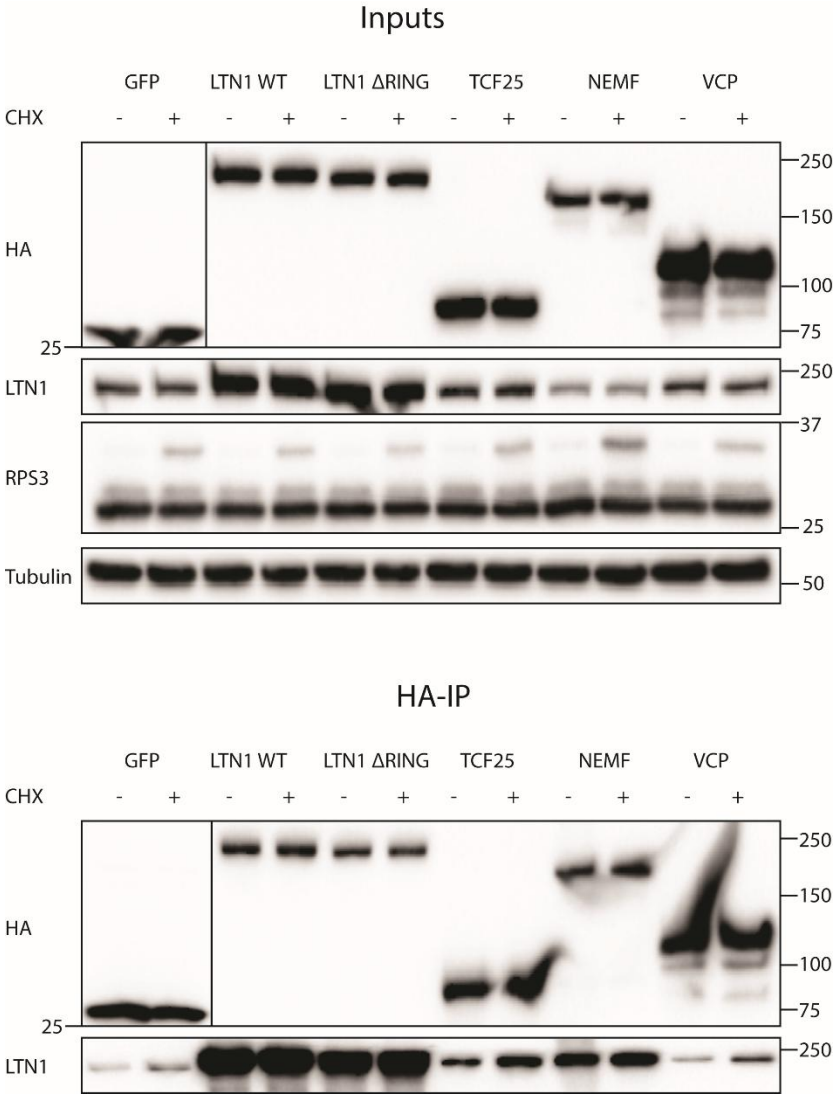


Figure 1.1: Listerin co-immunoprecipitates with mRQC factors with translational stalling
HEK293T cells inducibly expressing N-FLAG-HA GFP, Ltn1 WT, Ltn1 ΔRING, TCF25, NEMF, or VCP were treated with and without CHX (100μg/mL, 1hr). The top panel shows input blots, and the bottom panel shows IP blots following HA immunoprecipitation.

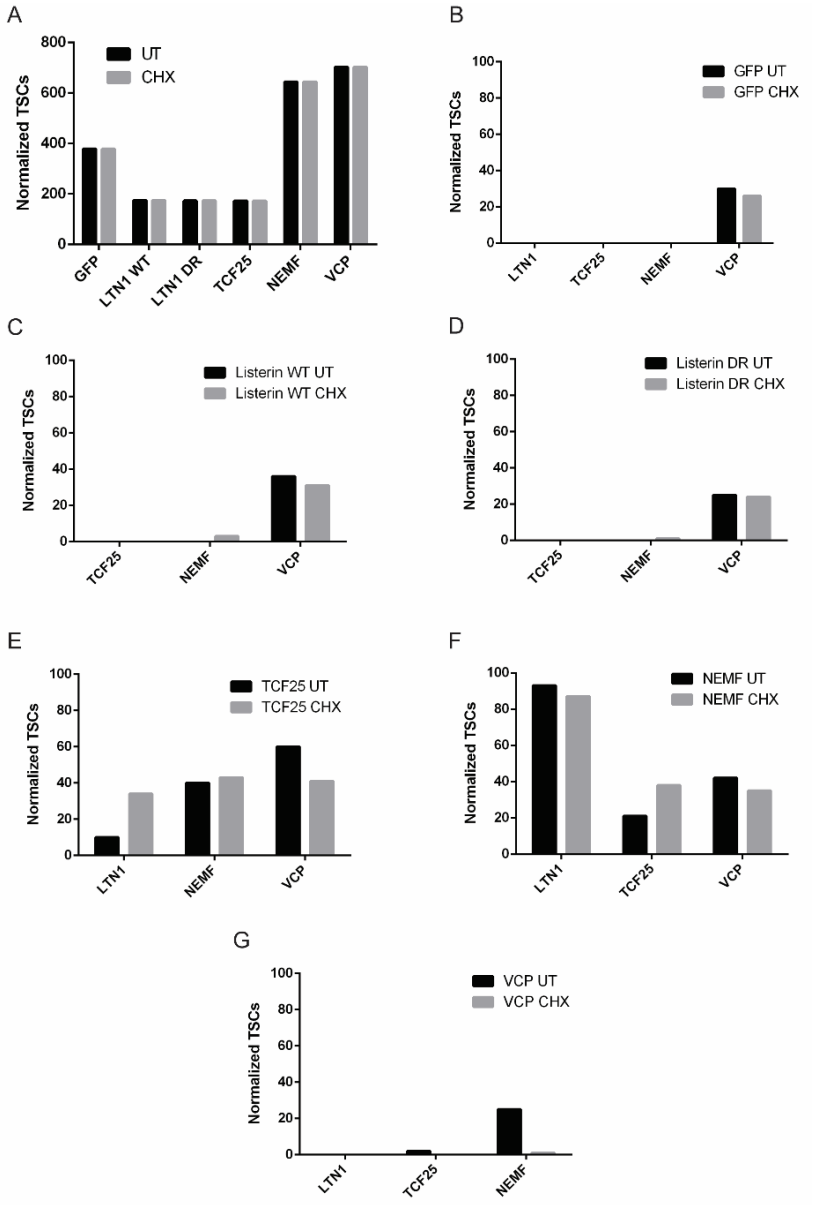


Figure 1.2: mRQC proteins co-immunoprecipitate variably with translational stalling

HEK293T cells inducibly expressing N-FLAG-HA GFP, Ltn1 WT, Ltn1 ΔRING, TCF25, NEMF, or VCP were treated with and without CHX (100μg/mL) for 30 min. Lysates were subjected to HA-IP and eluates were analyzed using LC-MS/MS. A.) Total spectral counts (TSCs) were normalized to bait abundance for each untreated/treated pair. Normalized TSCs were then counted for mRQC and ribosome splitting factors, using the bait C.) wild type Listerin, D.) ΔRING Listerin, E.) TCF25, F.) NEMF, and G.) VCP.

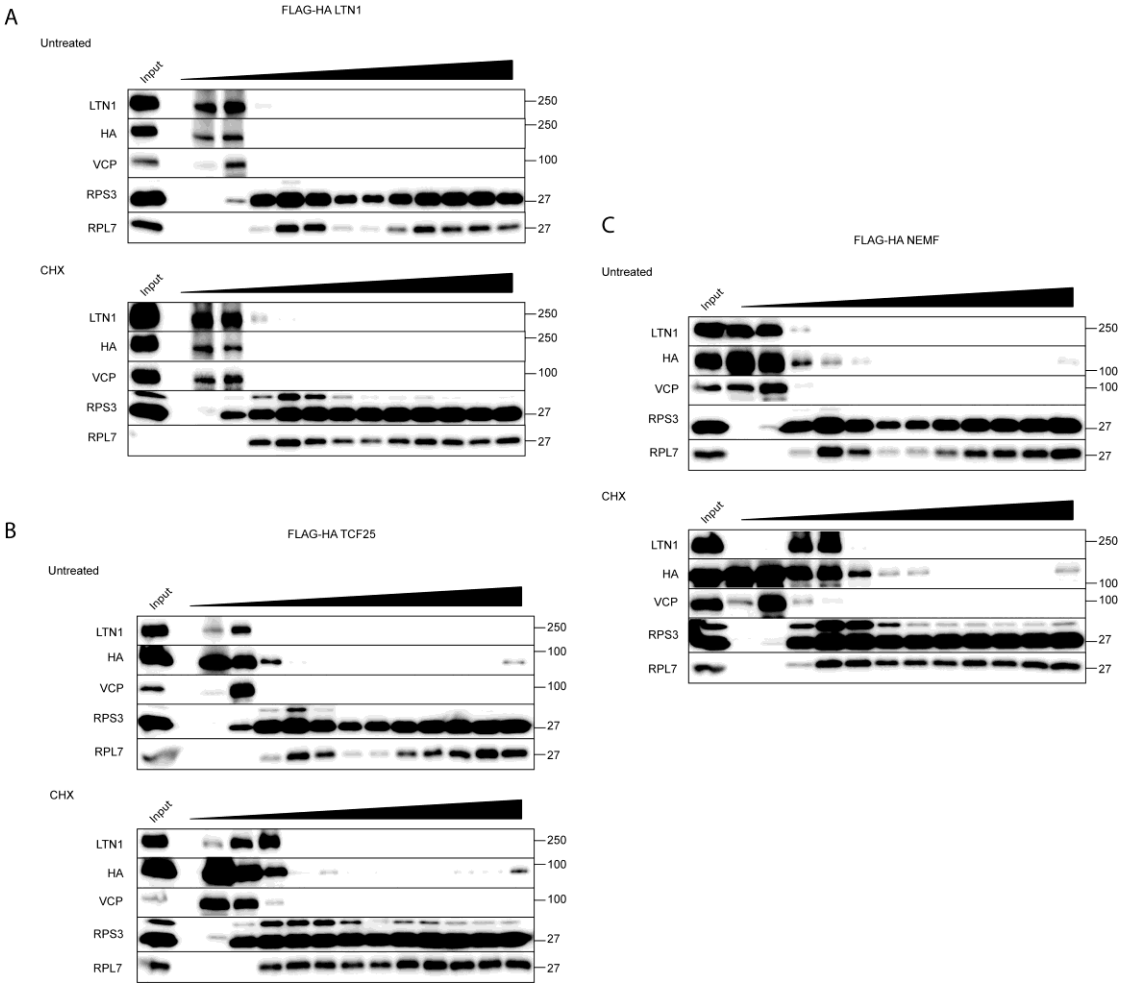


Figure 1.3: mRQC proteins shift to subunit-containing fractions with translational stalling

Lysates from HEK293T cells treated with or without CHX were analyzed using sucrose density centrifugation on a 10-50% gradient. These cell lines express FLAG-tagged A.) Listerin, B.) TCF25, or C.) NEMF, under a Tet-inducible promoter. Input fractions contain 1% of the total gradient protein load.

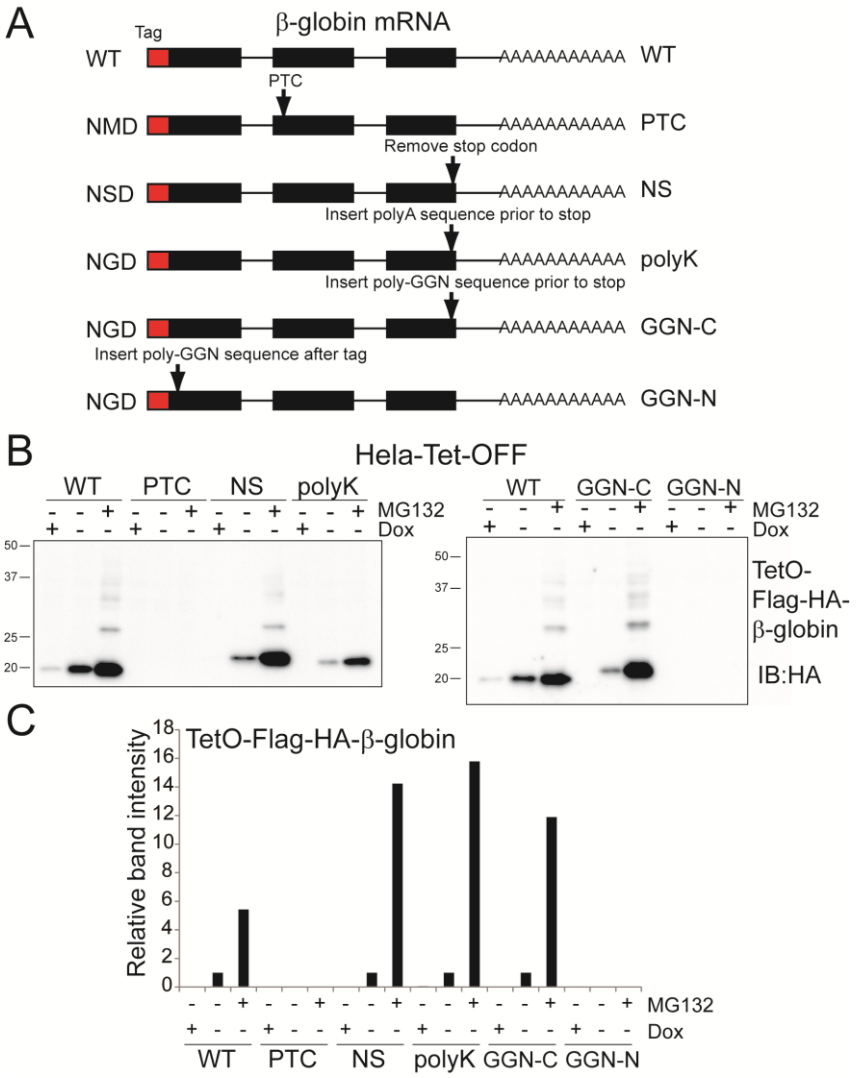


Figure 1.4: Expression and turnover of β-globin reporter constructs in HeLa cells β-globin based reporter mRNAs A.) Wild type (WT), containing a premature termination codon (PTC), lacking an in-frame stop codon (NS), encoding a polylysine (polyK) or polyglycine tract (GGN-C and GGN-N) are expressed using a Tet-OFF system. B.) These reporters are responsive to doxycycline repression. WT, NS, polyK, and GGN-C are stabilized by proteasome inhibition. C.) Quantification of the blots in (B.).

Materials and Methods

Cells, culturing conditions, and generation of expression lines

All mammalian cells were cultured in Dulbecco's Modified Eagle Medium (Thermo Fisher Scientific) supplemented with 10% FBS, and incubated at 37°C, 5% CO_2. Cells were grown until 70% confluency prior to subculture using 0.25% Trypsin-EDTA (Gibco).

Genes of interest were selected from the ORFeome (version 8.1), and cloning vectors were generated using Gateway recombination. Stable cell lines were generated using lentiviral transduction of target HEK293T or HeLa cells, followed by selection with 1μg/mL puromycin. All Flip-In cell lines were generated for inducible expression using the Tet-ON system, and were induced using doxycycline.

Lysis, immunoprecipitation, streptavidin pulldown

Cell pellets were incubated in mammalian cell lysis buffer (MCLB) at an approximate 2:1 (v:w) ratio per pellet. Protein from cleared lysates was quantified using a BCA protein assay. For HA immunoprecipitation (AP-MS), we incubated lysates with HA-conjugated agarose beads at 4°C overnight. Beads were washed thoroughly with cold PBS. Elution was performed using 150μL HA peptide. Proteins were precipitated using a final concentration of 20% TCA. Dried precipitates were digested with 1μg of Promega Sequencing Grade Modified Trypsin. After sample cleanup using C-18 filtration, samples were analyzed via LC-MS/MS.

Western analysis

For each sample, 10-20μg protein diluted in 4x Laemmli sample buffer containing β-mercaptoethanol were loaded onto hand-cast SDS-PAGE gels. Transfer

was performed using the semi-dry BioRad TransBlot Turbo Transfer System. The primary and secondary antibodies used are notated in the supplementary materials and methods section.

Sucrose Gradient

Cell lysis for the sucrose gradient analysis was performed using digitonin lysis buffer (DLB): 25mM HEPES pH 7.4, 125mM KAc, 15mM $MgAc_2$, 100µg/mL digitonin, supplemented with 40U/mL RNase inhibitor, 50µg/mL CHX, 1mM DTT, and protease inhibitor tablets. ~1.5mg protein was loaded onto a 5-45% sucrose gradient. Ultracentrifugation was performed using a Beckman Coulter SW41 rotor at 35,000rpm for 2.5hrs.

1mL fractions were collected manually from the top of the gradient. RNA absorbance were taken manually per fraction via Nanodrop. Proteins were precipitated using a final concentration of 20% TCA, washed with 10% TCA, followed by acetone wash. Dry precipitates were resuspended in 1x Laemmli sample buffer. For Western blots, 10µL was run per sample. Input lanes were loaded using 15µg of the original lysate (~1% of the total sucrose gradient load). All Western blots were done using hand-cast 12% SDS-PAGE gels.

Knockdown and overexpression assays

All knockdowns using shRNA were performed using Lipofectamine 2000 Reagent (Thermo Fisher Scientific) containing 1.5-2.5µg shRNA in Opti-MEM Reduced Serum Medium (Thermo Fisher Scientific). Cells were harvested after 48hrs. All overexpression assays were performed using Lipofectamine 2000 (Thermo Fisher Scientific) with 1.5-2.5µg of plasmid DNA.

Mass spectrometry data analysis

All datasets were exported as .RAW files, and imported into a proprietary data analysis suite. Peptide identification was performed through SEQUEST using the UniProt database. Peptide matches were filtered using Linear Discriminant Analysis using a false discovery rate (FDR) of 1%. Protein IDs were filtered using Protein Sieve with a 1% FDR, and clustered using the Protein Assembler algorithm.

Data analysis for the AP-MS experiments in Figures 1.1 and 1.2 was performed manually using total spectral count (TSC) values. For each untreated and CHX-treated pair, TSC values were bait normalized and rounded up to the next whole number.

Author Contributions

For the experiments in Chapter 1, all experiments were conducted by Nathan Zuzow (N.Z.), except Figure 1.4, which was the work of Eric Bennett (E.J.B.). Mass Spectrometry data analysis was performed by E.J.B. and N.Z.

Chapter 2: Testing proximity-labeling Methods to Identify the RQC and Transient E3-Substrate Associations

Abstract

E3-substrate interactions are often difficult to detect using the traditional affinity purification and mass spectrometry (AP-MS). Recently-developed proximity-based detection methods have been shown to be a powerful strategy for detection of protein-protein interactions. We utilized two proximity-based proteomic approaches, BioID and APEX, along with traditional AP-MS to evaluate each for their ability to detect potential E3/substrate interactions. This marks the first use of these three methodologies in parallel to the study E3 ubiquitin ligase interactors.

Using VCP as a proof of principle, we revealed the strength of BioID in identifying known protein-protein interactions. Applying these methods to Listerin, we observed that BioID was the only of our chosen methods capable of reproducibly identifying the single documented Listerin interacting protein NEMF. Furthermore, our BioID data revealed a list of putative interacting proteins which were reproducibly identified in a replicate experiment. Here we describe the discovery of the first-identified endogenous substrates of the E3 ligase Listerin.

Introduction/Results

Ltn1 is a RING-finger E3 ubiquitin ligase that is well studied for its role in ribosome-associated protein quality control in *S. cerevisiae*. This E3 is known to be involved in the destruction of nascent polypeptides resulting from ribosome stalls. Features of the mRNA transcript which trigger nonstop decay and no-go decay pathways for the destruction of the aberrant transcript also signal the recruitment of

protein quality control factors. These factors respond through ribosome splitting, nascent peptide ubiquitylation, and extraction of the peptidyl tRNA from the 60S ribosome.

Importantly, Ltn1 appears to be crucial to protein homeostasis, as a significant fraction of newly synthesized proteins on stalled ribosomes are ubiquitylated by this E3. Studies of Ltn1 function in yeast are limited to model reporter systems in which features are engineered to trigger ribosomal stalling, and no studies have yet characterized endogenous Ltn1 substrates. Though most studies of this protein focus on its role in ribosome-associated quality control, it is unknown whether Ltn1 performs the bulk of its cellular function at the ribosome.

E3 ubiquitin ligases have been implicated in the pathology of a number of neurodegenerative diseases, and serve as important pharmacological targets in their treatment. While it is tempting to speculate that the ribosome-associated quality control function of Listerin is responsible for the *Listerin* mouse phenotype, it may be the case that Listerin is targeting other key factors or signaling pathways important to neurons. In Chapter 1, we set out to understand the cellular function of Listerin by detecting its involvement in the ribosomal quality control complex *in vivo*. However, while the affinity-purification techniques we used allowed us to observe stable interactors with Listerin, they were insufficient at providing a reliable list of potential candidate substrates for the ligase.

E3 ligase substrates may contain sequence elements, such as the phosphoserine motif recognized by SCFβ-^{TrCP}, which are common to particular substrates (*92*). However, motif analysis does not explain all E3 substrate targeting (*93*). For the

degradation substrates of Listerin, the requirement for targeting may be more stringent than motif recognition. Peptidyl-tRNA nascent chains trapped at the 60S ribosomal subunit are specifically addressed by this ligase. It is possible that stall-inducing polybasic tracts in the nascent chain may serve as degrons for Listerin targeting, though this E3 has also been shown to respond to non-polybasic stalls (*72*).

Because this Listerin may have functions outside of ribosomal quality control, genetic and biochemical approaches for the detection of ligase-substrate interactions are crucial to understanding the substrate pools targeted by Listerin. Such approaches include variants on yeast-two-hybrid assays (*94*), co-immunoprecipitation (*95*), protein microarray (*96*), and ubiquitin ligase trapping techniques (*97, 98*). While these techniques have proven to be individually useful, the localization, interactions, and activities of E3 ligases are diverse, and no single method for substrate identification clearly outperforms all others. Comprehensive studies of E3 interactions require the use of orthogonal approaches for identification of substrates and interaction partners.

Affinity purification and mass spectrometry (AP-MS) is a common technique for the identification of E3 interacting proteins. However, interactions between E3 ubiquitin ligases and their degradation substrates are particularly difficult to detect due to their transient nature as well as the instability of the ubiquitylated protein. The ability of AP-MS to capture ubiquitylated substrates is diminished due to the short half-life of these proteins. Additionally, because AP-MS is affinity based, the weak E3-substrate interaction must be preserved throughout the duration of lysis and immunoprecipitation. Further complicating detection, E3 substrates may reside in

cellular compartments that are inaccessible using the non-denaturing lysis methods required for maintenance of protein-protein interactions.

We used AP-MS to study the binding interactions and substrates of Listerin. However, to overcome the aforementioned difficulties in detecting such interactions, we adapted two proximity-based proteomic methods, BioID and APEX, for use in parallel with AP-MS (Figure 2.1). Both BioID and APEX are in which the bait protein is fused in-frame with a biotin-activating domain, allowing for the covalent modification of nearby proteins with biotin.

While BioID and APEX are suitable for the detection of stable protein-protein interactions, the permanent nature of biotinylation makes these methods particularly useful in the detection weak and transient interactions, such as those between E3 and substrate. However, it is important to draw the distinction between BioID and APEX, and highlight the advantages and disadvantages of these techniques when compared to traditional AP-MS.

