

Regulation of proteasomal assembly by intrinsic factors in bacteria and extrinsic factors in eukaryotes

by

Anjana Suppahia

B.S., Devi Ahilya Vishwavidyalaya, 2010
M.S., Sardar Patel University, 2013

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Division of Biology
College of Arts and Sciences

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2019

Abstract

Proteasomes are large, multimeric proteases that are found in all eukaryotes, archaea and some bacteria. They function in a highly regulated manner to coordinate the recognition, unfolding and degradation of cellular proteins. At the center is the core particle (CP), a highly conserved dyad-symmetric structure composed of 28 subunits, that harbors the active sites in its cylindrically shaped structure. While the subunit complexity of CP in prokaryotes is low, with generally two unique subunits, alpha and beta, the eukaryotes possess a large number of paralogs and contain 14 unique subunits, seven different alpha and seven different beta subunits. The efficient and accurate assembly of the eukaryotic CP complex also depends on five different chaperones. In contrast, the prokaryotic CP assembles autonomously into active CPs. The work in this dissertation focuses on understanding the regulation of bacterial assembly pathways through intrinsic factors, using *Rhodococcus erythropolis* as a model. The bacterial beta subunits possess an N-terminal propeptide sequence that has previously been described to function as an intrinsic chaperone that promotes the formation of alpha-beta heterodimers early in the assembly. Our results, however, indicate additional functions for the propeptide in assembly. Our data indicate that a flexible region of propeptide regulates the dimerization of larger assembly intermediates called the half-proteasomes (HP). The dimerization of HPs results in the formation of an inactive form of CP which undergoes a conserved autocatalytic mechanism to cleave the propeptide sequences to form active CPs. Our data showed a role of the N-terminal region of propeptide in regulating this activation process of assembled CP. Based on the results of *in vitro* approaches, we propose a model where propeptide regulate the activation process through a cooperative mechanism involving the cleavage of propeptides.

Eukaryotic 26S proteasomes consist of a CP capped on one or both ends by regulatory particle (RP). The assembly of CP and RP complexes requires ten unique assembly chaperones which are subsequently removed during the formation of RP-CP complexes. When studying the effects of the absence of the CP chaperone Ump1, that shares functionality with the bacterial propeptides described above, we observed the

association of RP chaperones as well as Ecm29, a proteasome associated protein, on the proteasomes. Our data suggest a function of Ecm29, in preventing the release of chaperones from RP-CP complexes. Our results showed that the upregulation or overexpression of Ecm29 even in wildtype cells stabilizes RP chaperones on mature, functional 26S proteasomes. We also observed that the nucleotide state of specific ATPases found in the RP complex influences the stabilization of chaperones on 26S proteasomes. The ATPase mutants defective in ATP hydrolysis suggested that the hydrolysis of ATP is essential for the release of chaperones from RP-CP complexes. Our results imply a novel functional role of Ecm29 in recruiting or stabilizing RP chaperones on 26S thereby either assisting the RP base assembly or regulating the RP-CP interaction.

Regulation of proteasomal assembly by intrinsic factors in bacteria and extrinsic factors in eukaryotes

by

Anjana Suppahia

B.S., Devi Ahilya Vishwavidyalaya, 2010
M.S., Sardar Patel University, 2013

A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Division of Biology
College of Arts and Sciences

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2019

Approved by:

Major Professor
Dr. Jeroen Roelofs

Copyright

© ANJANA SUPPAHIA

2019.

Abstract

Proteasomes are large, multimeric proteases that are found in all eukaryotes, archaea and some bacteria. They function in a highly regulated manner to coordinate the recognition, unfolding and degradation of cellular proteins. At the center is the core particle (CP), a highly conserved dyad-symmetric structure composed of 28 subunits, that harbors the active sites in its cylindrically shaped structure. While the subunit complexity of CP in prokaryotes is low, with generally two unique subunits, alpha and beta, the eukaryotes possess a large number of paralogs and contain 14 unique subunits, seven different alpha and seven different beta subunits. The efficient and accurate assembly of the eukaryotic CP complex also depends on five different chaperones. In contrast, the prokaryotic CP assembles autonomously into active CPs. The work in this dissertation focuses on understanding the regulation of bacterial assembly pathways through intrinsic factors, using *Rhodococcus erythropolis* as a model. The bacterial beta subunits possess an N-terminal propeptide sequence that has previously been described to function as an intrinsic chaperone that promotes the formation of alpha-beta heterodimers early in the assembly. Our results, however, indicate additional functions for the propeptide in assembly. Our data indicate that a flexible region of propeptide regulates the dimerization of larger assembly intermediates called the half-proteasomes (HP). The dimerization of HPs results in the formation of an inactive form of CP which undergoes a conserved autocatalytic mechanism to cleave the propeptide sequences to form active CPs. Our data showed a role of the N-terminal region of propeptide in regulating this activation process of assembled CP. Based on the results of *in vitro* approaches, we propose a model where propeptide regulate the activation process through a cooperative mechanism involving the cleavage of propeptides.

Eukaryotic 26S proteasomes consist of a CP capped on one or both ends by regulatory particle (RP). The assembly of CP and RP complexes requires ten unique assembly chaperones which are subsequently removed during the formation of RP-CP complexes. When studying the effects of the absence of the CP chaperone Ump1, that shares functionality with the bacterial propeptides described above, we observed the

association of RP chaperones as well as Ecm29, a proteasome associated protein, on the proteasomes. Our data suggest a function of Ecm29, in preventing the release of chaperones from RP-CP complexes. Our results showed that the upregulation or overexpression of Ecm29 even in wildtype cells stabilizes RP chaperones on mature, functional 26S proteasomes. We also observed that the nucleotide state of specific ATPases found in the RP complex influences the stabilization of chaperones on 26S proteasomes. The ATPase mutants defective in ATP hydrolysis suggested that the hydrolysis of ATP is essential for the release of chaperones from RP-CP complexes. Our results imply a novel functional role of Ecm29 in recruiting or stabilizing RP chaperones on 26S thereby either assisting the RP base assembly or regulating the RP-CP interaction.

Table of Contents

List of Figures	x
List of Tables	xii
Acknowledgements	xiii
Dedication	xiv
Chapter 1 - Introduction	1
Chapter 2 - Cooperativity in proteasome core particle maturation	20
Abstract	21
Introduction	22
Experimental Procedures	25
Plasmids used	25
Protein expression and purification	26
In vitro reconstitution assays	27
Native gels and two dimensional (2D) assays	27
Multiple sequence alignment of bacterial species	27
Root Mean Square fluctuations (RMSF) of the propeptide in MD simulations	27
Results	29
Bacterial propeptide can be divided into three evolutionarily conserved regions	29
Propeptide region III regulates HP dimerization	32
Region I's role in autocatalytic processing	35
Cooperativity in maturation	38
Propeptide trimming by neighboring beta subunits	39
Analysis of the partially processed band formed during CP activation	41
Propeptide cleavage across HPs	43
Discussion	47
Author contributions	51
Chapter 3 - Nucleotide dependent stabilization of RP assembly chaperones on 26S proteasomes by Ecm29	52
Abstract	53
Experimental Procedures	57

Yeast Strains and Plasmids	57
Antibodies	60
Yeast growth conditions	60
Yeast cell lysis by cryo grinding and Native gels and Two-Dimensional (2D) gels using purified proteasomes	61
Imaging	61
Fluorescence Microscopy	61
Proteasome purification	61
Recombinant chaperone purification	62
Results.....	63
Ecm29 stabilizes interactions of the chaperones Hsm3 and Nas6 with RP-CP complexes..	63
Nucleotide state of RP ATPases has no significant effect on the interaction of chaperones with Ecm29-RP-CP complexes.....	68
Mutation affecting nucleotide hydrolysis by RP ATPases has no effect on Ecm29 dependent recruitment of chaperones <i>in vivo</i>	71
Mutation affecting nucleotide binding of RP ATPases destabilizes Ecm29 dependent recruitment of chaperones <i>in vivo</i>	74
<i>In vitro</i> analysis confirms the role of Ecm29 in stabilizing chaperones on RP-CP complexes	76
Ecm29 causes stabilization of proteasomes under oxidative stress conditions	78
Discussion.....	82
Author contributions	85
Chapter 4 - Discussion	86
Role of bacterial propeptides in the maturation.....	88
Ecm29 mediated interaction between RP and CP	90
References- Chapter 1 to 4.....	95
Appendix A - Supplementary information for chapter 1	106
Appendix B - Supplementary information for chapter 3	109

List of Figures

Figure 1-1 Proteasomes and associated activators in bacteria, archaea and eukaryotes.....	3
Figure 1-2 Schematic representation of CP assembly pathways	7
Figure 1-3 Conserved residues in actinomycete propeptide sequence.	10
Figure 1-4 Ecm29 dependent changes nucleotide occupancy of RP ATPases	19
Figure 2-1 The distribution of electron density in beta propeptide is evolutionarily conserved. .	31
Figure 2-2 Propeptide region of beta subunit can regulate the dimerization process through its flexible loop.	33
Figure 2-3 N-terminal truncations in beta subunit affects half proteasome dimerization rate.	36
Figure 2-4 Cooperativity in the activation of CP.....	39
Figure 2-5 Active beta subunits can cleave the propeptide of a neighboring beta subunit during CP activation process.....	41
Figure 2-6 Analysis of the partially processed band formed during CP activation	42
Figure 2-7 Calculation of distance between active sites in assembled CP.	43
Figure 2-8 Assembly of proteasomes can be regulated by reconstitution at different temperatures.	44
Figure 2-9 Active beta subunits can cleave the propeptide of a beta subunit present across dimerizing HP during CP activation process.	45
Figure 3-1 Ecm29 dependent rescue of RP base assembly defects.	64
Figure 3-2 Ecm29 upregulation or overexpression recruit chaperones on 26S.....	67
Figure 3-3 Ecm29 stabilizes chaperones on 26S independent of the nucleotide form available during purification process.	69
Figure 3-4 ATP-bound state of ATPases promotes stabilization of chaperones.	72
Figure 3-5 Nucleotide state of Rpt5 is critical for the cellular physiology under heat stress.	73
Figure 3-6 In vitro reconstitution suggests the stabilization of proteasomes by Ecm29 in ATP bound state.	77
Figure 3-7 Ecm29 stabilizes proteasomes under oxidative stress.....	79
Figure 3-8 Analysis of old and new proteasomes and Ecm29 by RITE method.....	81
Figure 4-1 Role of propeptides in regulation of HP dimerization in prokaryotes is orthologous to the role of Ump1 in eukaryotes.....	89

Figure 4-2 Structural changes in the 26 S proteasome at the Ecm29 binding region.	92
Figure 4-3 Rescue of RP-base assembly by Ecm29.	93

List of Tables

Table 2-1 Plasmids and primers.....	25
Table 3-1 Strains	57
Table 3-2 Primers.....	58

Acknowledgements

First and foremost, I would like to extend my sincere gratitude to my PhD advisor Dr. Jeroen Roelofs. I consider myself very fortunate to have had the chance to work under his guidance. I am grateful for his constant feedback, support and encouragement to help me hustle through grad school and grow as a researcher. Thank you, for being the best mentor one could wish for. And a special thanks for always putting up with the last minute reviews for almost everything.

I would also like to extend gratitude to my committee members Dr. Stella Lee, Dr. Timothy Durrett and Dr. Lorena Passarelli, for their constructive feedback and comments. The committee meetings have contributed greatly to my progress as a grad student.

To the current lab members- Akeem, Alicia and Gabrielle, thank you for all the weekly discussions and suggestions. Also, thanks for not only being the most supportive lab mates but for also being great friends. I would specifically like to thank Faith and Alex, for being excited about working in our lab and helping with my research. You guys are beyond best! To all the past lab members, Prashant, Alina and Ranjit, thank you for all the wise suggestions and guidance. To all the undergraduate students, thanks for all the ways you have contributed to the Roelofs lab.

