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Charisma Enam

Ubx cofactors are required in San1 dependent nuclear protein quality control degradation

Charisma Enam

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Reading Committee:
Richard Gardner, Chair
Edith Wang
Ning Zheng

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Abstract

Ubx cofactors are required in San1-dependent nuclear protein quality control degradation

Charisma Enam

Chair of Supervisory Committee:

Associate Professor Richard Gardner

Department of Pharmacology

Accumulation of misfolded proteins in the cell are linked to many human diseases. To ensure optimal cellular physiology, the cell has compartmentalized the protein quality control system (PQC). As a compartment, the nucleus is unique in two important ways. First, the nucleus is the home of our genome. Second, unlike the cytoplasm, the nucleus is a location for post synthetized proteins. Since several key activities such as DNA synthesis and repair occur in the nucleus, an important aspect of maintaining optimal nuclear function is to reduce the burden of misfolded proteins when they arise in the nucleus. Using the *Saccharomyces cerevisiae* as our model organism, we previously identified San1 as the E3 ubiquitin ligase which targets misfolded proteins for degradation. Our past studies also identified the AAA-ATPase Cdc48 to be another essential player that degrades nuclear misfolded protein in the San1 pathway. In the current study, my thesis work explores the players that assist Cdc48 to degrade nuclear misfolded proteins in the San1 pathway. We found Ubx1, Ubx4 and Ubx5 to regulate nuclear protein homeostasis. Our experiments reveal the Ubx cofactors to play distinct functional roles in maintaining a healthy nucleus.

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Dedication

I dedicate my thesis to Sami Iqram. Words are not enough to articulate what your love has meant in the last few years. This journey would not reach completion without you at my side.

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LIST OF ABBREVIATIONS

DAPI	4',6-diamidino-2-phenylindole
EDTA	Ethylenediaminetetraacetic acid
GFP	Green Florescent Protein
MOPS	3-(*N*-morpholino) propane sulfonic acid
NEM	n-ethylmaleimide
PGK1	3-PhosphoGlycerate Kinase
PMSF	Phenylmethylsulfonyl fluoride
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SUMEB	SDS, Urea, MOPES, EDTA and Bromophenol Blue
TUBE	Tandem Ubiquitin Binding Entity

CHAPTER ONE:

1.1 INTRODUCTION TO GENERAL PROTEIN QUALITY CONTROL

From birth at the ribosome to death by the proteasome or at the lysosome/vacuole, proteins encounter chemical and physical stresses that can damage their structure and lead to misfolding. Proteins can undergo misfolding even without an external stress as mutations or errors in transcription or translation can alter a primary protein sequence and change its ability to fold properly. As structure often dictates function, protein misfolding can lead to Loss-Of-Function (LOF) in the cell, which would perturb essential signaling, metabolic, transport, and architectural functions. Misfolded proteins can also acquire dominant-negative functions that lead to Gain-Of-Function (GOF) toxic aggregation. The deleterious consequences of an aggregation-prone state are thought to underlie numerous proteinopathies including Alzheimer's, Parkinson's, Huntington's, Amyotrophic Lateral Sclerosis (ALS), and prion disorders like Creutzfeldt-Jakob (1).

HOW DOES PROTEIN MISFOLDING CAUSE CELLULAR DYSFUNCTION?

There are two prevailing hypotheses in the field that seek to explain how protein misfolding can lead to cellular dysfunction. The first hypothesis is that misfolded proteins cause specific cellular pathways to become compromised. This might explain the selective vulnerability of particular neurons in diseases like Parkinson's and Huntington's (2,3). The second hypothesis is that the accumulation of misfolded proteins may lead to an overall burden that exceeds the capacity of the cell's defense mechanisms over time (Figure 1.1). This might be the case for the collapse of proteostasis observed in aging (4). As a corollary to this second hypothesis, the cell's defense mechanisms are themselves composed of proteins, which are susceptible to misfolding as well, thus an increasing burden of misfolded proteins could also be concomitant with a general reduction in overall capacity to manage the burden. It is important to note that these two hypotheses are not mutually exclusive and may actually happen concurrently in protein conformational diseases and aging. The specific effects of a misfolded protein on the cellular environment will depend on the particular compartment it arises and the capacity of the protective machinery of that organelle.

The complex web of when, where, and how the cell deals with protein aggregation, remains an overarching question in the cellular proteostasis field. It underscores the importance of deciphering how the cell normally deals with misfolded proteins and what happens when this process breaks down in aging and disease.

UBIQUITIN-MEDIATED PROTEIN DEGRADATION IN CELLULAR QUALITY CONTROL

Eukaryotic cells employ multiple Protein Quality Control (PQC) mechanisms to survey the existing proteome and manage misfolded proteins as they arise (Figure 1.2). Ongoing surveillance of cellular proteostasis relies on chaperones that assist proteins to maintain their appropriate structural conformations, and on the Ubiquitin-Proteasome System (UPS) that eliminates proteins recalcitrant to folding or permanently misfolded. Because a protein's structure is constantly in a dynamic state and since misfolding can occur anywhere in the cell, vigilant oversight of the cellular proteome would seemingly require organelle-specific PQC systems. In fact, compartmentalization of UPS-mediated PQC degradation has been reported in most of the eukaryotic organelles, suggesting that this is a common feature for maintaining proteostasis (5).

Most proteins are subjected to PQC shortly after emerging from the ribosome. In the cytosol, nascent peptides are usually folded with the aid of ribosome-associated chaperones (6). At the same time, co-translational insertion of nascent peptides to the Endoplasmic Reticulum (ER) results in their folding at the ER lumen with the aid of ER-resident chaperones (7). However, if folding fails, misfolded proteins are sent for UPS-mediated degradation with help from chaperones and ubiquitin-protein ligases (5,8). In addition, environmental changes, such as elevated temperature, altered pH, or exposure to chemicals, may trigger unfolding of mature proteins in their final cellular site. These proteins are ubiquitinated by designated UPS components at their specific cellular location such as the cytosol, the ER, the outer membrane of the mitochondria, the plasma membrane and the nucleus (5). In this chapter, we focus on what is currently known about PQC degradation in the nucleus and discuss future questions

and issues related to the nuclear PQC degradation field. For brevity, we wish to dub nuclear PQC degradation as NucPQCD.

1.2 THE UNIQUE FEATURES OF THE NUCLEUS

The nucleus is the main information repository of the eukaryotic cell – housing the genetic blueprint that must be copied, transcribed, and packaged appropriately. As such, the nucleus is organized into regions that accommodate molecules with distinct nuclear functions, including actively transcribed and silent chromatin, processing of RNA, splicing of immature mRNA, ribosome biogenesis, nuclear transport, and overall nuclear architecture and shape (Figure 1.3). The unique organization, structure, and biomolecular composition of the nucleus will dictate the properties of NucPQCD systems necessary to maintain optimal proteostasis.

THE MANY COMPARTMENTS OF THE NUCLEUS

The nucleus is surrounded by a double-layered membrane called the Nuclear Envelope (NE) that provides a physical barrier between the nucleoplasm and the cytosol (9). In metazoans, the NE interacts with the nuclear matrix through the nuclear lamina, which is associated with the Inner Nuclear Membrane (INM). The nuclear lamina provides a platform for spatial arrangement of nuclear molecules and the chromatin (10), and it forms a scaffold that offers mechanical support for nuclear shape and integrity (11). The nucleus also contains several spatially dynamic nuclear bodies that play integral roles in nuclear functions (10). The most prominent nuclear body is the nucleolus, which is the home to ribosome biogenesis and signal recognition particle assembly, among other functions (9). Additional significant structures in the nucleus are Cajal bodies that participate in ribosome biogenesis, regulation of telomeres and in spliceosomal snRNPs assembly, and ProMyelocytic Leukemia (PML) bodies that are involved in multiple genome maintenance pathways (12). All nuclear subcompartments are subjected to spatial and temporal surveillance and crosstalk between QC systems in the different compartments is common and necessary to orchestrate basic nuclear functions.

IMPORTANCE OF THE NUCLEAR ENVELOPE IN NucPQCD

Nuclear Pore Complexes (NPCs) span the outer and inner nuclear membrane to allow proper communication with the cytosol. They facilitate protein and mRNA trafficking to and from the nucleus (9). NPCs form a flexible barrier to passive diffusion that diminishes with increasing molecular mass (13). Macromolecules and large complexes can still shuttle between the cytosol and the nucleus via active transport that entails their interaction with importins or exportins (14). Thus, in terms of NucPQCD, it is important to consider that, although the nucleus and cytosol communicate with one another, they are separate compartments with distinct PQC requirements.

However, during mitosis, the nuclear and cytosolic compartments have the chance to intermingle. In metazoans, when open mitosis takes place (15), the nuclear envelope breaks down and misfolded nucleoplasmic proteins, generated during G1, can be managed by cytosolic PQC systems. Thus, in dividing metazoan cells, there is no critical need for NucPQCD-specific systems. Exit from mitosis prompts the NE to reform, separating the nucleus from the cytosol for the entire life of the somatic cell. In that case, NucPQCD-specific systems become important and they are likely to play key roles in cells that are especially long-lived such as neurons, cardiomyocytes, and inner cells of the eye. Conversely, many single-celled eukaryotes undergo closed mitosis in which the nuclear envelope remains intact (16). In these rapidly dividing organisms, NucPQCD-specific systems become essential for maintaining nuclear proteostasis during the entire cell's lifetime. This is why, for experimental reasons, NucPQCD systems in simple organisms like budding yeast may represent the NucPQCD capabilities of nondividing metazoan cells like neurons.

Mitosis is not the only time the nuclear and cytosolic compartments can be exposed to each other in an unregulated fashion. Loss of NPC selectivity or NE integrity can lead to unwarranted intermingling. With cell age, loss of NPC selectivity triggers leakiness through the NPC wherein cytosolic components have greater access to the nucleus (17). This provides an opportunity for cytosolic misfolded proteins to enter the nucleus,

and a need for vigilant control of NucPQCD to maintain proteostasis in the nucleus. The NE can also burst (18,19), which has consequences for NucPQCD, as the NE reforms and incorporates cytosolic components. In both cases, NucPQCD is a key mechanism to combat the potential damage evoked by cytosolic proteins outside their native compartment.

TO TRANSLATE OR NOT – A POTENTIAL NECESSITY FOR NUCLEAR PQCD

The translation of mRNAs generally occurs in the cytoplasm after their nuclear export. Thus, one aspect of general PQC that the nucleus does not need to manage is the misfolding of nascent peptides during synthesis, which constitute a considerable portion of cytoplasmic and ER PQC systems (6). The negligible translation in the nucleus enables nuclear PQC systems to dedicate their function to the post-production management of protein misfolding. In this regard, it is conceivable that nuclear PQC systems evolved specifically to manage damage-induced misfolding that might confer toxicity in the nucleus.

Despite the canonical view of translation occurring predominantly in the cytosol, limited nuclear ribosomal activity has been reported to take place, mainly in the nucleolus (20). It has been proposed that this phenomenon serves a QC step to assess the translation capacity of newly synthesized ribosomes prior to their export from the nucleolus (20). Although we find this unlikely, since ribosomes go through key maturation steps once they are exported from the nucleus (21), it is possible that the byproducts of such a QC test will need to be purged from the nucleus. If this phenomenon occurs, it suggests a nucleolar PQC mechanism for the complicated and highly orchestrated assembly and function of ribosomes, which is currently not well understood.

THE IMPORTANCE OF HOLISTICALLY CONSIDERING NUCLEAR QC

As described above, the unique nuclear milieu, which comprises a large variety of biomolecules that can all be subjected to damage, requires specialized QC mechanisms. QC studies aimed at understanding the integrity and fidelity of DNA and RNA have been a major focus in the literature for decades (22-24). However, QC

studies focusing on the nuclear proteome have only come to light in the last decade (5). We would like to posit that understanding all nuclear QC systems holistically is imperative because it represents the ultimate circle of cellular protection (Figure 1.4). Failure to maintain or transcribe the genetic code properly will lead to failures in protein function. In turn, a failure in protein function can lead to a failure in maintaining and transcribing the genetic code. While it remains important to explore each area of nuclear QC separately to understand the specific mechanisms, we think it is imperative to consider the overall unity of nuclear QC in maintaining key nuclear functions since any imbalance in QC can escalate cell dysfunction, resulting in a disease state.

1.3 NucPQCD SYSTEMS

In the nucleus, several ubiquitin-protein ligase complexes are responsible for the removal of misfolded proteins via NucPQCD. The activity the majority of these ubiquitin-protein ligases is restricted to nuclear substrates, though there are examples for extranuclear activity of one or more enzymes (25). The existence of multiple NucPQCD ubiquitin-protein ligases hints at non-overlapping functions. The need for such diverse pathways in NucPQCD is not clear. Yeast has been the primary organism for discovering NucPQCD pathways, so here we introduce the different yeast NucPQCD ubiquitin-protein ligases, their nuclear distribution (Figure 1.5), and what is known about their mode of operation. In metazoans, not much is known about NucPQCD systems, so illumination of the yeast NucPQCD systems should be informative for more general mechanisms that are likely to occur in metazoan nuclei.

THE San1 PATHWAY

San1 was the first ubiquitin-protein ligase shown to function explicitly in NucPQCD (26). San1's role in NucPQCD was not obvious from its initial discovery in 1989. It was found to be a gene that, when mutated, suppressed a temperature-sensitive form of the nuclear protein Sir4, which is involved in gene silencing (27). Based on this, the gene was dubbed a Silencing ANtagonist or *SAN*. A subsequent report in 1993 revealed that mutations in *SAN1* caused suppression of a temperature-sensitive form of the nuclear protein Cdc68 (Spt16), which is involved in nucleosome rearrangement (28). These

observations, concerning disparate nuclear functions, were initially confounding for understanding San1 function. A likely explanation for the temperature-suppressor effects of *san1* mutants is that normal San1 activity reduces the functional levels of the mutant misfolded proteins by increasing their degradation. San1's specific role in NucPQCD was first demonstrated by experiments showing that it is nuclear-localized and that it facilitates the ubiquitination of many mutant nuclear proteins, but not their normal counterparts (26).

Protein chaperones play a key role in the majority of PQCD pathways studied so far (29). They are also involved in San1-mediated NucPQCD (30-32), but do not appear to be directly engaged with San1 (33). Most of the evidence for chaperone involvement came from studies examining cytosolic misfolded proteins that have been found to enter the nucleus and become San1 substrates (30-32,34). These substrates require members of the Hsp70 *SSA* chaperone family, one of the Hsp40 chaperones Ydj1 or Sis1, and the Hsp110 chaperone Sse1 for San1-mediated degradation (30-32,34,35). While the exact role of chaperones in San1-mediated degradation remains unclear, there are important possibilities that need to be explored in the future. For example, it is conceivable that chaperones are necessary to maintain the solubility of highly aggregation-prone misfolded proteins, which San1 does not appear to detect once they aggregate (33). Alternatively, chaperones may aid in shuttling certain misfolded degradation substrates through the NPCs, as proposed for Sis1 (35). These options are not mutually exclusive, and chaperone involvement in San1-mediated degradation will likely be complex.

Currently, there is no clear San1 homolog or analog in metazoans. While a primary sequence search does not identify a San1 homolog in metazoans, there are some metazoan ubiquitin-protein ligases that match the meta-sequence requirements of yeast San1 (36). None have yet been shown to function analogous to San1 in metazoan NucPQCD. But, this is a worthy pursuit to understand how NucPQCD operates in mammals and if there are pathways analogous to the San1 pathway in yeast.

THE Doa10 PATHWAY

The endoplasmic reticulum/nuclear envelope (ER/NE) localized ubiquitin-protein ligase Doa10, ubiquitinates substrates that are either ER/NE resident, nucleoplasmic, or cytosolic. As such, Doa10 has the widest compartmental involvement of all identified NucPQCD ubiquitin protein ligases and it most likely acts as a central hub of PQC degradation in the eukaryotic cell. Doa10 was originally identified in 1994 by a mutation that suppressed the temperature-sensitive allele of the nuclear protein Rna14, which is involved in RNA processing (37). Accordingly, the *DOA10* gene was initially dubbed *SSM4*, which stands for Suppressor of mRNA Stability Mutant 4 (37). Doa10 was subsequently found to be a ubiquitin-protein ligase in a genetic screen for factors involved in the degradation of Matα2, a yeast mating-type transcription factor (38,39). This was unusual because, at the time, it was a mystery as to how a transcription factor could gain access to an ER-localized ubiquitin-protein ligase. A seminal study in 2006 demonstrated that Doa10 can transport laterally to the INM, where it ubiquitinates Matα2 (40). This was the first report demonstrating that Doa10 operates at both the cytosolic and nuclear leaflets of the ER/NE.