BioID

Since its introduction in 2012, BioID has been used to study the interactome of proteins important in numerous cellular processes (*99*), and has been used specifically to study the ubiquitin proteasome system. In conjunction with AP-MS, BioID was able to identify interacting proteins and substrates for the cullin-RING ligase adaptor β-TRCP (*100, 101*). Mechanistically, BioID differs from traditional AP-MS in that it covalently modifies target proteins through biotinylation. This allows for the use of denaturing lysis and IP conditions, which provide access to cellular compartments inaccessible by non-denaturing lysis. Additionally, covalent substrate modification

removes the requirement for substrate-bait interaction to be maintained throughout the immunoprecipitation step.

BioID consists of an in-frame fusion between the bait protein of interest and an *E. coli* biotin-ligase (BirA), which has been mutated to decrease its binding affinity for the activated biotin product (*102*). In the presence of biotin, the mutant domain (BirA R118G, or BirA*) generates activated biotinoyl-AMP, which diffuses and conjugates to nearby amine groups, particularly lysine residues (*100*).

The typical labeling period for the BioID method is 24hrs. The labeling radius for BioID is estimated to be on the order of 10-30nm for a tethered fusion protein, but the sample space of freely-diffusing cytosolic proteins may be much larger (*103, 104*), as the 24hr labeling period allows for considerable mobility of both bait and substrate. Biotinylated proteins can then be enriched using streptavidin beads and identified by mass spectrometry. To distinguish between endogenously-biotinylated proteins, and those specifically modified through BioID, it is important to include a non-specific negative control bait, such as GFP.

APEX

Similarly, APEX is a method for substrate identification through proximity-based biotinylation. This approach makes use of an in-frame fusion between the bait protein and a mutated (A134P or APEX2) 28kDa monomeric peroxidase reporter derived from plant sources (*105*). Though functionally similar to horseradish peroxidases, this reporter can be expressed *in vivo* in reducing cytosolic conditions without loss of activity (*106*).

Cells expressing APEX2 fusion-tagged proteins are incubated with biotin-phenol for a short period (~1hr), and then treated with H_2O_2 (~1min) to generate a short-lived biotin-phenoxyl radical which covalently tags nearby proteins. In contrast to BioID, the bio-phenol radical targets electron-dense side chains such as tyrosine, but possibly tryptophan, cysteine, and histidine as well (*107*). These biotinylated substrates can then be captured and identified using mass spectrometry in a manner identical to that of BioID.

Applications of APEX2 include targeted mapping of spatially-restricted organelles using live-cell proteomics and electron microscopy (*105, 107*). The biotinylation radius for the APEX2 method has not been reported, and the use of compartment-specific SILAC labeling schemes is recommended for a quantitative study of protein-protein interactions using this method (*107*). The short labeling period of APEX2 makes this approach particularly appealing for the study of dynamic cellular states and short-duration pharmacological treatments.

Comparison of AP-MS, BioID, and APEX

APEX has the advantage of requiring a very brief labeling time (~1min vs. overnight/16hr for BioID), which makes it more applicable for testing short-lived cellular states or acute drug responses than BioID (*107*). While the BioAMP generated by the BioID method has a half-life of minutes in water, APEX generates the highly-reactive biotin-phenoxyl radical, which has a much shorter half-life (<1ms) (*107*). It is also suggested that the long incubation time of BioID may lead to the accumulation of a potentially toxic biotinylated proteome (*107*). To date, published studies of APEX have been limited to membrane-tethered bait proteins or sampling of entire subcellular

compartments (*105-108*), while BioID has been tested and validated as a tractable approach for the study of E3 substrates.

Together, BioID and APEX methods are powerful tools for detecting protein-protein interactions, however AP-MS still provides a number of advantages over these methods. AP-MS is much more specific than the proximity-based identification methods, since protein-protein interactions must be preserved through lysis and immunoprecipitation. Additionally, unlike BioID, which includes a long (~16hr) biotin incubation step, this method provides the ability to probe the rapid response of the cell—for instance to short-term pharmacological inhibition of translation.

While BioID has been seen to outperform AP-MS at identification of E3 substrates, it is unclear whether this will hold true for Listerin substrates. The performance of the biotinylation-based approaches is dependent on the stability of the chimeric bait protein. BioID and APEX both involve the addition of a ~30kDa domain to the bait protein. Particularly in the case of proteins with hidden termini harboring the ligase domain, these bulky additions may disrupt regular molecular function.

Because of the distinct advantages and disadvantages of each method, AP-MS, BioID, and APEX should be used in parallel when using proteomics to study a protein's interactome. Prior to beginning our study of Listerin and its interacting proteins and substrates, we benchmarked the three methods through a comparative study of their relative effectiveness at capturing known interactions with a well-characterized protein, VCP.

Benchmarking the Three Methods with VCP/p97

Because Listerin is a low-abundance protein with only a single reported interactor, it was important for us to test the effectiveness of each proteomic strategy using a protein that does not suffer from these disadvantages. For our first comparison of AP-MS, BioID, and APEX, we used VCP: a highly-abundant, multifunctional protein with a large number of confirmed interactors.

Valosin-containing protein, VCP/p97, is involved in a number of independent cellular processes and plays a critical function in cellular homeostasis (*109*). VCP is a molecular chaperone, and can specifically target ubiquitylated proteins. It forms a hexameric ring which uses ATP hydrolysis to unfold associated client proteins, aiding in translocation or transfer of terminal proteins to the proteasome. This protein functions in a number of different cellular contexts, including ERAD, stress granule clearance, and cell cycle regulation (*78*). It also plays a direct role in ribosome-associated quality control, extracting ribosome-bound substrate proteins targeted by Listerin and Ubr1 (*49*).

For our benchmarking assay, we generated HEK293T cell lines expressing VCP engineered for use in our three platforms (Figure 2.2A). Expression was driven from a single locus, under the control of a doxycycline inducible (Tet-ON) promoter. As a negative control, we used GFP, which is not endogenously expressed in human cells, and has no known interacting partners. The engineered expression lines are listed in Table 2.1. We tested these cells for inducible expression. Additionally, we used biotin incubation and Western blot for Streptavidin-HRP to detect whether these

cell lines were capable of inducing biotinylation (Figure 2.2C). Notably, GFP and VCP expression levels are similar in this assay.

Stable Isotope Labeling with Amino Acids in Cell Culture (SILAC) is a useful method for unbiased quantitative proteomics. Long-term incubation of a population of cells with isotopically heavy lysine and arginine (K8R10) leads to stable incorporation of these amino acids in a way that does not impact the physiology of the cell, but can be detected via mass spectrometry as a subtle mass shift. Peptide pairs are identified through mass spectrometry, and can be normalized and used as an indicator of relative protein abundance (Figure 2.2B). We implemented this approach for quantitative interaction proteomics to for identification of specific VCP interacting partners.

We cultured heavy-labeled 293T cells expressing GFP engineered for use in each of the three paradigms: AP-MS, BioID, and APEX. We grew engineered VCP lines in light medium (K0R0). For BioID and APEX, we activated biotinylation prior to harvesting and lysis. We then mixed the lysates from the heavy-light pairs, and immunoprecipitated the FLAG tag (AP-MS), or used streptavidin-conjugated beads to pull down biotinylated proteins. All precipitates were subjected to on-bead trypsin digestion, and then analyzed using tandem mass spectrometry. This experiment was performed in biological duplicate, and datasets were analyzed independently.

We first analyzed the entire population of proteins identified in each of our pull-downs for VCP-specific interactors. Following data normalization, we used the $\log_2$ transformation of the heavy/light ratio (GFP/VCP) as a metric to represent relative protein abundance in each of our experiments. Specific VCP interacting proteins are identified in the 3rd quadrant of the scatter plots depicted in Figure 2.3.

For our AP-MS experiment, the tight distribution of hits centered at the origin represents the superior specificity of this method compared to BioID and APEX, which produced a number of false positives in the 2nd quadrant. Importantly, known VCP interactors were identified in each of the three experiments.

Examination of specific VCP interacting proteins reveals the differences between the three methods (Figure 2.4). Proteins found in stable complex with VCP, such as co-factors UFD1L, NPLOC4, and UFD1L, are represented in three methods (Figure 2.4A). Two proteins, UBXN7 and VCPIP1, were only identified in the BioID experiment. One explanation for this is that these proteins may only have a transient or cell-state specific interaction with VCP, and this interaction is labile. In terms of enrichment of known interactors, BioID outperformed both FLAG-IP and APEX methodology.

We then compared the number of positive identifications for VCP across the three platforms (Figure 2.4B). Using the cutoff of 2-fold enrichment over GFP control, BioID captured 92 positive IDs, while APEX and FLAG-HA captured 40 and 45, respectively. Of these proteins, BioID detected a greater number of known VCP interactors (data not shown) than the other two methods.

The BioID method appears to be more effective in capturing known VCP substrates than the other tested methods. However, because we did not perform any validation experiments, we cannot claim with any certainty that BioID is more effective at capturing novel interactions. Because the list of high-confidence interacting proteins for each approach had little overlap with the others, we opted to use the three methodologies together for the study of the E3 ligase Listerin.

Application of the Three Methods to the Study of Listerin Interacting Partners and Substrates

Identification of Listerin interactors poses an added level of difficulty over our preliminary work with VCP. Although we were able to identify known stable and transient interactors with VCP (*110*), Listerin has only one verified interactor: NEMF. It is unknown whether the other RQC interactions identified in *S. cerevisiae* are conserved in mammals.

We engineered cell lines for Listerin in each of the three enrichment paradigms in a manner identical to those generated for VCP and GFP in the previous experiment (Figure 2.5A). Instead of using SILAC as we did in the previous experiment, we opted to use a label-free proteomic approach (Figure 2.5B). After constructing these cell lines, we assayed for expression of the tagged Listerin or GFP control (Figure 2.5C). Note that Listerin expression is comparatively much lower than the expression of GFP across all three methods. Listerin produces a weak biotinylation signal compared to GFP as well.

We cultured Listerin and GFP expression lines for each of the platforms under identical media conditions. For the BioID and APEX cell lines, we activated biotinylation prior to harvesting and lysis. As before, the samples for AP-MS were immunoprecipitated using the FLAG tag, and the BioID and APEX samples were subjected to streptavidin pulldown. The captured proteins were subjected to on-bead trypsin digestion, and analyzed in technical triplicate using mass spectrometry.

Analysis of our label-free results differs somewhat from the SILAC results obtained from the previous VCP experiment. After normalizing for bait abundance, we

compared total spectral counts (TSCs) between Listerin and GFP for each of the three platforms. While non-specific interactors are not enriched in either the Listerin or GFP samples, Listerin-specific interactors are measured as those enriched two-fold over GFP (Example in Figure 2.5B). We used this approach to analyze the interaction between Listerin and other known ribosome-associated quality control factors. As expected, we detected a specific interaction between Listerin and NEMF (Figure 2.6A). It is important to note that this interaction was only detectable using BioID.

Using these three methodologies, we found no evidence that VCP is an interacting protein of Listerin (Figure 2.6B), which is consistent with our findings in Chapter 1. Pelota and Hbs1L (Dom34 and Hbs1 in yeast, respectively) are known termination factor-like proteins which act upstream of Listerin in response to ribosome stalling. While we did not observe any specific interaction between Hbs1L and Listerin, we were able to detect some enrichment for the Pelota-Listerin interaction in the AP-MS experiment only.

We then performed an analysis using a scoring algorithm which sorts interactors across replicate runs based on reproducibility, uniqueness, and abundance. We used this metric to identify high confidence interacting proteins (HCIPs) for Listerin in each of the three platforms (Figure 2.7A). AP-MS was able to detect almost twice as many HCIPs as BioID, and ~15-fold more than APEX. While the list of proteins detected by AP-MS was large, we were unable to identify NEMF using this method, which is consistent with our previous AP-MS results for steady state interactions between the two proteins. APEX did not capture this interaction either.

The BioID approach identified NEMF and the highest scoring interacting protein for Listerin (Figure 2.7B). We performed further analysis of our BioID dataset, using a normalized interaction score in which 2-fold enrichment over GFP signifies a strong interaction. Our dataset revealed four members of a hetero-pentameric tubulin glutamylation complex (Figure 2.7B, Red), and the protein RSK1 (Figure 2.7B, Green). Notably, we also detected more endogenously biotinylated proteins in our Listerin BioID than in GFP control.

We performed a replicate experiment in which Listerin BioID was once again compared to GFP control (Supplementary Figure 2.1). In this experiment, we were able to again detect NEMF, members of the tubulin glutamylation complex, and RSK1. Additionally, we were able to detect another RSK protein family member, RSK1, and the remaining tubulin glutamylation enzyme TTLL1 (data not shown).

Discussion

Challenges in Detecting Quality Control Substrates of Listerin

Listerin is suspected to have a variety of quality control substrates in response to translational stalling. Because the mRQC function of Listerin requires it to bind to the 60S-tRNA-nascent chain, this E3 may target any protein trapped in the exit tunnel, regardless of sequence specificity. This may indeed be triggered by specific sequences in the mRNA, as in the case of NSD and NGD, but may also arise from other varieties of translational stalls.

Generation of a comprehensive list of endogenous Listerin quality control substrates is beyond the scope of this project. We do not have evidence of enrichment for nascent chain polypeptide substrates in our analysis. In order to specifically

identify newly-synthesized Listerin substrates, a modified version of our label-free technique can be employed, using pulse SILAC to metabolically label newly-synthesized proteins. These likely low-abundance, partially-stochastic quality control events would be a significant challenge to detect.

In our early experiments using BioID, we suspected that expression of a Listerin fusion protein containing the BirA* domain at the C terminus, more proximal to the peptide exit tunnel, would likely promote the enrichment of ribosome-bound substrates. However, we did not observe a significant difference in the protein IDs obtained with either the N-terminal or C-terminal variant (data not shown).

Post-hoc Comparison of the Three Methods

We analyzed the interactors of VCP using AP-MS, BioID, and APEX. Our analysis included a list of proteins that are known VCP interactors—many of which were identified using traditional affinity-enrichment techniques. This biases our list of known interactors towards those which form a stable complex with VCP. Another disadvantage of the proximity-based methods is the promiscuity of biotinylation, which produces a significant breadth of false positives compared to AP-MS (Figure 2.3, upper-right quadrant). This appears to be due to unexpectedly specific GFP interactions within the proteome.

Despite these disadvantages, BioID appeared to outperform the other two approaches in terms of detection of known VCP interactors. All three proteomics approaches provided us with substantial numbers of high-confidence interacting proteins (HCIPs). However, the lack of significant overlap amongst HCIPs (Figure

2.4B) suggests that these three methods should be used in parallel as complementary methods for the detection of protein-protein interactions.

Detection of Listerin Substrates

BioID revealed novel associations between Listerin and the MAPK signaling kinases RSK1 and RSK2, as well as with tubulin glutamylation complex (TGC) components—TPGS1, TPGS2, LRRC49, NICN1 and, to a lesser extent, TTLL1. These interactions were identified in an independent BioID experiment (Supplementary Figure 3.1), supporting our previous finding that Listerin comes in close proximity with, and may interact directly with, RSK proteins and the TGC. To study whether Listerin functionally interacts with these proteins, we used activity-based assays to assess the impact of Listerin on tubulin polyglutamylation and RSK signaling. These experiments are detailed in Chapter 3.

Tubulin polyglutamylation plays a role in microtubule stability, which is key in neural development and function. One enticing hypothesis regarding the Listerin mouse phenotype is that dysregulation of tubulin polyglutamylation can lead to increased microtubule instability, thereby contributing to neurodegeneration. Evidence of this is supported by the phenotype of the *pcd* mouse, which harbors an inactivating mutation in a key tubulin deglutamylase (CCP1). Whether this is a contributor to the Listerin phenotype is yet to be demonstrated using functional studies.

RSK1 and RSK2, members of the RSK protein family, operate in the MAPK signaling pathway, which integrates extracellular growth factor signals, and promotes protein synthesis directly at the ribosome, or through crosstalk with regulators of mTOR signaling. RSK is a crucial hub for pro-growth and pro-survival signaling. One

hypothesis is that Listerin influences the production of faulty proteins through RSK signaling. Loss of RSK turns off pro-growth signaling—thus, if Listerin keeps RSK levels low, it suppresses growth-factor stimulated protein production. Hence, this ligase may directly influence protein production at a fundamental level.

Detecting Interacting Partners of Listerin

Detection of NEMF specifically through BioID may be attributed to the close proximity of the N-terminal BirA* domain and known NEMF-Listerin contact points (*40*). Lack of detection of this strong interaction via AP-MS is consistent with the notion that this interaction is labile and likely difficult to detect at steady state. This interaction may require a stimulus such as CHX to generate detectable levels of the complex, which is supported by our previous results with Listerin AP-MS (Figure 1.2C,D). However, using NEMF as bait in our AP-MS experiment, we observed a significant interaction with Listerin that is not induced by CHX. It is possible that the association between these two proteins is dependent on the abundance of NEMF.

In this and our previous AP-MS studies, we were not able to detect a Listerin-VCP interaction above negative control (Figures 1.1, 1.2, 2.6). This may suggest one of two things: either these two proteins do not interact, or the use of GFP, which is prone to nonspecific binding interactions, as a negative control diminishes our ability to detect bonafide interactions. Based on our data, we were unable to draw a definitive conclusion about this interaction. Our results highlight the importance of using a negative control protein which makes few nonspecific interactions, and is expressed at a level similar to that of Listerin.

Of our three approaches, AP-MS may be best suited for studies of co-interaction between components of the mRQC. However, in addition to capturing the Listerin-NEMF interaction, BioID appears to be the most powerful approach for detecting transient ligase-substrate interactions. Listerin BioID generated a reproducible list of interacting partners (Figure 2.7B, Supplementary Figure 2.1). However, while this list contains NEMF, we were not able to significantly detect other mRQC components.

Two potential confounds may limit the detection of mRQC interactions using BioID. First is the use of the GFP control, which generated an abundance of background interactions, potentially masking bonafide interactions. Second is the incompatibility of the BioID protocol with the use of CHX to stall translation and potentially induce mRQC formation. APEX initially appeared to be a solution to this problem, offering a promising combination of the strengths of proximity labeling and the temporal versatility of AP-MS. However, APEX yielded very few HCIPs, and was unable to capture the Listerin-NEMF interaction, which served as our standard for judging the effectiveness of the method.

Table 2.1: HEK293T cell lines generated for VCP and Listerin proteomics experiments using the AP-MS, BioID, and APEX approaches

	GFP	VCP	Listerin
AP-MS	FLAG-HA GFP	FLAG-HA VCP	FLAG-HA Listerin
BioID	FLAG-BirA* GFP	FLAG-BirA* VCP	FLAG-BirA* Listerin
APEX	FLAG-APEX2* GFP	FLAG-APEX2 VCP	FLAG-APEX2 Listerin

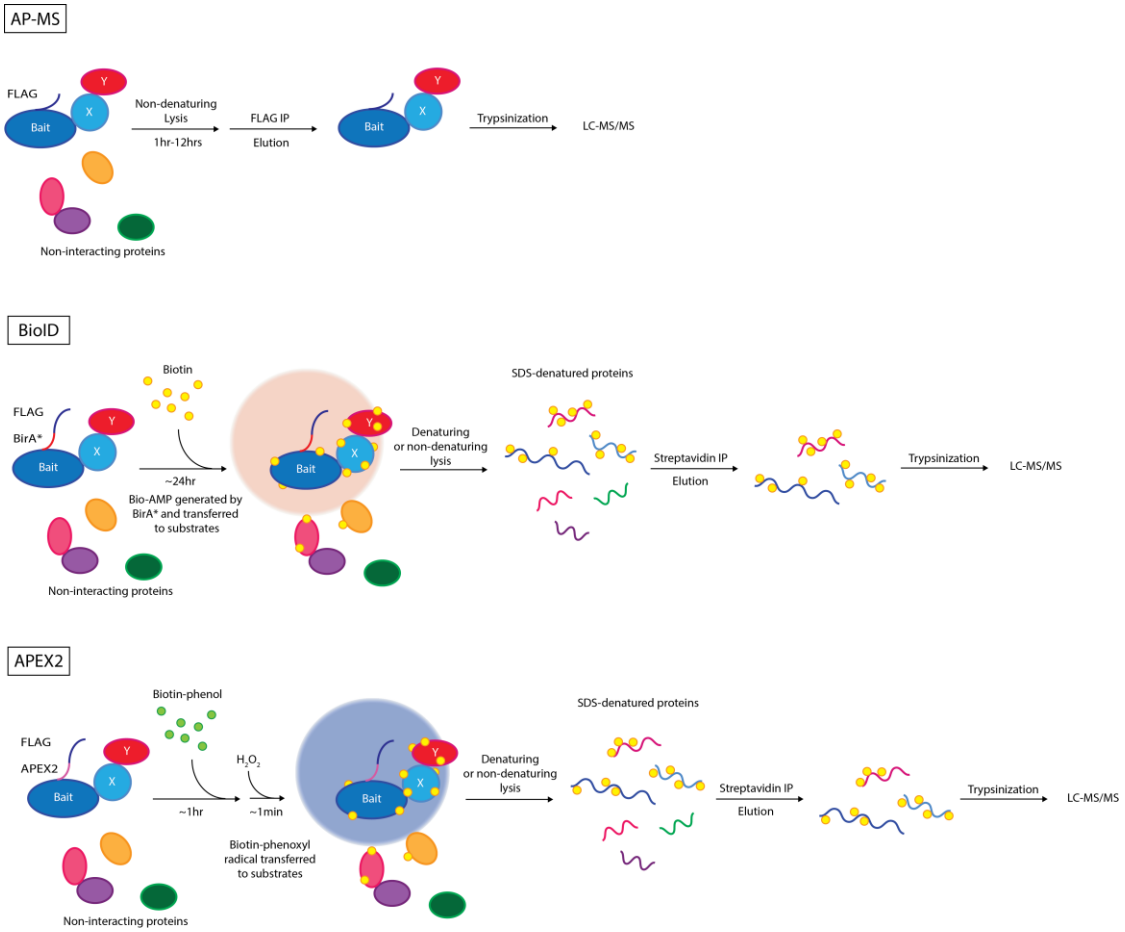


Figure 2.1: Schematic for AP-MS, BioID, and APEX proteomics approaches
A depiction of AP-MS, BioID, and APEX proteomics workflows.