I would also like to thank the Division of Biology and the Biology Graduate Student Association for their efforts in maintaining a great and inclusive environment to help every graduate student grow.

To all the friends I have made in last five years, thank you for making this phase of my life in Manhattan, a happy one.

I would like to thank my parents, Bikram Singh and Anita Suppahia for allowing me to stay more than 7000 miles away from home in pursuit my dreams. And my brother, Ajay Singh Supahiya, none of this would have been possible without your support and your belief in me. I would also like to thank all my other family members Vijay Singh Suppahia, Sunita Suppahia, Akshay Suppahia and Sonal Suppahia for their constant love and support. To my grandparents, I owe everything to you!

Lastly, but in no way the least, I would like to thank my fiancé Abhinav Ananthreddigari, for being my family away from home and for supporting me in a million ways.

Dedication

This work is dedicated to my guardian angel, the man I look up to, my grandfather, Capt. Rikhi Ram Suppahia. I miss you!

Chapter 1 - Introduction

Proteostasis

Protein synthesis, folding, and degradation are essential cellular processes required for maintaining protein homeostasis (proteostasis). These processes operate in a highly regulated and coordinated manner to meet the functional requirements of a cell. Defects in any of these fundamental processes or their regulation can lead to several pathological conditions including aging, cancer, and several neurodegenerative disorders. Thus, proper functioning of these processes is important to maintain a healthy intracellular proteome. Misfolding of proteins can lead to the exposure of hydrophobic residues in the cytosol. This can trigger the accumulation of non-native protein aggregates which have a negative impact on various cellular processes. Several intracellular factors help in the maintenance of proteostasis including the molecular chaperones that are found in the cytosol and other organelles like the nucleus, mitochondria and endoplasmic reticulum. Chaperones assist in protein folding during translation or post translationally, but also help in the formation of quaternary protein complexes and their sub cellular localization.

Degradation of proteins is as important as protein synthesis and folding for the maintenance of proteostasis. This is illustrated by the variability of protein half-lives. Some proteins are degraded almost as fast as they are synthesized with half-lives of just a few minutes, while other proteins are stable for hours or days. This shows that the degradation process is highly regulated and requires an input of energy. In eukaryotes, proteins targeted for degradation can be cleared by two different systems. The first is autophagy, which is a catabolic pathway that involves lysosomes and the second is the ubiquitin proteasome system (UPS). The UPS is the pathway that maintains proteostasis by selectively degrading proteins targeted for degradation (Finley, 2009). Here, the degradation machinery is a protein complex known as the proteasome, which is described in detail below. To be degraded by this complex, substrates are generally marked with ubiquitin moieties that are recognized by the proteasome. Ubiquitin is a 78 amino acid polypeptide that, through a cascade of enzymes, can be covalently attached to lysine residues of the substrate. Generally, several ubiquitin moieties are required, either

attached as single units on several lysine residues of the substrate or as poly-ubiquitin chains on a single lysine. The poly-ubiquitin chain is generated by subsequent ubiquitin units being attached to one of seven lysine residues found in ubiquitin itself (Streich & Lima, 2014). Ubiquitin modifications are reversible, as de-ubiquitinating enzymes can remove the modification. This deubiquitination can happen independent of the proteasome, but the proteasome itself also has deubiquitinase activity, which functions to recycle the ubiquitin prior to substrate degradation. In order for substrates to be degraded, they must first be unfolded into a linear polypeptide before they can reach the proteasome's proteolytic active sites. Access to these active sites is through a narrow channel that does not allow entry of folded proteins. This controlled degradation of targeted proteins is essential for the viability of the cell as it regulates the levels of proteins involved in essential cellular processes, such as cell growth, the cell cycle, DNA-damage repair, transcriptional regulation, translational regulation, stress response and programmed cell death.

Archaea and bacteria possess a number of self-compartmentalized ATP-dependent and independent proteases like ClpP, Lon, Hslv and proteasomes that perform a number of diverse functions (Butler et al., 2006). All these proteases share common architecture where the proteolytic active site is masked from the cellular environment to regulate selective degradation of proteins. Like eukaryotes, unfolding of protein substrates in prokaryotes is done by ATPases like HslV, ClpA, ClpC and ClpX that associate with various proteases. The proteasomes found in archaea are known to associate with PAN (proteasome-activating nucleotidase) or Cdc48 ATPases, and also contain a ubiquitin-like protein conjugation system termed SAMPylation (Maupin-Furlow, 2014; Maupin-Furlow et al., 2003). Similarly, the bacterial proteasomes are known to associate with ATPases, like the ARC/Mpa of actinobacteria, which is discussed later in detail.

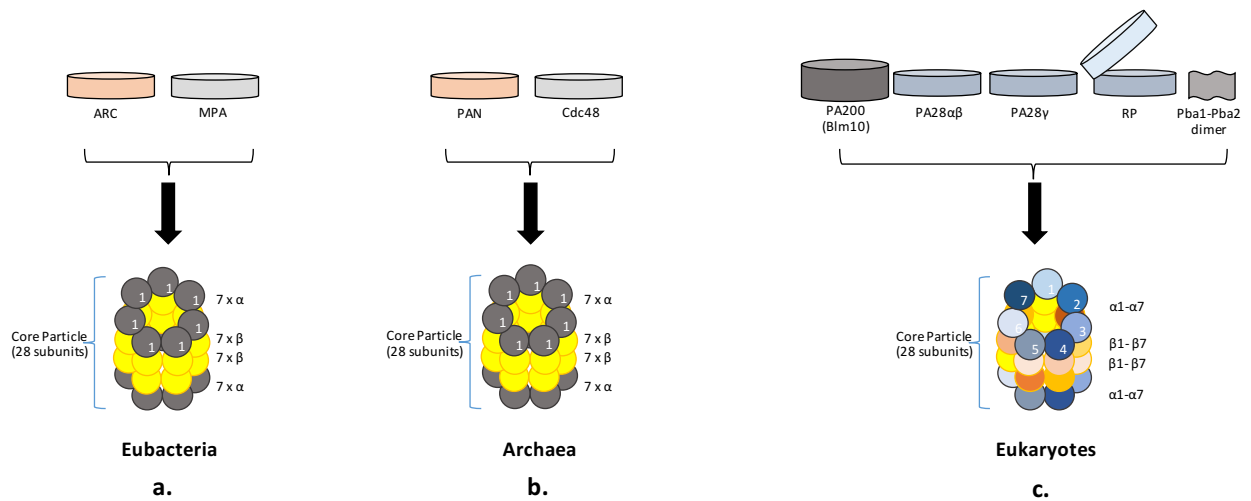
Ubiquity of proteasomes

Proteasomes are found in all three domains of life as they can be found in eukaryotes, archaea and a subset of bacteria. The function of proteasomes is conserved and is responsible for the degradation of the majority of proteins not required by the cell.

However, there is a substantial difference in their subunit complexity and is described as follows. The eukaryotic 26S proteasome holoenzyme, commonly referred to as the 26S proteasome, is a 2.5 mega-Dalton protease complex that can be found in the cytosol, associated with membrane structures like the ER, and in the nucleus. It consists of a total of 66 subunits that are distributed amongst two sub-complexes, the 20S core particle (CP) and the 19S regulatory particle (RP). The 28 CP subunits form a barrel shaped structure consisting of four stacked rings that contain the proteolytic active sites. The two outer rings are composed of seven distinct alpha subunits and the two inner rings are composed of seven distinct beta subunits organized as $\alpha_{1-7}\beta_{1-7}\beta_{1-7}\alpha_{1-7}$. The RP contains 19 subunits, organized as RP lid (10 subunits) and RP base (9 subunits). The RP base and lid function together to recognize, deubiquitinate and unfold proteasome substrates before they enter the catalytic CP chamber. The base is responsible for hydrolyzing ATP in order to produce the energy needed for substrate unfolding. The RP can cap one or both ends of CP (Coux & Goldberg, 1996). Several other ATP dependent or independent factors are known to alternatively cap the CP. These interacting protein are also referred to as proteasome activators that can bind to the CP and form alternate proteasome complexes (Stadtmueller & Hill, 2012; Tomko & Hochstrasser, 2013).

Similar to the UPS of eukaryotes, all archaeal species identified so far have genes encoding the proteasome and a ubiquitin-like protein conjugation system called SAMPylation. However, unlike the ubiquitous presence of proteasomes in eukaryotes and archaea, proteasomes have only been found in bacterial orders of *Actinomycetales* and *Nitrospirales* (Darwin, 2009; Jastrab & Darwin, 2015). Archaeal and eubacterial proteasomes are much less complex and generally contain only one form of alpha and one form of beta subunit each forming the rings of the core particle. Several ATPase and non-ATPase proteasome activators have also been identified in bacteria that help unfold proteins for their degradation by the catalytic core. Known proteasomes across all domains and their associated ATPases are summarized in figure 1.1).

Figure 1-1 Proteasomes and associated activators in bacteria, archaea and eukaryotes.



(a) Bacterial CP consists of homo-heptameric α and β rings. The two best characterized bacterial CP activators: ARC (AAA-ATPase forming ring shaped complexes) is found in *Rhodococcus erythropolis* and MPA (mycobacterial proteasome ATPase) found in *Mtb*. **(b)** Archaea CP also consists of homo-heptameric α and β rings. PAN (proteasome-activating nucleotidase) and cdc48 are two characterized ATPase activator present in archaea. The proteasomes can be associated with PAN or Cdc48 or both on one end each. Archaea identifies and degrades substrates modified by SAMPylation (similar to the ubiquitination in eukaryotes) **(c)** The eukaryotic CP consists hetero-heptameric α and β rings. A functional eukaryotic 26S proteasome consists of CP capped on both ends by regulatory particle (RP). RP is an AAA-ATPase and recognizes ubiquitinated substrates, whereas all others are non ATPases and do not recognize ubiquitin.

Bacterial proteasomes

Unlike eukaryotic proteasomes, bacterial proteasomes perform a range of physiological functions and are not essential for cell viability. One of the first roles of proteasomes was characterized in *Mycobacterium tuberculosis* (Mtb) in an attempt to find new anti-mycobacterial drug targets. It is shown that proteasomal activity is essential for the survival and pathogenicity of Mtb under oxidative and nitrosative (presence of mildly acidified nitrite) stress induced by macrophages in infected mice (Darwin & Ehrt, 2003; Gandotra et al., 2011).

Due to the absence of ubiquitin or ubiquitin like modifiers (UBLs) in bacteria, it has been unclear how substrates are marked and recognized for degradation by the proteasome. One hypothesis is that the proteins are targeted for degradation in a fashion that does not depend on a tag, but that instead involves signals within the substrate that lead to its recognition (Darwin, 2009). Using a two hybrid system specifically developed to identify components of the proteasomal degradation system in Mtb has led to the

identification of a small-protein modifier called Pup (prokaryotic ubiquitin-like protein), previously referred to as Rv2111c. Pup interacts with proteasome activators Mpa (*Mycobacterium* proteasome-associated ATPase) in Mtb, and ARC (AAA-ATPase forming ring-shaped Complexes) in *Rhodococcus erythropolis* (R.e.) to target substrates for ATP dependent degradation by the proteasome (Pearce et al., 2009). Thus, in the bacterial system there is also a specific post translational covalent modification to target substrates to the proteasome.