The degradation of all tested Doa10 substrates requires the assistance of chaperones and therefore they may be considered as integral components of the Doa10 degradation machinery. With regards to NucPQCD, degradation of a mutant yeast kinetochore protein Ndc10-2 by the Doa10 pathway requires members of the Hsp70 *SSA* chaperones as well as the Hsp40 chaperone Sis1 (41). While Sis1 is absolutely required for Doa10-dependent ubiquitination, the *SSA* chaperones are not. Similar findings were shown for the degradation of the PQCD substrate ∆ssCPY* (42), indicating a general mechanism for the Hsp70s in the maintenance of substrate solubility and/or in shuttling the misfolded protein to the proteasome.

There is a known Doa10 homolog in metazoans named TEB4 (43). Like Doa10, TEB4 recognizes misfolded proteins at the ER and it is also involved in the regulation of cholesterol biogenesis (44). The high conservation of Doa10/TEB4 function hints at their importance in regulating ER homeostasis. However, it is yet to be determined if similar

to Doa10, TEB4 is also engaged in NucPQCD at the INM and in the elimination of CytoPQCD substrates.

THE Asi PATHWAY

The INM-localized Asi complex forms a functional NucPQCD ubiquitin-protein ligase that controls the degradation of several membrane-associated proteins (45,46). Components of the Asi complex were originally identified in a yeast selection for spontaneous suppressing mutations in the SPS (Ssy1-Ptr3-Ssy5) amino acid-sensing pathway (47), and were shown later to alter the stability of latent forms of transcription factors that activate SPS-sensor regulated genes (48). All three proteins forming the Asi complex (Asi1, Asi2 and Asi3) are integral proteins of the INM (49,50). Asi1 and Asi3 are related proteins, forming a conserved ubiquitin-protein ligase with RING domains at their extreme C-termini (47). Independent studies support the role of the Asi complex as a ubiquitin-protein ligase at the INM that targets mutant proteins as well as mislocalized integral membrane proteins for degradation in the nucleus (45,46).

THE Tom1 PATHWAY

Tom1 (Trigger of Mitosis 1) was originally identified from a genetic screen searching for temperature-sensitive mutants that caused growth arrest at the G2/M transition of the cell cycle (51). Tom1 was subsequently found to be a HECT-type ubiquitin-protein ligase that activates transcription via the ubiquitination of components of the histone acetyltransferase ADA complex (52), and is involved in the degradation of excess (non-chromatin bound) histones (53). More recent studies revealed a critical role of Tom1 in the removal of excess ribosomal proteins in the nucleolus (54). Detergent-insoluble proteins are greatly increased in *tom1* mutant cells including those of both the 60S and 40S ribosomal subunits, which comprised the major class of aggregating proteins (54). A combination of active-site mutations of *TOM1* with the deletion of factors involved in ribosome biosynthesis, caused a severe imbalance in ribosome production and substantial synthetic growth defect, likely indicating proteostasis collapse (54). Importantly, a similar function, mediated by the mammalian Tom1 homolog HUWE1, restricts the accumulation of overexpressed ribosomal subunit hRpl26 in human cells

(54). Overall, these recent findings imply an essential and conserved role for Tom1 in NucPQCD.

THE Slx5/Slx8 PATHWAY

Slx5 and Slx8 belong to a family of six genes that are synthetic lethal with Sgs1, a DNA helicase, which is required for the maintenance of genome integrity in *S. cerevisiae* (55). They interact with each other to form a heterodimeric ubiquitin-protein ligase complex (55,56). The Slx5/Slx8 complex is thought to direct ubiquitination of substrates that have been modified with the Small Ubiquitin-like MOdifier (SUMO) (57-59), and is therefore named a SUMO-targeted ubiquitin-protein ligase or STUbL. Support for Slx5/Slx8 functioning in NucPQCD comes from a study showing that a temperature-sensitive variant of the transcription regulator Mot1 is targeted to proteolysis via Slx5/Slx8-mediated ubiquitination in a SUMO-dependent manner (60). In addition, loss of Slx5/Slx8 was shown to increase the accumulation of nuclear inclusions (61). In mammals, the STUbL RNF4 is analogous to yeast Slx5/Slx8 and has been similarly shown to target sumoylated misfolded proteins for NucPQCD (62). Whereas in yeast the SUMO-protein ligase that sumoylates misfolded proteins for Slx5/Slx8 ubiquitination has not yet been identified, in mammalian cells the PML protein appears to play such a role (62). Overall, SUMO-directed ubiquitination of misfolded proteins is an emerging, evolutionarily conserved theme in NucPQCD.

1.4. CURRENT ISSUES CONCERNING NucPQCD

The unique nature of the nucleus raises issues that have been brought to light in the current literature. Some controversial issues include the site of degradation for nuclear proteins (such as the PQC interplay between the nucleus and the cytosol), the degree of interaction between different nuclear PQC systems, and how each NucPQCD system targets its set of substrates. These are important concepts to consider as the NucPQCD field moves forward.

CELLULAR LOCALIZATION OF ACTIVE PROTEASOMES

Individual proteasome subunits are synthesized in the cytosol and assembled first into the 20S catalytic core and the 19S regulatory particle, which then associate to form the 26S proteasome (63). Although 26S proteasomes are present in the cytosol, they are predominantly found in the nucleus and this localization is evolutionarily conserved (64). Coincident nuclear localization of 26S proteasomes and ubiquitin-protein ligases would ensure the efficient coordination of misfolded protein ubiquitination and degradation critical for expeditious NucPQCD, and would also facilitate the precise and timely regulated degradation of normally folded nuclear proteins involved in key nuclear functions like transcription, chromosome replication and cohesion, and cell-cycle progression.

How 26S proteasomes enter the nucleus through the NPCs is still not completely understood and would appear to be a challenge for the cell due to the large size of the 26S proteasome that would seemingly restrict its ability to cross the NPCs (63). Based on this size dilemma, it has been postulated that subcomplexes of the proteasome are initially transported through the NPCs, then assembled into 26S proteasomes in the nucleus (64). However, a recent study using quantitative live-cell imaging in yeast revealed cytosolic assembly of the 26S proteasome that is transported into the nucleus through the NPCs as one complex (65). These findings imply that active 26S proteasomes might shuttle between the nucleus and the cytosol without a prerequisite of disassembly. Once the 26S proteasome is in the nucleus, it can associate with the NE via N-myristoylation of the Rpt2 subunit and this is important to protect the cell from proteotoxic stress (66). Thus, while both cytosolic and nuclear 26S proteasomes exist in cells, nuclear-localized 26S proteasomes appear to have an important role for cellular PQCD.

The nuclear localization of the 26S proteasomes would indicate that they are catalytically active within the nucleus. However, a recent study suggested that nuclear-localized proteasomes have drastically reduced, if any activity in relation to cytosolic proteasomes (67). This reasoning was based on cell fractionation experiments examining the proteolytic activity of proteasomes in nuclear and cytosolic fractions.

Curiously, much less proteasome subunits overall were found by immunoblotting in the nuclear fractions relative to the cytosolic fractions (67), which is contrary to what is observed *in vivo* (64) and may have led to a drastic underrepresentation of nuclear proteasome activity. It is important to acknowledge the difficulty in trying to follow the site of the proteasome activity using biochemical means, and that comparing *in vitro* biochemical data to *in vivo* studies examining proteasome localization and site of activity can be complex due to substantial differences in the technical approaches. Nevertheless, the contrarian studies by Dang and colleagues (67) open a door for future discussions as to the functional activity of nuclear proteasomes.

SUBSTRATE SPECIFICITY IN NucPQCD

Degradation signals (degrons) that trigger the breakdown of their cognate proteins by the proteasome are highly diverse (68). Degrons involved in regulatory processes typically employ simple motifs, of which the most common studied is the attachment of phosphate group to specific site(s) that alter the charge of the degron (69). Degrons involved in PQCD are more complicated and elusive because they are based on changes in the structure of the protein, where misfolding can occur by mutations, errors in production, and post-production damage. Furthermore, post-translational modifications can be variable and complicate PQCD degron identification. Nevertheless, PQCD ubiquitin-protein ligases “know” how to specifically identify misfolded proteins from the rest of the intact folded proteome. This task is likely to be accomplished by employing diverse strategies and will depend upon the nature of the misfolded substrate and the ubiquitin-protein ligases involved.

It is generally assumed in the field that PQCD ubiquitin-protein ligases recognize exposed hydrophobicity in their misfolded protein substrates (68). Hydrophobic residues would normally be buried within the core of normally folded proteins, and their exposure would signal misfolding. This idea originated from studies of Hsp70 chaperones that are known to recognize short hydrophobic stretches that become buried once the client protein is folded (70). Exploration of PQCD degron sequences seems to confirm the

idea that exposed hydrophobicity serves as a degron for recognition by PQCD ubiquitin-protein ligases (71,72). We would like to posit that exposed hydrophobicity is an exceptionally broad term, and it does not explain why there is a need for distinct PQCD ubiquitin-protein ligases in the nucleus to perform essentially the same task. A likely possibility is that each NucPQCD ubiquitin-protein ligase recognizes very distinct types of degrons and/or is responsible for the ubiquitination of substrates in specific nuclear subcompartments. Most of our knowledge about substrate specificity in NucPQCD emerges from studies of Doa10 and San1 (72-78), and we will therefore focus on the details currently known about these NucPQCD ubiquitin-protein ligases. The recently discovered role of Tom1 in the PQCD of ribosomal proteins provides new opportunities for studying ubiquitin-protein ligase specificity (53), since the majority of yeast ribosomal proteins and their interactions with ribosomal RNA (rRNA) are mapped at great detail (79). Accordingly, we will also include what is known about Tom1 recognition of substrates in NucPQCD.

Doa10 substrates generally share a strikingly similar degron, which is composed of an exposed amphipathic helix (71,73-75). Importantly, there is no sequence similarity between these degrons, which implies an importance for the context of the exposed hydrophobic patch within the amphipathic structure (74). However, not all Doa10 substrates rely on an amphipathic helix as a degron. For example, the exposed hydrophobic hairpin of Pgc1 (80) and the exposed hydrophobic transmembrane helix of Sbh2 (81) are non-amphipathic hydrophobic elements that trigger Doa10-mediated degradation. Notably, results from several high-throughput screens in yeast aimed at identifying Doa10 degrons demonstrated a prevalence for hydrophobic residues, but not a clear consensus for sequence (71,76,82).

San1 also recognizes exposed hydrophobicity (72,78), but the type of exposed hydrophobicity is different than Doa10. An extensive analysis of a broad substrate collection revealed the general recognition principles for San1, which targets a specific type of exposed hydrophobicity that is at or above the threshold for aggregation in the nucleus (72,78). San1's mode of substrate recognition appears direct (33), and the

binding to misfolded proteins entails the presence of intrinsically disordered domains within San1 that contains interspersed hydrophobic elements (33).

A major question left to be explored is whether Doa10 and San1 can recognize each other's hydrophobic degrons if they were targeted to the subnuclear compartments in which they operate. This would solve the compartment-specific issue. However, our anticipation is that targeting of Doa10 substrates to the nucleoplasm or San1 substrates to the INM is unlikely to change the rules of NucPQCD substrate recognition by these NucPQCD ubiquitin-protein ligases. This hypothesis is based in part on recent findings by the Ravid lab demonstrating that, upon deletion of Doa10, proteins containing the Ndc10 degron are stabilized in the nucleoplasm despite the presence of San1 (41).

As mentioned earlier, studies of nucleolar PQCD revealed a role for Tom1 in the removal of unassembled ribosomal subunits (54). When expressed in excess, ribosomal subunits can no longer bind to their stoichiometric partners, resulting in the exposure of regions at the interface that are normally inaccessible for contact or buried/hidden from contact. How Tom1 recognizes a large variety of ribosomal proteins, yet manages to maintain specificity for only their unassembled forms, is an open question. Taking advantage of the available crystal structure of the yeast ribosome (79), this dilemma was addressed by Sung and co-workers (54). Substrate recognition by Tom1 entails the exposure of positively-charged residues that are normally buried in the ribosome (54). This is analogous to what is seen with Doa10 and San1, as the Tom1 system is geared to recognize exposure of features that should normally be buried in a normally folded and assembled protein.

Each of the nuclear ubiquitin-protein ligases involved in NucPQCD exemplified above employ distinct strategies for recognizing substrates. The advantage of this strategy appears obvious. It ensures a broad capacity for the cell to eliminate misfolded proteins in the nucleus in a spatially and temporally controlled manner, thus ensuring efficient NucPQCD. However, it is still not clear what the overall complexity of substrate recognition for NucPQCD systems might be. While progress has been made in the last

few years to delve into NucPQCD pathway complexity, more work will be needed to more precisely define the ensemble of degrons for all NucPQCD pathways.

OVERLAPPING FUNCTIONS AND COMPARTMENT CROSSTALK OF NucPQCD – A COOPERATIVE SYSTEM?

As discussed in the previous section, experiments aimed at revealing the specific types of degrons recognized by NucPQCD pathways have led to the idea that they target distinct features in their substrates that are specific to the NucPQCD pathway. However, there are examples of substrates that are recognized by more than one NucPQCD pathway (54,83), suggesting potential overlapping functions for NucPQCD pathways in recognizing the same substrate. In these cases, do misfolded proteins expose multiple PQCD degrons that allow recognition by distinct ubiquitin-protein ligases of the same compartment or even in different compartments? Accordingly, crosstalk between PQCD ubiquitin-protein ligases in different compartments has emerged as an intriguing issue for general PQCD. For example, a number of supposed cytosolic PQCD substrates are not managed in the cytosol, but instead primarily localized to the nucleus where they are managed by NucPQCD systems (30-32,34). This strategy seems to be counterintuitive because misfolded proteins can have deleterious effects in the nucleus compared with the cytosol (33, 77). Overall, these general ideas are important when considering the overall potential interplay between PQCD pathways in a single compartment or multiple compartments.

Studies of the yeast transcription repressor Matα2 shed some light on the interplay between PQCD systems in the nucleus. As mentioned earlier, Matα2 harbors a degron composed of an amphipathic helix, which is recognized by Doa10 (73). However, Matα2 turnover is also controlled by the Slx5/Slx8 complex via non-overlapping degron elements (84). What is the benefit for Matα2 possessing two, or possibly more, non-overlapping degrons? In yeast haploid MATα cells, Matα2 is situated on a MATa-specific gene operator, where it is bound by cofactors that protect it from degradation. Because Matα2 represses expression of genes for the MATa mating type, its rapid turnover is required for timely mating-type switching from MATα to MATa cells.

Simultaneous exposure of both degrons, upon the release of Matα2 from the DNA, may provide a critical protection mechanism to ensure the complete removal of Matα2 under wide-ranging cellular conditions (83).

Non-overlapping degrons are likewise important for the regulation of ribosomal biogenesis (53). Binding of the ribosomal protein Rpl26a to Tom1 entails the exposure of two positively-charged clusters that normally interact with a nearby ribosomal protein. Mutations that alter this binding interface led to poor association with Tom1, yet the protein was still degraded by the proteasome. Apparently upon mutagenesis, Rpl26a became a substrate of the Doa10 pathway (54). This implies that Rpl26a and likely other ribosomal proteins can have multiple degrons to be recognized by distinct NucPQCD ubiquitin-protein ligases. As with Matα2, the two distinct degrons ensure that unassembled Rpl26a does not reach the cytosol, which may be deleterious to the cell.

Preventing leakage of unassembled ribosomal protein from the nucleus could be one way for cells to protect themselves from the potentially harmful effects of misfolded proteins in the cytosol. Another possible way for cytosolic protection is routing them to the nucleus to be degraded. In yeast, several supposedly cytosolic misfolded proteins are enriched in the nucleus where they are targeted for San1-mediated ubiquitination and proteasomal degradation (30-32,34,85,86). Why or how these proteins that lack an obvious NLS transit to the nucleus is unclear. In one case, the Hsp40 chaperone Sis1 appears to be important for nuclear import (35). In other cases, the size of the misfolded protein dictates whether it remains in the cytosol or transits to the nucleus (86). For those versions that remain in the cytosol, their degradation appears to be dependent upon the ubiquitin-protein ligase Ubr1 (86). This was an unusual finding because Ubr1 was originally discovered as a ubiquitin-protein ligase that binds its substrates with high selectivity based on the characteristics of the side chain of their N-terminal residue, known as the N-end rule (87). Ubr1-dependent degradation of misfolded proteins is independent of its mode of binding N-end rule substrates (30), suggesting a novel mode of targeting for Ubr1's function in misfolded protein degradation. As might be expected from their roles in different compartments, Ubr1 and San1 appear to act independently

as their combined loss of function is additive in the effects on substrate ubiquitination and degradation (30-32,34). It remains to be determined if the degradation of cytosolic proteins by San1 is merely a backup plan for misfolded proteins that initially evade Ubr1 inspection and accidentally find themselves in the nucleus because they are below the passive size diffusion limit of the NPCs, or if there is a concerted effort between the nuclear and cytosolic compartments as part of a broader mechanism to optimize intracellular PQCD. Regardless of the mode of operation, we wish to emphasize that, for clarity, it is important to characterize the type of PQCD according to the compartment within which it occurs. Accordingly, we propose that the degradation of cytosolic PQC substrates by San1 should be considered as part of NucPQCD.