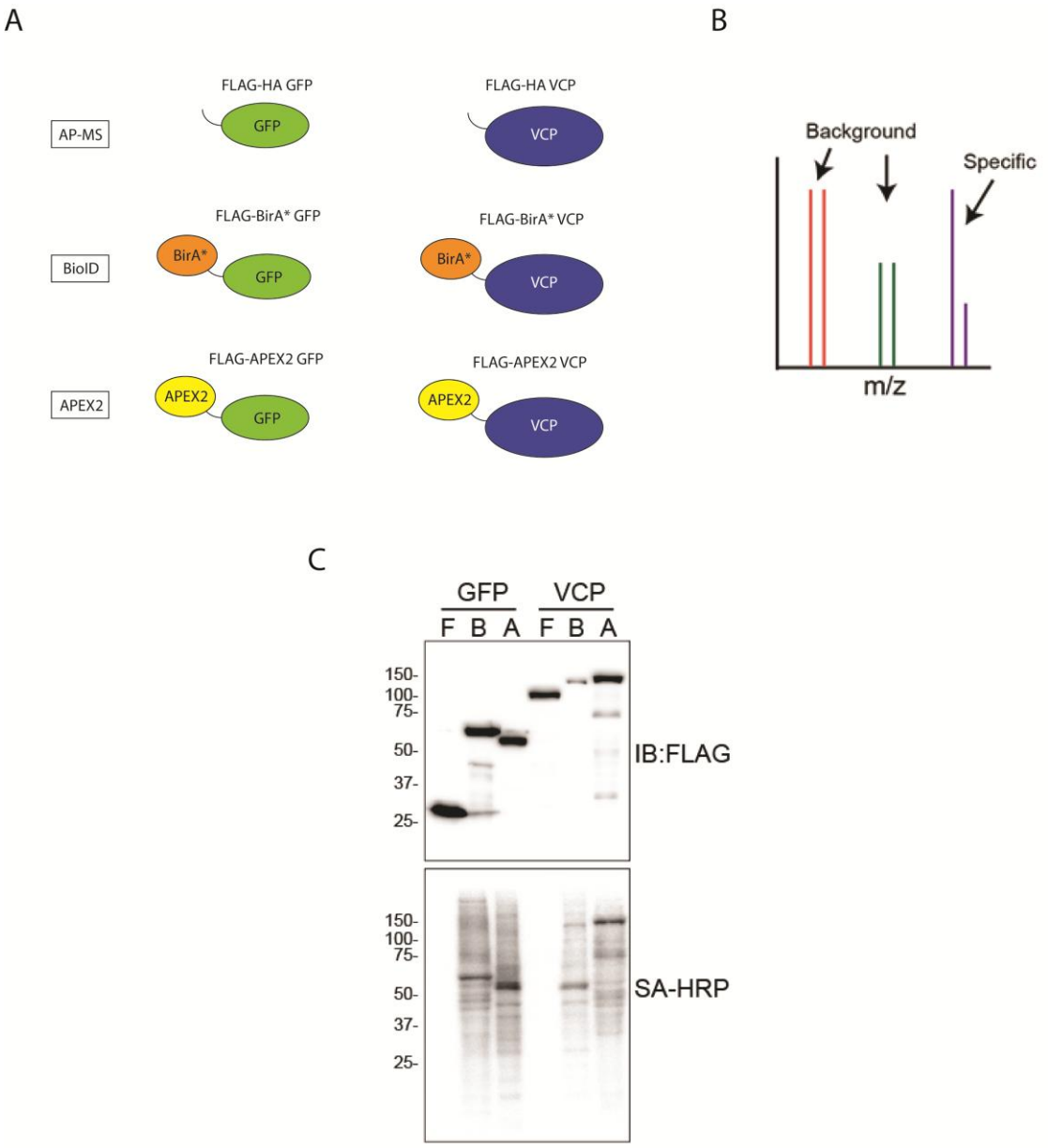


Figure 2.2: Schematic and expression of transgenic cell lines for VCP SILAC proteomics experiment

A.) Reporter design schematic depicting VCP protein products of FLAG-HA (F), BioID (B), and APEX (A), as well as GFP negative control. B.) Generalized chromatogram depicting peak intensity profiles for background and VCP-specific interacting proteins. C.) Inducible expression of the GFP and VCP reporters in HEK293T cells. Bottom Western blot shows streptavidin-HRP signal.

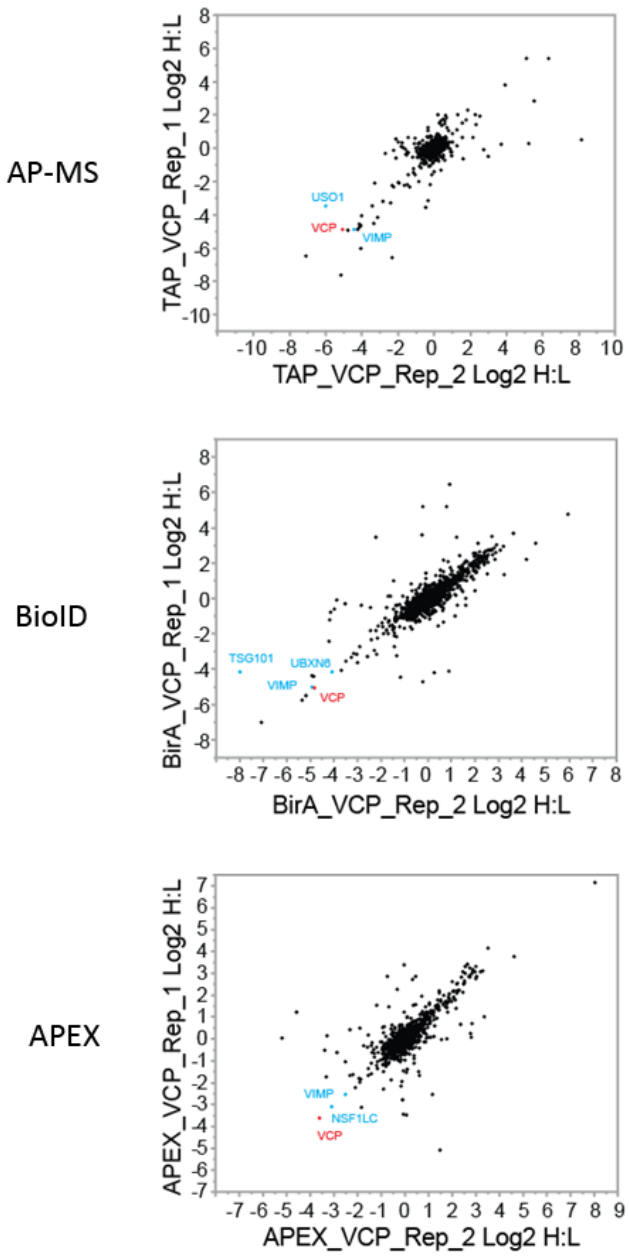


Figure 2.3: VCP SILAC proteomics data for two biological replicate experiments
Scatterplots representing AP-MS, BioID, and APEX experiments performed in biological duplicate. For each proteomic approach, heavy-labeled (K8 R10) HEK293T cells expressing GFP were mixed 1:1 with light (K0 R0) HEK293T cells expressing VCP, post-lysis. Heavy/light (H:L) ratios were taken for all quantified peptides and then transformed by $\log_2$. Bait protein is indicated in red, and known interactors are indicated in blue.

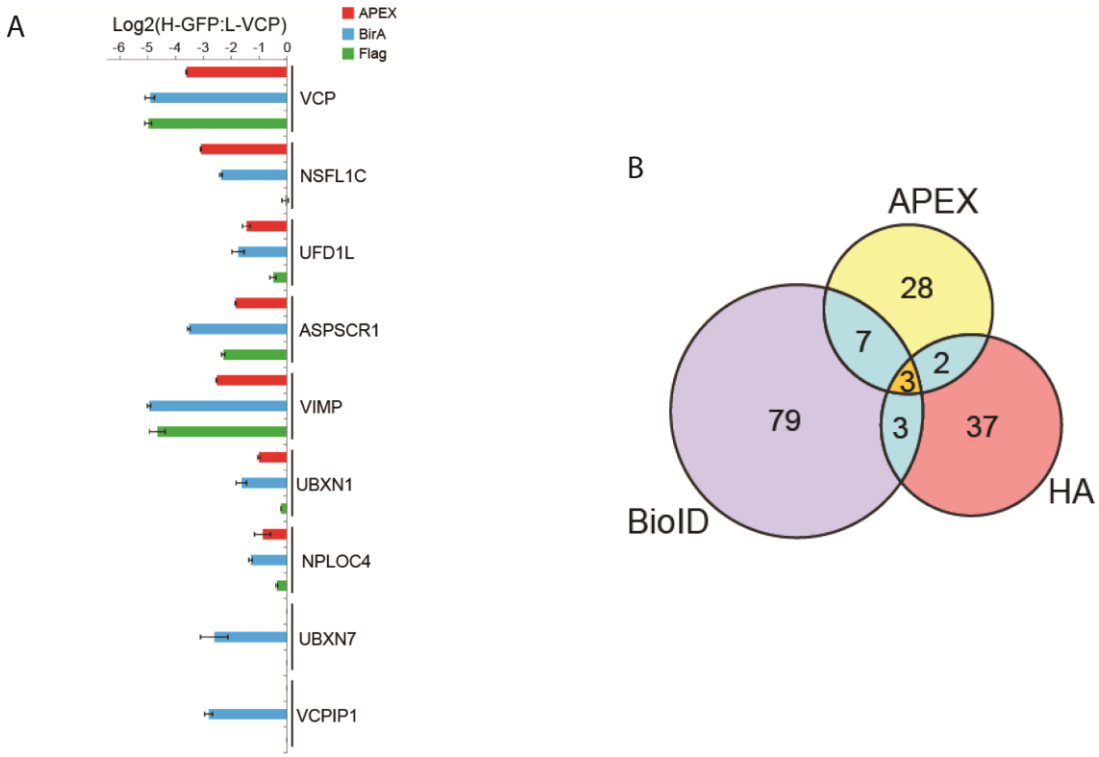


Figure 2.4: VCP proteomics identifies known and unknown VCP interactors
AP-MS, BioID, and APEX experiments were performed in biological duplicate. For each proteomic approach, heavy-labeled (K8 R10) HEK293T cells expressing GFP were mixed 1:1 with light (K0 R0) HEK293T cells expressing VCP, post-lysis. A.) Log2 Heavy/light (H:L) ratios of known VCP-interacting proteins. B.) Venn diagram depicting the number of high-confidence interacting proteins identified by each method.

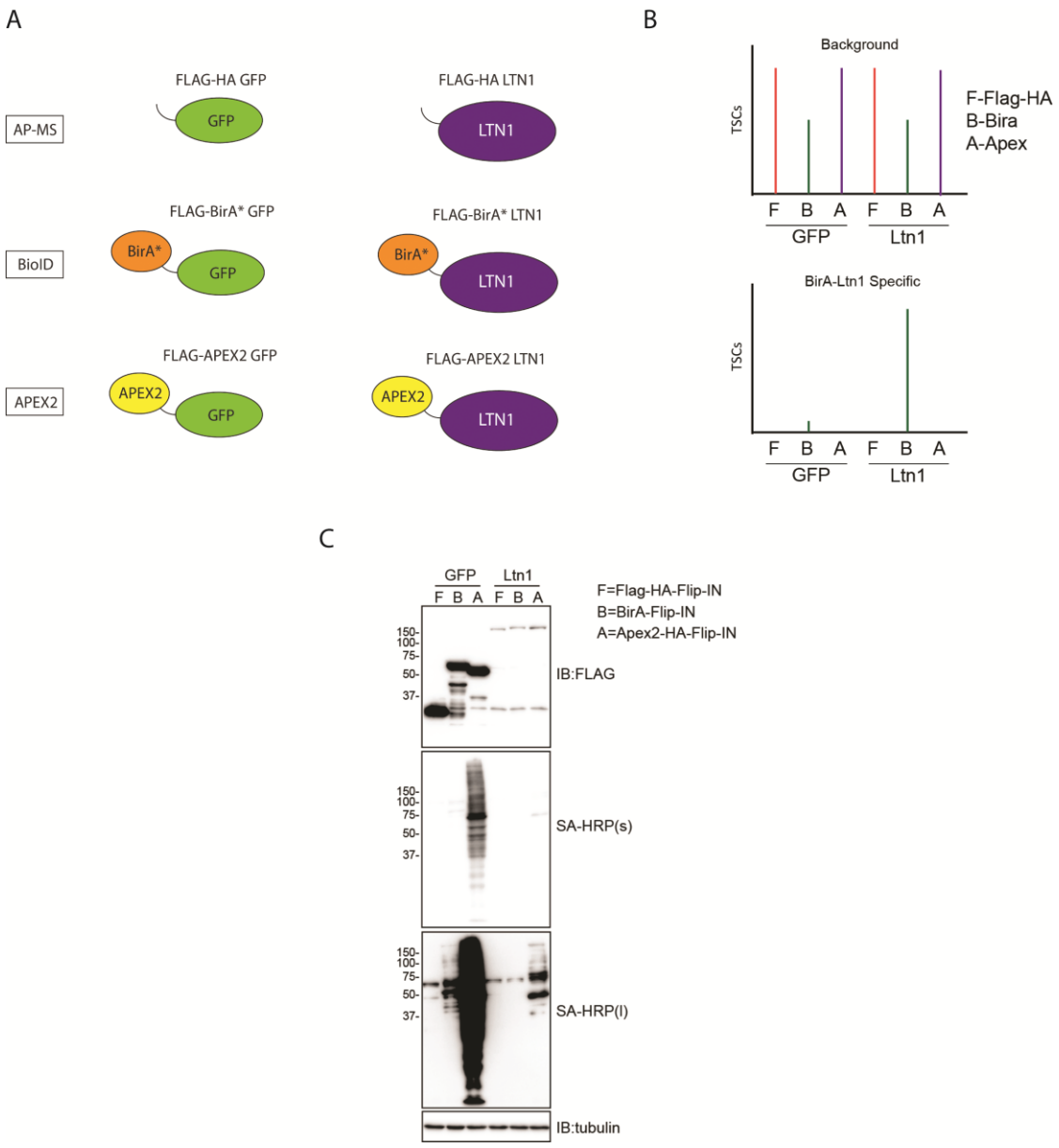


Figure 2.5: Schematic and expression of transgenic cell lines for Listerin proteomics experiment

A.) Reporter design schematic depicting Listerin protein products of FLAG-HA, BioID, and APEX, as well as GFP negative control. B.) Generalized chromatogram depicting peak intensity profiles for background and Listerin-specific interacting proteins. C.) Inducible expression of the GFP and Listerin reporters in HEK293T cells. Middle and bottom Western blots show streptavidin-HRP signal.

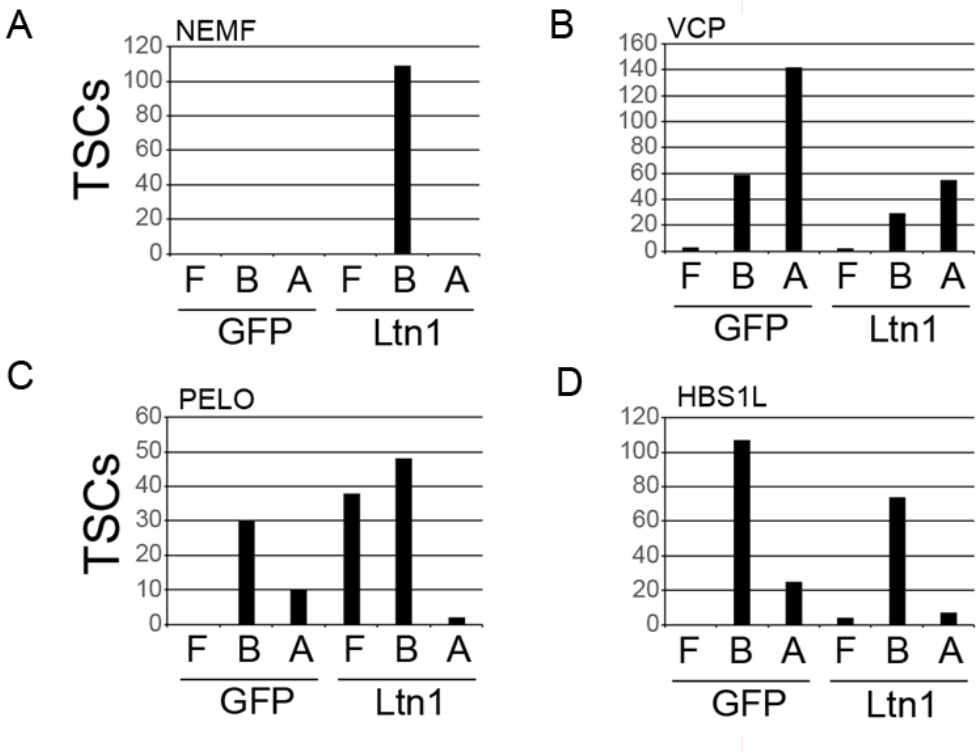


Figure 2.6: Listerin proteomics identifies mRQC interactions
Total spectral counts for the AP-MS (FLAG, F), BioID (B), and APEX (A) proteomics experiments using either Listerin or GFP as bait. Specific interactions detected for mRQC proteins A.) NEMF, B.) VCP (note: TCF25 was not identified in any of our experiments), and ribosome splitting factors C.) Pelota, and D.) Hbs1L

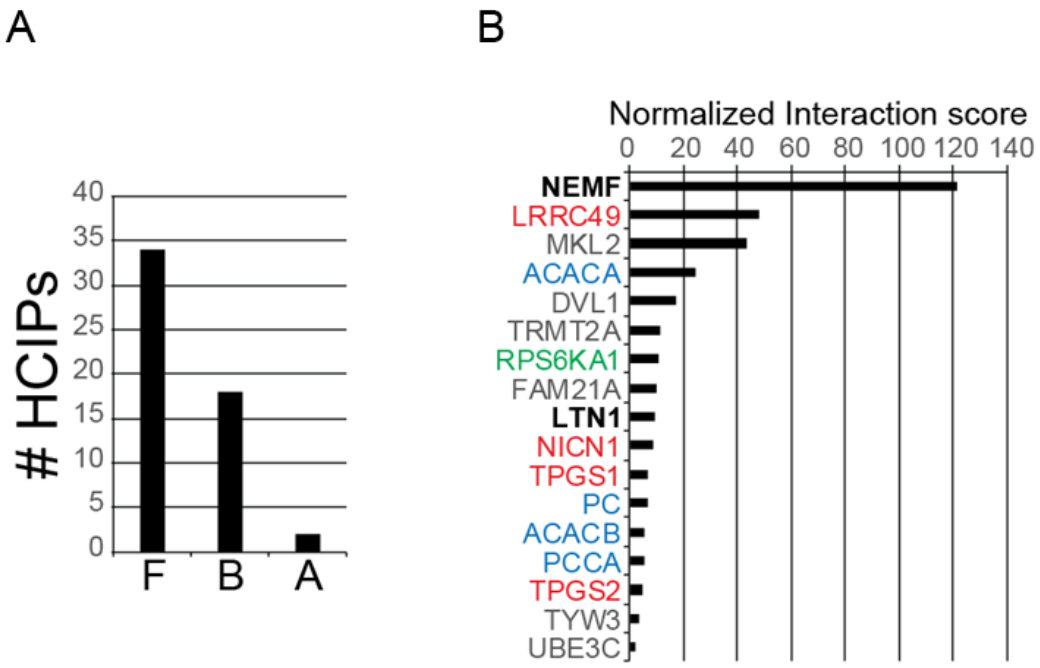


Figure 2.7: Detection of high-confidence interacting proteins, and top Listerin interactors identified in the BioID dataset
A.) Histogram of high-confidence interacting proteins (HCIPs) across the three methods, The normalized interaction score for the BioID experiment was taken using the ratio of WD scores (Listerin BioID/GFP BioID). B.) A curated list of proteins with a normalized interaction score above two. In red are tubulin glutamylation complex proteins, in green is the RSK family protein RSK1, and in blue are endogenously-biotinylated proteins.

Materials and Methods

Cells, culturing conditions, and generation of expression lines

HEK293T and HeLa cells were cultured in Dulbecco's Modified Eagle Medium (Thermo Fisher Scientific) supplemented with 10% FBS. Vectors containingr VCP and Listerin were selected from the ORFeome, and cloning vectors were generated using Gateway recombination. Stable cell lines were generated using lentiviral transduction of target HEK293T cells, followed by selection with 1μg/mL puromycin. All Flip-In cell lines were generated for inducible expression using the Tet-ON system, and were induced using doxycycline (5μg/mL). For serum starvation, cells were cultured in DMEM without FBS.

Western analysis

For each sample, 10-20μg protein diluted in 4x Laemmli sample buffer containing β-mercaptoethanol were loaded onto hand-cast SDS-PAGE gels. Transfer was performed using the semi-dry BioRad TransBlot Turbo Transfer System. The primary and secondary antibodies used are notated in the supplementary materials and methods section.

Lysis, immunoprecipitation, streptavidin pulldown

Cell pellets were incubated in mammalian cell lysis buffer (MCLB) at an approximate 2:1 (v:w) ratio per pellet. Protein from cleared lysates was quantified using a BCA protein assay. For FLAG immunoprecipitation (AP-MS), we incubated lysates with FLAG-conjugated agarose beads at 4°C overnight. Streptavidin pulldown was performed using streptavidin-conjugated agarose beads with overnight inversion at 4°C. Beads were washed thoroughly with IP buffer and cold PBS. On-bead trypsin

digestion was performed using 1µg of Promega Sequencing Grade Modified Trypsin in 50mM ammonium bicarbonate/10% acetonitrile overnight at 37°C. Peptide purification steps were performed using homemade pipette tip C18 columns (Empore SPE Extraction Disks).

BioID and APEX mass spectrometry

BioID: HEK293T cells expressing the BirA R118G (BirA*) domain fused in-frame with FLAG-tagged bait protein were co-treated with doxycycline and biotin (50µM) overnight. Cell pellets were harvested using trypsin, and stored at -80°C until lysis. Lysis was performed using MCLB supplemented with proteasome inhibitor (Roche, EDTA-free). Protein concentrations were determined using a BCA protein assay. The lysates are divided according to protein concentration: 600µg for Western blot (100µg for whole cell lysates, and 500µg for streptavidin pulldown followed by Western blot).

APEX: HEK293T cells expressing the APEX2 domain fused in-frame with FLAG-tagged bait protein were treated with doxycycline overnight. Prior to harvest, cells were incubated with biotin-phenol (500µM) for 1hr, and 30% H_2O_2 for ~1 min. Further processing was identical to BioID samples.

SILAC and label-free proteomics

SILAC: GFP-expressing HEK293T cells labeled with heavy arginine and lysine (K8 R10) were treated and harvested identically to VCP-expressing HEK293T cells cultured in light (K0 R0) growth medium. These cell populations were harvested and lysed as above, and lysates were mixed 1:1 prior to FLAG-IP or streptavidin pull-down for mass spectrometry and Western blot. **Label-free:** GFP- and Listerin-

expressing HEK293T cells were cultured in normal growth medium and processed in parallel.

Mass spectrometry data analysis

All datasets were exported as .RAW files, and imported to a proprietary data analysis suite. Peptide identification was performed through SEQUEST using the human UniProt database. Peptide matches were filtered using Linear Discriminant Analysis using a false discovery rate (FDR) of 1%. Protein IDs were filtered using Protein Sieve with a 1% FDR, and clustered using the Protein Assembler algorithm.

VCP analysis: Fold change is represented as $\log_2$(H/L). Using this as a scoring metric, the threshold for high-confidence interacting proteins (HCIPs) was set at ≥2. Listerin analysis: TSCs were normalized to GFP control with respect to bait abundance. Fold change was represented as the $\log_2$ ratio of experimental to control TSCs, and all zero denominator counts were treated as single matches. The CompPASS WD score obtained from this analysis was used as a scoring metric. The threshold for HCIP identification was set at ≥2 ($WD_{Listerin}/WD_{GFP}$).

Author Contributions

N.Z. and E.J.B. performed the molecular cloning and tissue culture work for the generation of stable cell lines. The VCP study and analysis were conducted in full by E.J.B. The Listerin study and analysis were completed through the joint efforts of E.J.B. and N.Z.

Chapter 2 is currently being prepared, along with Chapter 3, for submission for publication. Zuzow, Nathan; Leonard, Marilyn; Liao, Jeffrey; Bennett, Eric J. The author of this dissertation was the primary investigator and author of this material.

Chapter 3: Biochemical validation of novel Listerin interactions

Abstract

The E3 ubiquitin ligase Listerin is known for its role in ribosome-associated protein quality control. However, little is known about the endogenous targets of Listerin. In Chapter 2, we demonstrated the usefulness of BioID for identification of E3 ubiquitin ligase substrates, and presented a number of putative Listerin targets that were specifically identified using the BioID method. Those proteins include the p90 Ribosome S6 Kinases, RSK1 and RSK2, as well as members of the tubulin glutamylation complex (TGC).

Here, we reveal the first functionally validated substrates of Listerin in mammals. We show that Listerin-mediated ubiquitylation of RSK and TGC proteins does not decrease the stability of these proteins, indicating that this modification is regulatory in nature. The interaction between Listerin and RSK directly impacts downstream effectors of RSK, and this novel function of Listerin appears to be at least partially independent of the ribosomal quality control complex. Our findings present a new role for Listerin as a regulator of growth signaling and cellular metabolism through its regulatory association with RSK.