Mpa and ARC have functions analogous to the hexameric ring of the base sub-complex of the 19S RP of eukaryotic proteasomes (Braun et al., 1999). The attachment of pup to substrates is similar to ubiquitination where a covalent bond is formed between the C-terminal residue of Pup and the ϵ -amino group of a lysine residue on the substrate (Lys173 in FabD, a bacterial proteasome degradation substrate) (Pearce et al., 2009). Mass-spec analysis has revealed that Pup contains a C-terminal Gly-Gly-Gln motif, where the side chain of the terminal glutamine residue is deamidated to form glutamate prior to the formation of a covalent bond with the lysine residue on the substrate (Pearce et al., 2009). The deamidation reaction is carried out by an enzyme named Dop (deamidase of Pup) that leads to the conversion of Pup_{Gln} to Pup_{Glu} (Imkamp et al., 2010). An enzyme homologous to Dop, named PafA (proteasome accessory factor A) then phosphorylates the Glu residue on Pup_{Glu} and prepares it for nucleophilic attack by the ϵ -amino group of a specific Lys residue on the substrate (Striebel et al., 2009). Intriguingly, despite similar roles in modifying substrates for recognition by proteasomes, Dop and PafA are not homologous to E1, E2 and E3 enzymes found in eukaryotes. Another striking difference in ubiquitination and pupylation is that a single moiety of pup is sufficient to target proteins for degradation in bacteria, whereas in eukaryotes, poly-ubiquitination is required to target substrates to the proteasome (Burns et al., 2010; Thrower et al., 2000).

Structure of bacterial proteasomes

The existence of proteasomes in bacteria is considered to be a result of lateral gene transfer events from higher organisms (Lupas et al., 1997). The bacterial beta subunits exhibit an average of 29% sequence identity with that of eukaryotic and archaeal beta

subunits (Gille et al., 2003). On the other hand, alpha subunits show only 15% identity to the alpha subunits found in other domains (Gille et al., 2003). Genes encoding bacterial CP subunits, proteasomal activators and cofactors are encoded in an operon found on the bacterial chromosome. This operon includes the *PrcA* (proteasome core A), *PrcB* (proteasome core B) and *pup* (prokaryotic ubiquitin-like protein) genes. Other genes found near this operon encode for proteasome activator *MPA* (mycobacterial proteasome ATPase), *Dop* (deamidase of Pup, also known to de-pupylate substrates prior to degradation) and homologous enzyme *pafA* (proteasome accessory factor A) (Jastrab & Darwin, 2015). The 20S CP is a barrel shaped complex ~150Å long and ~115Å in diameter (Kwon et al., 2004; Tamura et al., 1995). It is composed of 28 subunits stacked together in the form of four heptameric rings organized as $\alpha_7\beta_7\beta_7\alpha_7$ (Groll et al., 1997; Tamura et al., 1995). In bacteria, the two inner rings are generally formed by only one isoform of the beta subunit, *Prcβ*. Similarly, the outer rings are composed of *Prcα*. However, some exceptions exist. For example, *R.e.* has two isoforms of both alpha and beta; *Prcα*₁, *Prcα*₂ and *Prcβ*₁, *Prcβ*₂. The two isoforms of α and β found in *R.e.* are known to be interchangeable and CP can be formed with only one alpha and one beta type (Zühl et al., 1997).

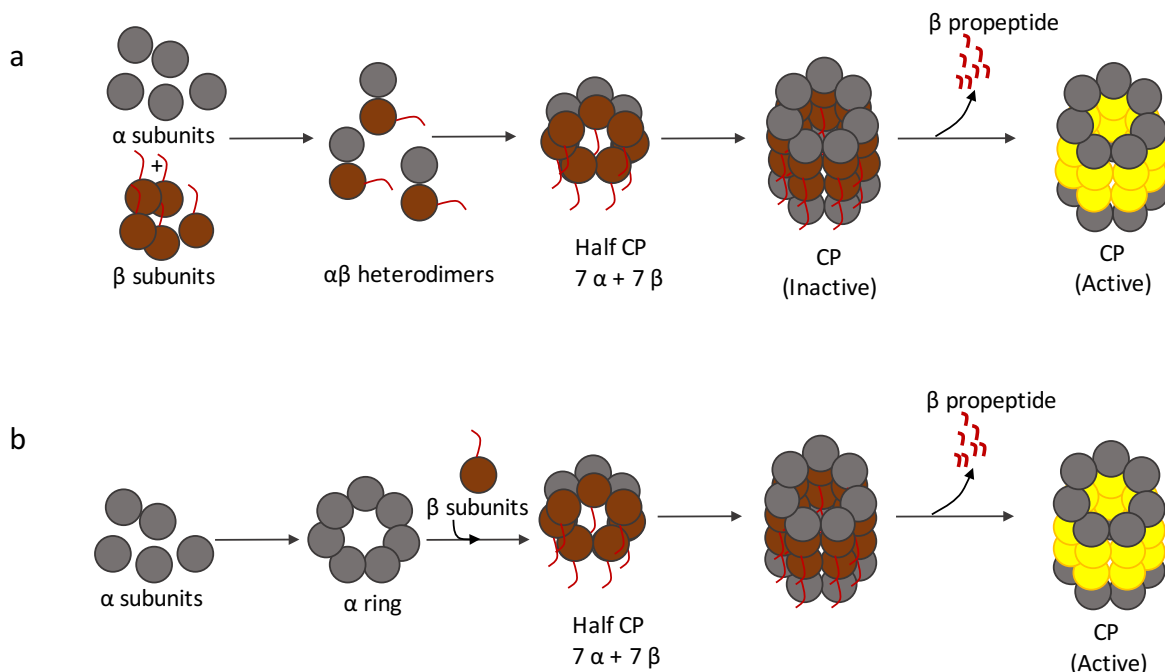
The inner cavity of CP contains three chambers, one between each of the adjoining α and β rings, known as the antechambers with a height of 40Å and a width of 50Å, and a third, larger chamber with a height of 40Å and a width of 55Å, that encompasses the active sites. These are formed by the N-terminal threonine residues of the beta subunits found at the center of the complex between the two beta rings which is discussed in detail in the following section (Kwon et al., 2004). All 14 beta subunits present in bacterial CP are believed to be catalytically active as it is a homo-heptamer. This is in contrast to the seven unique beta subunits of eukaryotes, which have only three active sites.

Assembly and activation process

The efficient assembly of the 20S CP containing the 28 subunits requires the assistance of five different chaperones in eukaryotes. Studies on eubacterial CP, done by expressing and purifying *R.e.* *PrcA* and *PrcB* genes in *E. coli*, has revealed an assembly process

independent of any such assembly chaperones (Zühl et al., 1997). Purified PrcA and PrcB, when reconstituted *in vitro*, also assembled readily to form active 20S CP without the need of any external factors (Zühl et al., 1997). These studies revealed that α and β first come together to form an $\alpha\beta$ heterodimer. This heterodimer formation is facilitated by the central box region of the propeptide sequence present on the beta subunits (Kwon et al., 2004). This process is different from the alpha ring dependent assembly pathway seen in archaea (Figure 1.2). These dimers then come together to form a seven fold, symmetrical, double heptameric-ring structure known as the half proteasome (HP). Two HPs then dimerize to form another assembly intermediate called the pre-holoproteasome. This intermediate is an inactive form of CP that needs to undergo the removal of the propeptide sequence from all 14 beta subunits in order to form an active CP, also referred to as a holoproteasome (Zühl et al., 1997). The beta subunits are synthesized in a precursor form that contains a propeptide sequence in front of the active site threonine 1 (Thr1). Thr1 facilitates the autocatalysis of the propeptide during the CP maturation process by hydrolyzing the peptide between Thr1 and the -1 residue. Once the propeptide is removed, the Thr1 also mediates the hydrolysis of substrates entering the catalytic chamber of CP. Throughout evolution the active site was conserved with residues commonly referred to as the catalytic triad which includes Thr, lysine (Lys) and aspartate (Asp)/glutamate (Glu). Together, these residues hydrolyze the peptide bonds of targeted substrates (Huber et al., 2016; Mc Cormack et al., 1997).

Figure 1-2 Schematic representation of CP assembly pathways



(a) α -ring independent pathway seen in bacteria. **(b)** α -ring dependent pathway seen in archaea. Note: Eukaryotes also follow the same assembly pathway, formation of α ring happens autonomously where as incorporation of β subunits requires assistance of CP assembly chaperones.

The propeptide sequence varies in length in different bacterial species. While the short length propeptide found in *Thermoplasma* or other archaea are known to not be important for CP assembly, longer propeptides like the one found in *R.e.* has several functions in the assembly process (Zühl et al., 1997). In fact, the propeptides found in eukaryotic Doa3 beta subunit is also essential during the assembly process and is known to facilitate the incorporation of Doa3 in CP (Chen & Hochstrasser, 1996). Electron microscopy studies on the *R.e.* 20S showed that only a small percentage (20%) of the total CP assembles correctly in the presence of a beta mutant lacking its propeptide sequence. This suggests that propeptide sequences, although not essential *in vivo*, are required for the efficient assembly of CP (Zühl et al., 1997). This same mutant also revealed that the activation of CP not only depends upon the autocatalytic removal of the propeptide sequence, but also requires an additional interaction between the propeptides of dimerized HPs in the pre-holoproteasome. Chapter 2 focuses on how cross processing of propeptides among beta subunits in a pre-holoproteasome can facilitate the activation of CP. The propeptides are intramolecular chaperones that help with the proper folding of beta subunits and also play a role in the assembly and maturation of CP. The role of

propeptides in proper folding of beta subunits was identified by monitoring the curve of unfolding-refolding experiments of proteasomal subunits (Zühl et al., 1997). On the other hand, propeptides in Mtb have been shown to negatively affect CP formation. PrcA and PrcB from Mtb, when co-expressed in *E. coli*, formed active CPs at 37 degrees, whereas the same reaction was inhibited at 30 degrees and required a deletion of the propeptide sequence from all beta subunits to undergo completion (Lin et al., 2006).

Upon the autocatalytic removal of propeptides during CP maturation, the active sites of beta subunits are exposed in the central chamber of the CP. The catalytic activity of a protease depends upon the amino acid residues lining the substrate binding pocket of the active site. The binding pockets in the active CP of *R.e.* is formed by non-polar amino acid residues and hence exhibits only chymotrypsin like activity (Tamura et al., 1995). However, crystal structure of Mtb has revealed that the substrate binding pockets formed by homo-heptameric beta rings are lined by polar amino acids on one side and non-polar on the other. This resembles that of eukaryotic proteasomes which possess trypsin, chymotrypsin and caspase like activities that allow them to hydrolyze substrates after non-polar, acidic or basic amino acids (Hu et al., 2006; Lin et al., 2006).

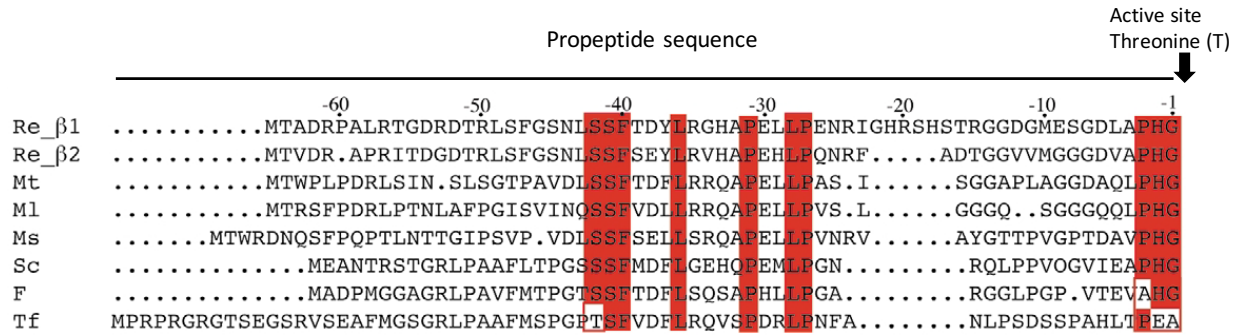
Evolution of proteasomes and proteasomal subunits

Long before the discovery of 20S proteasomes in bacteria, the protease most commonly known in bacteria was the proteasome like heat shock named HslV (Bochtler et al., 2000). Like proteasomes, HslV has an active site threonine and thus belongs to the N-terminal nucleophile hydrolase family (Groll et al., 1997). Other than bacteria, HslV was also found in protists (single cell eukaryotic microorganisms) which were known to have acquired it from the bacterial progenitor of mitochondria by horizontal gene transfer. Owing to the sequence similarities and presence of conserved residues among the proteasomal subunits in different organisms from all domains of life, the proteasomes are thought to have become more diverse over the course of evolution. Bacterial HslV protease named ClpQ, is a double ring structure, and like 20S CP, it interacts with an activator ATPase, ClpY and is known to be a phylogenetic precursor of the 20S proteasome (Bochtler et al., 1999). Not all bacteria have 20S proteasomes, it was first identified in high G+C gram

positive, nocardioform actinomycete bacteria *R.e.*, strain NI86/21 (Tamura et al., 1995). With the identification of ubiquitin like small protein modifier Pup in bacteria, as described earlier, it was clear that the protein degradation system like the ubiquitin-proteasome system was evolutionarily conserved.