Studies of misfolded protein degradation typically focus on a limited set of substrates and thus are often biased towards a narrow range of PQCD pathways. This limits our ability to examine nuclear-to-cytosolic crosstalk at the system level and draw general conclusions about how this compartmental crosstalk occurs. To overcome this limitation, we recently used an unbiased systematic approach in which we identified thousands of putative degron sequences that are either recognized in both the nucleus and the cytosol or are compartment specific (71). Moreover, the shared nuclear-cytosolic degrons are degraded at different rates depending on their cognate ubiquitin-protein ligase. At this point, it is not clear what determines general degron potency, but it is reasonable to anticipate that there are levels of complexity and regulation that extend beyond simple sequence composition that need to be addressed at a system level. From this initial approach, we learned that crosstalk between the nuclear and cytosolic PQCD systems is likely to be much more complex than initially thought.

4.4. REGULATION OF NucPQCD PATHWAYS UNDER STRESS

One way to explain the existence of multiple NucPQCD ubiquitin-protein ligases is that each is activated under distinct conditions, and therefore has its own regulatory pattern. Evidence for this exists in the literature through global transcript studies after yeast cells have been exposed to different stress conditions (88,89). In examining these expression datasets, it is readily observable that the eight genes coding the different NucPQCD

protein-ubiquitin ligases (*SAN1*, *DOA10*, *ASI1*, *ASI2*, *ASI3*, *SLX5*, *SLX8*, *TOM1*) have different expression patterns across different stress conditions (Figure 1.6). For example, expression levels of *DOA10* and the *ASI* complex increase following stresses that induce the Unfolded Protein Response (UPR). Furthermore, as anticipated by its role in amino-acid sensing (47), depletion of amino acids increases expression of the *ASI* complex. By contrast, expression of *SAN1*, *SLX8,* and *TOM1* is elevated upon DNA damage, as predicted by their role in nuclear foci formation during MMS treatment (60). Overall, global expression analyses show that NucPQCD pathways have evolved to cope with changing conditions in a manner consistent with their function.

1.5 CONSEQUENCES FOR THE FAILURE OF NucPQCD

An imbalance in any key nuclear process – DNA replication, transcription, ribosome biogenesis, etc – can make the nucleus vulnerable to protein misfolding. What happens when the NucPQCD machinery fails to manage misfolded proteins? In various circumstances this can lead to the accumulation of toxic and disease-causing protein aggregates.

BASICS OF NUCLEAR AGGREGATE FORMATION

One way the eukaryotic cell generally counters an incapacity of PQCD pathways is to concentrate misfolded proteins into compartment-specific inclusion bodies. These inclusion bodies sequester aggregation-prone misfolded proteins from the rest of the compartment (8). In the yeast nucleus, the major inclusion body is known as the INQ/JUNQ. The inclusion bodies were named JUNQ due to a visual JuxtaNuclear position Quality control compartment relative to the chromatin (90). More recent studies presented the inclusion as the INtranuclear Quality control compartment (INQ) due to its enclosure by the NE (91). Hence, the INQ/JUNQ may either represent a dynamic compartment that spans the NE or two distinct foci of PQC (92), an issue that is currently under study. The INQ/JUNQ can be visualized after overexpression of a misfolded protein in the presence of an intact NucPQCD pathway, but it becomes more prominent when NucPQCD is compromised (72,93), indicating the INQ/JUNQ is a dedicated subnuclear compartment that forms to isolate misfolded proteins until

NucPQCD can manage them. A failure to appropriately handle aggregation-prone misfolded proteins in the nucleus can lead to an eventual failure of nuclear proteostasis and the progression of nuclear proteinopathies.

FAILURE OF NucPQCD CAN LEAD TO NUCLEAR PROTEINOPATHIES

Neurodegenerative diseases are a common consequence of a failure to manage aggregation-prone proteins. The basic principles of misfolded protein aggregation and cytotoxicity are conserved in all eukaryotes including humans. Protein aggregation diseases that cause toxicity include the well-known neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson's disease (PD), and Huntington's disease (HD). Amongst known proteinopathies, there are ≥15 human diseases linked to protein aggregation and inclusion formation in the nucleus (94). Commonly known nuclear proteinopathies include the polyQ-expansion diseases such as HD, 6 spinal-cerebellar ataxias (SCAs), spinal-bulbar muscular atrophy (SBMA), and dentatorubral-pallidoluysian atrophy (DRPLA). Among polyA-expansion diseases, oculopharyngeal muscular dystrophy (OPMD) is known to also occur in the nucleus. Non-tract expanding diseases such as neuronal intranuclear inclusion disease (NIID), neuronal intermediate filament inclusion disease (NIFID), multiple system atrophy (MSA), and inclusion body myopathy with early-onset Paget disease and frontotemporal dementia (IBMPFD) are also known to occur in the nucleus.

How do protein aggregation diseases cause toxicity in the nucleus? One hypothesis is that they "soak up" NucPQCD members and other proteins in their inclusions. This can be seen in HD inclusions, which not only deplete essential chaperones from the system, but also induce aggregation of other misfolded proteins (95). In a yeast model, polyQ-expanded huntingtin caused terminally misfolded proteins to stabilize due to sequestration by the chaperone machinery (35). This phenomenon is not unique to HD diseases. For instance, aggregation of mutant SOD1 in ALS also sequesters the chaperone machinery and increases accumulation of aggregation-prone proteins in cells (96). Other ways aggregated proteins in the nucleus could induce toxicity are by interfering with DNA replication, repair, transcription, ribosome biogenesis and

chromatin organization. Interference in any key cellular functions by misfolded protein toxicity could have widespread effect throughout the system. Apart from the known loss of some chaperones, another open question is whether there are key NucPQCD ubiquitin-protein ligases whose loss further aggravates this process or whether the accumulation of misfolded proteins slowly impair NucPQCD pathways. These are potential questions we need to answer as we uncover how protein aggregation affects NucPQCD (Figure 1.1).

CANCER VIEWED THROUGH THE LENS OF NucPQCD

In addition to nuclear proteinopathies, we think it is also important to consider how NucPQCD might be involved in other diseases, such as cancer. While the link between NucPQCD and cancer is still under investigation, LOF phenotypes related to cancer mutations could be mediated through unwarranted NucPQCD. An example of this is the DNA mismatch recognition and repair protein Msh2, in which mutations are known to cause common hereditary colon cancer (97). Many of the cancer-causing mutations in Msh2 result in NucPQCD by San1 when they are introduced into yeast, even though these mutant proteins still function in DNA mismatch recognition and repair (97).

We would like to consider the seminal findings with Msh2 as applicable to other cases wherein mutations also cause LOF phenotypes, such as in the case of the tumor suppressor p53. Considered a guardian of the human genome, p53 plays many essential nuclear functions and it has many known cancer-causing mutations (98). Loss of p53 function in the nucleus leads to unregulated cell growth and is found to be mutated in more than 50% of human cancer cells (98). Although yet to be explored experimentally, we posit that some mutations in p53 may trigger NucPQCD, resulting in its elimination, even when the mutant p53 protein may be functional. Interestingly, some mutations in p53 are considered aggregative (99), suggesting that they can affect p53 folding. Is it possible that misfolded p53 variants that evade NucPQCD due to aggregation lead to cancerous outcomes? A new view of how cancer-causing mutations in nuclear proteins affect folding and stimulate their recognition by NucPQCD pathways might help refresh an understanding of cancer progression.

One of the multiple ubiquitin-protein ligases that regulate p53 levels is the Tom1 homolog HUWE1. This HECT-type enzyme is dysregulated in various cancer types; however, its function remains controversial (100). Mechanistic investigation revealed that HUWE1 can regulate p53 and c-Myc transcription complexes, thereby to function as a tumor suppressor (101,102). As such, HUWE1 may represent a novel therapeutic target for prevention or intervention of various cancers. Whether the tumor suppressor activity of HUWE1 is linked to its function as NucPQCD ubiquitin-protein ligase has yet to be determined.

AGING AND NucPQCD

One generally acknowledged aspect of aging is a progressive deterioration of an organism's proteome. Thus, we think it is worth considering that aging may lead to a loss of function in NucPQCD pathways and to an increase in the burden of misfolded protein toxicity in the nucleus (Figure 1.1). Because mammalian NucPQCD have yet to be identified and characterized, it is not yet known if depletion of a NucPQCD ubiquitin-protein ligase and/or chaperone contributes to aging. However, alterations in proteasome activity may be instructive as to what might be expected from NucPQCD pathways as cells age. It has been shown that neurons have a reduction in proteasome activity and enhanced accumulation of aggregated proteins as they age (103,104). The loss of degradative capacity and its effects on aging are also highlighted in fibroblasts, where inhibition of the proteasome decreases replicative lifespan and induces senescence (105). Conversely, overexpression of proteasome subunits increases lifespan in worms (106,107). Identification of mammalian NucPQCD pathways will be an important step in understanding their roles in the aging process.

POTENTIAL THERAPIES FOR NUCLEAR PROTEINOPATHIES

The existence of nuclear proteinopathies underscores a potential need to target NucPQCD for therapeutic purposes. Can NucPQCD pathways be targeted to treat misfolded protein diseases? While no therapies have been documented in the literature concerning NucPQCD, we think it is important to understand how current approaches

targeting the UPS might be applied to ameliorate nuclear proteinopathies. In this section, we discuss three primary ways in which NucPQCD could be manipulated in the clinic: (**1**) inhibition of the proteasome, (**2**) modulation of NucPQCD ubiquitin-protein ligases, and (**3**) targeting specific substrates.

Proteasome inhibitors such as Bortezomib and Carfilzomib are in clinical use and have shown efficacy in treating multiple myeloma and several related cancers (108). Although targeting the proteasome seems counterintuitive as it would affect multiple key regulatory and PQCD pathways in the cell, the therapeutic use of proteasome inhibitors to treat particular cancers may be effective in cases where mutations in key tumor suppressor proteins cause NucPQCD. We do have a healthy skepticism as to whether proteasome inhibitors are ideal because proteasome inhibition is not target specific and can increase the bulk of aggregated proteins (109), which is a hallmark of many neurodegenerative disorders. It remains to be seen if proteasome inhibition could be a viable therapy of choice for cancers that result from excessive NucPQCD. However, targeting the proteasome will not aid in treating nuclear protein aggregation diseases like HD.

Since ubiquitin-protein ligases are the most specific components of the UPS, many efforts are underway to modulate their function for therapeutic purposes. There are three principal strategies to target a ubiquitin-protein ligase: (**1**) inhibiting its enzymatic activity, (**2**) inhibiting the interaction with its substrate, or (**3**) altering its expression levels (110). Each strategy has its own challenges. Of the NucPQCD ubiquitin-protein ligases described, only the HECT-domain Tom1 is directly involved in catalysis and can be a target for active site inhibitors. Thus, it is possible to design enzymatic inhibitors for Tom1 and other HECT-domain proteins, but it is not a viable route for RING-domain NucPQCD ubiquitin-protein ligases. For RING-type ubiquitin-protein ligases, inhibition of substrate binding is a more feasible option, which entails the design of small molecules that bind specifically to the interface between the substrate and the ubiquitin-protein ligase. This approach requires structure-based studies to inform the development of small molecules. Small molecules that already show a clinical potential are nutlins that

insert into the binding interface between p53 and MDM2, preventing p53 ubiquitination and degradation, thus restoring p53 function as a tumor suppressor (111,112). Indeed, clinical trials of nutlins are underway and thus far they show a promising potential (113). Alternatively, an emerging concept put forward the use of small molecules as 'molecular glue' that enhances the interaction of a ubiquitin-protein ligase with its substrates (114). For example, in plants, binding of auxin hormone to the ubiquitin-protein ligase Tir1 strengthens the interaction between Tir1 and its substrates (115). Similarly, identification of small molecules that can help NucPQCD ubiquitin-protein ligases better 'stick' to misfolded proteins and target them for degradation could be a potential avenue for new therapies.

Upregulating the expression of NucPQCD ubiquitin-protein ligases could be another way for treating nuclear protein aggregation diseases. As shown earlier, NucPQCD ubiquitin-protein ligases are differentially regulated under various stress conditions (Figure 1.6). Is it possible to actively and specifically increase their expression levels? The accumulation of aggregation-prone proteins can be ameliorated by upregulating stress-signaling pathways using small molecules like Celastrols (116). Although not known for upregulating PQCD pathways, Celastrols can increase the production of chaperones involved in NucPQCD to match the proteostasis needs of the cell (116). It is important to note that upregulating stress pathways can be beneficial in removing aggregation-prone proteins, but diseases like cancer often show an increase in chaperone activity that manages the burden of misfolded proteins caused by rampant DNA mutation (Figure 1.4) (117). Thus, modulation of stress-responsive systems likely needs stringent testing before advancement to pre-clinical trials.

Targeting of selected substrates for degradation is an additional potential way for treating nuclear proteinopathies. Currently, two novel approaches are being tested for this purpose: PROteolysis TArgeting chimeras (PROTACs) and HYdrophobic Tagging (HyT) (118). PROTACs are dual functioning small molecules that contain a substrate binding module, connected by a short linker to a ligand specific for a desired ubiquitin-protein ligase. PROTACs were originally conceived in 2001 (119), but were not

considered as a viable therapeutic approach at the time due to general difficulties in their synthesis and cell permeability. These have been largely solved with recent developments in synthetic chemistry. Similar to PROTACs, HyTs can also be used to target a selected protein to a ubiquitin-protein ligase (118). The substrate-binding ligand in HyTs is a hydrophobic moiety that binds to exposed hydrophobicity, and therefore may be considered as a more general option to induce NucPQCD (120). By using HyTs, misfolded nuclear proteins that evade NucPQCD and accumulate as toxic aggregates are potential targets for the NucPQCD ubiquitin-protein ligase. We envision that future development of PROTAC or HyT ligands that target aggregation-prone proteins to NucPQCD ubiquitin-protein ligases will open promising new avenues to treat nuclear proteinopathies.

1.6 CONCLUDING REMARKS

The UPS is a central mechanism for protein degradation in the eukaryotic cell, especially in controlling regulatory pathways. It is therefore not surprising that a key role of the UPS is also in the maintenance of cellular proteostasis. Indeed, studies from the past two decades have taught us the instrumental role of PQCD in the viability and survival of eukaryotes. NucPQCD has largely been studied in yeast, where we have made considerable progress in identifying pathways and understanding targeting mechanisms. The discovery of San1 as a NucPQCD ubiquitin-protein ligase in 2005 highlighted the importance of UPS-mediated protein degradation for the maintenance of nuclear proteostasis and function. Since then, several yeast NucPQCD pathways have been identified, each of which recognizes a distinct feature in their misfolded protein substrates. A focal point of this chapter was to address why there is a need for multiple NucPQCD ubiquitin-protein ligases to essentially perform the same task. By presenting current mechanistic findings on ways to eliminate protein dysregulation in the nucleus, we reached a conclusion that each NucPQCD pathway has a unique contribution for nuclear proteostasis. It is also likely that the presence of several NucPQCD pathways enables spatial and temporal handling of protein misfolding in the nucleus, since each of

the NucPQCD ubiquitin-protein ligases respond to stress in a unique expression pattern. We also touched base on how a failure of NucPQCD can lead to protein accumulation and cellular harm that can be observed in certain nuclear proteinopathies, and explored how current therapies targeting the UPS system could aid in remedying these diseases. We think that understanding each component of this system in addition to a systematic view of its interconnectivity and complexity will lead to significant insight relevant for mammalian NucPQCD pathways and future therapies.

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Enam, C., Geffen, Y., Ravid, T. and Gardner, R. G. 2018. Protein quality control degradation in the nucleus. *Annual Review of Biochemistry* 87: 725-749.

FIGURE LEGENDS

Figure 1.1: The balance between PQC capacity and misfolded protein burden changes with age. One of the parameters that determine a proteome's health is the ratio of PQC capacity and the burden of misfolded proteins, which correlates with the age of the cell. When cells age, the burden of misfolded proteins may exceed the PQC capacity, and misfolded proteins can accumulate in aggregates. This may lead eventually to proteostasis collapse and cell death.

Figure 1.2: Protein folding is essential for nuclear function. Synthesis errors, post synthesis damage, stress, or mutations can trigger protein misfolding. If a protein's structure is beyond repair, it is sent for degradation via the UPS or the autophagy pathways.

Figure 1.3: The mammalian nucleus is divided into subcompartments. Each of the subcompartments plays a unique role in maintaining distinct nuclear functions.

Figure 1.4: Quality control is essential for basic cellular functions, including the maintenance of proteins of the PQC. Proteins are the cell's workhorses-from playing a role in maintaining DNA replication, transcription and translation to catalyzing enzymatic reactions and building key cellular structures. The overarching functions of proteins in the cell indicate that if proteins functions are not guarded by the PQC system, it will lead to severe decline in all cellular activity.