Introduction/Results

An appreciation for the importance of Listerin as a quality control ligase has grown over the past decade. While much of the function of this E3 has been studied in *S. cerevisiae,* and its functional output limited to a class of reporters engineered to cause ribosome stalling, the role of Listerin in general protein homeostasis is likely a large one. In mammals, nearly 4% of all nascent polypeptides are trapped in stalled

ribosomal complexes which are polyubiquitylated and degraded by the proteasome (*32*). Yeast Ltn1 is known to target these proteins at the 60S ribosome *in vivo*, and biochemical reconstitution assays using mammalian Listerin show that this ligase is capable of targeting trapped substrates at the 60S (*40*). As of yet, there have been no studies, in mammals or in yeast, that identify endogenous targets of Listerin.

As mentioned in Chapter 2, identification of E3 ubiquitin ligase targets is a difficult task in molecular biology. Substrates of degradative ubiquitylation are destroyed shortly after making transient contact with the E3. BioID overcomes a number of the drawbacks of the traditional affinity purification mass spectrometry (AP-MS) approach by covalently modifying substrates at lysine residues that may otherwise be modified by the ligase. Using this technique, transient interactions are captured with greater fidelity than AP-MS. This opens up the possibility of detecting novel ligase-substrate interactions that would ordinarily go undetected.

We describe in Chapter 2 the use of three parallel proteomics approaches for the unbiased identification of novel interacting partners and substrates of the E3 ubiquitin ligase Listerin. Of the three paradigms tested, BioID was the only one capable of identifying the single documented Listerin interactor, NEMF (Figure 2.6A). We leveraged the BioID approach for the identification of novel interacting partners of this E3, and were able to reproducibly detect two new classes of Listerin interactors: members of the 90 kDa ribosomal s6 kinase (RSK) family, and the tubulin glutamylation complex (TGC) (Figure 2.7B, Supplementary Figure 2.1).

These two classes of interacting proteins represent markedly different functions in cellular homeostasis. Protein kinases RSK1 and RSK2 function as key

regulators of pro-growth signaling as part of the MAPK pathway, and have a significant impact on mTOR signaling. The tubulin glutamylation complex is important for promoting the dynamic instability of microtubules, which has a direct influence on transport, cell polarity, and centrosome dynamics.

Validation of our list of putative Listerin interactors required us to first recapitulate our findings. In an independent BioID experiment, we observed the same RSK and TGC interactions as prior (Supplementary Figure 2.1). This BioID experiment expanded our list of Listerin interactors by the inclusion of RSK1 and TTLL1. Detection of these components reinforced the notion that Listerin may have a function in both MAPK signaling and tubulin dynamics.

Reciprocal BioID for TGC and RSK Components

As additional validation of these putative interactions, we conducted a reciprocal BioID experiment using TGC and RSK proteins as bait. We constructed HEK293T cell lines expressing the engineered TGC and RSK variants indicated in Table 2.1 in a manner identical to that used for our VCP and Listerin BioID lines. After generating these inducible expression lines, we confirmed doxycycline-inducible expression (Figure 3.1). We confirmed that these cell lines are also capable of biotinylation via the BirA* domain, as confirmed via streptavidin blot (Data not shown).

After validating our expression lines, we proceeded to run a label-free BioID experiment using the TGC and RSK baits, as well as negative control GFP (Figure 3.2). This experiment was conducted using the same protocol used for the Listerin BioID experiment detailed in Chapter 2. Following bait normalization to GFP, we

calculated fold change relative to GFP control for each of the putative interactors (Figure 3.2). Bait abundance is represented using total spectral counts for the BirA* domain (Figure 3.2A).

As a positive control, we included a third, independent biological replicate for our Listerin BioID experiment. Listerin was able to capture all previously-detected interactions at levels similar to those in our previous work (Supplementary Figure 2.1). All Listerin-TGC interactions were observed to be at least 1.5-fold enriched over control. Interactions with RSK1 and RSK2 are over 30-fold enriched over control using Listerin BioID (Supplementary Figure 3.1).

Our BioID data show that TGC components clearly interact with other members of the same complex (Figure 3.2B-D). However, TGC members NICN1 and TTLL1 show only a 1.5-fold enriched interaction with Listerin over GFP control (Figure 3.2B-D). LRRC49 BioID reveals an interaction with both Listerin and RQC component TCF25 (Figure 3.2B). Further strengthening the likelihood of this interaction being bonafide, LRRC49 was identified as an interactor in all three of our Listerin BioID experiments (Figure 2.7, Supplementary Figure 2.1, Supplementary Figure 3.1).

NICN1 and TTLL1 also both appear to interact with other TGC proteins (Figure 3.2C, D). Consistent with our previous results showing a subtle interaction with Listerin, NICN1 and TTLL1 are not enriched to the extent of LRRC49. In total, we were able to detect Listerin using LRRC49 BioID, further reinforcing the notion that Listerin interacts with at least one member of the tubulin glutamylation complex.

RSK2 BioID confirms an interaction with Listerin that appears to be 100-fold enriched over control (Figure 3.2E). However, because RSK proteins share a high level of homology (*111*), we cannot rule out the possibility that the protein IDs for RSK1 are false positives arising from overexpressed RSK2. We were also able to observe an interaction between RSK2 and Listerin, mirroring our results from the complementary Listerin BioID from this set (Figure 3.2E, Supplementary Figure 3.1). Interestingly, in our previous Listerin BioID results, RSK2 was not detected (Figure 2.7).

Biochemical validation experiments

After confirming reciprocal interactions between Listerin and our candidate proteins, we proceeded to run a series of validation experiments to determine whether Listerin is able to ubiquitylate and affect downstream signaling of these proteins *in vivo*. Additionally, we tested whether the contribution of Listerin is dependent on the mRQC.

We generated constructs for transient overexpression of our TGC and RSK proteins, as well as Listerin in both its wild-type and catalytically-inactive ΔRING form. To explore whether Listerin polyubiquitylates these proteins *in vivo* we expressed either wild-type or ΔRING Listerin in combination with the TGC and RSK proteins (Figure 3.3). Additionally, we expressed HA-tagged ubiquitin for direct immunoprecipitation of ubiquitin-modified proteins.

Our results reveal that TTLL1 and the two RSK proteins are ubiquitylated by Listerin in a RING-dependent manner. Listerin-specific ubiquitylation of NICN1 was not detected. We observed RING-dependent ubiquitylation of RSK1 and RSK2, as

well as another protein identified in our Listerin BioID assay, TYW3. This suggests that Listerin specifically interacts with and ubiquitylates these substrates, and this modification requires the catalytic RING domain.

One hallmark of degradative polyubiquitylation is increased substrate stability when the modification is blocked. In contrast to this, we observed decreased substrate abundance with loss of Listerin, which is an indicator of regulatory polyubiquitylation. Taken together, these data suggest that Listerin ubiquitylates RSK proteins and the TGC component TTLL1, but not NICN1, and this modification does not target the protein for degradation and rather serves a regulatory function.

Exploring the Role of Listerin in MAPK and mTOR Signaling via RSK

Mitogen-activated protein kinase (MAPK) signaling is involved in cell proliferation, survival, growth, and differentiation (*112*). MAPK signaling is initiated by sensing of extracellular cues, such as serum and phorbol esters, by membrane receptors. These signals are relayed to initiate a signaling cascade which includes activation of extracellular signal-related kinases 1 and 2 (ERK1/2). ERK1/2 directly phosphorylates proteins in the RSK family (*111*).

The RSK protein family is involved in a number of important cellular functions, including regulation of transcription, the cell cycle, and pro-survival signaling (*111*). Loss of RSK2 function in humans is the genetic root of Coffin-Lowry syndrome, which is an X-linked disorder characterized by severe growth and cognitive disorders, and significantly reduced lifespan (*113*). RSK1 and RSK2 also directly promotes activation of translation by phosphorylating ribosomal protein S6, and promote cap-dependent translation via the phosphorylation of eIF4B (*111*).

Importantly, RSK is an important hub for crosstalk between MAPK signaling and the insulin signaling pathway through regulation of mTOR, the master regulator of cell growth and metabolism. RSK can perform this function directly, promoting the activity of mTORC1 through phosphoactivation of RAPTOR. RSK can also modulate mTOR signaling indirectly, through site-specific inhibitory phosphorylation of TSC2 (*114*).

One hallmark of nutrient starvation is a high intracellular concentration of AMP, indicating that energy production via ATP hydrolysis is greater than ATP production. High AMP levels lead to the activation of TSC1/2 to repress pro-growth signaling by mTOR (*115*). In conditions of serum starvation, both insulin signaling and EGFR-mediated MAPK signaling are decreased.

Phorbol ester, or PMA, can specifically stimulate MAPK signaling. Use of PMA following serum starvation is a useful approach for the targeted study of the MAPK signaling pathway without involving mTOR signaling. We took advantage of this method of deconvoluting MAPK signaling from mTOR activity to specifically study the role of Listerin on RSK signaling.

In HEK293T cells, we examined the effect of Listerin knockdown on RSK signaling. We used three RNAi variants to ablate the expression of Listerin. Cells were then serum starved overnight to reduce MAPK and mTOR signaling and then the MAPK signaling pathway was activated by addition of phorbol ester (Figure 3.4). We observed the hallmarks of MAPK activation, ERK1/2 phosphorylation, and RSK1 phosphoryation, in response to PMA. TSC2 phosphorylation is significantly increased upon stimulation of MAPK signaling in the absence of Listerin. Phosphorylation of

S6K, a target of mTOR, is also increased. We see a marginal increase in the phosphorylation of RPS6, which is both a direct target of RSK, as well as a target of mTOR.

Our results provide important evidence that regulatory ubiquitylation of RSK by Listerin mediates the activity of this kinase. We are able to observe a modulation of RSK function, as read out by phosphoinhibition of TSC1/2 and increased mTOR signaling through activated S6K, as well as a direct effect on RPS6 phosphorylation. Importantly, the increase in TSC2 phosphorylation following Listerin knockdown is ablated using RSK inhibitor (Supplementary Figure 3.2). However, the increase in phosphorylation of S6K, an mTOR target, is largely independent of the regulation of RSK.

We determined that the serine 1798 residue on TSC2 is an important phosphosite for the readout of RSK activity. Modification at this site is ablated in the absence of functional RSK, but not in response to mTOR inhibition (Supplementary Figure 3.3). This provides us with a method to detect RSK activity at TSC2 in a site-specific manner. Interestingly, phosphorylation of RPS6 was significantly decreased but not ablated by both mTOR and RSK inhibition, suggesting that RPS6 integrates signals from both of these kinases.

Because increased phosphorylation of TSC2 at S1798 by RSK is dependent on regulatory signaling by Listerin, we wanted to determine if this activity is RING domain-dependent. We employed the use of siRNA resistant Listerin, allowing us to knock down endogenous Listerin and express an engineered wildtype or ΔRING (DR)

variant (Supplementary Figure 3.4). Our results indicated that the E3 catalytic RING domain is important for the regulation of RSK signaling.

We were interested to see whether the activation state of mTOR affects the phospho-specific activity of RSK. Using Torin to inhibit mTOR function, we determined that RSK phosphorylates TSC2 in a site-specific manner independent of a functional mTOR complex, and loss of Listerin enhances this modification (Figure 3.4). This provides evidence that Listerin directly regulates RSK function, and has a direct impact on mTOR signaling through site-specific phosphorylation of TSC2.

Because Listerin is known for functioning as a ribosome-associated quality control ligase, we wanted to test whether the effect of Listerin on RSK signaling is mediated through the RQC. We used siRNA to knock down ribosome-associated co-factor NEMF (Figure 3.6). While we were able to observe similar activation of RSK signaling via TSC2 S1798 after Listerin knockdown, loss of NEMF did not result in the same effect.

Our findings suggest a new layer of regulatory control on an important growth factor signaling hub, RSK. Here, we show that Listerin polyubiquitylates RSK1 and RSK2 via its E3 catalytic RING domain, and that this modification is regulatory in nature. We also show that Listerin may perform this function independently of its ribosomal co-factor NEMF. In total, this study has demonstrated a novel function for this E3 that expands upon its known role in protein homeostasis to include regulation of a signaling hub in cellular metabolism.

The Tubulin Glutamylation Complex

Tubulin regulation plays a critical role in the survival and function of mitotic and post-mitotic cells. In actively-dividing cells, tubulin dynamics are crucial for ciliary beating and motility, as well as mitotic spindle integrity (*116*). In post-mitotic neurons, tubulin stability is required for proper neuronal transport along microtubules (MTs) (*88*). Though tubulin exhibits stable abundance in the proteome, its assembly into MTs is dynamically regulated by post-translational modifications (PTMs).

One such modification is glutamylation, in which polyglutamate (polyE) α-chains are extended from the γ-carboxyl group of glutamate residues in the primary sequence of the exposed carboxy-terminal tails of tubulin after MT assembly. C-terminal polyglutamylation creates a strong negative charge that favors interaction with microtubule-associated proteins (MAPs), including those involved in MT disassembly (*117*). Induction of long glutamate side chains induces MT severing in HeLa cells, and leads to an overall loss of MT mass through activation of severing MAPs spastin, katanin p60, and possibly fidgetin (*118*).

The TTLL protein family is crucial in regulating the function of axonemal microtubules involved in ciliary and flagellar functions, and is functionally conserved from protozoa to mammals (*87*). TTLL1 is accepted to be the major brain polyglutamylase. Mice homozygous for the *Ttll1* mutation exhibit pronounced and diverse ciliopathies, including male infertility, abnormal development and maturation of spermatozoa, respiratory exudate accumulation, and other gross pathologies representative of primary ciliary dyskinesia (PCD) and Kartagener syndrome in humans (*119*).

TPGS1 and 2 are found in the same multiproteincomplex with TTLL1, and *Pgs1*-null mice exhibit strong phenotypic similarities to *Ttll1*-deficient mice (*119*). In human patients, copy number variant loss of TTLL1 was associated with poor relapse-free survival and poor overall survival in patients with neuroblastoma (*115*). Mutations in CCP deglutaminases have also been correlated with human ataxia (*120*).

Exploring the functional link between Listerin and polyglutamylation

We have previously shown that the Listerin E3 ligase association with the members of the tubulin glutamylation complex is robust and reproducible using Listerin as bait (Figure 2.7, Supplementary Figure 2.1, Supplementary Figure 3.1). A reciprocal TGC BioID experiment was also effective at capturing these interactions, though to a lesser degree (Figure 3.2A-C). When we tested these substrates for RING-dependent ubiquitylation by Listerin, we observed a response by factor TTLL1 but not NICN1 (Figure 3.3).

To determine if Listerin is influencing the activity of this complex independently of the mRQC, we knocked down Listerin or NEMF, and used immunoblot to detect tubulin glutamylation levels (Figure 3.7). The polyE antibody detects polyglutamylation on α- and β-tubulin subunits, while the GT335 antibody detects both mono- and polyglutamylation on these subunits.

For each knockdown multiple siRNAs were used. Levels of the targeted proteins were decreased in a manner that was consistent across the siRNA variants. NEMF knockdown was determined by RT-PCR to be 10-40% (data not shown). Knockdown of Listerin and NEMF had no detectable effect on the levels of polyglutamylase TTLL1, or the TGC complex member TPGS1. As expected from our

previous findings, levels of RSK1 were unchanged following Listerin or NEMF knockdown.

For both the Listerin and NEMF knockdowns, polyglutamylation was affected inconsistently across siRNA variants. Using siLTN1-3 to knock down Listerin, we saw a specific increase in monoglutamylation of α-tubulin (Figure 3.7, GT335 top band). This is consistent with an increase in TTLL1 glutamylase activity. However, although siLTN1-1 and siLTN1-2 did decrease Listerin protein levels to the same extent as siLTN1-3, tubulin glutamylation was not increased in these samples.

We used NEMF knockdown to determine if disruption of the mRQC affects tubulin glutamylation. We did not detect tubulin glutamylation at levels above the negative control. This may, however, be a result of the poor knockdown efficiency of our siRNAs against NEMF.

Discussion

Listerin as a Regulatory Ligase

Here, we verify the identities of the first known endogenous substrates of Listerin in mammals. Biochemical validation supports RSK2 as a *bonafide* regulatory substrate of Listerin. This broadens our knowledge of the molecular function of Listerin, and casts a new role for this E3 in regulation of MAPK signaling hub RSK. While Listerin is known to be involved in the destruction of aberrant proteins that arise from ribosome stalling, our work uncovers a new role for Listerin as a regulator of the RSK signaling hub identifies this ligase as a potential influencer of translational control.

One hypothesis is that Listerin can signal translational stress to the ribosome and mTOR through RSK. In this scenario, Listerin senses translational stalling and responds by decreasing protein translation through inhibition of RSK (Figure 3.8). Since Listerin contains a polybasic sequence, its levels are likely kept low by the mRQC itself. When translational stress passes a certain threshold, the function of the mRQC becomes saturated and switches from favoring ubiquitylation to favoring aggregation, thereby permitting Listerin to be diverted to other cellular functions. Listerin may then directly suppress RSK levels, thereby affecting global translation rates, as well as cell survival through mTOR.

Tubulin Glutamylation and the Role of Listerin

We tested the function of Listerin with tubulin glutamylation. Listerin knockdown has an inconsistent effect on tubulin mono- and polyglutamylation. While knockdown of NEMF appears to increase α-glutamylation, these results were mild and fairly inconsistent across knockdowns. If NEMF is required for the polyglutamylation phenotype, it is possible that Listerin and NEMF act in concert to regulate the TGC. It will be important for future studies to determine whether the mRQC itself has an influence on tubulin glutamylation.

Our identification of the tubulin glutamylation complex protein TTLL1 as a regulatory substrate of Listerin is promising. However, our inconsistent results with both our biochemical and functional assays indicate that this topic warrants further study. Because tubulin glutamylation is critical for microtubule stability and therefore neuronal development and homeostasis, further elucidation of the role of Listerin in

microtubule dynamics will contribute to our understanding of the *Lister* phenotype in mice.

Post hoc Analysis of BioID Results

Based on our results comparing the efficacy of the three proteomics approaches in Chapter 2, we concluded that BioID is advantageous for the capture of both transient and stable protein-protein interactions. Stable interactors for bait proteins Listerin, RSK2, and TGC were readily identified in each of our BioID experiments (Figure 3.2, Supplementary Figure 3.1). Detection of what we have verified in this chapter to be bonafied ligase-substrate interactions was variable across biological replicate experiments (Figure 2.7, Supplementary Figure 2.1, Figure 3.2). Together, our data reinforces that the use of BioID for the detection of transient and weak interactions requires replicate experiments in conjunction with biochemical validation to confirm any putative interactions.

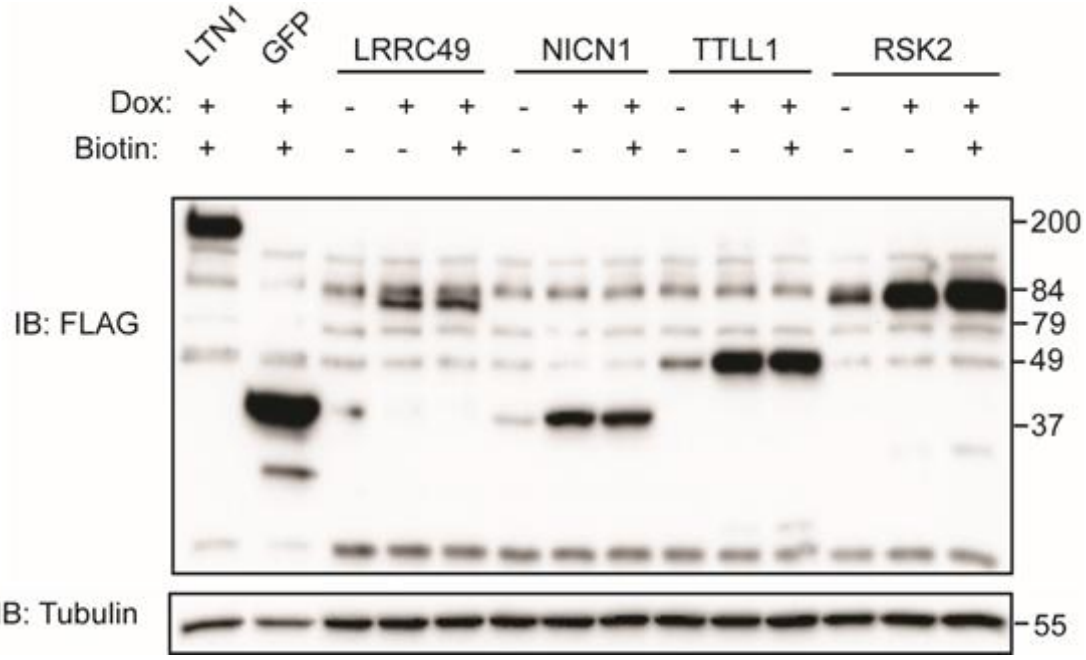


Figure 3.1: Expression of transgenic cell lines for TGC and RSK BioID experiment
HEK293T cells expressing N-FLAG-BirA* tagged TGC or RSK proteins under a Tet-ON promoter were treated with or without doxycycline and biotin. Also included in this experiment are Listerin, and GFP as a negative control.

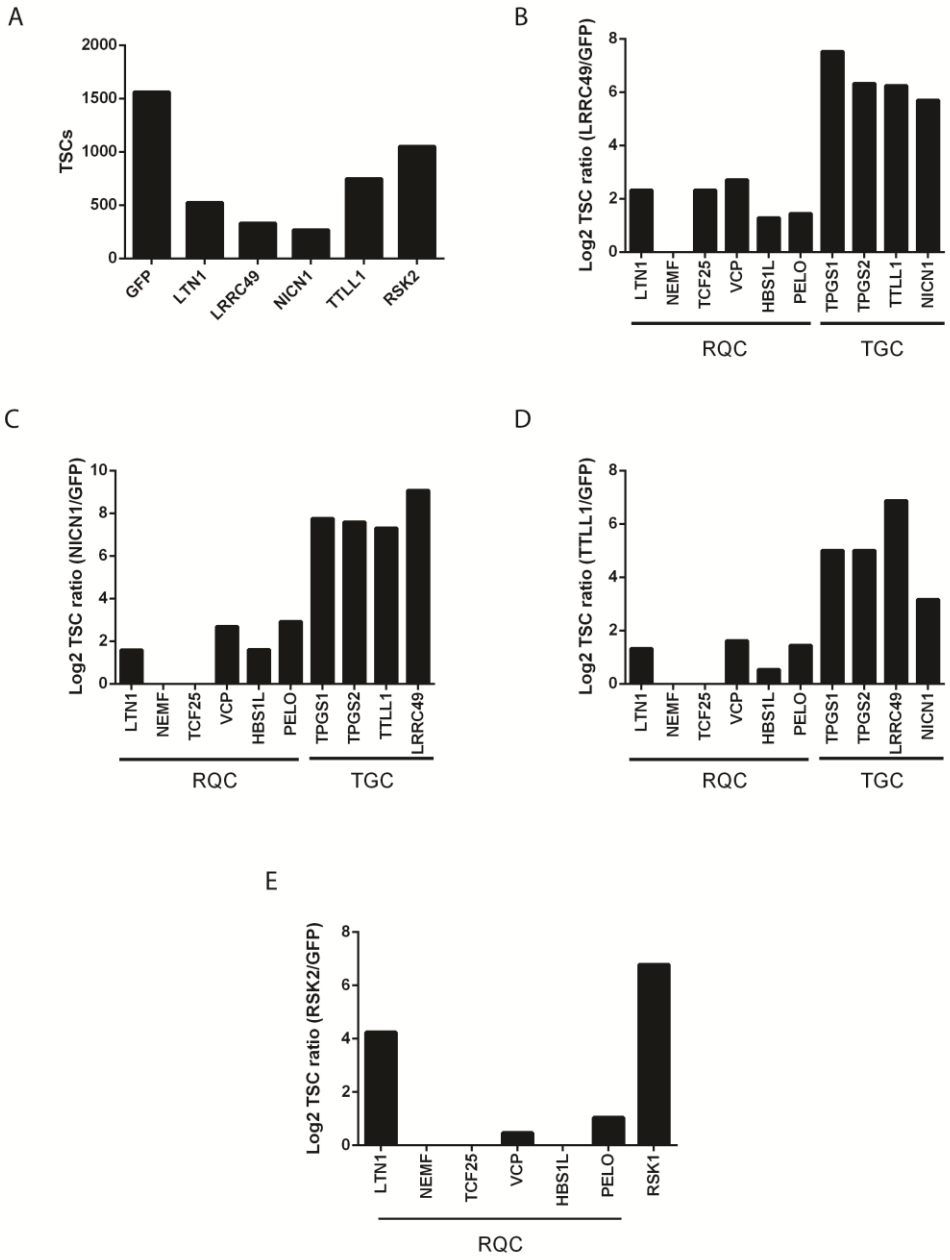


Figure 3.2: TGC and RSK BioID reveals interactions between Listerin and putative substrates LRRC49 and RSK2

Fold change of total spectral counts (TSCs) over GFP control from a BioID experiment using Listerin, LRRC49, NICN1, and TTLL1, or RSK2 as bait. A.) total spectral counts (TSCs) were normalized. Interactions were quantified using the $\log_2$ TSC ratio of bait to control for TGC proteins B.) LRRC49, C.) NICN1, and D.) TTLL1, as well as E.) RSK2. Proteins labeled "RQC" are known to be part of the mRQC, or the upstream ribosomal splitting factors. "TGC" proteins are members of the tubulin glutamylation complex.