The propeptide sequence in eukaryotic Doa3 subunits has been shown to be essential for their incorporation into the CP. Since eubacterial beta subunits also possess propeptide sequences and the 20S CP assembly does not require any external chaperones, we investigated the chaperone like role of propeptides in bacterial CP assembly. The sequence alignments of bacterial beta subunits have suggested the presence of several conserved residues suggesting an important functional significance of the propeptide sequence (Figure 1.3). The propeptide sequence of bacterial propeptides has been assigned a number of roles and has also been considered to function as an intra-molecular chaperone that helps in proper folding of beta subunits. Most of these roles have been assigned by using *in vitro* approaches using different mutants of beta either lacking the propeptide sequence, inactive mutants incapable of cleaving the propeptide sequence, or by using N-terminally truncated forms of beta. The precise mechanism of how the amino acid residues of propeptides can regulate the assembly process is not completely understood. Chapter two includes our studies regarding the role of bacterial propeptides in the assembly process. Secondly, chapter two also includes our studies regarding the maturation of the 20S CP. Although the activation of beta subunits in general is known to be obligatorily autocatalytic, we show that the propeptide sequence also has an important role in the activation and maturation of the CP.

Figure 1-3 Conserved residues in actinomycete propeptide sequence.



β propeptide sequences aligned from the following: *β*1 and *β*2 type subunits from *Rhodococcus erythropolis* (Re_β1 and Re_β2), *Mycobacterium tuberculosis* (Mt), *M. leprae* (Ml), *M. smegmatis* (Ms), *Streptomyces coelicolor* (Sc), *Frankia strain ACN14a* (F), and *T. fusca* (Tf). Conserved residues are highlighted in red. Active site threonine (T) residue is present downstream on the -1 glycine residue of all the aligned strains. The figure is adapted from (Kwon et al., 2004).

Eukaryotic proteasomes

The ubiquitin proteasome system (UPS)

The ubiquitin proteasome system (UPS) found in eukaryotes is responsible for the regulated degradation of the majority of proteins in a cell. Since the degradation of proteins is an expensive process in terms of energy requirements, the UPS ensures highly selective degradation of proteins. UPS substrates include a wide range of proteins, including short lived cellular proteins, misfolded proteins and non-native protein aggregates. Thus, the activity of the UPS is essential to ensure proper functioning of almost all cellular processes including post-translational regulation, stress responses, antigen presentation and the cell cycle.

A cascade of proteins recruit substrates to the proteasome by modifying them post-translationally with ubiquitin (Ub) tags. Ub is a small protein, 76 amino acid residues long, and is considered an important protein of proteostasis. Poly-ubiquitination (PolyUb) of proteins targets them to the 26S proteasome for degradation. This cascade includes three classes of ubiquitin modifying enzymes: E1, E2 and E3 ligases (Streich & Lima, 2014; Varshavsky, 2012). First, the E1 enzyme, also known as the Ub-activating enzyme, activates ubiquitin by adenylating the glycine (Gly) residue present on the C-terminus. This adenylation allows for the formation of a Ub-E1 thioester bond between the C-terminal glycine of Ub and a conserved active site Cysteine (Cys) on the E1 enzyme. A second round of adenylation leads to the formation of an E1 ternary complex. The Ub

moiety from the active site cysteine of E1-ternary complex is transferred to the E3 active site cysteine of the E2 Ub conjugating enzyme. In some cases, the E2-Ub can transfer Ub directly to the substrate, but this step usually involves an E3 ligase that binds both the E2-Ub and the substrate and mediates the conjugation of Ub to the substrate. The poly-ubiquitin chains are formed by linkages of ubiquitin to the substrate by several possible linkages. Linkages through Lys-43 leads to the recruitment of poly-ubiquitinated substrates to the 26S proteasome (Streich & Lima, 2014). Ubiquitin and the UPS are of high interest in the field of medicine considering their functional roles in maintaining proteostasis as well as the multitude of cellular processes that are affected when it malfunctions.

Structure

The 26S proteasome is a 2.5 MDa macromolecular protease composed of 33 canonical subunits arranged in the form of two sub-complexes called the 19S regulatory particle (RP) and the 20S core particle (CP). The RP, also known as PA700, can be further subdivided into two sub-complexes, the lid and the base (Glickman et al., 1998; Isono et al., 2007). The lid is composed of nine regulatory particle non-ATPase (Rpn) subunits: Rpn3, Rpn5-9, Rpn11-12, Rpn15 (Sem1), while the base is composed of 6 regulatory particle tri-phosphatase subunits: Rpt1-6 and 4 Rpn subunits: Rpn1-2, Rpn10 and Rpn13. The Rpn11 subunit of the RP-lid possesses intrinsic deubiquitinating (DUB) activity within the proteasome and helps in deubiquitinating a majority of substrates targeted for degradation by the proteasome. The Rpt1-6 subunits present in the RP-base are classified as AAA-ATPases (ATPases associated with various cellular activities) that chemically hydrolyzes ATP to harvest energy to unfold and channel the substrates for degradation by the catalytic core (Howell et al., 2017).

The 26S proteasome, with the help of its own set of receptors (Rpn1 and Rpn2), binds to the poly-ubiquitinated substrates, and then utilizes the ATPase ring of the RP base to unfold the substrate. The unfolded proteins are deubiquitinated by Rpn11, a metalloprotease deubiquitinating enzyme, before they are channeled into the catalytic chamber of the CP. The CP is a barrel shaped complex that harbors the proteolytic active

sites. It contains four hetero-heptameric rings stacked to form a dyad symmetrical complex. The outer rings are composed of seven distinct alpha subunits and the two inner beta rings formed by seven distinct beta subunits. The outer rings contain a gated entry channel formed by the N-terminal α -subunits tails. The catalytic center is formed by the beta subunits. Only three out of the seven beta subunits ($\beta 1$, $\beta 2$ and $\beta 5$) possess the active site catalytic triad which provide the eukaryotic proteasome with three distinct activities, namely trypsin ($\beta 2$), chymotrypsin ($\beta 5$) and caspase ($\beta 1$) like activity (Arendt & Hochstrasser, 1997; Heinemeyer et al., 1997). These distinct activities of the 20S CP help it in degrading a large population of diverse substrates. Additional beta subunit paralogs, $\beta 1i$, $\beta 2i$, $\beta 5i$, and $\beta 5t$, have been found in humans and are known to alter the proteolytic activity and function of proteasomes. The “i” and “t” in the names of alternative beta subunits stands for immuno- and thymo-, respectively, and the alternate forms of proteasomes are called immunoproteasomes (functions in antigen presentation) and thymoproteasomes (has role during the maturation of T-cells) (Gaczynska et al., 1993; Klotzel, 2004).

Assembly

The assembly of eukaryotic proteasome is strikingly different from the 20S proteasome assembly seen in bacteria where one or two paralogs of alpha and beta subunits can be assembled in any order in their respective heptamerical rings. Efficient assembly of the eukaryotic proteasomes is a complex process that involves several external chaperones to ensure efficient assembly. Each of the 66 subunit that are present in a 26S proteasome, need to acquire a designated spot in the assembled complex and every assembly process should culminate in the exact same relative positioning of these subunits. This becomes more critical during the assembly of CP since it contains seven closely related paralogs of alpha and beta subunits and during the assembly of RP-base that contains six similar trio-phosphatase Rpt subunits. It is important for the cell to ensure proper placement of these subunits which are highly similar in sequence and structure, in their designated site. An alternative arrangement of these subunits can lead to the formation of dead end complexes or dysfunctional proteasomes. In addition to the highly specific intra-ring placement of subunits, efficient proteasome assembly must also ensure the rings to be

placed in a specific orientation with respect to each other in a functional complex. The structure and assembly process of proteasomes is highly conserved in all eukaryotes, from yeast to humans. Studies so far have unveiled mechanistic insights into how different factors facilitate the assembly process of CP and RP and how these two sub-complexes interact to form a functional 26S proteasome. So far, ten unique assembly chaperones have been identified that ensures correct assembly of the 26S. As discussed in the following section, five of these chaperones assist the assembly of CP and the other five chaperones are specific for the RP. Interestingly, the assembly of lid appears to be completely autonomous and does not require any external factors to facilitate the process.

20S CP assembly

The assembly of eukaryotic CP is similar to the α -ring dependent assembly pathway seen predominantly in archaea (Figure 1.2) (Zwickl et al., 1994). Four heterodimeric chaperone complexes namely, Pba1-Pba2 (referred to as PAC1-PAC2 in humans and PbaA and PbaB in archaea) and Pba3-Pba4 (referred to as PAC3-PAC4 in humans) are known to facilitate the formation of the alpha ring (Tallec et al., 2007). Pba1-Pba2 contains a C-terminal HbYX motif (generally found in proteasome activators) that is required to interact with the alpha ring. Pba1 docks into the pocket formed by $\alpha 5$ - $\alpha 6$ and Pba2 binds to the $\alpha 6$ - $\alpha 7$ pocket (Kusmierczyk, et al., 2011). Pba1-Pba2 are together known to prevent premature RP-CP interactions by binding tightly to the immature CP complex and are expelled out during CP maturation following an affinity switch (Wani et al., 2015). Pba3-Pba4 also exists as a heterodimer where Pba3 binds to $\alpha 5$ near the axial channel of the α ring and the Pba3-Pba4 dimer together prevents the premature interaction with $\beta 4$ as seen upon completion of assembly. On the other hand, Pba4 interacts with $\alpha 4$ to promote its incorporation next to $\alpha 5$ (Kusmierczyk et al., 2008). Pba3-Pba4, unlike the Pba1-Pba2 heterodimer, are released early on in the assembly process (Li et al., 2007). Unlike the formation of the α -ring, where the precise sequence of the incorporation of alpha subunits is not known, single gene knockdowns in cultured mammalian cells have led to the identification of the sequential assembly process of beta rings (Hirano et al., 2008). Beta subunits are incorporated as $\beta 2$, $\beta 3$, $\beta 4$, $\beta 5$, $\beta 6$, $\beta 1$ and $\beta 7$, in that order. The alpha ring serves as the template for the formation of the β ring and is assisted by a chaperone

named Ump1 (referred to as hUMP1 or POMP in humans). In addition to Ump1, the N-terminal propeptide sequence of β 1, β 2, and β 5 subunits, as well as the long C-terminal tail of β 2 and β 7, are also known to function as intrinsic chaperones that guide the assembly process (Hirano et al., 2008). The N-terminal propeptide of β 5 (75 residues in length in yeast) is essential for assembly of CP. The N-terminal propeptide of β 5 and the C-terminal tail of β 7 are known to facilitate the dimerization of half-proteasomes. In addition to this, the β 5 propeptide interacts with Ump1 to promote propeptide auto cleavage during proteasome maturation (Chen & Hochstrasser, 1996; Hirano et al., 2008).