Figure 1.5: Ubiquitin protein ligases involved in NucPQCD are shown respective to their locations in the ER and nucleus. Higher concentration of the ubiquitin protein ligase is signified by darker green color and lower concentration is signified by lighter green color.

Figure 1.6: Distinct expression levels of the different Regulation of NucPQCD ubiquitin-protein ligases. Heat map showing the changes in expression of NucPQCD ubiquitin-protein ligases under various stress conditions.

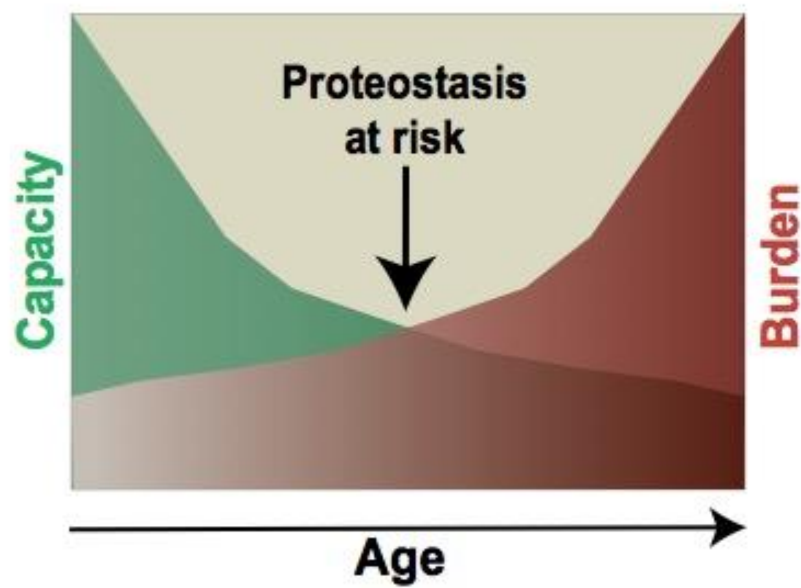

Figure 1.1: The balance between PQC capacity and misfolded protein burden changes with age.

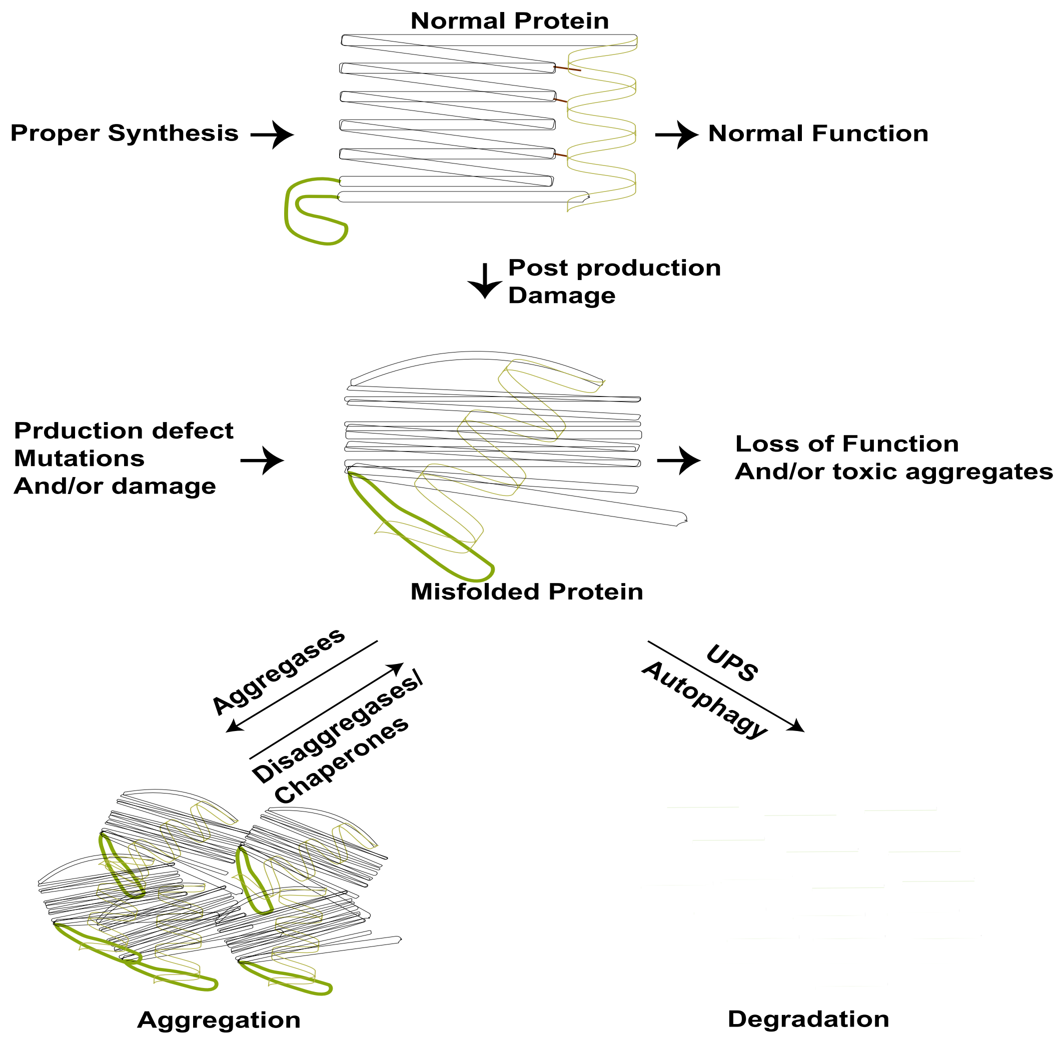

Figure 1.2: Protein folding is essential for nuclear function.

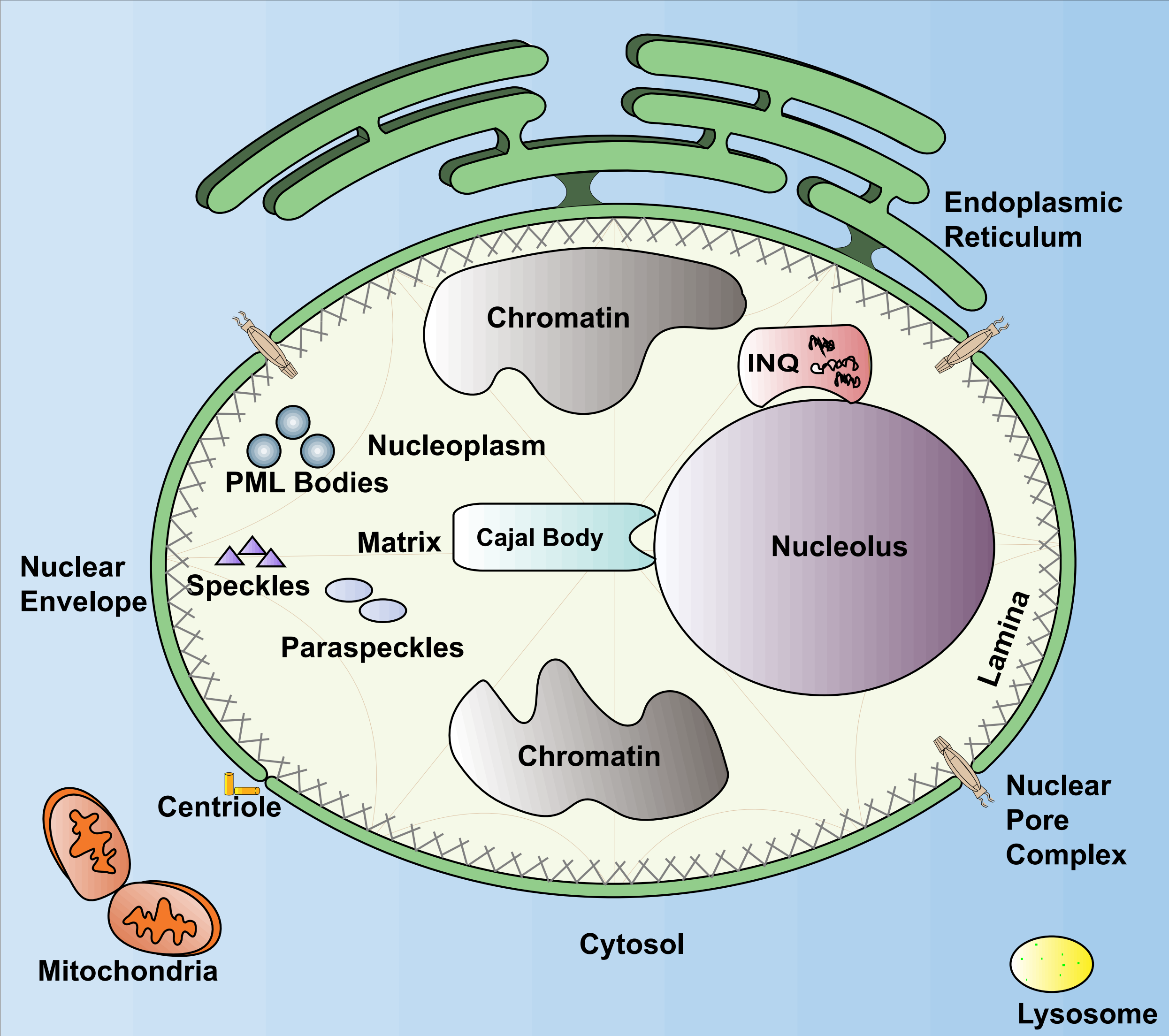

Figure 1.3: The mammalian nucleus is divided into subcompartments.

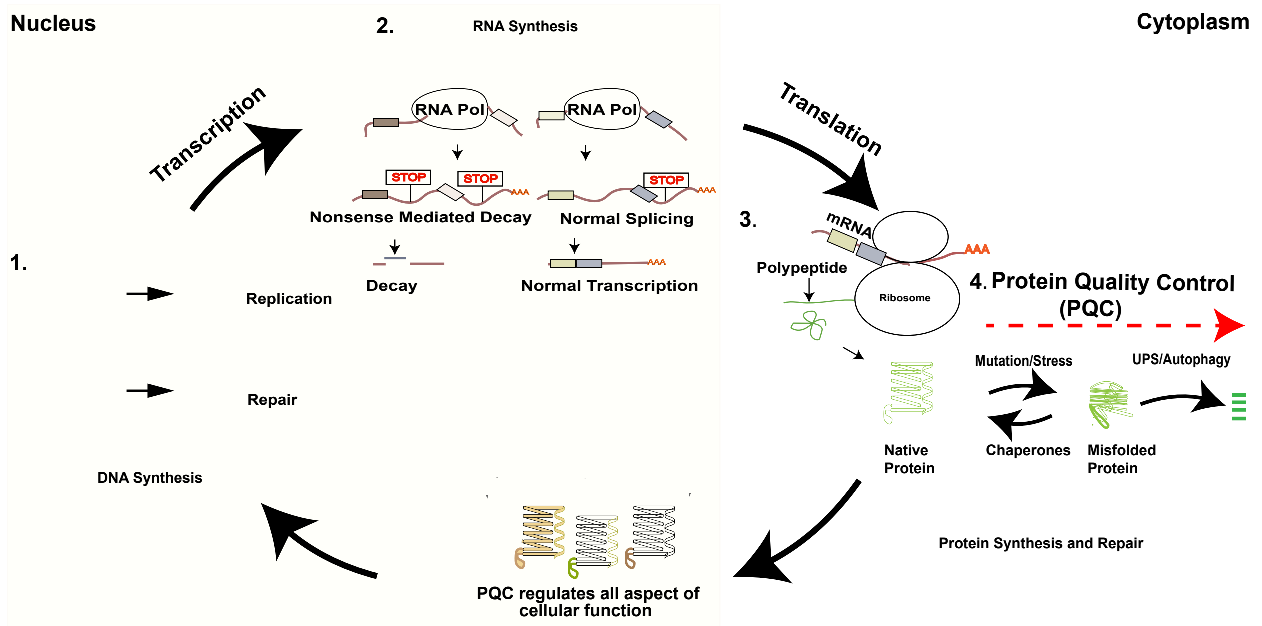

Figure 1.4: Quality control is essential for basic cellular functions, including the maintenance of proteins of the PQC.

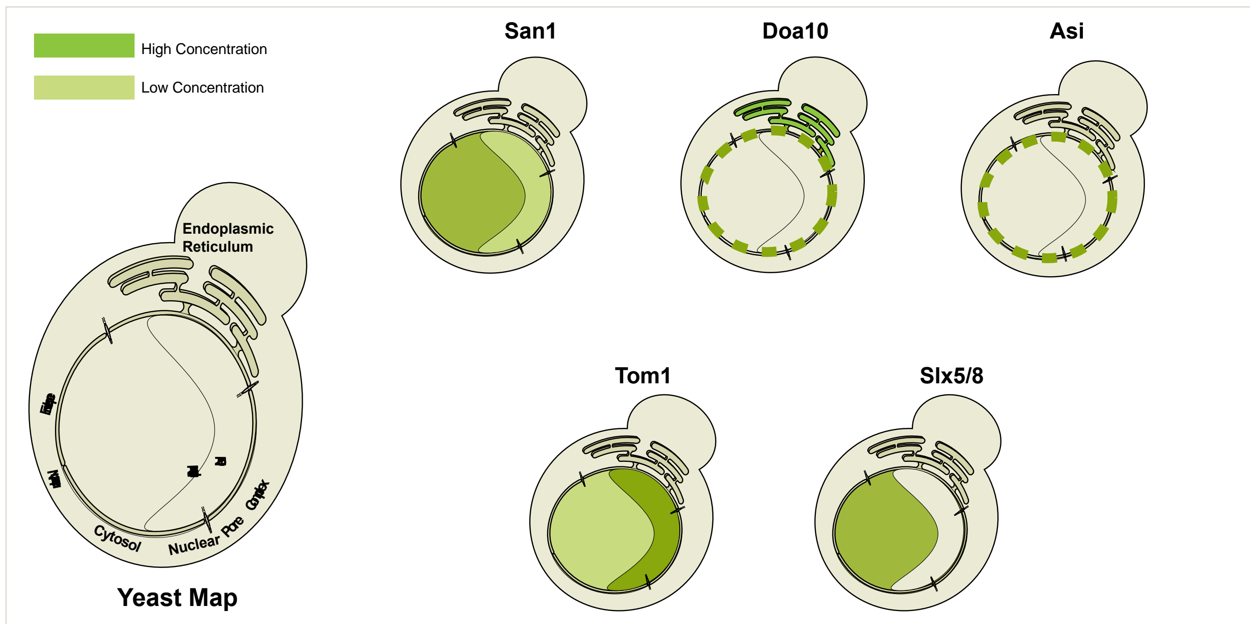

Figure 1.5: Ubiquitin protein ligases involved in NucPQCD are shown respective to their locations in the ER and nucleus.

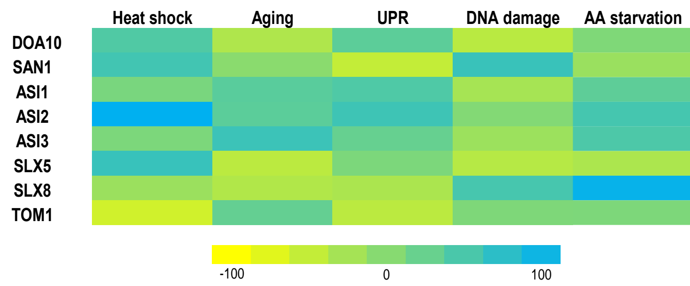

Figure 1.6: Distinct expression levels of the different Regulation of NucPQCD ubiquitin-protein ligases.

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CHAPTER TWO:

Cdc48 and Ubx cofactors play a role in protein degradation

2.1 Cdc48 plays an important role in cellular function:

The protein Cdc48 (known as p97 in mammals) is highly abundant and conserved from flies to mammals (1,2). Disruption of the Cdc48 gene in budding yeast was found to inhibit cell division, affect cell viability in flies and is lethal to mammalian embryos, thus establishing the key cellular functions of this protein (3,4,5). As a member of the AAA ATPase family, Cdc48 uses energy generated from ATP hydrolysis to work as a "segregase" or "unfoldase" that can separate or unfold misfolded proteins from membranes and complexes (6). Homohexamic in shape, each protomer of Cdc48 contains a N-domain, two ATPase domain (D1 and D2) that are stacked on each other and an unstructured C terminal domain (2). The D2 domain of this protein is mainly responsible for the mechanical force generated by ATP hydrolysis under physiological condition (7). Despite being an essential component for the cell, the exact mechanism by which Cdc48 separates proteins and/or unfolds them is still poorly understood.