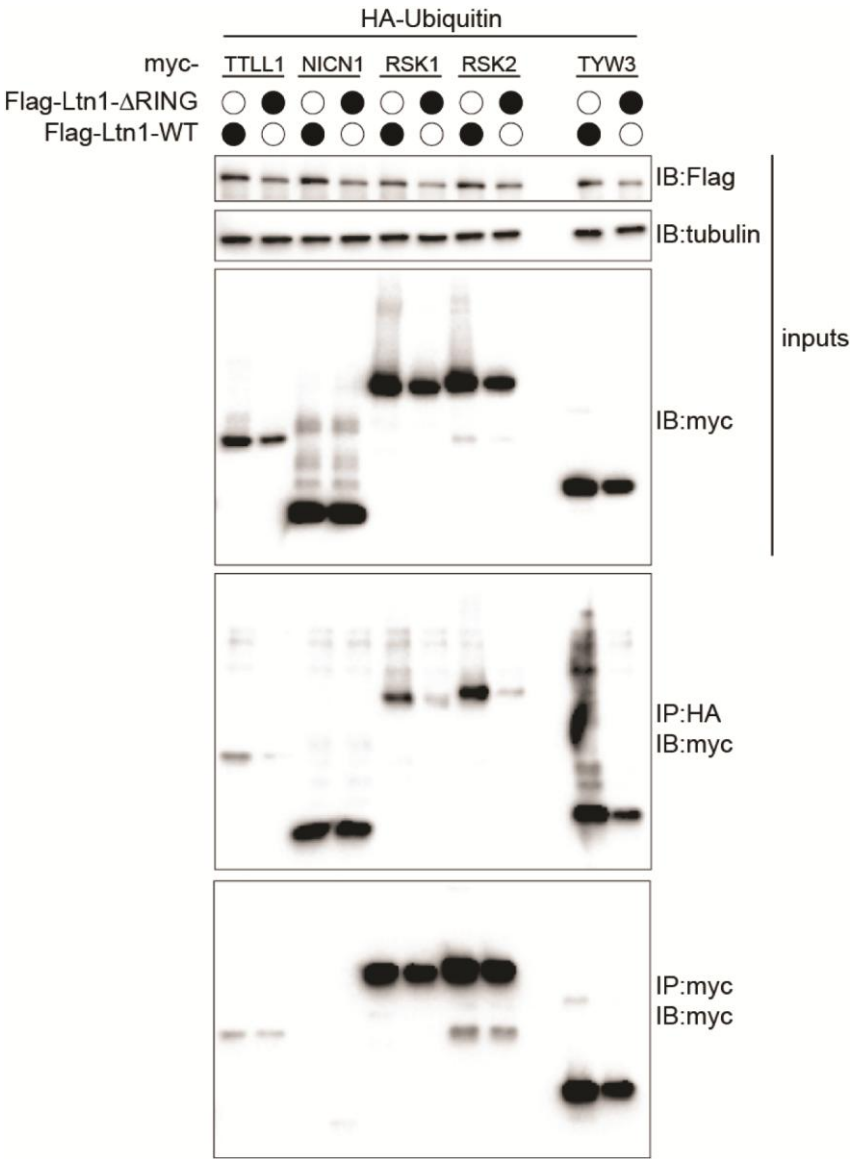


Figure 3.3: BioID candidate interactors undergo regulatory ubiquitylation by Listerin in a RING-dependent manner
HEK293T cells transfected with HA-Ubiquitin were additionally transfected with FLAG-Listerin WT or ΔRING, along with myc-tagged candidate interaction proteins. Immunoprecipitation was performed using either HA or myc antibody-conjugated resin.

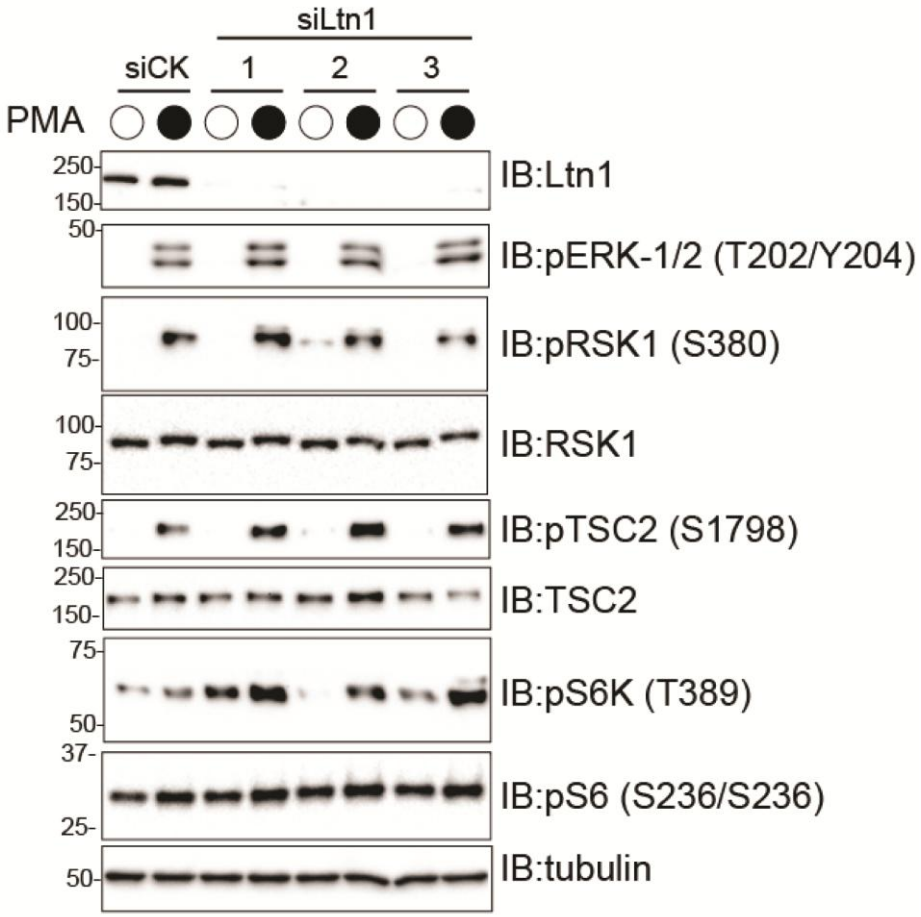


Figure 3.4: Listerin knockdown increases downstream activation of RSK targets
HEK293T cells transfected with siRNA against Listerin were serum starved and then treated with and without phorbol ester (PMA) to activate MAPK signaling.

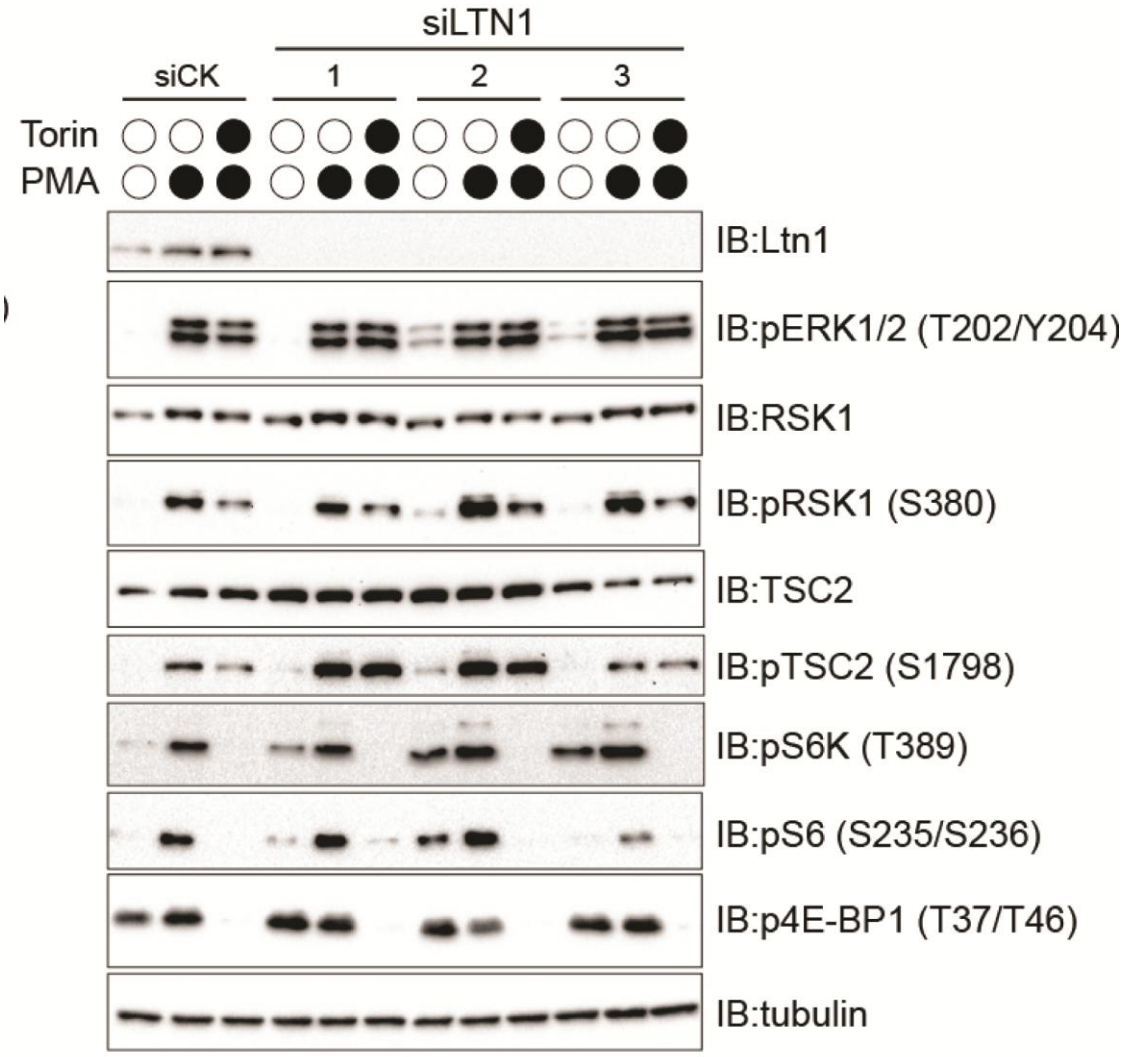


Figure 3.5: Increased RSK signaling is partially independent of mTOR activity
HEK293T cells transfected with siRNA against Listerin were serum starved and then treated with and without PMA to activate MAPK signaling. PMA-treated cells were also treated with or without mTOR inhibitor Torin.

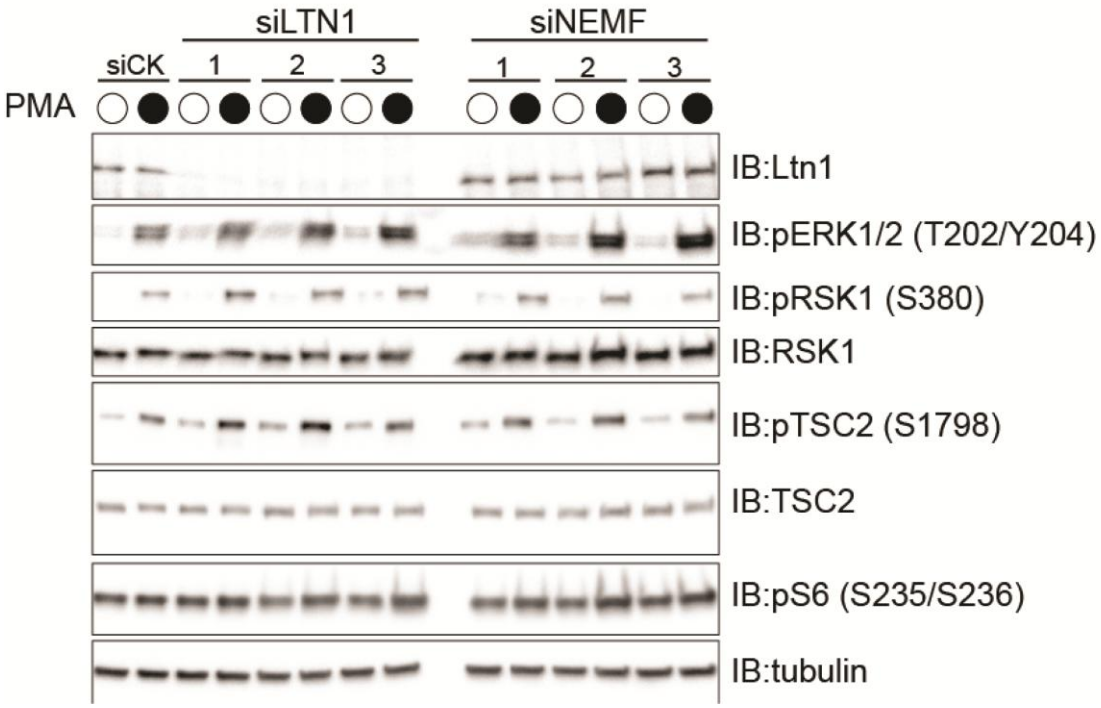


Figure 3.6: Listerin regulates RSK signaling largely independent of mRQC function

HEK293T cells transfected with siRNA against Listerin or NEMF were serum starved and then treated with and without phorbol ester PMA to activate MAPK signaling.

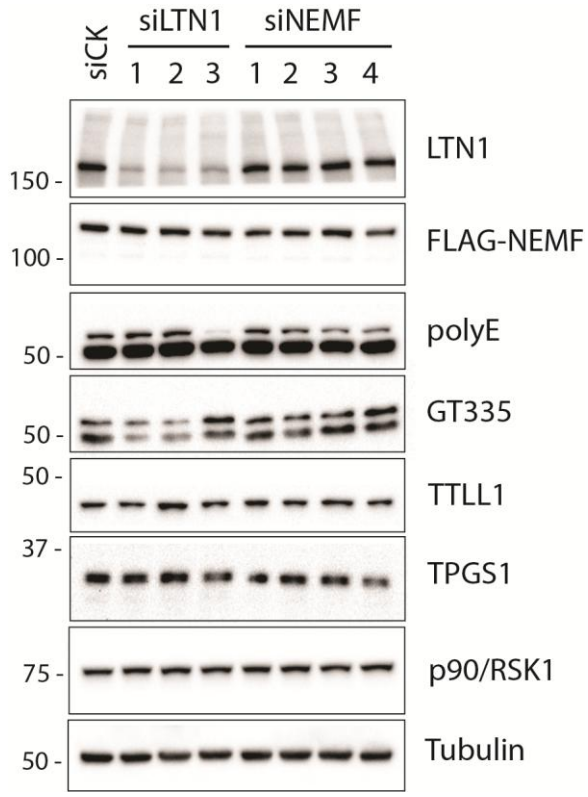


Figure 3.7: Listerin and NEMF knockdown have an inconsistent effect on tubulin polyglutamylation

HeLa cells expressing Tet-ON FLAG-BirA* NEMF were transfected with siRNA against either Listerin or NEMF. Lysates were analyzed using Western blotting for polyglutamylation markers polyE and GT335 (upper band: α-tubulin glutamylation; lower band: β-tubulin glutamylation), as well as TGC factors TTLL1 and TPGS1.

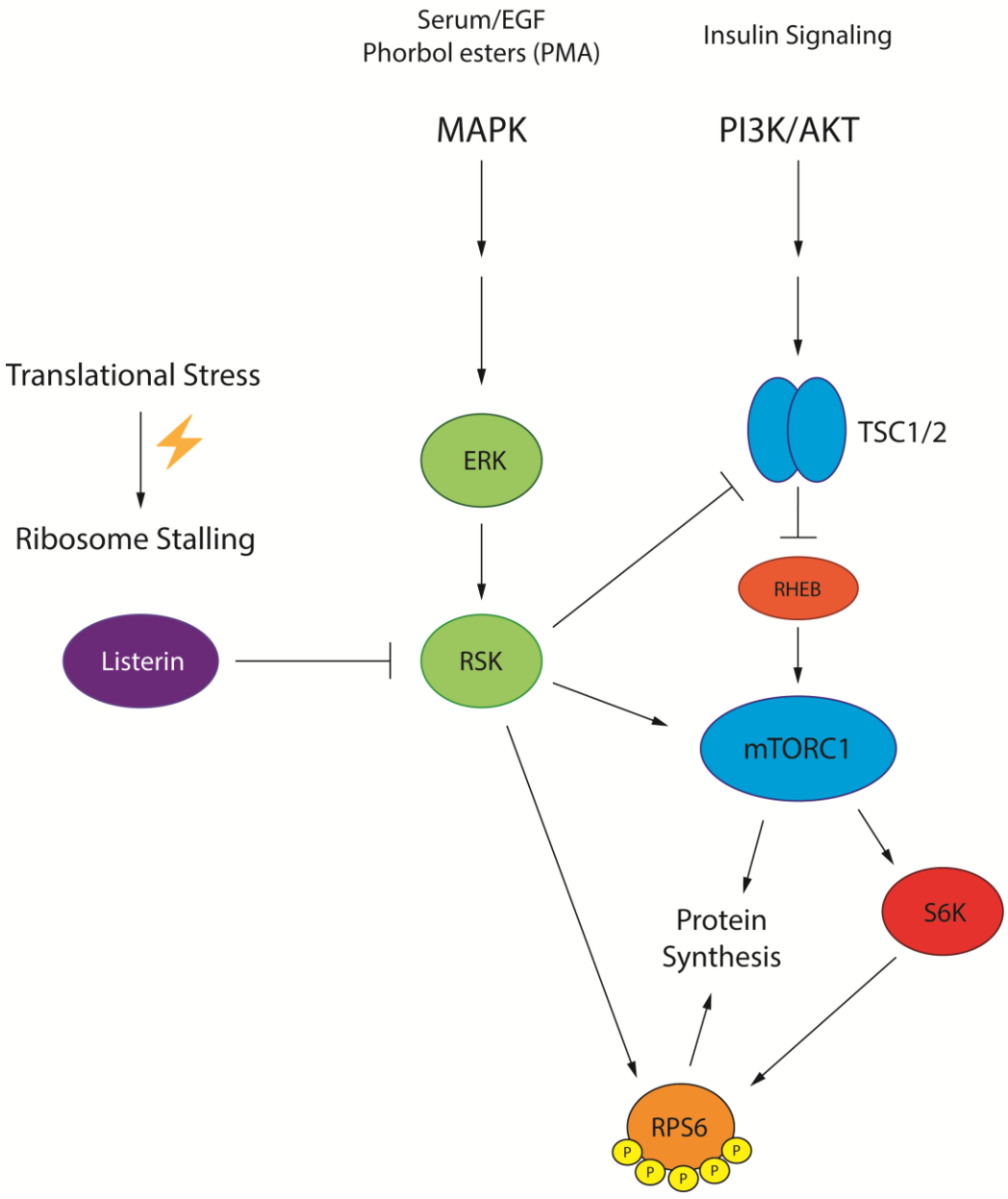


Figure 3.8: Model of Listerin-RSK interaction
Proteomic and biochemical data identify Listerin as a regulator of RSK activity.

Materials and Methods

Cells, culturing conditions, and generation of expression lines

HEK293T and HeLa cells were cultured in Dulbecco's Modified Eagle Medium (Thermo Fisher Scientific) supplemented with 10% FBS. Bacterial clones expressing Listerin, LRRC49, NICN1, TTLL1, RSK2, and TYW3 were taken from the ORFeome, and cloning vectors were generated using Gateway recombination. Stable cell lines were generated using lentiviral transduction of target HEK293T cells, followed by selection with 1μg/mL puromycin. All Flip-In cell lines were generated for inducible expression using the Tet-ON system, and were induced using doxycycline (5μg/mL). For serum starvation, cells were cultured in DMEM without FBS.

Treatments

The following reagents were used in the biochemical validation experiments shown in Figures 3.4-3.7 and Supplementary Figures 3.2-3.4: PMA – 1μM, Torin1 – 100nM, RSKi (BI-D1870) – 10μM.

RNAi experiments

All knockdowns using siRNA were performed using Lipofectamine RNAiMAX Reagent (Thermo Fisher Scientific) containing a final concentration of 10μM siRNA in Opti-MEM Reduced Serum Medium (Thermo Fisher Scientific). Cells were harvested after 72hrs.

Lysis, streptavidin pulldown

Cell pellets were incubated in mammalian cell lysis buffer (MCLB) at an approximate 2:1 (v:w) ratio per pellet. Protein from cleared lysates was quantified

using a BCA protein assay. Streptavidin pulldown was performed using streptavidin-conjugated agarose beads with overnight inversion at 4°C. Beads were washed thoroughly with cold PBS. Trypsin digestion was performed on the beads, using 1μg of Promega Sequencing Grade Modified Trypsin.

Western analysis

For each sample, 10-20μg protein diluted in 4x Laemmli sample buffer containing β-mercaptoethanol were loaded onto hand-cast SDS-PAGE gels. Transfer was performed using the semi-dry BioRad TransBlot Turbo Transfer System. The primary and secondary antibodies used are notated in the supplementary materials and methods section.

BioID mass spectrometry

HEK293T cells expressing the BirA R118G (BirA*) fused in-frame with FLAG-tagged bait protein were co-treated with doxycycline (5μg/mL) and biotin (50μM). Cell pellets were harvested using trypsin, and stored at -80°C until lysis. Protein concentrations were determined using a BCA protein assay. The lysates are divided according to protein concentration: 600μg for Western blot (100μg for whole cell lysates, and 500μg for streptavidin pulldown followed by Western blot).

Mass spectrometry data analysis

All datasets were exported as .RAW files, and imported to a proprietary data analysis suite. Peptide identification was performed through SEQUEST using the UniProt database. Peptide matches were filtered using Linear Discriminant Analysis using a false discovery rate (FDR) of 1%. Protein IDs were filtered using Protein Sieve with a 1% FDR, and clustered using the Protein Assembler algorithm.

The BirA* sequence was used as a bait identifier. All total spectral counts (TSCs) in our experimental BioID runs were normalized to GFP control with respect to BirA* abundance. Fold change was represented as the $\log_2$ ratio of experimental to control TSCs, and all zero denominator counts were treated as single matches.