The Ump1 chaperone was the first proteasome assembly chaperone discovered (Ramos et al., 1998). It is an intrinsically disordered chaperone, dedicated to the assembly of immature CP by assisting the incorporation of β subunits. The assembly of beta rings in yeast starts by association of Ump1 with alpha ring along with β 2, β 3 and β 4 (X. Li et al., 2007). In humans, hUmp1 binds to alpha ring and mediates the incorporation of β 2 (Hirano et al., 2008). In an immature CP, Ump1 extends into the central cavity interacting with α 1, α 4, β 4 and β 6. The N-terminal region of Ump1 is suggested to interact with the β 5 propeptide sequence as well as the binding surface between β 6 and β 7. The incorporation of β 7 completes the β ring assembly and promotes immature CP dimerization to form a pre-holoproteasome complex.

19S RP assembly

The RP is composed of 18 subunits assembled in the form of two sub complexes, the lid and the base. The lid and base complex assemble independently and then interact to form a functional RP. The function of RP is to receive proteasome substrates, deubiquitinate them and unfold them into the catalytic core. The assembly of RP-base requires five unique chaperones, the nine lid subunits can be reconstituted by recombinant yeast protein expression in *E. coli* in the presence of Hsp90 (Tomko et al., 2015). The role of Hsp90 in RP lid assembly is not known.

The assembly of lid does not depend on the RP-base or CP assembly. It starts with the formation of an Rpn8-Rpn11 heterodimer that further forms module 1 by joining Rpn5, Rpn6, and Rpn9 into the dimer (Ramnath et al., 2014). A second lid sub-complex named LP3 (lid particle 3) is formed by Rpn15 (Sem1), Rpn3 and Rpn7. Module 1 and LP3 together forms LP2. The LP2 sub-complex is joined by Rpn12 that completes the formation of RP-lid. Rpn12 binding to LP2 causes conformational changes in the structure of the lid that further facilitates the association of RP lid with base (Fukunaga et al., 2010).

The base is composed of 9 subunits, 6 homologous AAA-ATPase subunits named Rpt1-6 and 3 non-ATPases named Rpn1, Rpn2 and Rpn14. The Rpt subunits have an N-terminal α -helix followed by an oligonucleotide binding fold (OB), the AAA-ATPase domain, and a C-terminal helical domain (Djuranovic et al., 2009). Eukaryotic cells utilize five different chaperones that guide the base assembly process (Funakoshi et al., 2009; Kaneko et al., 2009; Roelofs et al., 2009; Saeki et al., 2009). These include Rpn14 (PAAF1 in mammals), Nas6 (gankyrin/p28 in mammals), Nas2 (p27 in mammals), and Hsm3 (S5b in mammals). Deletion of any of these chaperones does not affect RP-lid or CP assembly. The assembly of RP base is a step wise process where Rpt subunits form ATPase pairs which then associate with their aforementioned designated chaperones. These chaperones can bind to their respective Rpt subunit in the absence of other dimerized ATPase-Rpt subunits, making it unclear if the ATPase dimerization precedes their association with chaperones or if chaperones mediate this dimerization process (Roelofs et al., 2009). It is noteworthy that the formation of ATPase dimers does not depend upon the nucleotide state of the ATPase subunits, however it can influence the interaction of one ATPase dimer with another dimer (Kim et al., 2013). In chapter three, we have explored the effect of nucleotide state of Rpt subunits on their interaction with chaperones. We speculate that the binding of Ecm29, a proteasome quality control factor, can affect the nucleotide state of the Rpt subunits in the base which can promote their interaction with RP chaperones.

Three set of ATPase pairs, along with their RP assembly chaperones, form three different assembly modules. First is the Rpn14/Nas6 module composed of Rpn14-Rpt6-

Rpt3-Nas6, second is the Nas2 module of Rpt4-Rpt5-Nas2 and third is the Hsm3 module of Hsm3-Rpt1-Rpt2-Rpn1. These modules associate with one another, along with Rpn2 and Rpn13, and release the chaperones prior to the formation of the AAA-ATPase ring of the base. The fifth chaperone involved in the RP-base assembly is Adc17, which is known to interact with the N-terminus of Rpt6 to assist in the formation of the Rpt6-Rpt3 ATPase dimer prior to the formation of the Rpn14/Nas6 module (Hanssum et al., 2014). CryoEM structures have revealed that the Rpt subunits in a base ring are arranged in the form of two ring structures where the upper ring is composed of the oligonucleotide binding domain of Rpt subunits and the N-terminal coiled coil domains extending outwards and the lower ring is composed of the AAA-ATPase domain and the C-terminal helical domains that are docked into the α -pockets of CP (Smith et al., 2008). The C-terminal tails of Rpt2, Rpt3, and Rpt5 contain the conserved HbYX motif that assist in docking into α -pockets and opening the CP gate. The ATPase ring, when bound to a substrate, acquires a planar confirmation which aligns with the central chamber of CP. CryoEM structures have revealed that in the absence of any substrate the ATPase domains of the all Rpt subunits in a ring acquire a staircase like confirmation. In this confirmation, the Rpt subunits arranged at different planes relative to each other with Rpt3 at the top followed by Rpt4-Rpt5-Rpt1 and Rpt2 at the bottom closest to CP. Rpt6 is present in the same plane as Rpt5 filling up the gap created between Rpt3 and Rpt2 by this staircase like arrangement (Luan et al., 2016; Matyskiela et al., 2014). The RP subunits, Rpt3 and Rpt4, are known to be present on the top in a substrate free state, and have higher contribution in receiving and translocating the protein substrates.

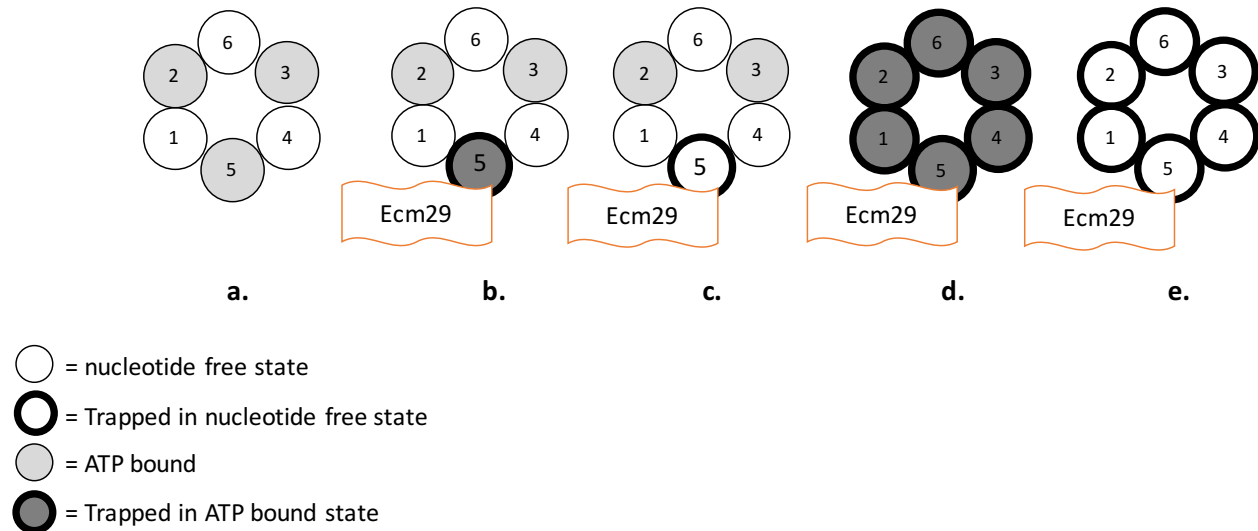
Rpn1 and Rpn2 are the non-ATPase subunits of the RP. They are also the largest proteasomal subunits. The large structure, with multiple proteasome/cyclosome repeats, is known to act as a scaffold during RP base assembly and also facilitates the transfer of substrates to the ATPase ring for unfolding (He et al., 2012). Some reports suggest Rpn1 to act as ubiquitin receptor. Rpn1 and Rpn2 are known to bind to the UBL domains in a number of proteins including Rad23 and Dsk2 (Elsasser et al., 2003).

Role of CP as a template for RP-base

The assembly of RP-base has been suggested to be independent of CP based on a study where reconstitution of a complete yeast RP was carried out in the absence of CP by expressing all the 6 AAA-ATPase subunits, the non-ATPases and four RP assembly chaperones in *E. coli* (Beckwith et al., 2013). An alternative pathway for the assembly of RP base is dependent on the alpha ring of assembled CP, which was proposed when RP base intermediates were seen to accumulate upon compromising the assembly of CP (Kusmierczyk et al., 2008; Park et al., 2011). Disruption of RP-CP interaction by a deletion in the C-terminal helical domain of Rpt2, Rpt3 and Rpt5 subunits that dock into the alpha ring of mature CP, also leads to an accumulation of RP intermediates bound with their respective chaperones (Lee et al., 2011; Park et al., 2009). This is consistent with the idea that the interaction of base with CP leads to an expulsion of chaperones from base to allow for the formation of stable RP-CP complex. In mammalian cells, deletion of the Rpn11 C-terminal tail lead to the accumulation of an RP-lid intermediate composed of module 1 (Rpn8-Rpn11-Rpn5-Rpn6-Rpn9) bound to base-CP complex (Yu et al., 2015). This complex suggested a role of base-CP interaction in the assembly of lid. It is still not completely understood if CP can act as a template for the assembly of RP. Although RP can be assembled in *E. coli* in the absence of CP, an alternate parallel mechanism involving CP for the efficient assembly of RP can still exist. So far, CP complexed with any RP-base assembly intermediate has not been reported.

In chapter three, we explore the role of Ecm29 to mediate the RP-base-CP interaction in certain conditions. We report the presence of RP assembly chaperones on 26S complexes under conditions that lead to an increased binding of Ecm29 with 26S proteasomes. This is interesting because the interaction of RP with chaperones or with CP is known to be mutually exclusive and the RP chaperones have never been reported to bind to the RP-CP complex. It still needs to be determined whether Ecm29 can recruit chaperones to RP-CP complexes or if they are some novel partially-assembled RP-CP intermediate complex captured by Ecm29. We explore the mechanism of how binding of Ecm29 to the Rpt5 subunit on base can affect the nucleotide state of the ATPases in RP base (Figure 1.4). This change in nucleotide state in turn has effects on the interaction of Rpt subunit dimers with respective chaperones and also with the CP.

Figure 1-4 Ecm29 dependent changes nucleotide occupancy of RP ATPases



(a) Model of RP base ATPase ring with heterogeneous nucleotide states of the ATPases under normal physiological conditions (Kleijnen et al., 2007). The following hypothesis were tested in chapter 3. **(b)** Binding of Ecm29 can arrest Rpt5 (binding site for Ecm29) in an ATP nucleotide bound state, but has no effect on the nucleotide state of other ATPases. **(c)** Binding of Ecm29 can arrest Rpt5 in an nucleotide free state, but has no effect on the nucleotide state of other ATPases. **(d)** Binding of Ecm29 can arrest Rpt5 and all other ATPases in an ATP nucleotide bound state. **(e)** Binding of Ecm29 can arrest Rpt5 and all other ATPases in a nucleotide free state.