Cdc48 was first identified in a genetic screen for cell cycle defects (8). It plays multiple roles in both proteolytic and non-proteolytic processes. The proteolytic functions of Cdc48 include retrotranslocating misfolded proteins during ERAD, DNA damage, DNA replication, ribosome-associated degradation and mitochondrial-associated degradation (2,9). In non-proteolytic processes, Cdc48 plays a role in nuclear, Golgi, and ER membrane fusion (10,11). The key to such diverse cellular functions lies in the multiple cofactors which interacts with Cdc48 during various cellular functions. These cofactors bind to Cdc48 and guide it spatially and temporally, thus specifying localization and function of Cdc48 in the cell.

2.2 Ubx cofactors interact with Cdc48:

The Ubx proteins are the largest family of cofactors known to interact with Cdc48 (2). They contain a domain called the UBX "ubiquitin regulatory X" domain, which has a similar tertiary structure to ubiquitin (β–β–α–β–β–α–β) (27). The UBX domain interacts by inserting the conserved loop between strand 3 and 4 of its β sheet into the hydrophobic pocket of the N-terminal domain of Cdc48 (13). There are 13 UBX proteins in mammals and 7 in yeast. Although all of the Ubx cofactors have been shown to bind Cdc48 directly, the cellular function of many of these Ubx cofactors are not well known (13,14,15).

Apart from the general UBX domain, some Ubx proteins also contain a ubiquitin-binding domain (UBA) which binds to ubiquitin (2). Ubx cofactors that have a UBA domain are thought to function as a recruiting platform, where the UBA domain binds to ubiquitinated substrates, and the Ubx domain binds to Cdc48, leading to the efficient interaction of Cdc48 with its ubiquitinated substrates. Other Ubx proteins not containing a UBA domain are thought to work as "substrate processing cofactors" (6,16). These proteins work downstream of the "substrates recruiting cofactors" and influence the final fate of the ubiquitinated substrates. Once recruited to Cdc48, these "substrate processing cofactors" help Cdc48 unfold ubiquitylated substrates before delivering them to the proteasome (6,11).

Along with the UBA and UBX domains, other domains within the Ubx cofactors such as the SHP domain in Ubx1, help in the oligomerization of the cofactor. Binding of the oligomerized cofactor to Cdc48 imposes stearic hindrance to the binding of other cofactors. For instance, when mammalian Ubx1 binds to Cdc48, the Ubx1 trimer imposes a conformational change in Cdc48 that inhibits the binding of other cofactors such as Npl4-Ufd1 (17). As a result, Ubx1 proteins control the orientation and spatial features of Cdc48, its interaction with ubiquitinated substrates, and prevent or enhance other cofactors from binding to Cdc48, thereby restricting Cdc48 to a specific pathway. Although the oligomerization status of other Ubx cofactors are not known, it can be

hypothesized that the particular conformational changes resulting from Ubx cofactors binding to Cdc48 tightly regulates its activity in specific cellular pathways.

2.3 Cellular functions of Ubx1-7:

Ubx cofactors directly interact with Cdc48, however their individual contribution in the functional pathways are not known in detail. Their importance in maintaining essential cellular functions have been demonstrated by the disruption of major cellular function in organism lacking functional Ubx cofactors. For example, absence of Ubx1 disturbs cell cycle division and results in severe growth phenotypes (15,18), and loss of Ubx1, Ubx2 and Ubx4 triggers yeast sensitivity to various kinds of stresses (15).

The mammalian Ubx1 is the most characterized of the UBX-domain proteins. First identified in the membrane fusion pathway (19), Ubx1 has been established to play a role in the nuclear envelope assembly, ER and Golgi membrane fusion and autophagosome biogenesis (20,21,22). In the nucleus, Ubx1 is required for turnover for Asi1 degradation in the inner nuclear membrane in yeast (23). Moreover, Ubx1 has also been shown to regulate Glc7, the Protein Phosphatase 1 in yeast and thus regulate cell division (24).

Ubx2 plays a key role in ERAD, where it recruits Cdc48 to the ER membrane and facilitates Cdc48's interaction with ubiquitinated substrates and ubiquitin ligases that leads to efficient degradation of misfolded proteins (25). Both Ubx3 and Cdc48 controls mono-ubiquitylation of histone H2B which is needed for chromatin organization during active gene transcription in the nucleus (26). Similar to Ubx2, Ubx4 also modulates Cdc48 in the ERAD pathway and plays a role in the release of ubiquitinated substrates from the Cdc48 complex (27). Moreover, Ubx4 plays a role in degrading mitotic regulators during anaphase proteolysis and redistributing the proteasome correctly in the nucleus (28). During DNA damage, both Ubx4 and Ubx5 recruit Cdc48 to the DNA lesion and facilitate the degradation of chromatin bound Rpb1 (RNA polymerase II) bound to the chromatin (29). Interestingly, apart from their direct interaction to Cdc48, the unique role of Ubx6 and Ubx7 in the cell are not yet known.

2.4: Understanding the role of Ubx cofactor can help mitigate Cdc48-related diseases:

Mutations in Cdc48 has received a lot of attention due to its causal link to the lethal inclusion body myopathy, Paget's disease of the bone, frontotemporal dementia (IBMPFD), amyotrophic lateral sclerosis (ALS), and even several types of cancer (30,31). Absence of Cdc48 leads to the accumulation of ubiquitinated proteins such as TDP-43, which is considered to be a neuropathological marker (32). Mutations in Cdc48 affect various functions including ERAD disruption (33), and impaired autophagosome biogenesis (34). Most of the known mutations reside in the N-terminal domain of Cdc48 and affect the conformation of Cdc48, thus perturbing the hydrolysis of ATP and resulting in high ATPase activity (7). Moreover, since many cofactors dock at the N domain, mutations in this part of the protein affect interaction of the cofactors with Cdc48. Recently, a study revealed that overexpression of Ubx1 reduced the mutant Cdc48 ATPase activity to baseline. This opens an exciting possibility where titrating Ubx1 can restore normal Cdc48 cellular function (35).

In order to design therapeutic strategies that modulate Cdc48 activity and improve human health, we need to elucidate three important mechanisms of action, (i) the interaction of Cdc48 with its cofactors, (ii) the interaction of cofactors with its substrates, and (iii) the interaction of Cdc48-cofactors-substrates in the context of their target pathway(s). The Gardner lab recently found that in absence of Cdc48, degradation of misfolded proteins is halted in the San1 pathway (36). My present work establishes Ubx1, Ubx4 and Ubx5 as the three cofactors required by Cdc48 to perform its function in the San1 pathway, and reveals their distinct role in degrading misfolded proteins in the nucleus.

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CHAPTER THREE:

Ubx cofactors regulate nuclear protein quality degradation

Introduction:

Protein homeostasis is essential for optimal cell function. Several diseases including Huntington's Disease (HD), Parkinson's Disease (PD) and cancer have been associated with aberrations in protein homeostasis (1). To ensure the degradation of misfolded and short-lived proteins in a timely manner, the cell coordinates chaperones and proteolytic systems to ensure vigilant protein quality control (PQC) (2). The ubiquitin proteasome system (UPS) is the major proteolytic route that degrades proteins before they aggregate and damage the cell. In the UPS, the misfolded proteins are tagged with ubiquitin in an ATP-dependent process and targeted to the proteasome for degradation. The selectivity of tagging misfolded proteins with ubiquitin are carried out by ubiquitin ligases (3).

As the home of essential processes such as DNA and RNA synthesis, failure to remove misfolded proteins from the nucleus can lead to failure in nuclear protein homeostasis (4). Compared to compartmental proteolytic systems in the cytoplasm and the endoplasmic reticulum (ER), protein quality control systems in the nucleus are less understood. Nuclear aggregation of misfolded proteins is connected to several neurodegenerative diseases such as HD and PD, which indicates the nuclear vulnerability towards toxicity (5). Thus, the need to understand nuclear protein quality control systems.

Our previous work has identified San1 as the central ubiquitin ligase that targets misfolded proteins in the nucleus of *S. cerevisiae* (6). Recently we also identified Cdc48 to be an essential chaperone of San1 that selectively degrades the more insoluble nuclear proteins (7). Cdc48 is a well-characterized AAA ATPase. By selectively separating ubiquitinated proteins from complexes and membranes, Cdc48 ensures specific protein degradation (8). Cdc48 is involved in a variety of processes, such as ER associated degradation (ERAD), DNA damage, and DNA replication (8). The versatility of Cdc48 lies in the large number of cofactors that form a complex and guide Cdc48 to interact with various substrates both spatially and temporally (9). Although the cofactors that

collaborate with Cdc48 during ERAD are well characterized, the cofactors that regulate Cdc48 to efficiently degrade misfolded proteins in the nucleus are under exploration to delineate their functional roles.

One of the largest family of Cdc48 cofactors are the Ubiquitin regulatory X (UBX) proteins. The UBX cofactors dock to Cdc48 and guide it temporally and spatially in PQC in various cellular function (10). The role of Ubx1 is well known in membrane fusion and autophagy (11,12). Both Ubx2 and Ubx4 are known players in ERAD mediated protein degradation (13,14). In the nucleus, Ubx1 was shown to degrade Asi1 in the inner nuclear membrane and Ubx4 is known to degrade mitotic regulators for anaphase (15,16). Moreover, during DNA damage, Ubx4 and Ubx5 were shown to play a role in degrading Rpb1, the largest subunit of RNA Polymerase II (17). However, the cofactor combination that helps Cdc48 carry out its function is not yet known in the San1 nuclear pathway.

In the present study we identified the Ubx cofactors that work with Cdc48 to degrade misfolded proteins in the nucleus. We reveal that the Ubx cofactors maintain solubility of misfolded proteins before they reach the proteasome, localize Cdc48 in the nucleus for efficient protein degradation, and reduce toxicity burden induced by misfolded protein.

Results:

Ubx cofactors are required for misfolded protein degradation: We characterized over 40 bonafide San1 substrates that included missense mutations, truncated proteins, and hydrophobic peptide degrons in our lab previously (18,19). We chose to study a few proteins from each category, to identify which of them were dependent on Cdc48 for degradation. We narrowed down two truncated proteins: GFP^{NLS}-Tef2* (translation elongation factor fused to GFP) comprising of residues 190-458, and GFP^{NLS}-Bgl2* (endo-b-1,3-glucanase fused to GFP) consisting of residues 20-313 (7,19). Deletion of key amino acids in both substrates is thought to trigger protein misfolding, resulting in San1 dependent protein degradation. These substrates are highly insoluble compared to the other San1 substrates we tested and required Cdc48 to maintain their solubility before proteasomal degradation (7). Since Ubx cofactors work alongside and assist

Cdc48 to degrade misfolded proteins, we chose to study both GFP^{NLS}-Tef2* and GFP^{NLS}-Bgl2* for Ubx mediated protein degradation (Figure 2.1 and 2.2).

All seven Ubx cofactors in *S. cerevisiae* are known to interact with Cdc48 via their Ubx domain (17,20,21). To establish which specific Ubx cofactors are required in the San1-dependent nuclear protein degradation pathway, we generated deletions of all *UBX* genes. Loss of Ubx1 (*ubx1∆)*, Ubx4 (*ubx4∆) and* Ubx5 (*ubx5∆)* resulted in the reduced degradation of the protein substrates GFP^{NLS}-Tef2* and GFP^{NLS}-Bgl2*(Figure 2.1). Loss of Ubx2, Ubx3, Ubx6 and Ubx7 did not affect the degradation of the substrates indicating these cofactors play no role in degrading misfolded proteins in the San1 pathway in the nucleus (Figure 2.2). Thus, our data reveals that all three Ubx cofactors are involved to different extent in nuclear mediated protein degradation in the San1 pathway.

Ubx cofactors work in the San1 pathway: From our protein degradation studies, we reasoned that perhaps Ubx1, 4, and 5 work in parallel pathways, since their single absence halted protein degradation only partially. A previous study observed that during DNA damage in the nucleus, Ubx4 and Ubx5 work in parallel pathway to degrade Rpb1 at the chromatin (17). Perhaps, Ubx1 and Ubx4 or Ubx1 and Ubx5 work in parallel, each assisting Cdc48 in different routes to degradation. We were also curious why three Ubx cofactors were required in a single protein degradation pathway. Perhaps they were redundant in their function, and acting as safeguard to ensure undisrupted and consistent PQC. To investigate, we generated *ubx1∆/ubx5∆* and *ubx4∆ubx/5∆* mutated *S. cerevisiae*. The protein degradation profile of the two substrates GFP^{NLS}-Tef2* and GFP^{NLS}-Bgl2* showed that there was no excess of stabilization of misfolded proteins in both *ubx1∆/ubx5∆* and *ubx4∆/ubx5∆* (Figure 2.3), suggesting that these proteins are not degraded via parallel pathways. Loss of both Ubx1 and Ubx4 resulted in synthetic lethality, thus we could not query into the redundancy of Ubx1 and Ubx4(Figure 2.4). Our data indicates that when misfolded proteins are over expressed in the nucleus, the Ubx cofactors all work in San1 dependent pathway, perhaps in a sequential manner.

Loss of Ubx cofactors impairs cell viability: Accumulation and impaired degradation of misfolded proteins leads to toxicity and hampers cell growth. Previous studies have indicated the importance of Ubx1, Ubx2, and Ubx4 to protect yeast under various stressful

conditions (13,21,22). Based on the observation that Ubx cofactors are necessary to clear misfolded proteins in the nucleus, we hypothesized that over expression of misfolded proteins in cells lacking expression of the Ubx1, Ubx4, and Ubx5 proteins will reduce cell viability. Under basal conditions, only the loss of Ubx1 resulted in a slower growth rate (Figure 2.5), which could be expected given that Ubx1 has been previously implicated in cell cycle control (22). Moreover, this growth can be rescued when the protein level of Ubx1 is restored thus confirming that the slow growth for *ubx1*Δ observed is only due to loss of the Ubx1 cofactor (Figure 2.5). Over expression of both GFP^{NLS}-Tef2* and GFP^{NLS}-Bgl2*, resulted in toxicity in strains lacking Ubx1 and Ubx4 cofactors (Figure 2.6-2.7). Thus, with overload of misfolded proteins in the nucleus, Ubx1 and Ubx4 likely play an important role in clearing the burden of misfolded proteins. In *ubx1Δ/ubx5*Δ and *ubx4Δ/ubx5*Δ strains, the burden of toxicity is even more, indicating the importance of cofactors in maintaining cellular health. Thus, Ubx1 and Ubx4 are important for maintaining the health of cells when misfolded proteins are expressed and absence of Ubx1 and Ubx5, or Ubx4 and Ubx5 exacerbates toxicity.

Ubx cofactors are required to maintain substrate solubility: Aggregation of misfolded proteins often block the narrow chamber in the proteasome, leading to insoluble inclusion, delaying degradation and enhancing toxicity in the cell (5). Cdc48 has been previously shown to prevent aggregation in vitro and play a role in maintaining solubility in vivo (7,23). We reasoned that the Ubx cofactors may play a similar role as chaperones in keeping the misfolded proteins soluble before they enter the proteasome. To query, we investigated the solubility of the GFP^{NLS}-Tef2* as our surrogate substrate in *ubx*Δ strains. In the absence of Ubx1 and Ubx5 cofactors, GFP^{NLS}-Tef2* partitions in the insoluble fraction more than soluble fraction, a pattern we also observe in *cdc48-3* (Figure 2.8). Thus, the substrates that are dependent on Cdc48 for maintaining their solubility are also equally dependent on the Ubx1 and 5 cofactors to keep them soluble before they are degraded by the proteasome. To our surprise, we found this was not the case with *ubx4*Δ cells, indicating that Ubx4 plays alternate roles to maintaining substrate solubility. Furthermore, our experiments revealed that in both *ubx1Δ/ubx5*Δ and *ubx4Δ/ubx5*Δ strains, there is no enhancement of insolubility of GFP^{NLS}-Tef2* compared to single *ubx*Δ,

indicating when maintaining solubility of misfolded proteins, Ubx1 and Ubx5 are redundant in function.

Absence of Ubx cofactors increases substrate ubiquitination: Our solubility assay suggests Ubx4 is participating in a separate role compared to Ubx1 and Ubx5. To investigate this hypothesis, we use GFP^{NLS}-Tef2* as our surrogate substrate and performed *in vivo* ubiquitination assays (Figure 2.9). We show that GFP^{NLS}-Tef2* was ubiquitinated in parent cells, absent in *san1*Δ cells and as previously described, this ubiquitination was enhanced in *cdc48-3* cells (7,17). Absence of Ubx1, Ubx4 and Ubx5 enhanced the ubiquitination of GFP^{NLS}-Tef2*, demonstrating that after a misfolded protein is ubiquitnated, Ubx cofactors further influences the level of substrate ubiquitination. Moreover, our data also reveals that Ubx1, Ubx4 and Ubx5 work downstream of San1 in the UPS pathway. Although, there was an increased amount of ubiquitinated substrates in all *ubx*Δ more than parents, and ubiquitin conjugates were of higher molecular weight in size (110 kDa and greater) in *ubx4*Δ compared to *ubx1*Δ and *ubx5*Δ strains. This increase in ubiquitin conjugate size is similar to *cdc48-3* mutant strains, indicating that perhaps absence of Ubx4 affects Cdc48's ability in processing the misfolded proteins before they enter the proteasome. We also queried at the *ubx1Δ/ubx5Δ* and *ubx4Δ/ubx5Δ* strains. Our results reveal that the ubiquitinated substrates have the same pattern in *ubx1Δ/ubx5Δ* compared to *ubx1*Δ and *ubx*Δ*5*. Furthermore, our results also hint that in the San1 pathway Ubx4 is probably working upstream in the pathway compared to Ubx1 and Ubx5.