Author Contributions

N.Z. and E.J.B. conducted and performed the analysis for the repeat BioID experiment in Figure 3.1. N.Z. and E.J.B. generated constructs and cell lines, and N.Z. conducted and performed the analysis for the reciprocal BioID experiment in Figure 3.2. E.J.B. conducted and analyzed the functional assays for RSK proteins (Figures 3.4-6), and N.Z. conducted and analyzed the functional assays for the tubulin glutamylation complex (Figure 3.7). A special thank you to Jeff Liao, who performed Western blotting for a number of the figures in Chapter 3.

Chapter 3 is currently being prepared, along with Chapter 2, for submission for publication. Zuzow, Nathan; Leonard, Marilyn; Liao, Jeffrey; Bennett, Eric J. The author of this dissertation was the primary investigator and author of this material.

Conclusions and Final Remarks

The pathology of a number of degenerative diseases can be attributed to the toxic accumulation of misfolded proteins. As an organism ages, progressive decline in protein homeostasis networks increases the incidence of misfolding events, making it even more important for the cell to maintain properly-functioning protein quality control systems. E3 ubiquitin ligases are particularly important in the clearance of these proteins before they can pose a significant threat to the cell.

Listerin is an important E3 ubiquitin ligase in co-translational protein quality control. Its essential function, targeting aberrant nascent polypeptides arising from no-go and non-stop decay, is conserved from yeast to humans. The implication of Listerin in detoxification of aberrant Huntingtin protein (*69*), and the prevention of ALS-like neurodegeneration in mice (*73*) underscore the importance of this ligase in general cellular homeostasis.

Listerin is primarily known for the severe neurodegenerative phenotype exhibited in hypomorphic mice, as well as the critical function of its yeast ortholog Ltn1 in the ribosomal quality control complex. While the proteins comprising the RQC are conserved from yeast to humans, their functional interaction in mammals has only been implied by biochemical and structural data.

In our work probing the composition and function of the mRQC, we were able to demonstrate that, at steady state, interactions mRQC proteins mirror those observed in yeast. Surprisingly, widespread translational inhibition did not induce mRQC proteins to form more robust interactions. Instead, we noted that mRQC proteins respond in a manner that is consistent with a shift in the modality of the mRQC

complex, from favoring the Listerin-mediated degradation pathway at steady state to favoring the NEMF-mediated CATylation/aggregation pathway under global translational stress.

We did observe mRQC proteins shifting to ribosome subunit-containing fractions following treatment with CHX, which is consistent with the notion that this complex addresses ribosome stalls. This is not inconsistent with our previous observations, however. It is possible that widespread translational stalling mediated by CHX saturates the mRQC, dampening its ability to mediate targeted degradation of aberrant nascent polypeptides.

While the contribution of Listerin to ribosomal protein quality control is evident, and we were able to confirm orthologous RQC interactions in mammalian cells, the endogenous substrates of Listerin remain elusive. Our attempts to functionally characterize the complex were hindered by errors in assay development and implementation. We became intrigued by the possibility that proximity-based proteomic methods would assist, not only in capture and identification of stable interacting partners with Listerin, but transient ligase-substrate interactions as well.

In our work dissecting the cellular function of Listerin through combined use of traditional affinity purification and proximity-dependent proteomics techniques, we discovered that BioID was uniquely useful for detecting the known Listerin-NEMF interaction. BioID also provided us with an array of potential novel interactors with Listerin, evidencing that this can be a powerful orthogonal approach for use alongside traditional AP-MS in the detection of E3 substrates and interacting partners.

Using BioID, we uncovered two new classes of putative interacting proteins for Listerin: RSK family proteins RSK1 and 2, as well as members of the tubulin glutamylation complex. Given the phenotype of the *Listerin* mouse, the tubulin glutamylation complex is alluring as a potetnial target of Listerin, since a single mutation in a tubulin deglutamylase enzyme is responsible for the severe neurodegenerative phenotype of *pcd* mice (*121*). However, our functional assays probing the effects of Listerin and NEMF perturbation on tubulin glutamylation were inconclusive.

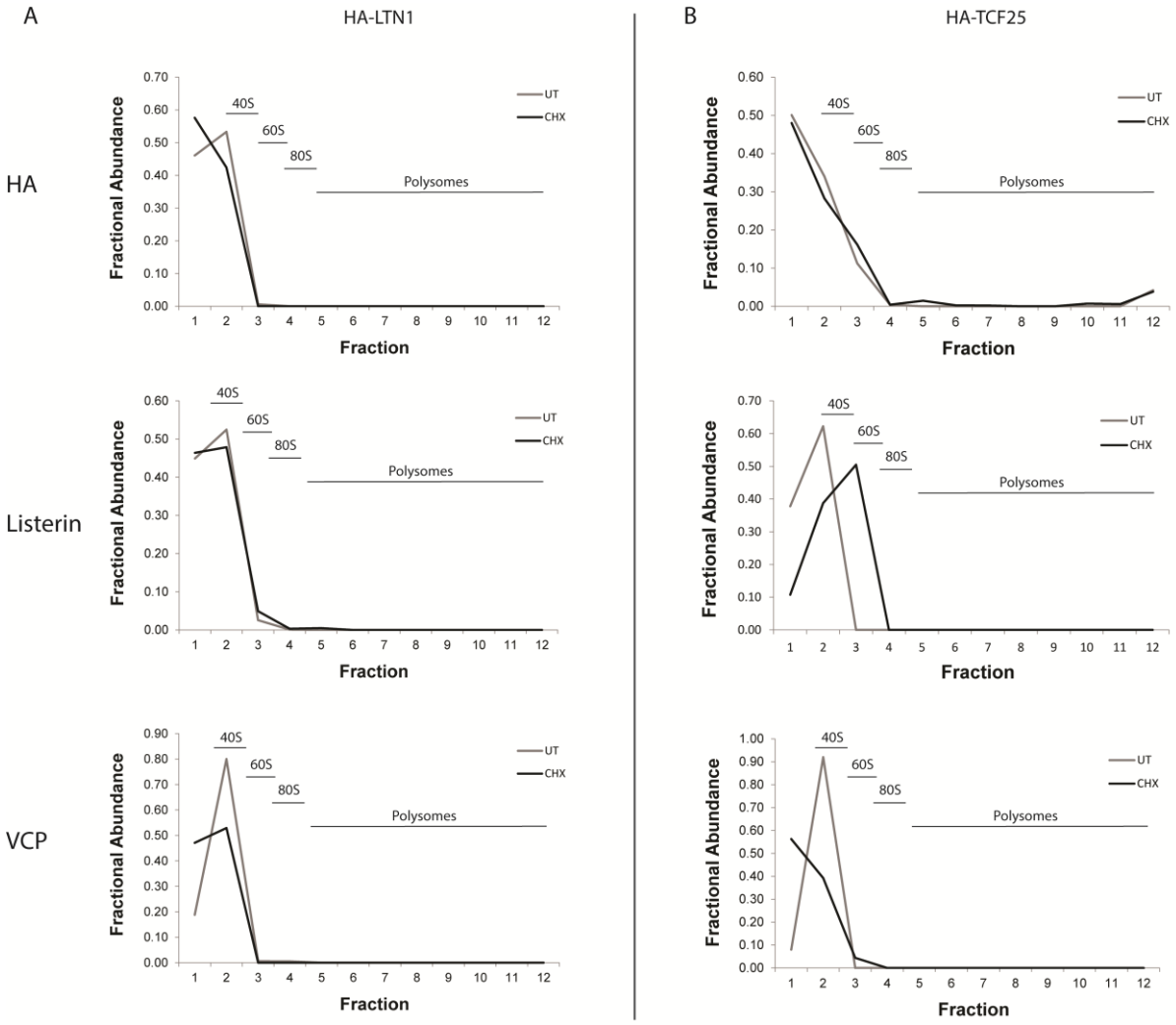
Equally alluring was the putative interaction between Listerin and the two ribosomal s6 kinases RSK1 and RSK2, which act downstream of MAPK as part of an important signaling hub for cell growth and survival. In our work probing the function of Listerin with respect to RSK proteins, we were able to demonstrate that Listerin ubiquitylates RSK1 and RSK2 in a RING dependent manner. Because neither of these proteins is stabilized in the absence of ubiquitylation by Listerin, we surmised that this modification is regulatory in nature.

Upon activation, RSK positively regulates translation directly through ribosomal protein S6 and mTOR, as well as indirectly through inhibition of TSC1/2. Using site-specific TSC2 phosphorylation and S6K phosphorylation as proxies for increased mTOR activity, were able to demonstrate that genetic knockdown of Listerin can have a stimulatory effect on signaling important to protein synthesis and cell survival. Taken together, our results establish that Listerin can act outside of its canonical function as the mRQC ligase to regulate proteins involved in protein synthesis and cellular survival.

In total, our studies of Listerin have led to the discovery of novel substrates for this essential ligase. The identification of RSK proteins as *bonafide* Listerin substrates opens the door to new research at the intersection of co-translational quality control and growth and survival signaling. The neurodegenerative phenotype of the *Listerin* mouse is suspected to arise from impairment of defective protein clearance, which points to Listerin as part of the mRQC. However, the novel functions we present here underscore the importance of using orthogonal proteomic approaches when studying E3-substrate interactions, and establish the importance of this ligase in general cellular homeostasis. Given the diverse nature of the substrates targeted by Listerin, further studies of this ligase may lead to its implication it in human pathologies in which its role has yet to be classified.

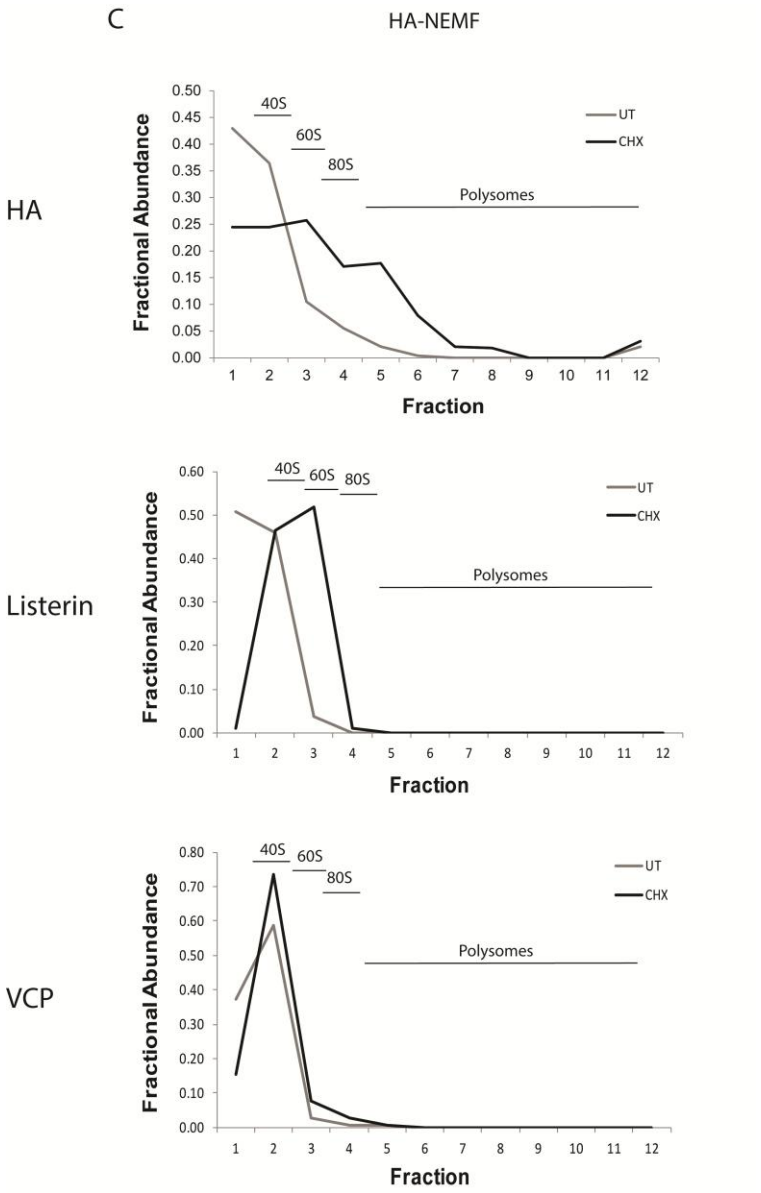
Appendix

Chapter 1 Supplementary Figures

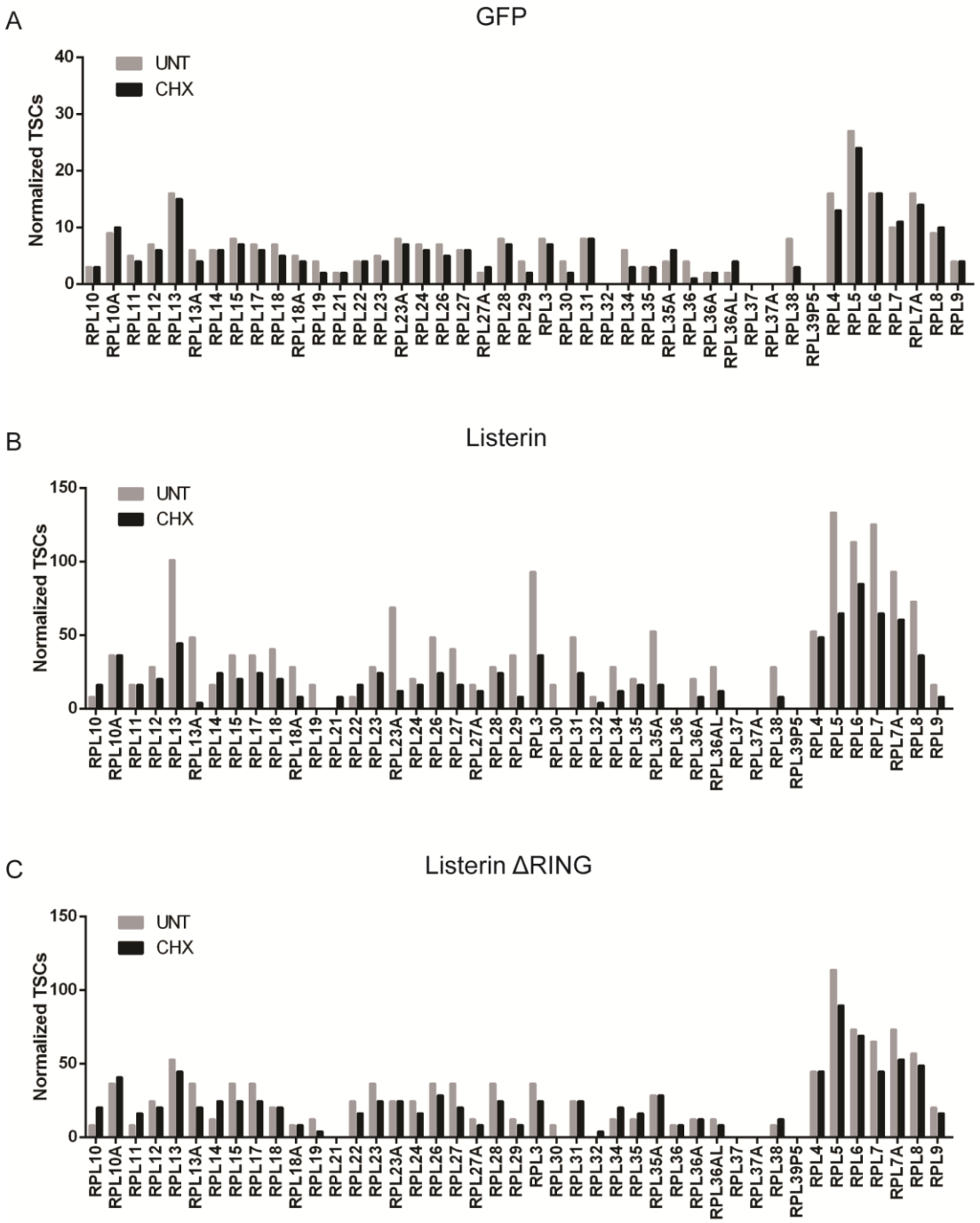


Supplementary Figure 1.1: mRQC proteins shift to ribosome-containing fractions following CHX treatment

Lysates from HEK293T cells treated with or without CHX were analyzed using sucrose density centrifugation on a 10-50% gradient. These cell lines express FLAG-tagged A.) Listerin, B.) TCF25, or C.) NEMF (see next page) under a Tet-inducible promoter. Fractional abundance of the proteins probed in our immunoblots is the ratio of band intensity for each fraction over total intensity across all fractions.

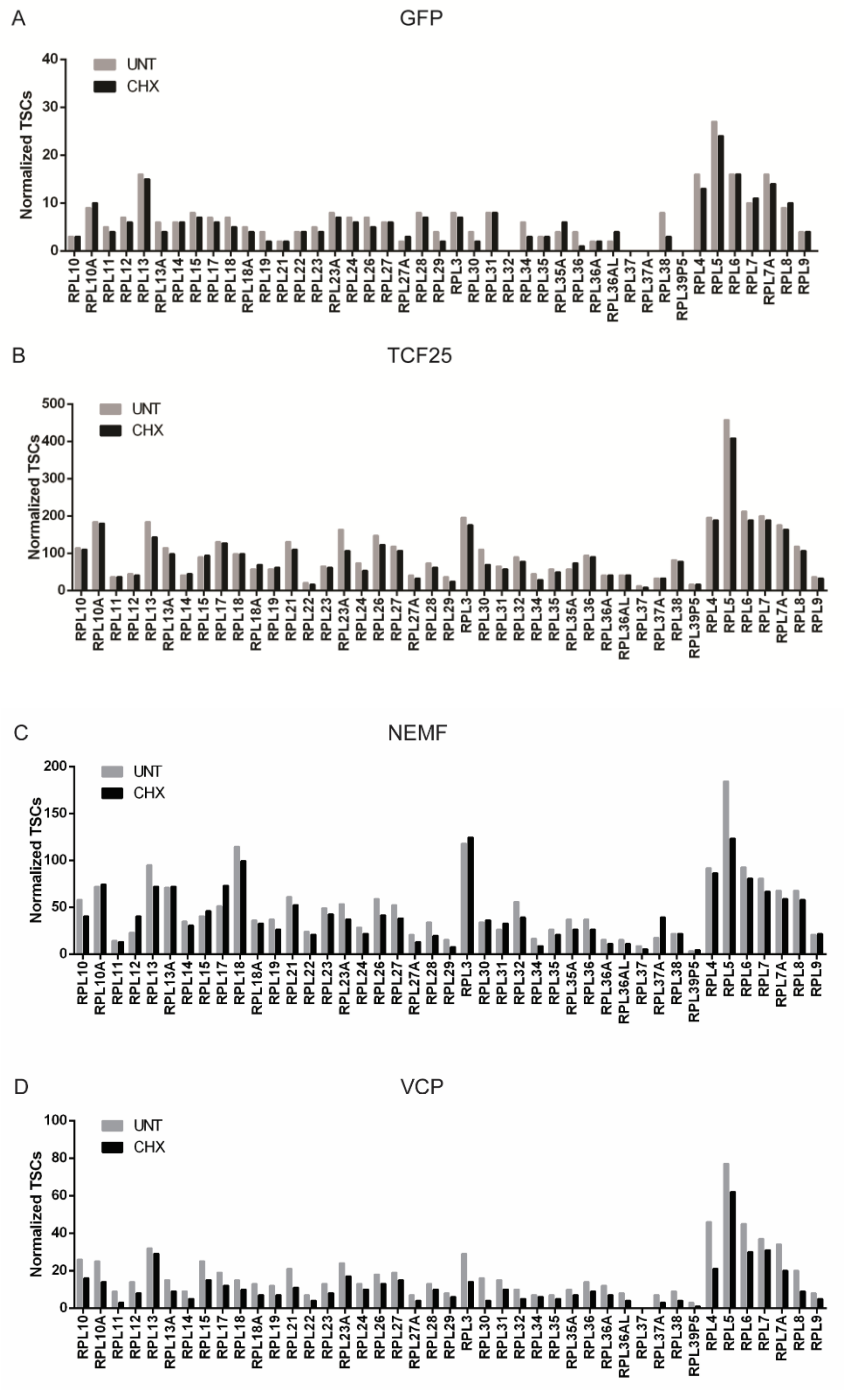


Supplementary Figure 1.1: mRQC proteins shift to ribosome-containing fractions following CHX treatment, continued



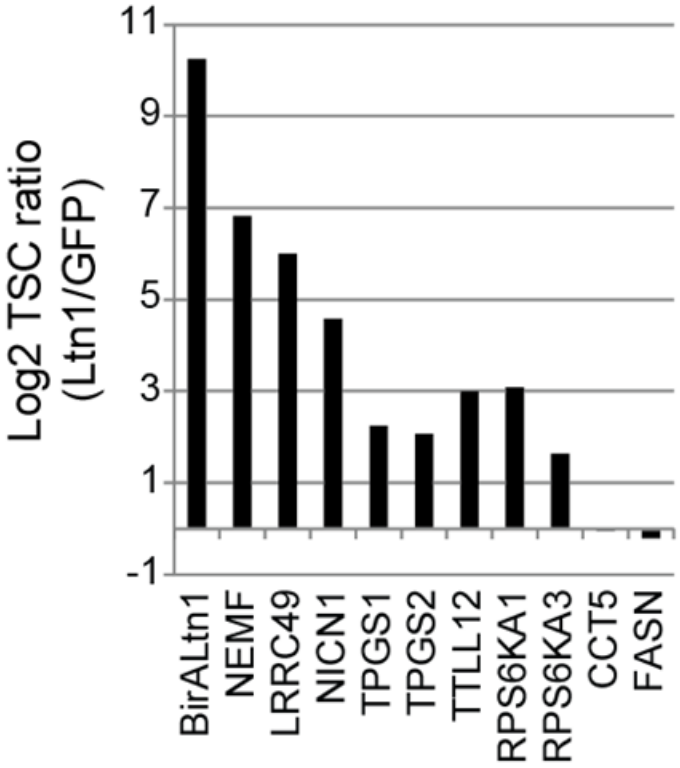
Supplementary Figure 1.2: CHX treatment does not increase association between Listerin and the 60S ribosome

HEK293T cells expressing FLAG-tagged A.) GFP, B.) wild type Listerin, or C.) ΔRING Listerin were treated with or without CHX. TSCs were normalized between each treatment pair, and then bait normalized to GFP. Reported are normalized TSCs for proteins comprising the 60S ribosome.



Supplementary Figure 1.3: CHX treatment does not increase association between TCF25, NEMF, and VCP and the 60S ribosome
HEK293T cells expressing FLAG-tagged A.) GFP, B.) TCF25, C.) NEMF, or D.) VCP were treated with or without CHX. TSCs were normalized between each treatment pair, and then bait normalized to GFP. Reported are normalized TSCs for proteins comprising the 60S ribosome.