Chapter 2 - Cooperativity in proteasome core particle maturation

Anjana Suppahia¹, Pushpa Ityagi^{2, 3}, Faith Mi Ge Kim¹, Alexander Vontz¹, Alicia Burris¹, Eric Deeds^{2, 3, 4}, Jeroen Roelofs^{1#}

1- Molecular, Cellular, and Developmental Biology Program, Division of Biology, Kansas State University, 338 Ackert Hall, Manhattan, Kansas 66506 USA

2- Center for Bioinformatics, University of Kansas, 2030 Becker Drive, Lawrence, KS 66047 USA

3- Department of Molecular Biosciences, University of Kansas, Lawrence, KS 66047 USA

4- Santa Fe Institute, Santa Fe, NM 87501 USA

Abstract

Proteasomes are multi-subunit protease complexes that are found in all three domains of life. Five unique assembly chaperones assist the formation of eukaryotic proteasome core particle (CP) which is further activated by the autocatalytic removal of the N-terminal propeptide from six beta subunits bearing proteolytic active sites. On the other hand, no such assembly chaperones have been identified in bacteria. How bacterial proteasomes undergo efficient assembly without the assistance of any extrinsic factors is not completely understood. As opposed to the six active sites found in eukaryotic CP, the activation of bacterial CP requires the removal of propeptide sequences from all of the fourteen beta subunits present in the homo-heptameric beta rings of bacterial CP. The precise mechanism of how all the active sites are exposed during the activation process is also not known. Bioinformatics analysis indicates that the propeptide region of beta subunit has an evolutionarily conserved distribution of electron density. In this paper we show that the propeptides can regulate the dimerization of process through the flexible loop. We also show that the N-terminal truncations in propeptide sequence enhance the half proteasome (HP) dimerization and decelerate the CP activation rate. As opposed to the strictly autocatalytic processing of propeptides in eukaryotes, our results indicate the existence of cooperativity phenomenon in the processing of the propeptides in bacterial CP. We show that the propeptide sequence can be partially processed by an exposed active site present on an adjacent beta subunit present in a CP, thus complementing the otherwise strictly autocatalytic mechanism. We suggest this cross processing as the underlying mechanism that leads to the observed cooperativity phenomenon. Our work thus provides useful insights into the bacterial CP assembly and activation process.

Introduction

Protein degradation is an essential cellular process required to maintain cellular homeostasis. Various proteases, including the 26S proteasome in eukaryotes, are involved in the clearance of proteins targeted for degradation. The proteasome core particle (CP) is found ubiquitously in eukaryotes, archaea, and some bacterial orders like Actinomycetales and Nitrospirales (reviewed in (Becker & Darwin, 2017)). The structurally conserved CP consists of four heptamerical rings stacked to form a hollow cylindrical protease complex. In eukaryotes, the rings consist of seven distinct α and β subunits. However, archaea and eubacteria mostly encode for only one paralog of the α and β subunit each and thus have homo-heptametrical α and β rings. *Rhodococcus erythropolis* (*R.e.*), a nocardioform actinomycete, is an exception as it produces two distinct but related α and β subunits that form their 20S CP (Tamura et al., 1995). Archaea can also encode additional variants of α and β subunits whose presence has been attributed to gene duplication events (Budenholzer et al., 2017).

In vitro studies have suggested the presence of two distinct assembly pathways for CP, which differ in the initial steps to form a half CP (HP). The α ring dependent assembly pathway, found mainly in eukaryotes and archaea, involves the formation of a seven membered α ring which then serves as a docking site for the seven β subunits, thereby creating a double ring structure, referred to as HP. However, bacterial α subunits do not form ring structures independent of β and assembly starts by β subunits interacting with α subunits to form $\alpha\beta$ heterodimers. This results in the rapid and simultaneous formation of HP consisting of α and β rings (Zühl et al., 1997; Zühl et al., 1997). The dominance of any of the two alternate assembly pathways seem to be driven, in part, by the stability of the different interactions. This feature correlates with the amount of interaction surface there is amongst the different subunits. For example, in the α ring, bacterial α subunits show much less interaction surface with each other as compared to archaea or eukaryotes, thus forming $\alpha\beta$ heterodimers as opposed to independently forming an initial α ring (Hu et al., 2006; Kwon et al., 2004). On the other hand, mutations in the α subunit of *Methanococcus maripaludis*, an archaea, can divert the assembly from

the predominant α ring dependent pathway to the $\alpha\beta$ heterodimer path, suggesting both can function in parallel (Panfair et al., 2015). Once HP is formed, two HPs dimerize with the beta rings forming interactions to create a full CP with an $\alpha_7\beta_7\beta_7\alpha_7$ topology. The dimerization coincides with proteolytic processing of some (eukaryotes) or all (archaea and bacteria) of the β subunits to form a proteolytically active CP (Becker & Darwin, 2017; Budenholzer et al., 2017; Kunjappu & Hochstrasser, 2014; Sharon et al., 2007).

The assembly in eukaryotes is more complex as the heterogeneous CP consists of fourteen different polypeptides. The assembly, probably in part due to the complexity, is guided by five distinct chaperones. Some of these chaperones have orthologs in archaea (Kusmierczyk et al., 2011), but no dedicated chaperones have been identified for the assembly of bacterial CP. Several archaeal and bacterial CPs have been reconstituted from α and β subunits heterologously expressed in *E.coli*, indicating chaperones are not essential here (Budenholzer et al., 2017). Apart from chaperones, the propeptides of beta subunits have been shown to function as “intrinsic chaperones”. For example, in yeast, the propeptide of Doa3 (β_5 subunit of the CP) is required for its incorporation into the CP (Chen & Hochstrasser, 1996). Similarly, propeptides in actinobacteria are also essential for their proper folding and incorporation into higher complexes during assembly (Zühl et al., 1997). The bacterial propeptides bind to α subunits and assist in the formation of $\alpha\beta$ heterodimers during HP assembly. That said, the short propeptides of *Mycobacterium tuberculosis* (Mtb) are dispensable for CP formation *in vitro*, but are known to help maintain the stability of β subunits. (Kaczowka & Maupin-Furlow, 2003; Lin et al., 2006; Zwickl et al., 1994). Some archaea have longer propeptides that are known to facilitate the 20S assembly by preventing the N-terminal acetylation of the active site threonine, as seen in yeast (C. S. Arendt & Hochstrasser, 1999; Soppa, 2010). In all, there are a variety of proposed evolutionarily diverged roles of propeptides. Furthermore, some of the propeptide functions might have been conserved through the chaperones that assist in CP assembly in eukaryotes. For example, Ump1 in eukaryotes is an intrinsically disordered protein, which remains associated with immature CP preventing its premature dimerization and is degraded along with other propeptides as the first substrate of an assembled and active CP (Ramos

et al., 1998). A better understanding of the bacterial propeptides will help to elucidate these fundamental evolutionary aspects and reveal the critical core functions of propeptides. Moreover, this may also reveal differences in the process that might be exploited in the development of drugs, specifically against bacterial CP assembly. For example, specifically targeting the *Mtb* CP could be valuable because this complex is essential for the survival of this pathogen. It is thus important to fully understand the function of propeptides in bacterial CP assembly.

In this paper, a bioinformatics approach has been used to define three distinct regions in bacterial β subunit propeptides. To determine a role for these regions in the assembly process of CP, we used *in vitro* reconstitution experiments to analyze the effect of specific mutations and truncations of the β subunit from *R.e.* We identified a role for the N-terminal region of the propeptide in regulating the speed of HP dimerization and the autocatalytic activation of CP. The N-terminal mutants of β subunits, also allowed us to observe a previously elusive intermediate complex called pre-holoproteasome, which is an assembled CP with unprocessed propeptides. This indicates that dimerization and activation are distinct independent processes. Furthermore, we identified the third region as a flexible loop based on molecular dynamics simulations and showed it strongly influences the dimerization rate of HPs. Interestingly, our results show that an unprocessed propeptide can be processed by the active site of a second beta subunit present in either the same beta ring or the beta ring of the adjoining half CP. We thus propose a novel mechanism for the activation of CP that involves cooperativity in the processing of propeptides between beta subunits present in a CP.

Experimental Procedures

Plasmids used

R.E. PrcA gene (pJR 662) and PrcB gene (pJR659) was obtained from the lab of Dr. Eric J. Deeds (University of Kansas, Lawrence, KS). Plasmids used in this paper are listed in Supplementary Table 1. The accuracy of the plasmids generated was confirmed by sanger sequencing (Genewiz).

Table 2-1 Plasmids and primers

Plasmid	Template	Primers	Protein expressed
pJR681	pJR659	FP: pRL437 (5'-GCCGCACGGCgcgACCATTGTGGCGCTGAC-3') RP: pRL438 (5'-GCCAGGTCACCGCTCTCCATG-3')	N-(PrcB1 _{TtoA} ORF)-6xHis-C (β _{TtoA})
pJR770	pJR659	FP: pRL519 (5'-gaaagcgggGACCTGGCGCCGCACGGC-3') RP: pRL520 (5'-gccaccgccACCGATACGGTTTTCCGGCAGCAG-3')	N-(PrcB1 _{EDM2} ORF)-6xHis-C (β _{EDM2})
pJR771	pJR659	FP: pRL521 (5'-ggcgggGACCTGGCGCCGCACGGC-3') RP: pRL522 (5'-accgccACCGATACGGTTTTCCGGCAGCAG-3')	N-(PrcB1 _{EDM3} ORF)-6xHis-C (β _{EDM3})
pJR772	pJR659	FP: pRL523 (5'-ggccgtggcgggtgatGGCATGGAGAGCGGTGAC-3') RP: pRL524 (5'-gtcacctccgcgaccATCGCCACCACGGGTGCT-3')	N-(PrcB1 _{EDM4} ORF)-6xHis-C (β _{EDM4})
pJR773	pJR659	FP: pRL525 (5'-cgggtatggagcgcgggtggagatGGCATGGAGAGCGGTGAC-3') RP: pRL526 (5'-ccacggccgctcacctccgcgaccATCGCCACCACGGGTGCT-3')	N-(PrcB1 _{EDM5} ORF)-6xHis-C (β _{EDM5})
pJR775	pJR779	FP: pRL463 (5'-GCCGCACGGCACCACCATTGTGGCGCTGAC-3') RP: pRL438 (5'-GCCAGGTCACCGCTCTCCATG-3')	N-(Δ18 PrcB1 ORF)-6xHis-C (β Δ18)
pJR776	pJR780	FP: pRL463 (5'-GCCGCACGGCACCACCATTGTGGCGCTGAC-3') RP: pRL438 (5'-GCCAGGTCACCGCTCTCCATG-3')	N-(Δ23 PrcB1 ORF)-6xHis-C (β Δ23)
pJR777	pJR781	FP: pRL463 (5'-GCCGCACGGCACCACCATTGTGGCGCTGAC-3') RP: pRL438 (5'-GCCAGGTCACCGCTCTCCATG-3')	N-(Δ25 PrcB1 ORF)-6xHis-C (β Δ25)
pJR779	pJR681	FP: pRL441 (5'-TTTGGCAGCAATCTGAGCAGC-3') RP: pRL449 (5'-CATATGTATATCTCCTTCTTAAAGTTAAACAAAATTATTCTAGAGGG-3')	N-(Δ18 PrcB1 _{TtoA} ORF)-6xHis-C (β Δ18 _{TtoA})

pJR780	pJR681	FP: pRL442 (5'- AGCAGCTTTACCGACTACC-3') RP: pRL449 (5'- CATATGTATATCTCCTTCTTAAAGTTAAACAAAATTATTTCTAGAGGG- 3')	N-($\Delta 23$ PrcB1 T _{10A} ORF)- 6xHis-C (β $\Delta 23$ T _{10A})
pJR781	pJR681	FP: pRL443 (5'- TTTACCGACTACCTGCGTGGTC-3') RP: pRL449 (5'- CATATGTATATCTCCTTCTTAAAGTTAAACAAAATTATTTCTAGAGGG- 3')	N-($\Delta 25$ PrcB1 T _{10A} ORF)- 6xHis-C (β $\Delta 25$ T _{10A})
pJR659		Provided by Dr. Eric J. Deeds (University of Kansas, Lawrence, KS)	N-(PrcB1 ORF)-6xHis-C (β_{wt})
pJR662		Provided by Dr. Eric J. Deeds (University of Kansas, Lawrence, KS)	pTBSG-PrcA (α)