Ubx4 localizes Cdc48 into the nucleus to mediate efficient protein degradation: Although Cdc48 is localized throughout the cell under basal condition, we observe more Cdc48 to localize to the nucleus during expression of misfolded nuclear proteins (7). Previous function of Ubx2 in ERAD revealed its importance in localizing Cdc48 with its complex at the ER for efficient protein degradation. Could the Ubx cofactors also play a role in localizing Cdc48 to the nucleus? To investigate, we express GFP^{NLS}-Tef2* and GFP^{NLS}-Bgl2* as our substrates and GFP^{NLS} as our control. We observed that in *ubx1Δ, ubx4*Δ and *ubx5Δ strains*, Cdc48-Mcherry enter the nucleus upon expression of both GFP^{NLS}-Tef2* and GFP^{NLS}-Bgl2*, similarly to parent strain (Figure 3.0). However, this localization is less pronounced in absence of Ubx4, indicating a possible role of Ubx4 in

keeping the Cdc48 present in the nucleus (Figure 3.0-3.1). Our results indicate that Ubx1 and Ubx5 are dispensable in shuttling Cdc48 to the nucleus. One explanation for this could be that Ubx1 and Ubx5 play a redundant role in shuttling Cdc48 to the nucleus. Thus, in absence of both Ubx1 and Ubx5, we should see less Cdc48 in the nucleus. However, we observe that Cdc48 can localize to the nucleus in the *ubx1Δ/ubx5Δ* to the same extent as *ubx1Δ* and *ubx5Δ*, indicating that Cdc48 does not depend on Ubx1 and Ubx5 cofactors to shuttle it to the nucleus. Furthermore, our *ubx4Δ/ubx5Δ* strains also show less pronounced Cdc48 localization in the nucleus similar to absence of Ubx4, thus indicating Ubx4 cofactor to play an important role in recruiting Cdc48 to the nucleus compared to Ubx1 and Ubx5.

Discussion:

Role of Cdc48 Cofactors in Health and Disease: Mutant Cdc48 is causally linked to IBMPFD, ALS and cancer (9). Since Cdc48 plays different roles in the cell, targeting Cdc48 alone will affect all key cellular function and may not be desirable. Instead, identifying the particular combination of cofactors that work with Cdc48 in a particular pathway may help us to target Cdc48 more specifically. For example, mutant Cdc48 has high ATPase activity (24). A recent study revealed that overexpression of p47 (mammalian homologue to Ubx1) is able to reduce the high ATPase activity of mutant Cdc48 to normal. This opens up possibilities where modulating particular cofactors of Cdc48 will help us modulate Cdc48 more specifically in the cell (25).

Role of Ubx cofactors in Cdc48 mediated protein degradation: The role of Cdc48 in nuclear mediated protein degradation is well established (9,7,17). However, the cofactor combination that helps Cdc48 degrade the nuclear misfolded proteins in the San1 pathway was not known. In this study, we reveal Ubx1, Ubx4, and Ubx5 work with Cdc48 in degrading misfolded proteins in the nucleus.

One of the aftermaths of aggregated misfolded proteins in the cell is that it causes cell toxicity. Ubx1 and Ubx4 have been shown to help yeast cells survive through multiple stress (14,17). Our data here reveals Ubx1 and Ubx4 to play a role in maintaining nuclear

protein burden, because in their absence yeast cells are not viable under stress of accumulated misfolded nuclear protein. We also wanted to pursue if Ubx1 and Ubx4 play redundant role in keeping the nucleus free of misfolded protein aggregation, but loss of Ubx1 and Ubx4 together show synthetic lethality. Based on previous and our current studies, we reason that both Ubx1 and Ubx4 help cells survive through stress, and absence of both is detrimental for the cell survival. Moreover, Ubx1 is a well-known player in yeast cell division, and Ubx4 has been shown to also play an important role during anaphase by degrading mitotic regulators (15,22). Thus, we think it is possible that in the absence of both Ubx1 and Ubx4, the yeast cells fail to undergo error free cell cycle and is non-viable. Thus, these cofactors are not dispensable as their presence during cellular stress is important for the cell survival.

We previously discovered Cdc48 to be responsible for keeping misfolded proteins soluble before they enter the proteasome (7,26). This is important since aggregation of misfolded protein block the narrow channel of the proteasome and induce toxicity (27). We reasoned that the Ubx cofactors will play a similar role in reducing aggregation and maintaining solubility of misfolded proteins. We identified Ubx1 and Ubx5 to play a role in the keeping the misfolded proteins soluble before it reaches the proteasome. To our surprise we observed that Ubx4 plays no role in maintaining solubility. To parse this observation, we wanted to investigate if the Ubx cofactors were functioning before or after the San1 ubiquitination of misfolded proteins. Our previous study reveals Cdc48 to work after San1 ubiquitination, but before the proteasome. Since both Ubx1 and Ubx5 possess UBA domains, we reasoned that they may interact with misfolded proteins after San1 protein ubiquitination. Our data reveals that Ubx1, 4 and 5 all work after San1 pathway in the proteasome. However, the ubiquitination pattern of *ubx4*Δ is similar to *cdc48-3*, indicating that in absence of Ubx4, Cdc48 loses its ability to process ubiquitinated substrates in contrast to Ubx1 and Ubx5. Given that Cdc48's localization is perturbed in absence of Ubx4, we reason that Ubx4 plays an integral role in localizing Cdc48 in the nucleus. Thus, absence of Ubx4 affect's function of Cdc48 in processing its ubiquitinated substrates and degrading misfolded proteins.

Conclusion: For the first time, we show the particular combination of Ubx cofactors that regulate nuclear protein degradation in the San1 pathway. Functional consequence in absence of Ubx cofactors reveal their role in regulating toxicity, maintaining solubility and processing misfolded proteins in the nucleus. Importantly, our solubility and *in vivo* ubiquitination assay shows Ubx4 to play a distinct role in degrading misfolded proteins compared to Ubx1 and Ubx5. Specifically, the localization of Cdc48 in the nucleus is perturbed in absence of Ubx4, thus indicating Ubx4 to play an important role in localizing Cdc48 to the nucleus. Future studies on how Ubx4 localizes Cdc48 to the nucleus will be investigated to query this further.

Figure Legends:

Fig. 2.1: Ubx1, Ubx4 and Ubx5 cofactors regulate nuclear protein degradation in the San1 pathway. GFP^{NLS}-Tef2* and GFP^{NLS}-Bgl2* were expressed in parent, *san1*Δ and *ubx*Δ from GAL inducible promoter for 3 hours at 25˚C and shifted to 1 hour at 37˚C to inactivate *cdc48-3* before adding cycloheximide to the strains. Cycloheximide-chase assay was carried out for 0-3 hours. Anti-GFP antibody were used to detect the expression of substrates and anti-PGK-1 antibody was used to detect PGK-1 which has been used as a loading control.

Figure 2.2: Ubx2, Ubx3, Ubx6 and Ubx7 does not regulate nuclear mediated protein degradation. Cycloheximide assays were carried out to investigate GFP-GFP^{NLS}-Tef2* and GFP^{NLS}-Bgl2* substrate expression upon loss of Ubx2, Ubx3, Ubx6 and Ubx7. Substrates were induced for 3 hours and shifted for 37˚C to investigate *cdc48-3* before adding cycloheximide. Cycloheximide-chase assay was carried out for 0-3 hours. Anti-GFP antibody were used to detect the expression of substrates. Loading for all samples was determined by Ponceau S staining of the nitrocellulose blots after transfer (not shown)

Figure 2.3: Ubx1 and Ubx5 function in the San1 pathway. Cycloheximide-chase assays were performed on *ubx1*Δ*/ubx5*Δ and *ubx4*Δ*/ubx5*Δ to investigate stability of GFP^{NLS}-Tef2* and GFP^{NLS}-Bgl2*. Substrates were induced for 3 hours at 25˚C and shifted for 37˚C to inactivate *cdc48-3* before adding cycloheximide. Cycloheximide-chase assay was carried out for 0-3 hours. Anti-GFP antibody were used to detect the expression of substrates and anti-PGK-1 antibody was used to detect PGK-1 which has been used as a loading control.

Figure 2.4: Double deletion of Ubx1 and Ubx4 show synthetic lethality upon tetrad dissection. Spore growth on rich media from tetrad dissection are shown. Genotype is shown underneath the vertical strip of each tetrad.

Figure 2.5: Ubx1 cofactor rescues slow growth in *ubx1*Δ strain. Parent, *san1*Δ and *ubx*Δ were spotted at 10-fold dilution to measure grown on glucose medium. Ubx cofactors were added back to check if they can rescue the growth deficiency. Western blot indicates the expression of HA-tagged Ubx cofactors in *ubx*Δ strains. Anti-HA antibody was used to detect each of the Ubx cofactors tagged with HA.

Figure 2.6: Ubx1 and Ubx4 are essential for yeast to grow upon expression of misfolded nuclear proteins. Strains of parent, *san1*Δ, and *ubx*Δ were spotted at 10-fold dilution to measure growing efficiency in glucose, and in galactose to investigate growth upon expression GFP^{NLS}-Tef2* and GFP^{NLS}-Bgl2* at 30˚C.

Figure 2.7: Adding back Ubx1 and Ubx4 rescues growth in *ubx*Δ strains.10-fold serial dilution of parent, *san1*Δ and *ubx*Δ along with their Ubx cofactors were spotted at 10-fold dilution to measure growing efficiency in glucose, and in galactose to investigate growth upon expression GFP^{NLS}-Tef2* and GFP^{NLS}-Bgl2* at 30˚C.

Figure 2.8: Ubx 1 and Ubx5 regulate substrate solubility. Sedimentation assays were performed to investigate how GFP^{NLS}-Tef2* partition between soluble(S) and insoluble pellet(I) fraction in parent, *cdc48-3* and *ubx*Δ strains. All the strains were grown at 25˚C and shifted to 37˚C for 1 hour before lysis to inactivate *cdc48-3*. Total lysate(T) shows the total amount of GFP^{NLS}-Tef2* in cell lysate. Anti-GFP was used to probe for each of the substrates.

Figure 2.9: Loss of Ubx1, Ubx4 and Ubx5 enhances substrate ubiquitination. Ubiquitination assays were performed in each of the *ubx*Δ and *ubx*Δ*/ubx*Δ by immunoprecipitation via TUBE beads (Life Sensors). GFP^{NLS}-Tef2* was induced after adding galactose for 3 hours, and shifting to 1 hour at 37˚C to inactivate *cdc48-3*. Anti-GFP antibodies were used to detect immunoprecipitated substrates.

Figure 3.0: Loss of Ubx4 disrupts localization of Cdc48-Mcherry in the nucleus upon expression of GFP^{NLS}-Tef2*. Expression of GFP^{NLS}-Tef2* was induced by

addition of galactose for 6 hours in log phase. Cells were examined by fluorescence microscopy after fixing in 4% paraformaldehyde and stained with DAPI.

Figure 3.1: Loss of Ubx4 disrupts localization of Cdc48-Mcherry in the nucleus upon expression of GFP^{NLS}-Bgl2*. Expression of GFP^{NLS}-Bgl2* was induced by addition of galactose for 6 hours in log phase. Cells were examined by fluorescence microscopy after fixing in 4% paraformaldehyde and stained with DAPI.

Figure 3.2: GFP-NLS expression localizes Cdc48 uniformly throughout the cell. Expression of GFP^{NLS} was induced by addition of galactose for 6 hours in log phase. Cells were examined by fluorescence microscopy after fixing in 4% paraformaldehyde and stained with DAPI.

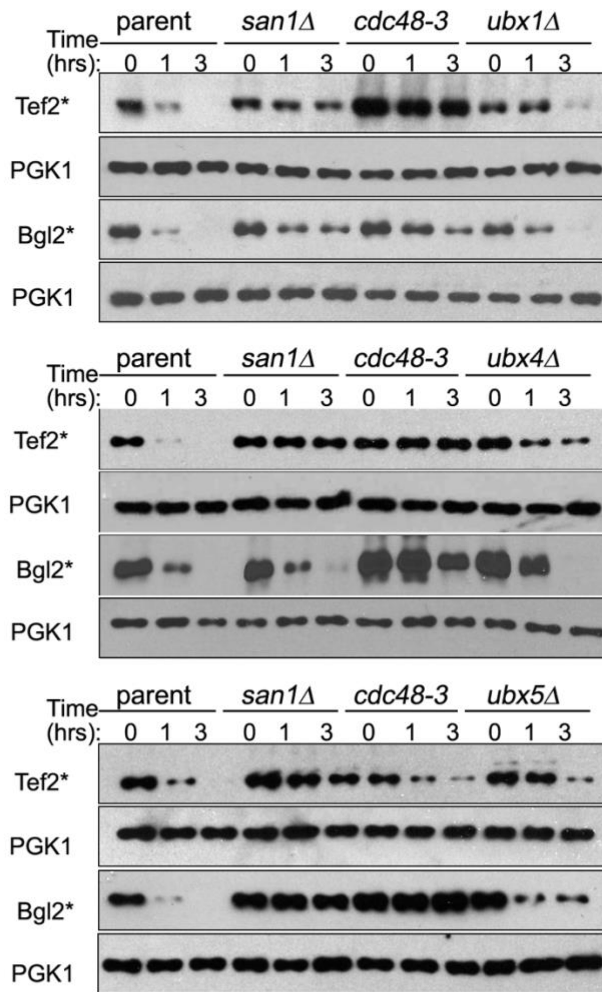

Fig. 2.1: Ubx1, Ubx4 and Ubx5 cofactors regulate nuclear protein degradation in the San1 pathway.

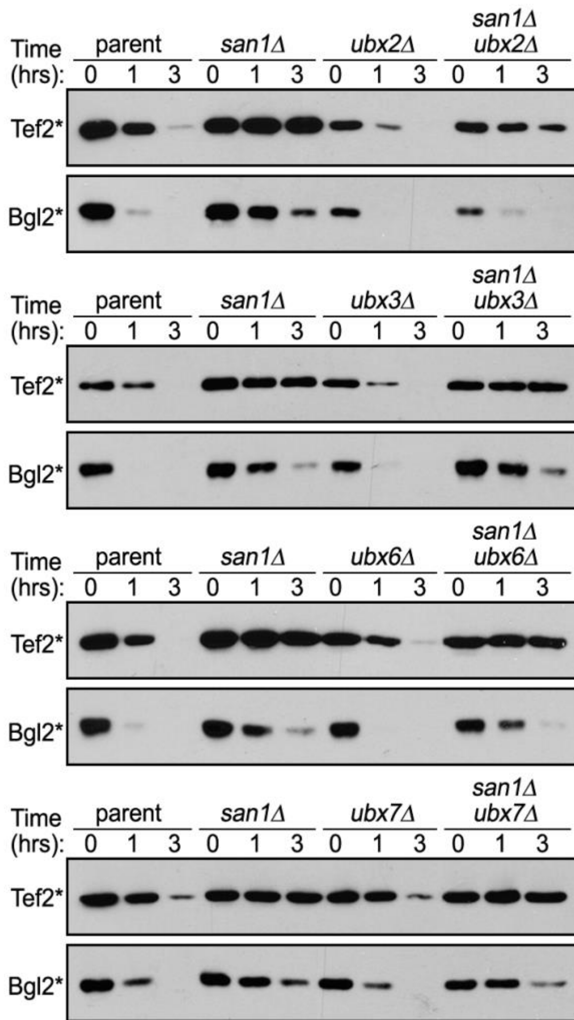

Figure 2.2: Ubx2, Ubx3, Ubx6 and Ubx7 does not regulate nuclear mediated protein degradation.