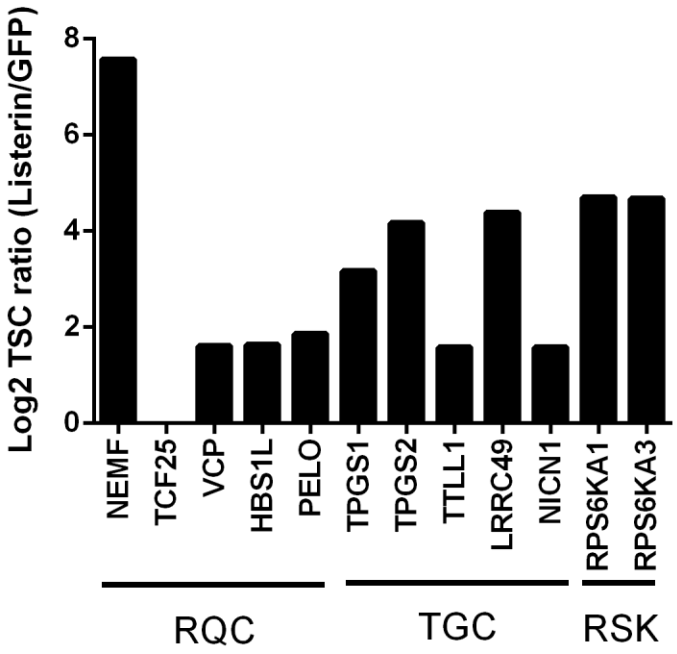
Chapter 2 Supplementary Figures



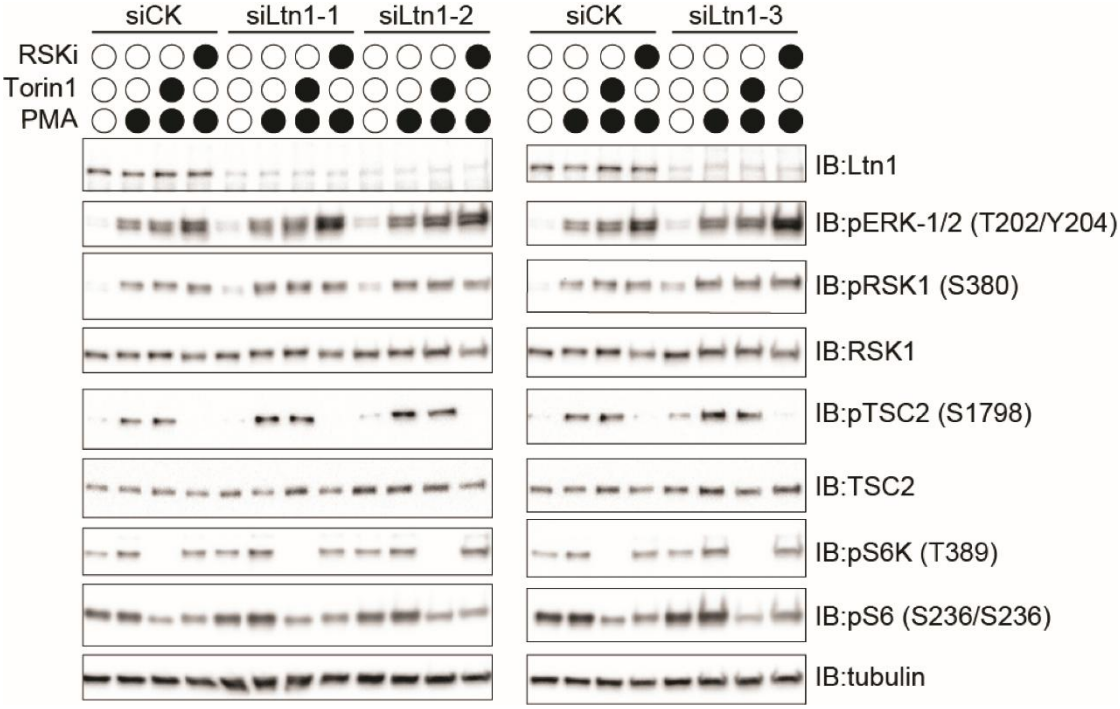
Supplementary Figure 2.1: Listerin BioID confirms associations with TGC and RSK proteins

A biological replicate of the Listerin BioID experiment shows that previously-identified interactions with Listerin are reproducible. Also included in the analysis are tubulin polyglutamylation factors TPGS1/2 and TTLL12, as well as deglutamylase CCT5. TSCs were bait normalized to GFP and interactions were quantified using the $\log_2$ TSC ratio of Listerin to GFP control.

Chapter 3 Supplementary Figures

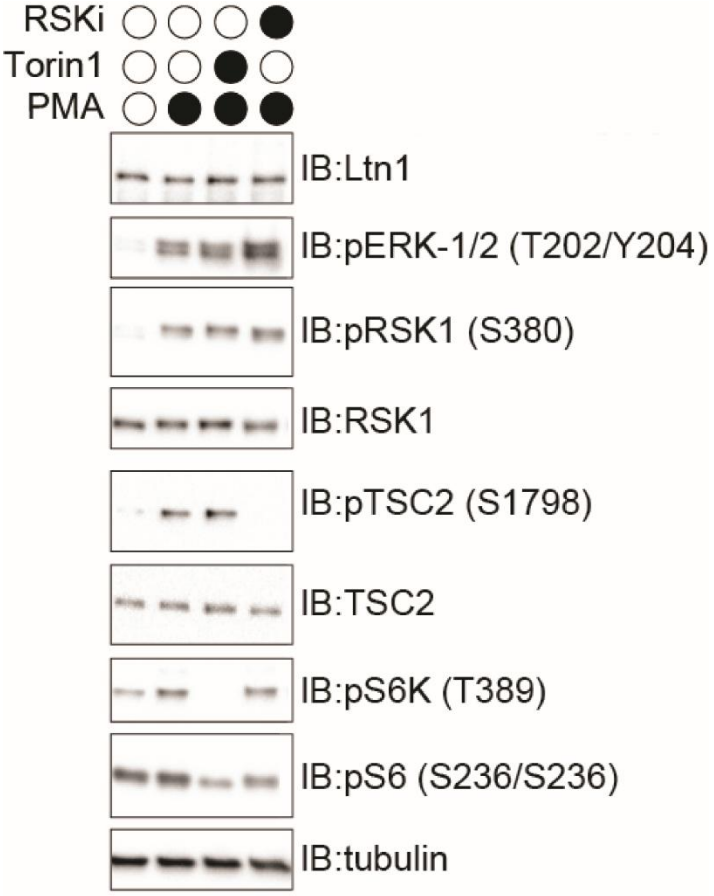


Supplementary Figure 3.1: Repeat Listerin BioID experiment confirms and expands the list of TGC and RSK interactors
HEK293T cells expressing N-FLAG-BirA* Listerin or GFP under a Tet-ON promoter were treated with doxycycline and biotin. Following a label-free proteomics experiment, TSCs for all proteins were normalized and quantified as the $\log_2$ transformation of the ratio of TSC abundance in the Listerin experiment vs. GFP control experiment.

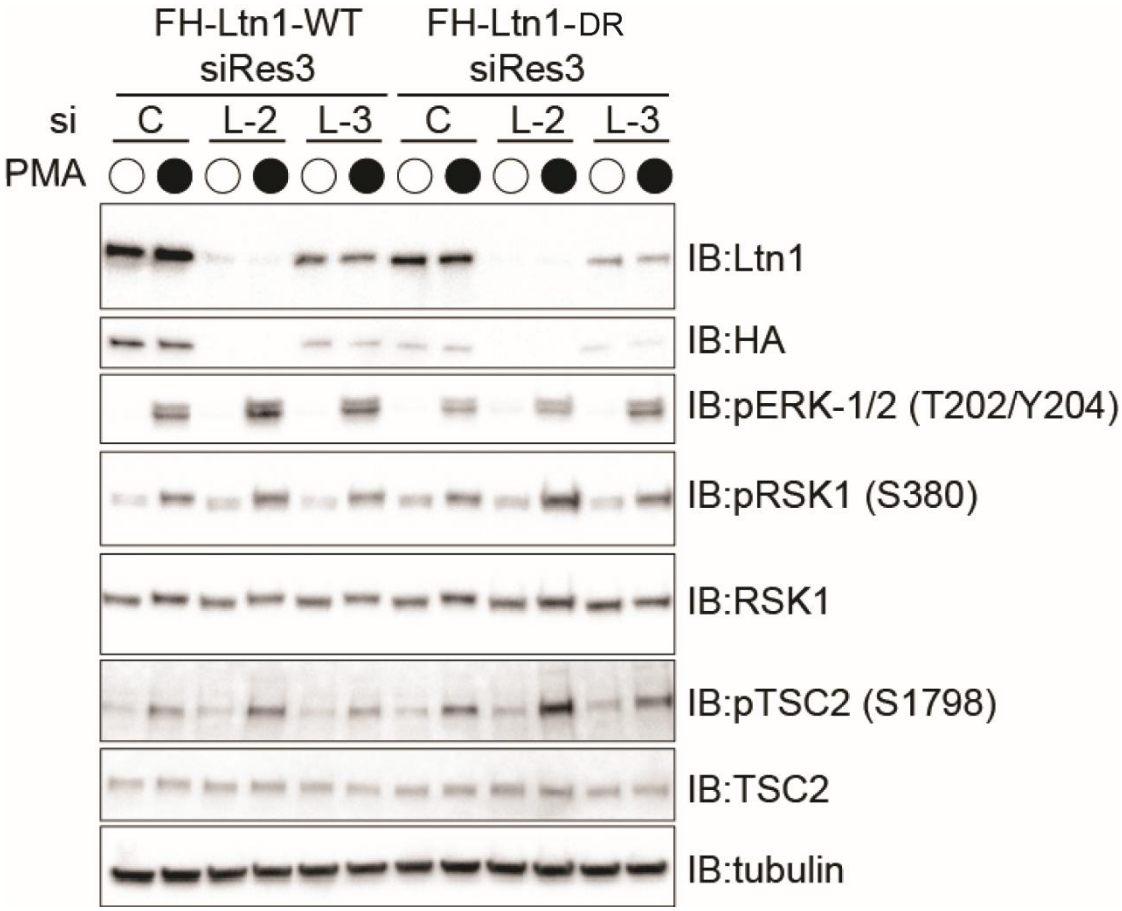


Supplementary Figure 3.2: RSK inhibition ablates increased RSK signaling induced by loss of Listerin

HEK293T cells transfected with siRNA against Listerin were serum starved and treated with or without phorbol ester. PMA treated cells were also treated with or without mTOR inhibitor Torin1, or RSK inhibitor



Supplementary Figure 3.3: Phosphorylation of TSC at S1798 is a specific marker of RSK activity following MAPK activation
HEK293T cells were serum starved and treated with or without PMA, along with either Torin1 or RSK inhibitor.



Supplementary Figure 3.4: Listerin suppresses RSK signaling in a RING domain-dependent manner
Wild type or siLtn1-3 resistant HEK293T cells were transfected with either FLAG-HA Listerin WT or ΔRING, as well as with siRNA against Listerin. Following serum starvation, cells were treated with or without PMA.

References

1. J. W. Hershey, N. Sonenberg, M. B. Mathews, Principles of translational control: an overview. *Cold Spring Harbor perspectives in biology* **4**, (2012); published online EpubDec (10.1101/cshperspect.a011528).

2. M. Vendruscolo, T. P. Knowles, C. M. Dobson, Protein solubility and protein homeostasis: a generic view of protein misfolding disorders. *Cold Spring Harbor perspectives in biology* **3**, (2011); published online EpubDec (10.1101/cshperspect.a010454).

3. W. E. Balch, R. I. Morimoto, A. Dillin, J. W. Kelly, Adapting proteostasis for disease intervention. *Science* **319**, 916-919 (2008); published online EpubFeb 15 (10.1126/science.1141448).

4. C. Janke, K. Rogowski, D. Wloga, C. Regnard, A. V. Kajava, J. M. Strub, N. Temurak, J. van Dijk, D. Boucher, A. van Dorsselaer, S. Suryavanshi, J. Gaertig, B. Edde, Tubulin polyglutamylase enzymes are members of the TTL domain protein family. *Science* **308**, 1758-1762 (2005); published online EpubJun 17 (10.1126/science.1113010).

5. J. Parker, Errors and alternatives in reading the universal genetic code. *Microbiological reviews* **53**, 273-298 (1989); published online EpubSep (

6. A. Tiessen, P. Perez-Rodriguez, L. J. Delaye-Arredondo, Mathematical modeling and comparison of protein size distribution in different plant, animal, fungal and microbial species reveals a negative correlation between protein size and protein number, thus providing insight into the evolution of proteomes. *BMC research notes* **5**, 85 (2012); published online EpubFeb 01 (10.1186/1756-0500-5-85).

7. D. A. Drummond, C. O. Wilke, The evolutionary consequences of erroneous protein synthesis. *Nature reviews. Genetics* **10**, 715-724 (2009); published online EpubOct (10.1038/nrg2662).

8. F. Chiti, C. M. Dobson, Protein misfolding, functional amyloid, and human disease. *Annual review of biochemistry* **75**, 333-366 (2006)10.1146/annurev.biochem.75.101304.123901).

9. C. M. Dobson, Protein misfolding, evolution and disease. *Trends in biochemical sciences* **24**, 329-332 (1999); published online EpubSep (

10. H. Koga, S. Kaushik, A. M. Cuervo, Protein homeostasis and aging: The importance of exquisite quality control. *Ageing research reviews* **10**, 205-215 (2011); published online EpubApr (10.1016/j.arr.2010.02.001).

11. A. G. Ryazanov, B. S. Nefsky, Protein turnover plays a key role in aging. *Mechanisms of ageing and development* **123**, 207-213 (2002); published online EpubJan (

12. N. P. Dantuma, L. C. Bott, The ubiquitin-proteasome system in neurodegenerative diseases: precipitating factor, yet part of the solution. *Frontiers in molecular neuroscience* **7**, 70 (2014)10.3389/fnmol.2014.00070).

13. R. J. O'Brien, P. C. Wong, Amyloid precursor protein processing and Alzheimer's disease. *Annual review of neuroscience* **34**, 185-204 (2011)10.1146/annurev-neuro-061010-113613).

14. A. Kumar, A. Singh, Ekavali, A review on Alzheimer's disease pathophysiology and its management: an update. *Pharmacological reports : PR* **67**, 195-203 (2015); published online EpubApr (10.1016/j.pharep.2014.09.004).

15. J. W. Lee, K. Beebe, L. A. Nangle, J. Jang, C. M. Longo-Guess, S. A. Cook, M. T. Davisson, J. P. Sundberg, P. Schimmel, S. L. Ackerman, Editing-defective tRNA synthetase causes protein misfolding and neurodegeneration. *Nature* **443**, 50-55 (2006); published online EpubSep 7 (10.1038/nature05096).

16. R. Ishimura, G. Nagy, I. Dotu, H. Zhou, X. L. Yang, P. Schimmel, S. Senju, Y. Nishimura, J. H. Chuang, S. L. Ackerman, RNA function. Ribosome stalling induced by mutation of a CNS-specific tRNA causes neurodegeneration. *Science* **345**, 455-459 (2014); published online EpubJul 25 (10.1126/science.1249749).

17. A. Stolz, W. Hilt, A. Buchberger, D. H. Wolf, Cdc48: a power machine in protein degradation. *Trends in biochemical sciences* **36**, 515-523 (2011); published online EpubOct (10.1016/j.tibs.2011.06.001).

18. J. S. Ju, R. A. Fuentealba, S. E. Miller, E. Jackson, D. Piwnica-Worms, R. H. Baloh, C. C. Weihl, Valosin-containing protein (VCP) is required for autophagy and is disrupted in VCP disease. *The Journal of cell biology* **187**, 875-888 (2009); published online EpubDec 14 (10.1083/jcb.200908115).

19. V. Gandin, I. Topisirovic, Co-translational mechanisms of quality control of newly synthesized polypeptides. *Translation (Austin)* **2**, e28109 (2014)10.4161/trla.28109).

20. A. N. Fedorov, T. O. Baldwin, Cotranslational protein folding. *The Journal of biological chemistry* **272**, 32715-32718 (1997); published online EpubDec 26 (

21. L. Ellgaard, N. McCaul, A. Chatsisvili, I. Braakman, Co- and Post-Translational Protein Folding in the ER. *Traffic* **17**, 615-638 (2016); published online EpubJun (10.1111/tra.12392).

22. C. J. Shoemaker, R. Green, Translation drives mRNA quality control. *Nature structural & molecular biology* **19**, 594-601 (2012); published online EpubJun (10.1038/nsmb.2301).

23. E. W. Wallace, J. L. Kear-Scott, E. V. Pilipenko, M. H. Schwartz, P. R. Laskowski, A. E. Rojek, C. D. Katanski, J. A. Riback, M. F. Dion, A. M. Franks, E. M. Airoldi, T. Pan, B. A. Budnik, D. A. Drummond, Reversible, Specific, Active Aggregates of Endogenous Proteins Assemble upon Heat Stress. *Cell* **162**, 1286-1298 (2015); published online EpubSep 10 (10.1016/j.cell.2015.08.041).

24. T. C. Squier, Oxidative stress and protein aggregation during biological aging. *Experimental gerontology* **36**, 1539-1550 (2001); published online EpubSep (

25. A. Koplin, S. Preissler, Y. Ilina, M. Koch, A. Scior, M. Erhardt, E. Deuerling, A dual function for chaperones SSB-RAC and the NAC nascent polypeptide-associated complex on ribosomes. *The Journal of cell biology* **189**, 57-68 (2010); published online EpubApr 5 (10.1083/jcb.200910074).

26. A. J. Weids, C. M. Grant, The yeast peroxiredoxin Tsa1 protects against protein-aggregate-induced oxidative stress. *Journal of cell science* **127**, 1327-1335 (2014); published online EpubMar 15 (10.1242/jcs.144022).

27. J. Verghese, J. Abrams, Y. Wang, K. A. Morano, Biology of the heat shock response and protein chaperones: budding yeast (Saccharomyces cerevisiae) as a model system. *Microbiology and molecular biology reviews : MMBR* **76**, 115-158 (2012); published online EpubJun (10.1128/MMBR.05018-11).

28. G. C. Turner, A. Varshavsky, Detecting and measuring cotranslational protein degradation in vivo. *Science* **289**, 2117-2120 (2000); published online EpubSep 22 (

29. F. U. Hartl, A. Bracher, M. Hayer-Hartl, Molecular chaperones in protein folding and proteostasis. *Nature* **475**, 324-332 (2011); published online EpubJul 20 (10.1038/nature10317).

30. U. Schubert, L. C. Anton, J. Gibbs, C. C. Norbury, J. W. Yewdell, J. R. Bennink, Rapid degradation of a large fraction of newly synthesized proteins by proteasomes. *Nature* **404**, 770-774 (2000); published online EpubApr 13 (10.1038/35008096).

31. R. M. Vabulas, F. U. Hartl, Protein synthesis upon acute nutrient restriction relies on proteasome function. *Science* **310**, 1960-1963 (2005); published online EpubDec 23 (10.1126/science.1121925).

32. F. Wang, L. A. Durfee, J. M. Huibregtse, A cotranslational ubiquitination pathway for quality control of misfolded proteins. *Molecular cell* **50**, 368-378 (2013); published online EpubMay 9 (10.1016/j.molcel.2013.03.009).

33. F. Buttgereit, M. D. Brand, A hierarchy of ATP-consuming processes in mammalian cells. *The Biochemical journal* **312 (Pt 1)**, 163-167 (1995); published online EpubNov 15 (

34. J. W. Yewdell, E. Reits, J. Neefjes, Making sense of mass destruction: quantitating MHC class I antigen presentation. *Nature reviews. Immunology* **3**, 952-961 (2003); published online EpubDec (10.1038/nri1250).

35. M. H. Fortier, E. Caron, M. P. Hardy, G. Voisin, S. Lemieux, C. Perreault, P. Thibault, The MHC class I peptide repertoire is molded by the transcriptome. *The Journal of experimental medicine* **205**, 595-610 (2008); published online EpubMar 17 (10.1084/jem.20071985).

36. L. C. Anton, J. W. Yewdell, Translating DRiPs: MHC class I immunosurveillance of pathogens and tumors. *Journal of leukocyte biology* **95**, 551-562 (2014); published online EpubApr (10.1189/jlb.1113599).

37. L. F. Lareau, A. N. Brooks, D. A. Soergel, Q. Meng, S. E. Brenner, The coupling of alternative splicing and nonsense-mediated mRNA decay. *Adv Exp Med Biol* **623**, 190-211 (2007).

38. N. J. McGlincy, C. W. Smith, Alternative splicing resulting in nonsense-mediated mRNA decay: what is the meaning of nonsense? *Trends in biochemical sciences* **33**, 385-393 (2008); published online EpubAug (10.1016/j.tibs.2008.06.001).

39. J. Lykke-Andersen, E. J. Bennett, Protecting the proteome: Eukaryotic cotranslational quality control pathways. *The Journal of cell biology* **204**, 467-476 (2014); published online EpubFeb 17 (10.1083/jcb.201311103).

40. S. Shao, R. S. Hegde, Reconstitution of a minimal ribosome-associated ubiquitination pathway with purified factors. *Molecular cell* **55**, 880-890 (2014); published online EpubSep 18 (10.1016/j.molcel.2014.07.006).

41. O. Brandman, R. S. Hegde, Ribosome-associated protein quality control. *Nature structural & molecular biology* **23**, 7-15 (2016); published online EpubJan (10.1038/nsmb.3147).

42. G. C. Atkinson, S. L. Baldauf, V. Hauryliuk, Evolution of nonstop, no-go and nonsense-mediated mRNA decay and their termination factor-derived components. *BMC evolutionary biology* **8**, 290 (2008)10.1186/1471-2148-8-290).

43. S. Lykke-Andersen, T. H. Jensen, Nonsense-mediated mRNA decay: an intricate machinery that shapes transcriptomes. *Nature reviews. Molecular cell biology* **16**, 665-677 (2015); published online EpubNov (10.1038/nrm4063).

44. Y. Harigaya, R. Parker, No-go decay: a quality control mechanism for RNA in translation. *Wiley interdisciplinary reviews. RNA* **1**, 132-141 (2010); published online EpubJul-Aug (10.1002/wrna.17).

45. A. A. Klauer, A. van Hoof, Degradation of mRNAs that lack a stop codon: a decade of nonstop progress. *Wiley interdisciplinary reviews. RNA* **3**, 649-660 (2012); published online EpubSep-Oct (10.1002/wrna.1124).

46. O. Brandman, J. Stewart-Ornstein, D. Wong, A. Larson, C. C. Williams, G. W. Li, S. Zhou, D. King, P. S. Shen, J. Weibezahn, J. G. Dunn, S. Rouskin, T. Inada, A. Frost, J. S. Weissman, A ribosome-bound quality control complex triggers degradation of nascent peptides and signals translation stress. *Cell* **151**, 1042-1054 (2012); published online EpubNov 21 (10.1016/j.cell.2012.10.044).

47. Q. Defenouillere, Y. Yao, J. Mouaikel, A. Namane, A. Galopier, L. Decourty, A. Doyen, C. Malabat, C. Saveanu, A. Jacquier, M. Fromont-Racine, Cdc48-associated complex bound to 60S particles is required for the clearance of aberrant translation products. *Proceedings of the National Academy of Sciences of the United States of America* **110**, 5046-5051 (2013); published online EpubMar 26 (10.1073/pnas.1221724110).

48. S. K. Doamekpor, J. W. Lee, N. L. Hepowit, C. Wu, C. Charenton, M. Leonard, M. H. Bengtson, K. R. Rajashankar, M. S. Sachs, C. D. Lima, C. A. Joazeiro, Structure and function of the yeast listerin (Ltn1) conserved N-terminal domain in binding to stalled 60S ribosomal subunits. *Proceedings of the National Academy of Sciences of the United States of America* **113**, E4151-4160 (2016); published online EpubJul 19 (10.1073/pnas.1605951113).

49. R. Verma, R. S. Oania, N. J. Kolawa, R. J. Deshaies, Cdc48/p97 promotes degradation of aberrant nascent polypeptides bound to the ribosome. *eLife* **2**, e00308 (2013)10.7554/eLife.00308).

50. K. von der Malsburg, S. Shao, R. S. Hegde, The ribosome quality control pathway can access nascent polypeptides stalled at the Sec61 translocon. *Molecular biology of the cell* **26**, 2168-2180 (2015); published online EpubJun 15 (10.1091/mbc.E15-01-0040).