Protein expression and purification

For expression of recombinant proteins, plasmid carrying the gene of interest was transformed into competent Rossetta cells (BL-21 DE3 derived strain). The transformed cells were then inoculated in 3ml Luria-Bertani (LB) broth supplemented with 100 μ g/mL ampicillin (Amp) and 34 μ g/ml chloramphenicol (Chlor) and incubated under shaking conditions at 37 degrees overnight. This culture was used to inoculate 100ml of LB broth supplemented with Amp and Chlor and allowed to grow to an O.D.₆₀₀ of 0.6 before induction with 0.1mM IPTG. The culture was then grown on shaker at 30 degrees for 3 hours. The cells were then centrifuged and pellets were stored at -80 degrees. For purification of the His-tagged proteins, the pellet was re-suspended in Lysis buffer (5mM Imidazole, 20mM Tris-pH8, 100mM NaCl, 0.75mM EDTA, protease inhibitors) and the bacterial cells were then lysed using French press at 900 psi. The total lysate was then cleared by centrifugation at 10,000 rpm for 20 minutes using SS34 rotor. The cleared lysate was then incubated with 100 μ l Roche complete His-Tag purification resin for 1 hour at 4 degrees. The resin was then collected by centrifugation at 500g and re-suspended in 5 ml wash buffer (15mM Imidazole, PBS, 0.1% TritonX-100, 0.02% azide). The wash step was repeated 3 times. The His-tagged proteins were eluted by re-suspending the resin in 150 μ l of elution buffer (50mM Tris (pH7.5), 100mM NaCl, 1mM EDTA, 0.25mM DTT) containing 60mM imidazole. The elution step was subsequently repeated four times with changing concentration of imidazole (120mM, 250mM, 400mM and 500mM respectively). The elutes containing most of the protein of interest were pooled and

dialyzed to separate imidazole from the purified protein using dialysis buffer (50mM Tris (pH7.5), 100mM NaCl, 1mM EDTA, 0.25mM DTT).

In vitro reconstitution assays

The reconstitution of the purified proteins and specified concentrations was done in the buffer containing 1M Tris pH7.5, 1M MgCl₂, 0.5M ATP. The total reaction volume for all assays was 25μl. The temperature used for the reconstitution of the subunits to form CPs was 30 degrees. The reconstitution of subunits to form HPs was done at 4 degrees.

Native gels and two dimensional (2D) assays

The reconstituted samples were analyzed on Native gels by first adding 1X native gel loading buffer (50mM Tris-HCl (pH 7.4), 50% glycerol and 60 ng/ml1 xylene cyanol). The samples were then loaded on a 3.6% native gel and analyzed for the activity using in-gel Suc LLVY-AMC assay as described previously (Elsasser et al., 2005). The gels were then stained with Coomassie brilliant blue. For 2D assay, the samples were run on a native PAGE to separate complexes formed and the lane containing our sample of interest was cut from the native gel and incubated with 1X SDS-Sample buffer for 10 minutes. The lane was then transferred and separated on a second dimension to separate the proteins based on their size using SDS-PAGE. After electrophoresis the gels were stained using Coomassie brilliant blue.

Multiple sequence alignment of bacterial species

The input query used to perform a BLAST search on all bacterial species in the database was the β subunit of R.e. including the propeptide. We performed a multiple sequence alignment of the selected bacterial sequences using CLUSTALW omega.

Root Mean Square fluctuations (RMSF) of the propeptide in MD simulations

The RMSF per residue is calculated for the two different proteasome simulations. First, using NAMD package with conventional MD we have 3 replicates each having about 70ns

time length, run on the KU cluster. Second, a replicate with a longer i.e. 2 μ s length, run on ANTON computer. Each replicate has RMSF values reported are for all the 7- β subunits since each subunit propeptide would have different fluctuations.

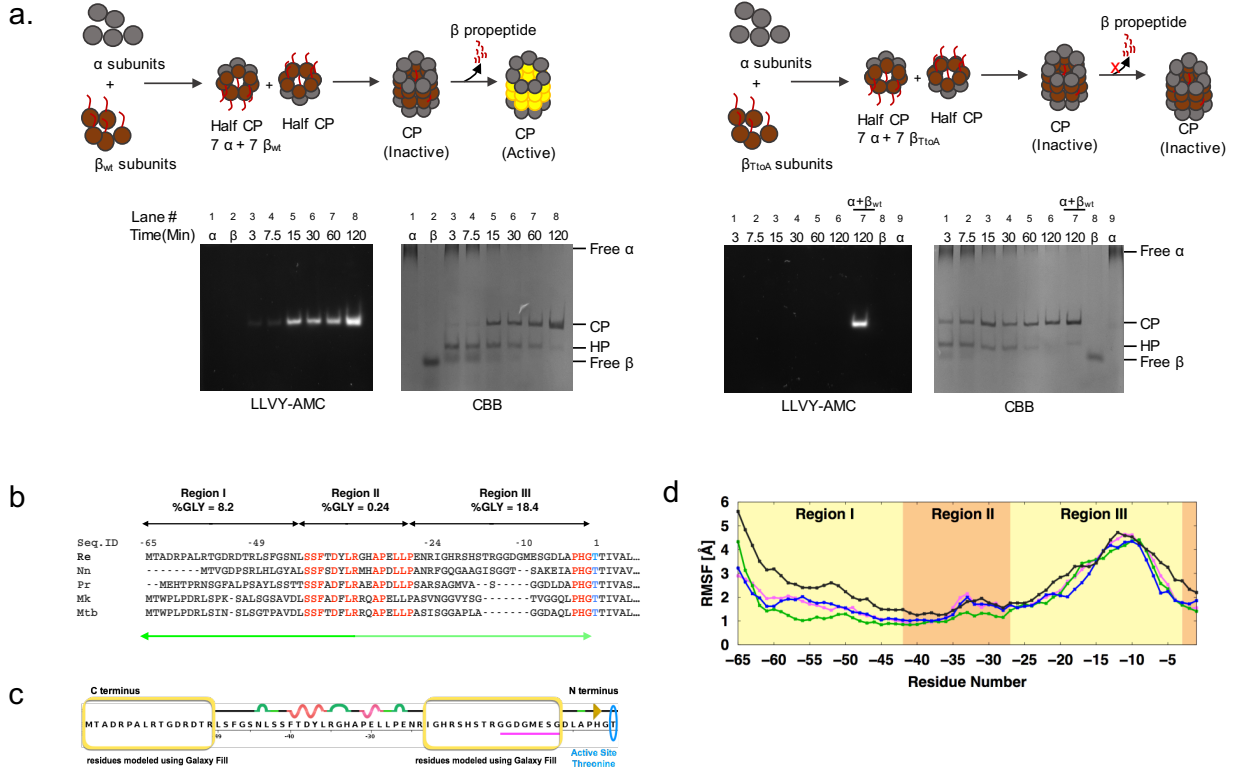
Results

Bacterial propeptide can be divided into three evolutionarily conserved regions

The assembly of proteasomes in eukaryotes is more complex than that of archaeal and bacterial proteasomes. Five different polypeptides have been identified that assist the assembly of seven unique α and β subunits into hetero-heptameric rings to form eukaryotic 20S core particles (CP). The genomes of bacteria and archaea normally encode one α and β subunit each (Maupin-Furlow et al., 2006). This results in a much less complex ring structure, as it eliminates the specific order of subunits within the α and the β ring that is observed in eukaryotes (Murata et al., 2009). It also simplifies how the rings are positioned relative to each other. Consistent with this lower complexity, the archaeal and bacterial CPs assemble more readily and without the need for specific chaperones *in vitro* or in *E.coli* (Becker & Darwin, 2017). Nevertheless, some chaperone homologs have been identified in archaea (Kusmierczyk et al., 2011; Sikdar et al., 2014). However, studies of the assembly and maturation of the eukaryotic 20S has identified specific functions for CP chaperones that expand beyond ensuring the proper placement of particular α or β subunits. Considering the absence of chaperones in bacteria and the reported role for the pro-peptides of the β subunits in assembly, we wondered if some of the functionality of the eukaryotic assembly chaperones have been retained in the propeptides of the eubacterial β subunits. To assess this, we focused on the propeptide of the *R.e.* 20S β subunit Prc β 1 (proteasome component B). Although *R.e.* is unusual amongst bacteria in that it has two paralogs of α and β each, it has been well characterized and shown that functional CP forms with only one α and β present. Furthermore, *R.e.* lacks 20S chaperones and has detailed structural and biochemical information available, allowing us to interpret our results in a structural context (see e.g. (Kwon et al., 2004; Witt et al., 2006). Finally, this system is amendable to *in vitro* reconstitution using *E. coli* purified α and β subunits. Making it a powerful model to study the assembly and maturation of CP when using native-polyacrylamide gel electrophoresis (PAGE) (Fig. 2.1a) (Zühl et al., 1997).

To assess potential conservation of properties within the bacterial propeptides, we compared the β subunits from different bacterial species identified based on homology to the *R.e.* complete β subunit Prc β 1. We selected 265 beta subunits assigned to different bacterial species that showed 61% or more sequence identity following BLAST analyses. Based on our analyses, we defined three distinct regions (named I to III) in the propeptide (Fig. 2.1b). Region II is largely identical to the previously described central box. This region has a well-defined crystal structure and functions in ring formation by allowing β to interact with two α subunits (Kwon et al., 2004). The region presents towards the N-terminal of Region II is called Region I. It has an average length of 22 amino acids, residues -65th to -43th in *R.e.* Prc β 1 (negative residues are those from the propeptide with -1 being just N-terminal of the propeptide cleavage site). Region III has an average length of 25 amino acids, running in *R.e.* from -26th to -1st. While alignments of Region III did not show any obvious conservation, our analyses indicate this region is highly enriched in glycine residues, suggesting there is some evolutionary pressure and function for this region. Nevertheless, biochemical and structural analyses to date have not identified any clear function for Regions I or III.

Figure 2-1 The distribution of electron density in beta propeptide is evolutionarily conserved.



(a) *In vitro* assembly of the bacterial 20S core particle. Purified α and β_{wt} subunits when mixed *in vitro* form half core particle (HPs) which then dimerizes to form full core particle (CP) which further gets activated by the removal of pro-peptide sequence from all of the beta subunits (activated β_{wt} subunits are shown in yellow color). The assembly was assessed by LLVY-AMC assay used to visualize active CPs and coomassie brilliant blue G250 dye (CBB stain) to visualize all the complexes present on Native PAGE. Similarly an inactive version of β_{T10A} was also used for reconstitution with α and analyzed by LLVY-AMC assay and CBB stain(right). As seen on CBB stained gels, free α subunits tends to form aggregates and run on top of gel, whereas beta subunits run farthest in the gel appearing as a band below HP. (b) Amino acid sequences of the N-terminal regions of the β – subunit from *Rhodococcus erythropolis* (Re), *Nocardia niwae* (Nn), *Prauserella rugosa* (Pr), *Mycobacterium kansasii* (Mk) and *Mycobacterium tuberculosis* (Mtb). The conserved residues are highlighted in red and the active site is highlighted in blue. The propeptide region is depicted by green arrow with the %GLY indicated on top. Note: This alignment is a representative subset of the 265 species MSA used for analysis. (c) Sequence view of the propeptide of β subunit of R.e. The residues with missing electron density are highlighted in yellow boxes. The flexible region of the propeptide (residues -7 to -14) is underlined in pink and the active site is shown in blue circle. (d) RMSF (Å) of the Re propeptide for the three replicates (magenta, blue and green color) and ANTON (black color) system. The background color indicates different regions: ordered region (orange), disordered region (yellow).