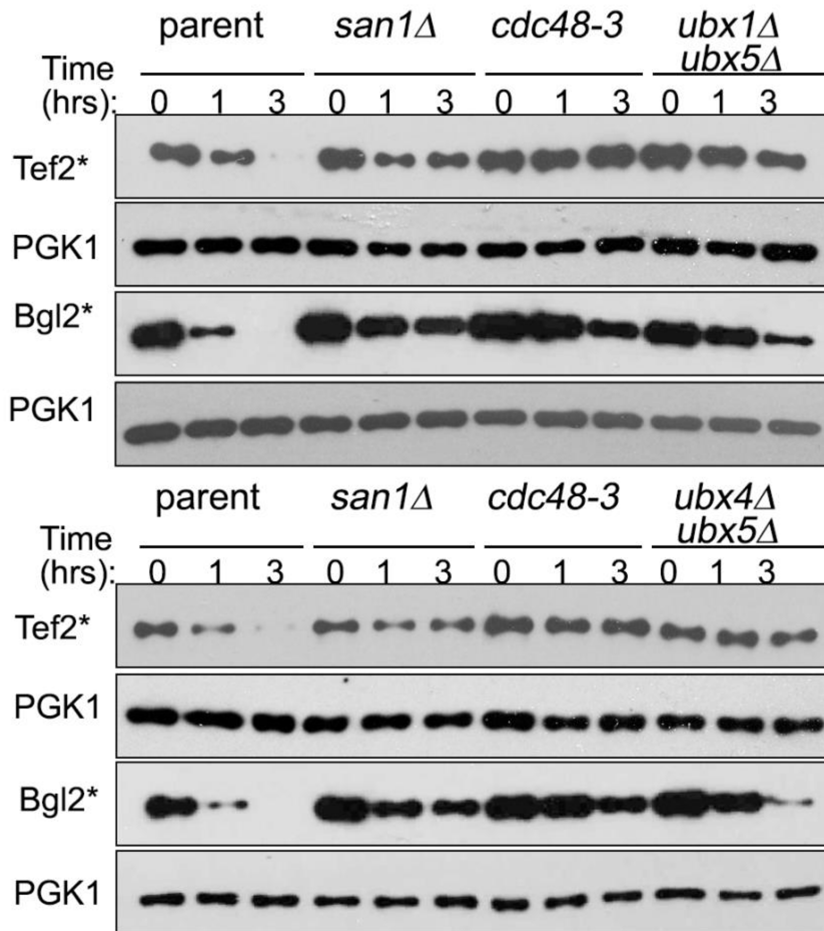

Figure 2.3: Ubx1 and Ubx5 function in the San1 pathway.

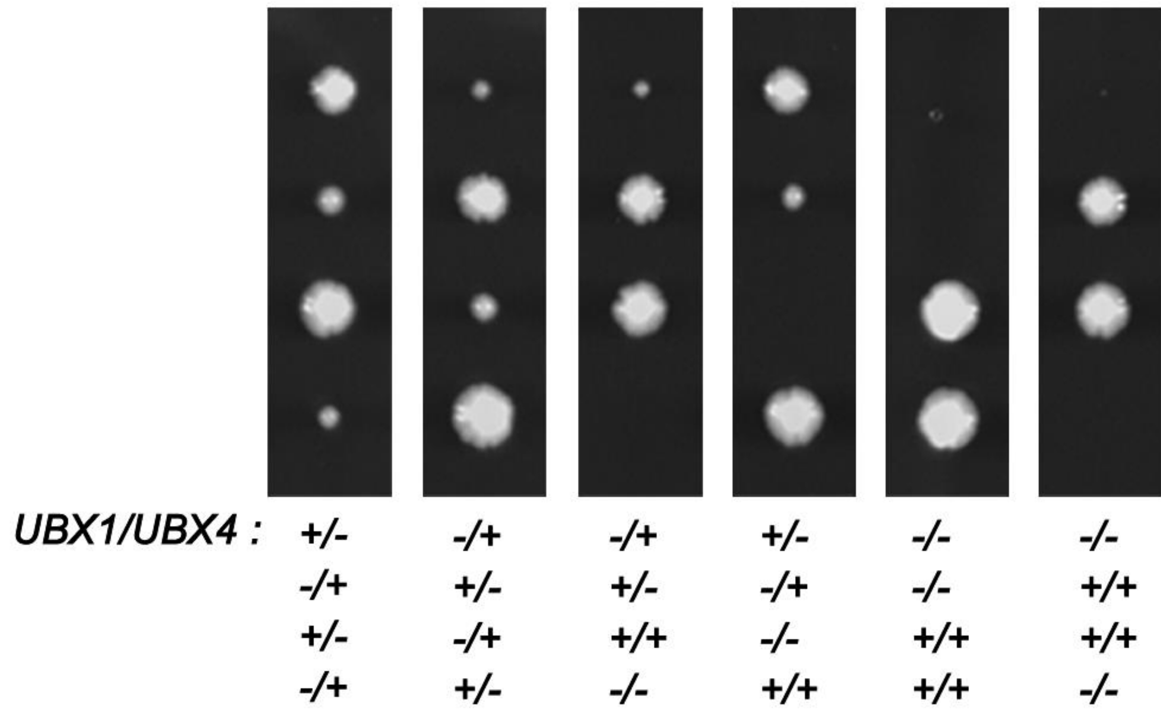

Figure 2.4: Double deletion of Ubx1 and Ubx4 show synthetic lethality upon tetrad dissection.

Figure 2.5: Ubx1 cofactor rescues slow growth in *ubx1*Δ strains.

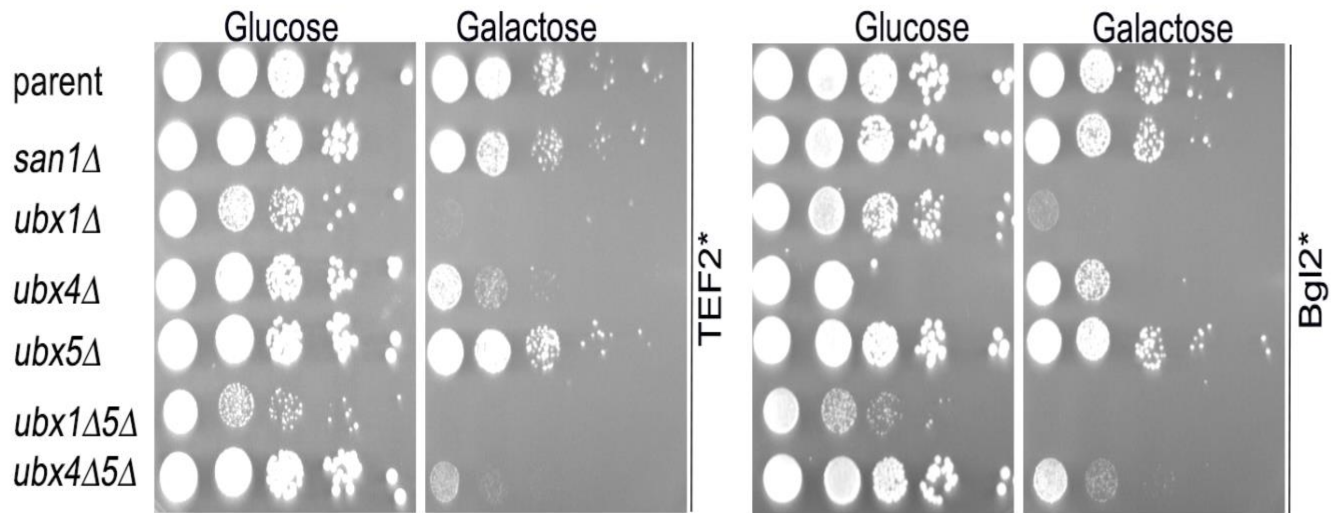

Figure 2.6: Ubx1 and Ubx4 are essential for yeast to grow upon expression of misfolded nuclear proteins.

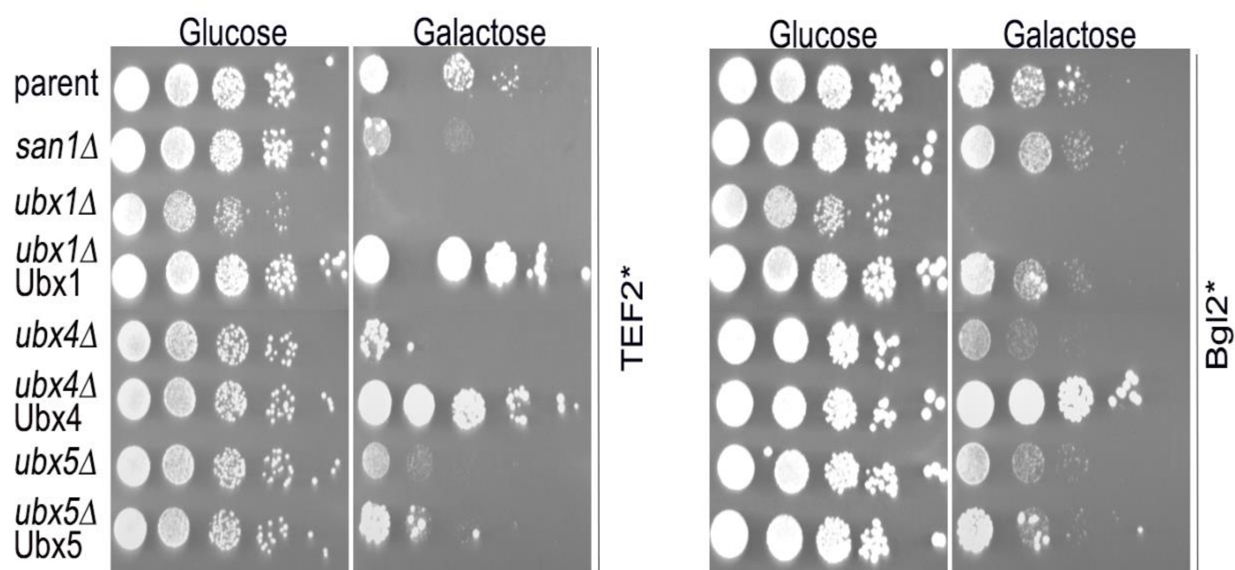

Figure 2.7: Adding back Ubx1 and Ubx4 rescues growth in *ubx*Δ strains.

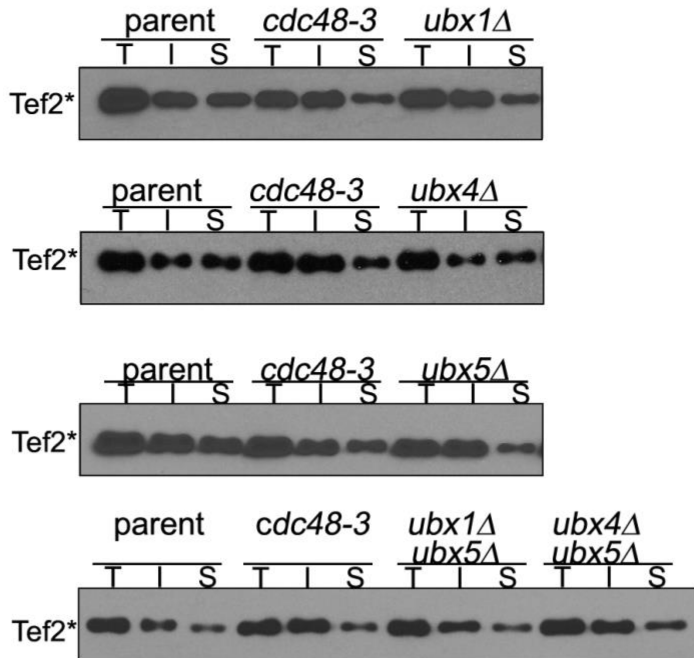

Figure 2.8: Ubx 1 and Ubx5 regulate substrate solubility.

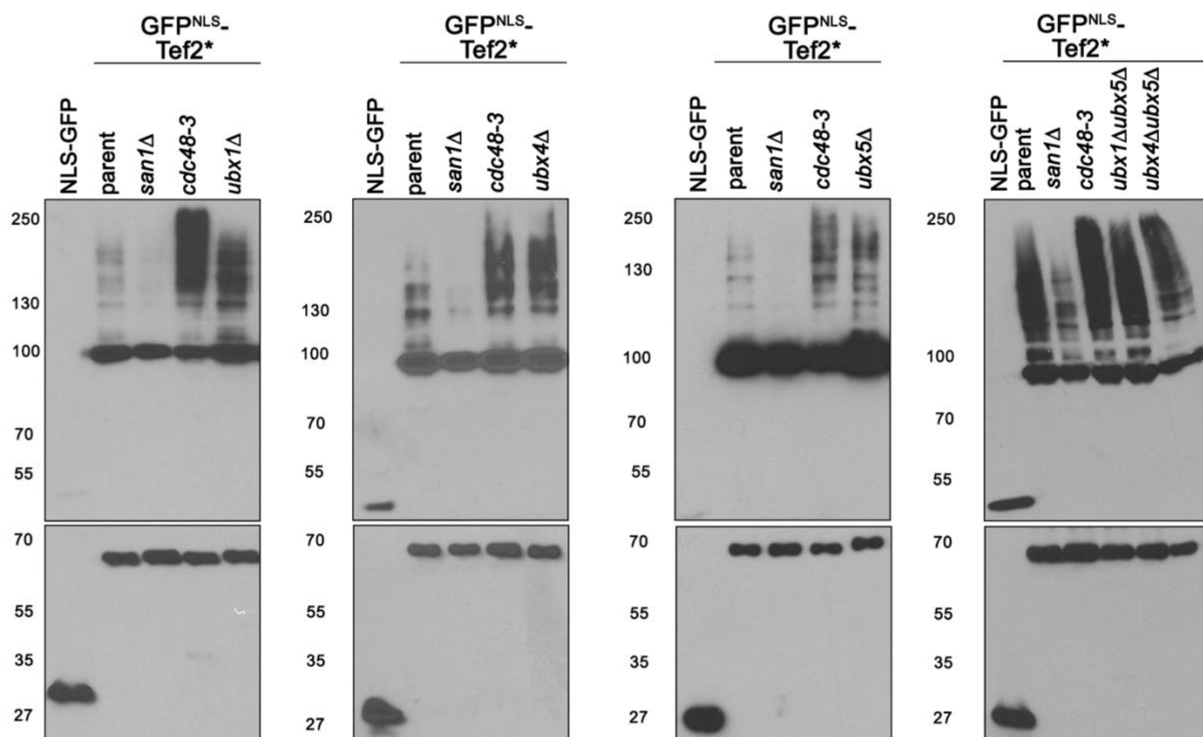

Figure 2.9: Loss of Ubx1, Ubx4 and Ubx5 enhances substrate ubiquitination.

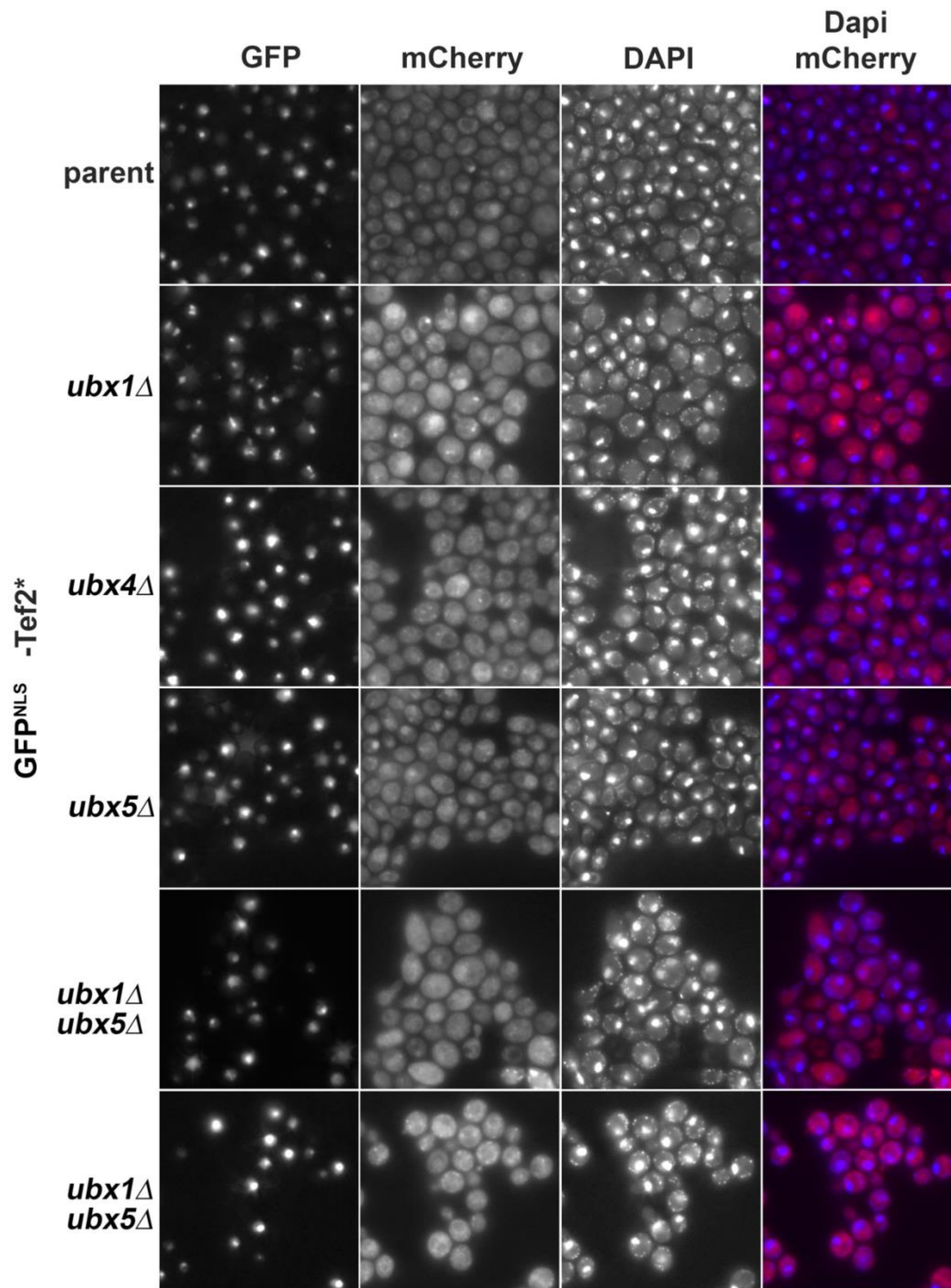

Figure 3.0: Loss of Ubx4 disrupts localization of Cdc48-Mcherry in the nucleus upon expression of GFP^{NLS}-TEF2*.