51. N. R. Guydosh, R. Green, Dom34 rescues ribosomes in 3' untranslated regions. *Cell* **156**, 950-962 (2014); published online EpubFeb 27 (10.1016/j.cell.2014.02.006).

52. M. K. Doma, R. Parker, Endonucleolytic cleavage of eukaryotic mRNAs with stalls in translation elongation. *Nature* **440**, 561-564 (2006); published online EpubMar 23 (10.1038/nature04530).

53. T. Tsuboi, K. Kuroha, K. Kudo, S. Makino, E. Inoue, I. Kashima, T. Inada, Dom34:hbs1 plays a general role in quality-control systems by dissociation of a stalled ribosome at the 3' end of aberrant mRNA. *Molecular cell* **46**, 518-529 (2012); published online EpubMay 25 (10.1016/j.molcel.2012.03.013).

54. D. Lyumkis, D. Oliveira Dos Passos, E. B. Tahara, K. Webb, E. J. Bennett, S. Vinterbo, C. S. Potter, B. Carragher, C. A. Joazeiro, Structural basis for translational surveillance by the large ribosomal subunit-associated protein quality control complex. *Proceedings of the National Academy of Sciences of the United States of America* **111**, 15981-15986 (2014); published online EpubNov 11 (10.1073/pnas.1413882111).

55. P. S. Shen, J. Park, Y. Qin, X. Li, K. Parsawar, M. H. Larson, J. Cox, Y. Cheng, A. M. Lambowitz, J. S. Weissman, O. Brandman, A. Frost, Protein synthesis. Rqc2p and 60S ribosomal subunits mediate mRNA-independent elongation of nascent chains. *Science* **347**, 75-78 (2015); published online EpubJan 2 (10.1126/science.1259724).

56. R. Yonashiro, E. B. Tahara, M. H. Bengtson, M. Khokhrina, H. Lorenz, K. C. Chen, Y. Kigoshi-Tansho, J. N. Savas, J. R. Yates, S. A. Kay, E. A. Craig, A. Mogk, B. Bukau, C. A. Joazeiro, The Rqc2/Tae2 subunit of the ribosome-associated quality control (RQC) complex marks ribosome-stalled nascent polypeptide chains for aggregation. *eLife* **5**, e11794 (2016)10.7554/eLife.11794).

57. Q. Defenouillere, E. Zhang, A. Namane, J. Mouaikel, A. Jacquier, M. Fromont-Racine, Rqc1 and Ltn1 Prevent C-terminal Alanine-Threonine Tail (CAT-tail)-induced Protein Aggregation by Efficient Recruitment of Cdc48 on Stalled 60S Subunits. *The Journal of biological chemistry* **291**, 12245-12253 (2016); published online EpubJun 3 (10.1074/jbc.M116.722264).

58. J. M. Harris, P. P. Nguyen, M. J. Patel, Z. A. Sporn, J. K. Hines, Functional diversification of hsp40: distinct j-protein functional requirements for two prions allow for chaperone-dependent prion selection. *PLoS genetics* **10**, e1004510 (2014); published online EpubJul (10.1371/journal.pgen.1004510).

59. Q. Wang, C. Song, C. C. Li, Molecular perspectives on p97-VCP: progress in understanding its structure and diverse biological functions. *Journal of structural biology* **146**, 44-57 (2004); published online EpubApr-May (10.1016/j.jsb.2003.11.014).

60. A. Deichsel, J. Mouysset, T. Hoppe, The ubiquitin-selective chaperone CDC-48/p97, a new player in DNA replication. *Cell Cycle* **8**, 185-190 (2009); published online EpubJan 15 (10.4161/cc.8.2.7356).

61. S. Ghaemmaghami, W. K. Huh, K. Bower, R. W. Howson, A. Belle, N. Dephoure, E. K. O'Shea, J. S. Weissman, Global analysis of protein expression in yeast. *Nature* **425**, 737-741 (2003); published online EpubOct 16 (10.1038/nature02046).

62. M. H. Bengtson, C. A. Joazeiro, Role of a ribosome-associated E3 ubiquitin ligase in protein quality control. *Nature* **467**, 470-473 (2010); published online EpubSep 23 (10.1038/nature09371).

63. B. Liu, Y. Han, S. B. Qian, Cotranslational response to proteotoxic stress by elongation pausing of ribosomes. *Molecular cell* **49**, 453-463 (2013); published online EpubFeb 7 (10.1016/j.molcel.2012.12.001).

64. J. Frydman, F. U. Hartl, Principles of chaperone-assisted protein folding: differences between in vitro and in vivo mechanisms. *Science* **272**, 1497-1502 (1996); published online EpubJun 7 (

65. S. Duttler, S. Pechmann, J. Frydman, Principles of cotranslational ubiquitination and quality control at the ribosome. *Molecular cell* **50**, 379-393 (2013); published online EpubMay 9 (10.1016/j.molcel.2013.03.010).

66. P. M. Douglas, A. Dillin, Protein homeostasis and aging in neurodegeneration. *The Journal of cell biology* **190**, 719-729 (2010); published online EpubSep 6 (10.1083/jcb.201005144).

67. W. Kim, E. J. Bennett, E. L. Huttlin, A. Guo, J. Li, A. Possemato, M. E. Sowa, R. Rad, J. Rush, M. J. Comb, J. W. Harper, S. P. Gygi, Systematic and quantitative assessment of the ubiquitin-modified proteome. *Molecular cell* **44**, 325-340 (2011); published online EpubOct 21 (10.1016/j.molcel.2011.08.025).

68. Y. J. Choe, S. H. Park, T. Hassemer, R. Korner, L. Vincenz-Donnelly, M. Hayer-Hartl, F. U. Hartl, Failure of RQC machinery causes protein aggregation and proteotoxic stress. *Nature* **531**, 191-195 (2016); published online EpubMar 10 (10.1038/nature16973).

69. J. Yang, X. Hao, X. Cao, B. Liu, T. Nystrom, Spatial sequestration and detoxification of Huntingtin by the ribosome quality control complex. *eLife* **5**, (2016)10.7554/eLife.11792).

70. S. Shao, A. Brown, B. Santhanam, R. S. Hegde, Structure and assembly pathway of the ribosome quality control complex. *Molecular cell* **57**, 433-444 (2015); published online EpubFeb 5 (10.1016/j.molcel.2014.12.015).

71. S. Shao, K. von der Malsburg, R. S. Hegde, Listerin-dependent nascent protein ubiquitination relies on ribosome subunit dissociation. *Molecular cell* **50**, 637-648 (2013); published online EpubJun 6 (10.1016/j.molcel.2013.04.015).

72. S. Saito, N. Hosoda, S. Hoshino, The Hbs1-Dom34 protein complex functions in non-stop mRNA decay in mammalian cells. *The Journal of biological chemistry* **288**, 17832-17843 (2013); published online EpubJun 14 (10.1074/jbc.M112.448977).

73. J. Chu, N. A. Hong, C. A. Masuda, B. V. Jenkins, K. A. Nelms, C. C. Goodnow, R. J. Glynne, H. Wu, E. Masliah, C. A. Joazeiro, S. A. Kay, A mouse forward genetics screen identifies LISTERIN as an E3 ubiquitin ligase involved in neurodegeneration. *Proceedings of the National Academy of Sciences of the United States of America* **106**, 2097-2103 (2009); published online EpubFeb 17 (10.1073/pnas.0812819106).

74. S. D. Brown, M. W. Moore, The International Mouse Phenotyping Consortium: past and future perspectives on mouse phenotyping. *Mammalian genome : official journal of the International Mammalian Genome Society* **23**, 632-640 (2012); published online EpubOct (10.1007/s00335-012-9427-x).

75. X. Bi, T. Jones, F. Abbasi, H. Lee, B. Stultz, D. A. Hursh, M. A. Mortin, Drosophila caliban, a nuclear export mediator, can function as a tumor suppressor in human lung cancer cells. *Oncogene* **24**, 8229-8239 (2005); published online EpubDec 15 (10.1038/sj.onc.1208962).

76. M. Castella, R. Pujol, E. Callen, J. P. Trujillo, J. A. Casado, H. Gille, F. P. Lach, A. D. Auerbach, D. Schindler, J. Benitez, B. Porto, T. Ferro, A. Munoz, J. Sevilla, L. Madero, E. Cela, C. Belendez, C. D. de Heredia, T. Olive, J. S. de Toledo, I. Badell, M. Torrent, J. Estella, A. Dasi, A. Rodriguez-Villa, P. Gomez, J. Barbot, M. Tapia, A. Molines, A. Figuera, J. A. Bueren, J. Surralles, Origin, functional role, and clinical impact of Fanconi anemia FANCA mutations. *Blood* **117**, 3759-3769 (2011); published online EpubApr 7 (10.1182/blood-2010-08-299917).

77. J. M. Muller, K. Deinhardt, I. Rosewell, G. Warren, D. T. Shima, Targeted deletion of p97 (VCP/CDC48) in mouse results in early embryonic lethality. *Biochemical and biophysical research communications* **354**, 459-465 (2007); published online EpubMar 9 (10.1016/j.bbrc.2006.12.206).

78. H. Meyer, C. C. Weihl, The VCP/p97 system at a glance: connecting cellular function to disease pathogenesis. *Journal of cell science* **127**, 3877-3883 (2014); published online EpubSep 15 (10.1242/jcs.093831).

79. B. Rauscher, F. Heigwer, M. Breinig, J. Winter, M. Boutros, GenomeCRISPR - a database for high-throughput CRISPR/Cas9 screens. *Nucleic acids research*, (2016); published online EpubOct 26 (10.1093/nar/gkw997).

80. I. Akinsheye, A. Alsultan, N. Solovieff, D. Ngo, C. T. Baldwin, P. Sebastiani, D. H. Chui, M. H. Steinberg, Fetal hemoglobin in sickle cell anemia. *Blood* **118**, 19-27 (2011); published online EpubJul 7 (10.1182/blood-2011-03-325258).

81. I. M. Adham, M. A. Sallam, G. Steding, M. Korabiowska, U. Brinck, S. Hoyer-Fender, C. Oh, W. Engel, Disruption of the pelota gene causes early embryonic lethality and defects in cell cycle progression. *Molecular and cellular biology* **23**, 1470-1476 (2003); published online EpubFeb (

82. G. Atkin, H. Paulson, Ubiquitin pathways in neurodegenerative disease. *Frontiers in molecular neuroscience* **7**, 63 (2014)10.3389/fnmol.2014.00063).

83. K. Mori, S. M. Weng, T. Arzberger, S. May, K. Rentzsch, E. Kremmer, B. Schmid, H. A. Kretzschmar, M. Cruts, C. Van Broeckhoven, C. Haass, D. Edbauer, The C9orf72 GGGGCC repeat is translated into aggregating dipeptide-repeat proteins in FTLD/ALS. *Science* **339**, 1335-1338 (2013); published online EpubMar 15 (10.1126/science.1232927).

84. T. Zu, Y. Liu, M. Banez-Coronel, T. Reid, O. Pletnikova, J. Lewis, T. M. Miller, M. B. Harms, A. E. Falchook, S. H. Subramony, L. W. Ostrow, J. D. Rothstein, J. C. Troncoso, L. P. Ranum, RAN proteins and RNA foci from antisense transcripts in C9ORF72 ALS and frontotemporal dementia. *Proceedings of the National Academy of Sciences of the United States of America* **110**, E4968-4977 (2013); published online EpubDec 17 (10.1073/pnas.1315438110).

85. D. Lyumkis, S. K. Doamekpor, M. H. Bengtson, J. W. Lee, T. B. Toro, M. D. Petroski, C. D. Lima, C. S. Potter, B. Carragher, C. A. Joazeiro, Single-particle EM reveals extensive conformational variability of the Ltn1 E3 ligase. *Proceedings of the National Academy of Sciences of the United States of America* **110**, 1702-1707 (2013); published online EpubJan 29 (10.1073/pnas.1210041110).

86. R. Higgins, J. M. Gendron, L. Rising, R. Mak, K. Webb, S. E. Kaiser, N. Zuzow, P. Riviere, B. Yang, E. Fenech, X. Tang, S. A. Lindsay, J. C. Christianson, R. Y. Hampton, S. A. Wasserman, E. J. Bennett, The Unfolded Protein Response Triggers Site-Specific Regulatory Ubiquitylation of 40S Ribosomal Proteins. *Molecular cell* **59**, 35-49 (2015); published online EpubJul 2 (10.1016/j.molcel.2015.04.026).

87. C. Regnard, E. Desbruyeres, P. Denoulet, B. Edde, Tubulin polyglutamylase: isozymic variants and regulation during the cell cycle in HeLa cells. *Journal of cell science* **112 (Pt 23)**, 4281-4289 (1999); published online EpubDec (

88. F. J. Baird, C. L. Bennett, Microtubule defects & Neurodegeneration. *Journal of genetic syndromes & gene therapy* **4**, 203 (2013); published online EpubDec 6 (10.4172/2157-7412.1000203).

89. L. N. Dimitrova, K. Kuroha, T. Tatematsu, T. Inada, Nascent peptide-dependent translation arrest leads to Not4p-mediated protein degradation by the proteasome. *The Journal of biological chemistry* **284**, 10343-10352 (2009); published online EpubApr 17 (10.1074/jbc.M808840200).

90. R. Matsuda, K. Ikeuchi, S. Nomura, T. Inada, Protein quality control systems associated with no-go and nonstop mRNA surveillance in yeast. *Genes to cells : devoted to molecular & cellular mechanisms* **19**, 1-12 (2014); published online EpubJan (10.1111/gtc.12106).

91. K. Kuroha, M. Akamatsu, L. Dimitrova, T. Ito, Y. Kato, K. Shirahige, T. Inada, Receptor for activated C kinase 1 stimulates nascent polypeptide-dependent translation arrest. *EMBO reports* **11**, 956-961 (2010); published online EpubDec (10.1038/embor.2010.169).

92. J. T. Winston, P. Strack, P. Beer-Romero, C. Y. Chu, S. J. Elledge, J. W. Harper, The SCFbeta-TRCP-ubiquitin ligase complex associates specifically with phosphorylated destruction motifs in IkappaBalpha and beta-catenin and stimulates IkappaBalpha ubiquitination in vitro. *Genes & development* **13**, 270-283 (1999); published online EpubFeb 1 (

93. P. K. Jackson, A. G. Eldridge, E. Freed, L. Furstenthal, J. Y. Hsu, B. K. Kaiser, J. D. Reimann, The lore of the RINGs: substrate recognition and catalysis by ubiquitin ligases. *Trends in cell biology* **10**, 429-439 (2000); published online EpubOct (

94. K. Iwai, K. Yamanaka, T. Kamura, N. Minato, R. C. Conaway, J. W. Conaway, R. D. Klausner, A. Pause, Identification of the von Hippel-lindau tumor-suppressor protein as part of an active E3 ubiquitin ligase complex. *Proceedings of the National Academy of Sciences of the United States of America* **96**, 12436-12441 (1999); published online EpubOct 26 (

95. M. Zhuang, S. Guan, H. Wang, A. L. Burlingame, J. A. Wells, Substrates of IAP ubiquitin ligases identified with a designed orthogonal E3 ligase, the NEDDylator. *Molecular cell* **49**, 273-282 (2013); published online EpubJan 24 (10.1016/j.molcel.2012.10.022).

96. R. Gupta, B. Kus, C. Fladd, J. Wasmuth, R. Tonikian, S. Sidhu, N. J. Krogan, J. Parkinson, D. Rotin, Ubiquitination screen using protein microarrays for comprehensive identification of Rsp5 substrates in yeast. *Molecular systems biology* **3**, 116 (2007)10.1038/msb4100159).

97. H. F. O'Connor, N. Lyon, J. W. Leung, P. Agarwal, C. D. Swaim, K. M. Miller, J. M. Huibregtse, Ubiquitin-Activated Interaction Traps (UBAITs) identify E3 ligase binding partners. *EMBO reports* **16**, 1699-1712 (2015); published online EpubDec (10.15252/embr.201540620).

98. B. Stynen, H. Tournu, J. Tavernier, P. Van Dijck, Diversity in genetic in vivo methods for protein-protein interaction studies: from the yeast two-hybrid system to the mammalian split-luciferase system. *Microbiology and molecular biology reviews : MMBR* **76**, 331-382 (2012); published online EpubJun (10.1128/MMBR.05021-11).

99. C. Schweingruber, P. Soffientini, M. D. Ruepp, A. Bachi, O. Muhlemann, Identification of Interactions in the NMD Complex Using Proximity-Dependent Biotinylation (BioID). *PloS one* **11**, e0150239 (2016)10.1371/journal.pone.0150239).

100. E. Coyaud, M. Mis, E. M. Laurent, W. H. Dunham, A. L. Couzens, M. Robitaille, A. C. Gingras, S. Angers, B. Raught, BioID-based Identification of Skp Cullin F-box (SCF)beta-TrCP1/2 E3 Ligase Substrates. *Molecular & cellular proteomics : MCP* **14**, 1781-1795 (2015); published online EpubJul (10.1074/mcp.M114.045658).

101. C. Yeh, E. Coyaud, M. Bashkurov, P. van der Lelij, S. W. Cheung, J. M. Peters, B. Raught, L. Pelletier, The Deubiquitinase USP37 Regulates Chromosome Cohesion and Mitotic Progression. *Current biology : CB* **25**, 2290-2299 (2015); published online EpubAug 31 (10.1016/j.cub.2015.07.025).

102. K. Kwon, E. D. Streaker, D. Beckett, Binding specificity and the ligand dissociation process in the E. coli biotin holoenzyme synthetase. *Protein science : a publication of the Protein Society* **11**, 558-570 (2002); published online EpubMar (10.1110/ps.33502).

103. D. I. Kim, K. C. Birendra, W. Zhu, K. Motamedchaboki, V. Doye, K. J. Roux, Probing nuclear pore complex architecture with proximity-dependent biotinylation. *Proceedings of the National Academy of Sciences of the United States of America* **111**, E2453-2461 (2014); published online EpubJun 17 (10.1073/pnas.1406459111).

104. K. J. Roux, D. I. Kim, M. Raida, B. Burke, A promiscuous biotin ligase fusion protein identifies proximal and interacting proteins in mammalian cells. *The Journal of cell biology* **196**, 801-810 (2012); published online EpubMar 19 (10.1083/jcb.201112098).

105. S. S. Lam, J. D. Martell, K. J. Kamer, T. J. Deerinck, M. H. Ellisman, V. K. Mootha, A. Y. Ting, Directed evolution of APEX2 for electron microscopy and proximity labeling. *Nature methods* **12**, 51-54 (2015); published online EpubJan (10.1038/nmeth.3179).

106. J. D. Martell, T. J. Deerinck, Y. Sancak, T. L. Poulos, V. K. Mootha, G. E. Sosinsky, M. H. Ellisman, A. Y. Ting, Engineered ascorbate peroxidase as a genetically encoded reporter for electron microscopy. *Nature biotechnology* **30**, 1143-1148 (2012); published online EpubNov (10.1038/nbt.2375).

107. V. Hung, N. D. Udeshi, S. S. Lam, K. H. Loh, K. J. Cox, K. Pedram, S. A. Carr, A. Y. Ting, Spatially resolved proteomic mapping in living cells with the engineered peroxidase APEX2. *Nature protocols* **11**, 456-475 (2016); published online EpubMar (10.1038/nprot.2016.018).

108. H. W. Rhee, P. Zou, N. D. Udeshi, J. D. Martell, V. K. Mootha, S. A. Carr, A. Y. Ting, Proteomic mapping of mitochondria in living cells via spatially restricted enzymatic tagging. *Science* **339**, 1328-1331 (2013); published online EpubMar 15 (10.1126/science.1230593).

109. H. Meyer, M. Bug, S. Bremer, Emerging functions of the VCP/p97 AAA-ATPase in the ubiquitin system. *Nat Cell Biol* **14**, 117-123 (2012); published online EpubFeb (10.1038/ncb2407).

110. L. Xue, E. E. Blythe, E. C. Freiberger, J. L. Mamrosh, A. S. Hebert, J. M. Reitsma, S. Hess, J. J. Coon, R. J. Deshaies, Valosin-containing protein (VCP)-Adaptor Interactions are Exceptionally Dynamic and Subject to Differential Modulation by a VCP Inhibitor. *Molecular & cellular proteomics : MCP* **15**, 2970-2986 (2016); published online EpubSep (10.1074/mcp.M116.061036).

111. R. Anjum, J. Blenis, The RSK family of kinases: emerging roles in cellular signalling. *Nature reviews. Molecular cell biology* **9**, 747-758 (2008); published online EpubOct (10.1038/nrm2509).

112. P. J. Roberts, C. J. Der, Targeting the Raf-MEK-ERK mitogen-activated protein kinase cascade for the treatment of cancer. *Oncogene* **26**, 3291-3310 (2007); published online EpubMay 14 (10.1038/sj.onc.1210422).

113. R. C. Rogers, F. E. Abidi, in *GeneReviews(R)*, R. A. Pagon, M. P. Adam, H. H. Ardinger, S. E. Wallace, A. Amemiya, L. J. H. Bean, T. D. Bird, C. T. Fong, H. C. Mefford, R. J. H. Smith, K. Stephens, Eds. (Seattle (WA), 1993).

114. M. C. Mendoza, E. E. Er, J. Blenis, The Ras-ERK and PI3K-mTOR pathways: cross-talk and compensation. *Trends in biochemical sciences* **36**, 320-328 (2011); published online EpubJun (10.1016/j.tibs.2011.03.006).

115. F. H. Khan, V. Pandian, S. Ramraj, M. Natarajan, S. Aravindan, T. S. Herman, N. Aravindan, Acquired genetic alterations in tumor cells dictate the development of high-risk neuroblastoma and clinical outcomes. *BMC cancer* **15**, 514 (2015)10.1186/s12885-015-1463-y).

116. K. Ikegami, S. Sato, K. Nakamura, L. E. Ostrowski, M. Setou, Tubulin polyglutamylation is essential for airway ciliary function through the regulation of beating asymmetry. *Proceedings of the National Academy of Sciences of the United States of America* **107**, 10490-10495 (2010); published online EpubJun 8 (10.1073/pnas.1002128107).

117. A. Roll-Mecak, F. J. Mcnally, Microtubule-severing enzymes. *Curr Opin Cell Biol* **22**, 96-103 (2010); published online EpubFeb (10.1016/j.ceb.2009.11.001).

118. B. Lacroix, J. van Dijk, N. D. Gold, J. Guizetti, G. Aldrian-Herrada, K. Rogowski, D. W. Gerlich, C. Janke, Tubulin polyglutamylation stimulates spastin-mediated microtubule severing. *The Journal of cell biology* **189**, 945-954 (2010); published online EpubJun 14 (10.1083/jcb.201001024).

119. P. Vogel, G. Hansen, G. Fontenot, R. Read, Tubulin tyrosine ligase-like 1 deficiency results in chronic rhinosinusitis and abnormal development of spermatid flagella in mice. *Veterinary pathology* **47**, 703-712 (2010); published online EpubJul (10.1177/0300985810363485).

120. T. Wang, J. I. Morgan, The Purkinje cell degeneration (pcd) mouse: an unexpected molecular link between neuronal degeneration and regeneration. *Brain research* **1140**, 26-40 (2007); published online EpubApr 6 (10.1016/j.brainres.2006.07.065).

121. K. Rogowski, J. van Dijk, M. M. Magiera, C. Bosc, J. C. Deloulme, A. Bosson, L. Peris, N. D. Gold, B. Lacroix, M. Bosch Grau, N. Bec, C. Larroque, S. Desagher, M. Holzer, A. Andrieux, M. J. Moutin, C. Janke, A family of protein-deglutamylating enzymes associated with neurodegeneration. *Cell* **143**, 564-578 (2010); published online EpubNov 12 (10.1016/j.cell.2010.10.014).