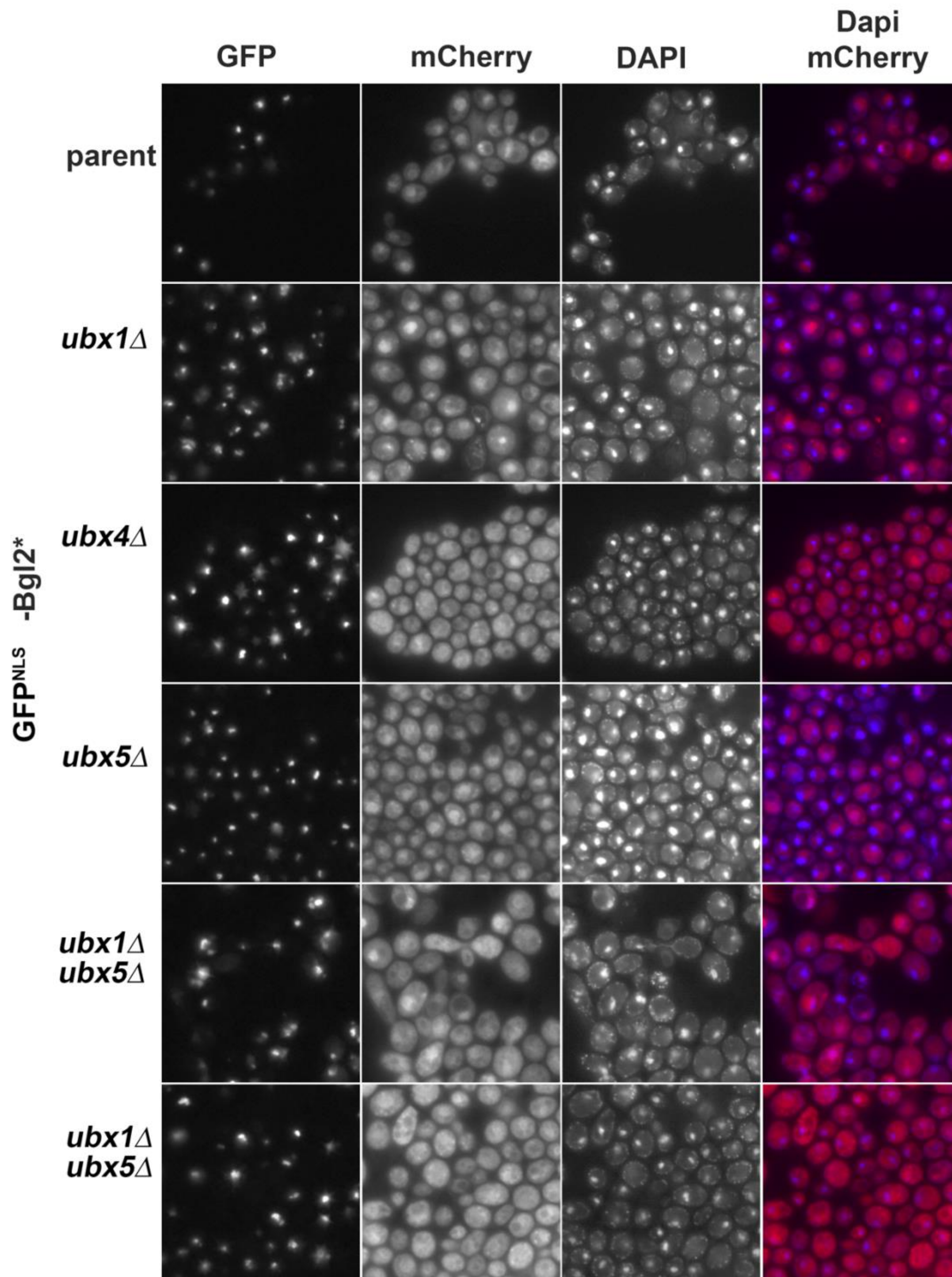

Figure 3.1: Loss of Ubx4 disrupts localization of Cdc48-Mcherry in the nucleus upon expression of GFP^{NLS}-Bgl2*.

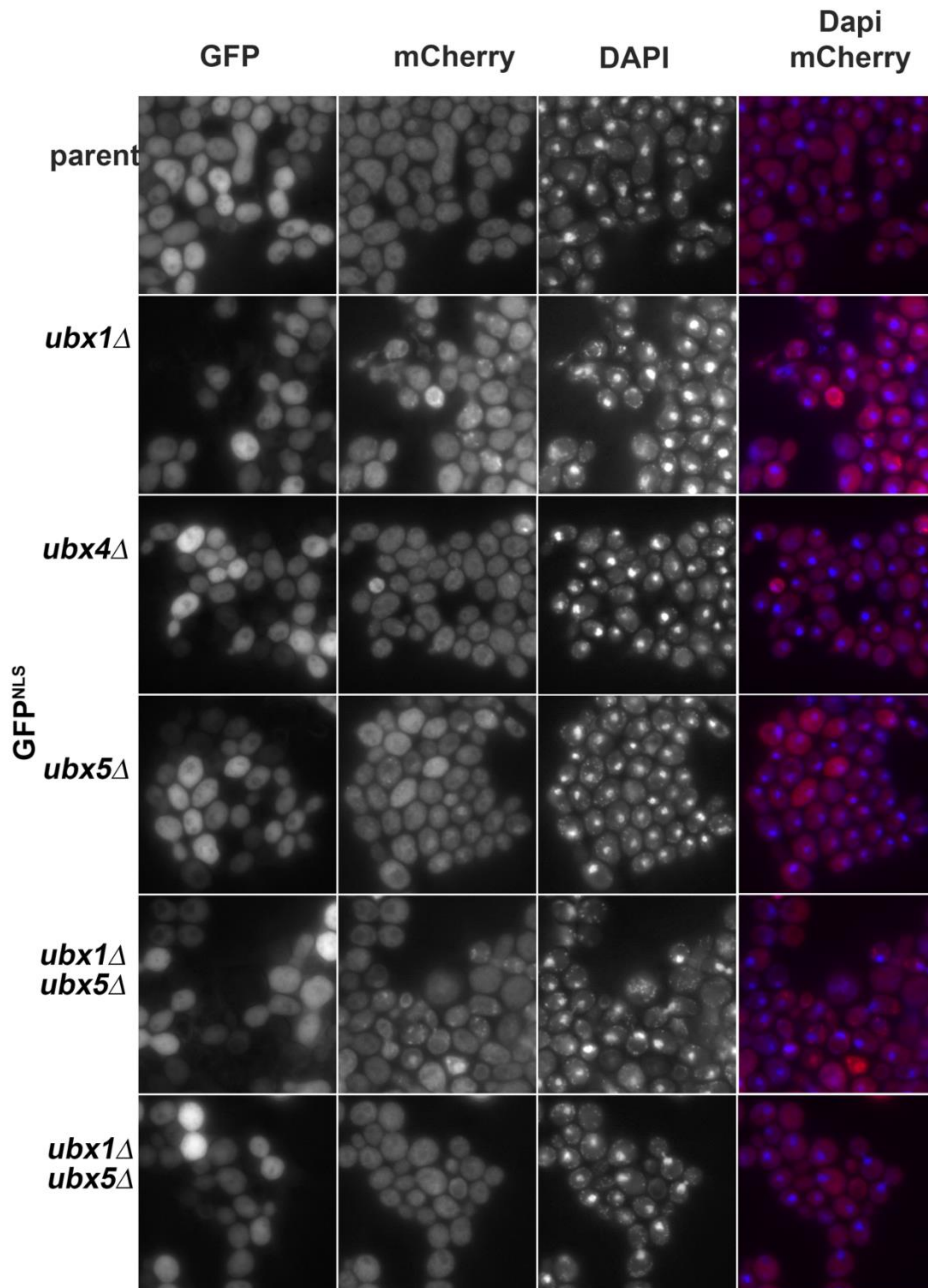

Figure 3.2: GFP-NLS expression localizes Cdc48 uniformly throughout the cell.

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CHAPTER FOUR:

CONCLUSIONS

Previous studies have implicated Cdc48 in playing essential roles in many cellular processes in different compartments in the cell, including the nucleus. Yet, the cofactor combination that helps Cdc48 degrade misfolded proteins in the nucleus has not yet been explored. I show that Ubx1, Ubx4 and Ubx5 play a role in San1 dependent protein degradation. Importantly, in my study I found that loss of Ubx1 and Ubx5 help to keep misfolded substrates more soluble. This is important because one of the ways misfolded proteins cause toxicity in cells is by forming inclusions and inhibiting the proteasomal degradation. Given that we see greater levels of ubiquitinated substrates in *ubx*Δ, we deduce that all the three Ubx cofactors are working after San1 ubiquitination pathway. Furthermore, the ubiquitination pattern in *ubx4*Δ is similar to *cdc48-3*, suggesting to us that Ubx4 works in similar way in degrading misfolded proteins. Following the solubility and ubiquitination pattern, my dissertation also shows that Ubx4 plays a role in localizing Cdc48 to the nucleus. To our knowledge, this is the first study that shows Ubx4 playing a role in Cdc48's localization to the nucleus. Perhaps when Ubx4 is lost, Cdc48 is no longer able to dock inside the nucleus more regularly, and able to efficiently degrade misfolded proteins.

Although we are ending the study with exciting and novel finding, our study here also points to several unknowns that will need to be resolved in the future. For example, we have not yet resolved how Ubx4 is recruiting Cdc48 to the nucleus. Since unlike Ubx1 and Ubx5, Ubx4 does not have a UBA domain, perhaps the Ubx domain is responsible for tethering Cdc48 inside the nucleus. Domain deletion experiments will be needed to understand if the Ubx domain is only responsible part for recruiting Cdc48 to the nucleus. One way Ubx4 cofactor could be recruiting Cdc48 into the nucleus is via controlling the NLS within Cdc48. If this is the case, it will be interesting to reroute Cdc48 into the nucleus, and investigate if we can reverse the reduction in protein degradation and high molecular size ubiquitinated substrates in *cdc48-3* and

*ubx4*Δ. Furthermore, although all the Ubx cofactors show direct binding to Cdc48, the exact cofactor hierarchy via which Ubx cofactors bind to Cdc48 in the nucleus has not yet been investigated.

The exquisiteness of UPS regulation on protein homeostasis can only happen upon the timely work of many players in the pathway. Dysregulation of UPS during cancer and neurodegeneration underlies the importance of understanding how the players in the system work to remove proteins and regulate protein homeostasis. The work in the Gardner lab identified San1 E3 ligase, and paved our way to understand how the nucleus manages nuclear protein burden. My work here has extended that understanding by adding the Ubx cofactor contribution to the pathway, thus enriching our understanding of the overall nuclear UPS.

APPENDIX I - METHODS

Cycloheximide assay: Cells were grown to 1*10^7 cells/ml in 3% raffinose. 3% galactose was added for 3 hours at 25˚C and transferred to 37˚C for 1hour before addition of cycloheximide at a concentration of 50μg/ml. Cells were incubated for 0-3 hours. Cells were lysed at 0hr,1hr, and 3hrs using 200μl of Lysis Buffer (8M Urea, 1% SDS, 10 mM MOPS, pH 6.8, 10 mM EDTA, 1 mM PMSF, 0.01% Bromophenol Blue). Cells were vortexed with 100μL of 0.5mm acid-washed glass beads (Biospec Products). Proteins were resolved on SDS-PAGE gels, transferred to nitrocellulose and immunoblotted with anti-GFP (Sigma), anti-PGK1(Abcam), and anti-HA(Sigma).

Sedimentation Assay: Cells were grown in 3% raffinose medium to 1*10^7 cells/ml before adding 3% galactose for 3hours at 25˚C and shifting to 37C to inactivate cdc48-3 for 1 hour. Cells were lysed using (100 mM Tris-HCl pH 7.5, 200 mM NaCl, 1 mM EDTA, 1 mM DTT, 5% glycerol and 0.1% Nonidet P40 and PMSF) for 20minutes at 4˚C using 0.5mm acid-washed beads. Unlysed cells were removed by centrifuging the lysates at 700g for 1minute at 4˚C. 50μl lysate was removed and added to 50μl of SUMEB and labelled “Total Lysate”. The remaining 100ul of lysate was centrifuged at 12,800 g for 15 minutes at 4˚C. The supernatant was collected and added to 100ul of SUMEB buffer. This is labeled as the “Soluble fraction”. To the pellet, 100μl of lysis buffer and 100μl of SUMEB were added and this represents the “insoluble fraction”. The samples were heated at 65˚C for 10minutes before running on 8% SDS-PAGE gel and immunoblotted with anti-GFP(Sigma).

Microscopy: Cells were grown in 3% raffinose medium to a concentration of 1*10^7cells/ml before 3% galactose was added for 6hours. Cells were fixed in 4% paraformaldehyde in 0.1M sucrose for 30minutes. Cells were washed in 1.2M sorbitol, and stained in DAPI in wash buffer containing 2% Triton X-100. Cells were washed 2 times before being imaged on Nikon Eclipse 90i with a 1003 objective (DIC N2 N.A. 1.4), filters for GFP (ET470/403, T495LP, ET525/50 m), Mcherry (ET560/403, T585LP, ET630/75 m) fluorescence, and a Photometrics Cool Snap HQ2 cooled CCD camera

with NIS-Elements acquisition software. Images have been processed using Photoshop CS (Adobe Systems Inc.).

Ubiquitination Assay: Cells were grown to 0.8*10^7cell/ml before adding galactose for 3hoursat 25˚C and then shifted to 37˚C for 1 hour to inactivate *cdc48-3*. Cells were then spun down and lysed in Lysis buffer: (50mM Tris 7.5, 150mM NaCl, 1mM EDTA, 10% Glycerol, and 1%NP40) in addition to 100mM PMSF and NEM. Lysates were clarified using centrifugation before 20ul of TUBE beads (Life Sensor) were added to each sample and incubated overnight. Next day, the TUBE beads were washed with 1ml of Lysis buffer without the NEM for 4 times. 25µl of SUMEB was added to elute the affinity purified proteins. The samples were heated in 65˚C, before resolving in 8% SDS-PAGE gels, and transferred to nitrocellulose membrane and immunoblotted with anti-GFP(Sigma).

APPENDIX II – STRAINS AND PLASMIDS

Yeast Strains	Genotype	Contribution
RGY4028	*ubx1Δ::NatMX*	Pam Gallagher
RGY4034	*ubx4Δ::NatMX*	Pam Gallagher
RGY4040	*ubx5Δ::NatMX*	Pam Gallagher
RGY 5555	*ubx5Δ::NatMX* *ubx1Δ::URA3*	Pam Gallagher
RGY 5174	*ubx4Δ::URA3* *ubx5Δ::URA3*	Pam Gallagher
RGY4461	*cdc48-3*	Pam Gallagher
RGY5950	Cdc48 –Mcherry *ubx4Δ::NatMX*	Enam, Charisma
RGY5951	Cdc48-Mcherry *ubx1Δ::NatMX*	Enam, Charisma
RGY5952	Cdc48-Mcherry *ubx5Δ::NatMX*	Enam, Charisma
RGY5975	Cdc48-Mcherry *ubx5Δ::NatMX* *ubx1Δ::URA3*	Enam, Charisma
RGY5976	Cdc48 tagged with *ubx4Δ::URA3* *ubx5Δ::URA3*	Enam, Charisma

Plasmids:

Plasmid	What is it	Contribution
pRG4246	*HIS3-Cdc48-Mcherry*	Enam Charisma
pRG4247	*URA UBX1-3HA 2 micron*	Enam, Charisma
pRG4248	*URA UBX4-3HA 2 micron*	Enam, Charisma
pRG4249	*URA UBX5-3HA 2 micron*	Enam, Charisma
pRG1289	P_{GAL1}-*NLS-GFP-TEF2(190-458)*	Richard Gardner
pRG1290	P_{GAL1}-*NLS-GFP-BGL2(20-313)*	Richard Gardner
pRG2342	P_{GAL1}-*NLS-GFP*	Richard Gardner