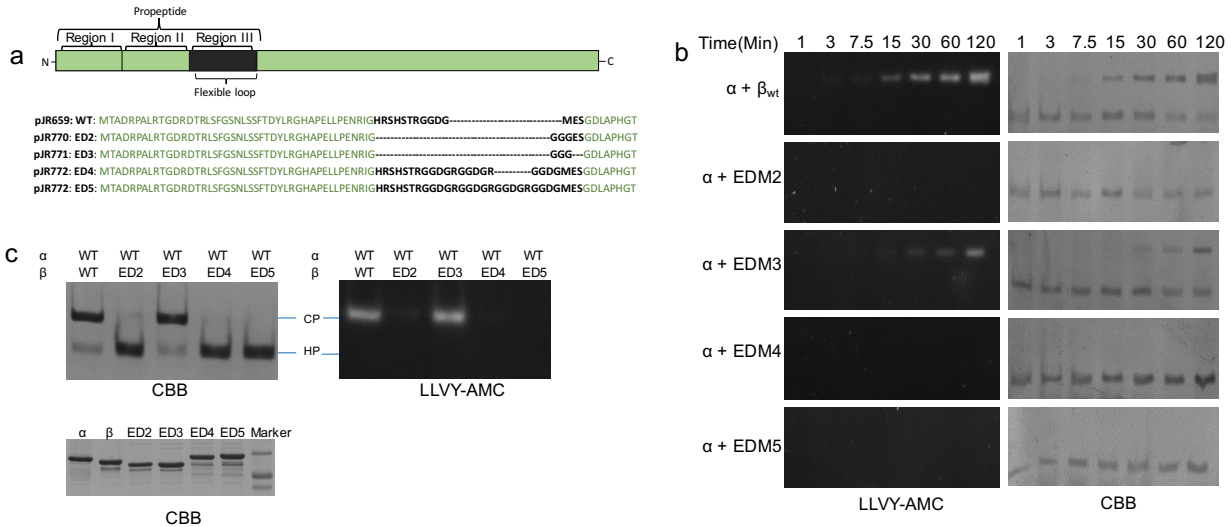
Propeptide region III regulates HP dimerization

Using the alignment of 265 bacterial β subunits, we calculated an average of 20.3% glycine residues for region III. This is a strong enrichment of glycine residues as compared to the other propeptide regions (Fig. 2.1b), complete β sequences or protein sequences in general. Glycine residues generally disrupt α -helices and β -sheets and are more common in loops between secondary structure elements and as flexible linkers between protein domains or regions (Imai & Mitaku, 2005; Levitt, 1978). Therefore, we postulated that there has been evolutionary pressure to generate a flexible disordered loop between the active site and the structured region (II) of the propeptide. Consistent with this, the region has ill-defined electron densities in the crystal structure (Kwon et al., 2004). To gain insight into the flexibility we used molecular dynamics (MD) simulations to determine fluctuations of the amino acid residues in *R.e.* by calculating RMSF for the backbone atoms (C, O, N and C α) in the HP. RMSF is a metric used for determining the atomic mobility per residue averaged over time (Fig. 2.1c and 2.1d). Interestingly, in the MD simulations the flexible loop was observed to reside for some extended time outside of the HP. The presence of propeptide density outside the HP potentially causes a steric clash when a second HP would need to occupy this space during dimerization. This is an intriguing phenomenon considering the reported role of several assembly factors in sterically blocking steps in the assembly process of proteasomes (Barrault et al., 2012; Ehlinger & Walters, 2014; Kock et al., 2015; Park et al., 2013; Roelofs et al., 2009; Satoh et al., 2014; Singh et al., 2014; Wani et al., 2015). Particularly interesting is the Ump1 chaperone, which in eukaryotes is known to assist CP assembly by inhibiting the premature dimerization of HPs (Li et al., 2007; Ramos et al., 1998).

To understand the role of region III in the assembly process, we designed mutants by altering the length of the flexible loop (Fig. 2.2a). Two mutants, EDM2 and EDM3, were created with shorter flexible loops based on the minimal theoretical length needed to span the distance between Region II and the remainder of β in the crystal structure (Kwon et al., 2004). Two other mutants, EDM4 and EDM5, were created with extended flexible loops with the idea that these loops would reside longer in the space outside the HP and

thus be more effective at sterically blocking HP dimerization (Fig. 2.2a). These mutants retained putative flexibility by including glycine in the modified loops. Reconstitution of each mutant with α subunits showed that all were able to quickly and efficiently form HP, indicating the flexible loop in region III is dispensable for the formation of HP. Furthermore, this shows that the mutants are not misfolded and that these mutations result in polypeptides that retain the ability to form HP's, and that region II is likely properly folded and positioned to allow the association of β and α subunits, which has been shown to promote HP formation (Kwon et al., 2004). However, all four loop mutants showed strongly reduced efficiency in the dimerization of HPs to form CP, suggesting there is some evolutionary optimization in the length or composition of this region (Fig. 2.2b). The short flexible loop as seen for EDM2 did not show any detectable levels of CP upon 2 hours of incubation. However, shortening the loop further by deleting two more amino acids resulted in partial rescue of the ability to form CP. Extending the loop also resulted in a reduced ability of HPs to dimerize, as observed for EDM4 and EDM5 mutants. Clearly, Region III appears to play a role in regulating dimerization of HPs.

Figure 2-2 Propeptide region of beta subunit can regulate the dimerization process through its flexible loop.



(a) Graphical representation of the region I, II and III of beta subunit (top), N-terminal sequence of the flexible loop mutants (bottom). (b) Equimolar amounts of α and respective flexible loop mutants were reconstituted in vitro for indicated time points and analyzed on native gels by LLVY-

AMC assay and CBB staining. (c) Indicated α and β mutants were reconstituted overnight and analyzed on Native gel for hydrolytic activity using AMC-LLVY followed by CBB stain(top). 4uM of respective subunits used for reconstitution was separated on a SDS-PAGE to serve as a control.

To understand if flexible loop mutations completely abrogated or severely attenuated the dimerization capability of these mutants, we extended the reconstitutions overnight at 30 degrees (Fig. 2.2c). Upon longer incubation, we detected a very small amount of active CPs for the EDM2, 4, and 5 mutants. The activity correlated with the amount of full CP formed. This suggests these three mutants are severely compromised in HP dimerization. Alternatively, dimerization per se is not compromised, but the mutant loops cannot be physically accommodated within a CP structure. To test the ability of these mutants form full CP, we mixed the EDM mutants with a form of beta with active site threonine substituted to alanine (β_{TtoA}) rendering the mutant catalytically dead (β_{TtoA} , Fig. A.1a). Here, any observed activity in CP must be derived from the EDM mutants and indicates their incorporation into CP and autocatalytic processing of the propeptides. When EDM2 was reconstituted with α and a small amount of β_{TtoA} (molar ratio of EDM2: β_{TtoA} = 7:1, data not shown), β_{TtoA} was not able to rescue CP formation for EDM2. However, we observed a substantial amount of active CP with a 1:1 ratio of EDM2: β_{TtoA} (Fig. A.1a). Conducting similar experiments with the extended loop mutants (EDM4 and EDM5) showed a different outcome (Fig A.1b). In a one to one ratio very little CP was formed for EDM4 and even less with the longest loop, EDM5. When reducing the relative amount of EDM4 or 5 mutant compared to β_{TtoA} (1:7) it yielded increased amounts of CP and activity, indicating these mutants dramatically inhibit dimerization. Thus, while the levels of β with active site residues was lower (i.e. EDM4 or 5), the CPs showed increased activity, which indicates that incorporated EDM4 or 5 mutants undergo efficient autocatalytic processing. In sum, Region III has no apparent role in HP formation or autocatalytic processing of β propeptide. However, changes in its length or composition have a dramatic effect on HP dimerization.

In the reconstitution experiments with an equimolar mixture of EDM4 and β_{TtoA} , the HPs that form will have a distribution in the number of EDM4 subunits versus β_{TtoA} which

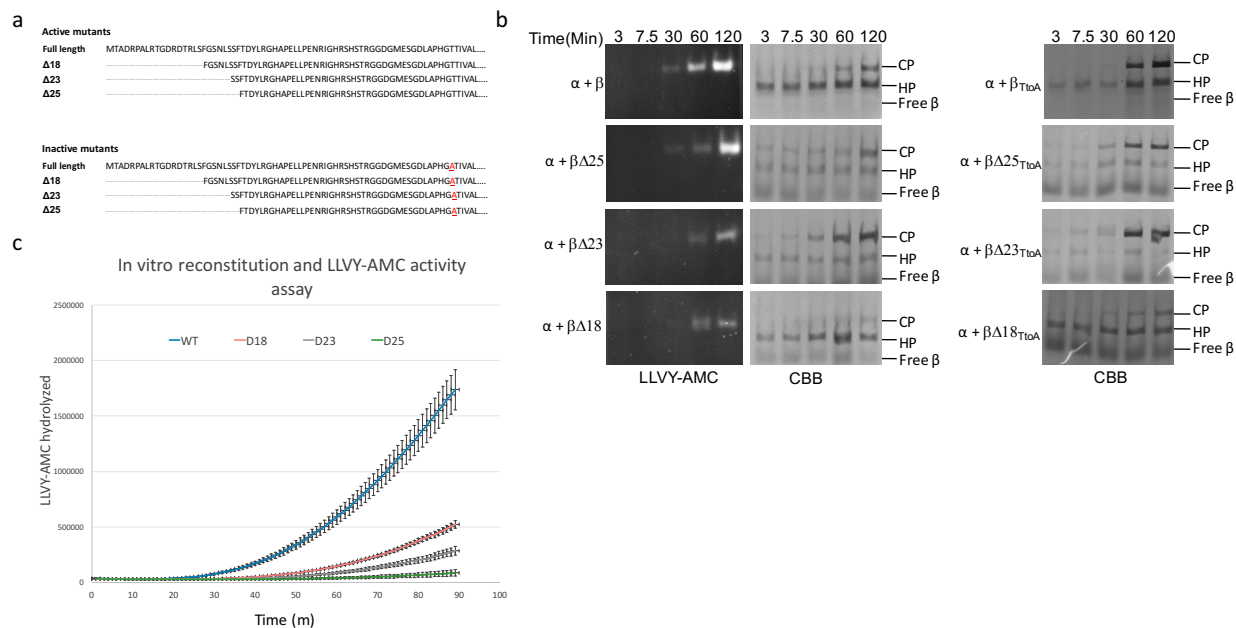
is centered around 50% if both are incorporated with the same efficiency into HP. Based on our model, the extended region III would interfere with dimerization. Thus, we would predict that after reconstitution, the HPs that failed to dimerize are those that are enriched in β s with an extended loop (EDM4 or EDM5). On the other hand, HPs with lower levels of, for example, EDM4 relative to β_{T10A} should be preferentially incorporated into CP. To test this, we performed a reconstitution using EDM4 in combination with $\beta\Delta 23_{T10A}$ (Fig. A.1c). The truncation on β_{T10A} allowed us to determine the ratios of the two different β s in CP and HP bands by using native-SDS-page 2-D gel analyses. The quantification of the relative abundance of $\beta\Delta 23_{T10A}$ to EDM4 or EDM5 mutant in HP and CP showed that indeed the HPs that failed to form CPs were enriched in the mutant with extended flexible loops (EDM4 and EDM5) (Fig. A.1c). As discussed later in more detail, reconstitution at low temperatures allowed us to form HPs that, despite being competent, do not dimerize. This provided us the opportunity to subsequently mix HPs with each other and monitor their dimerization at 30 degrees (Fig. A.1d). When we first allowed for the formation of HP that consisted of either EDM4 or EDM5 and reconstituted these with HPs that were formed exclusively from β_{T10A} , we observed the formation of active CP. Since EDM4 or 5 forms little to no CP by itself (Fig. A.1d, lane 3) and $\beta\Delta 23_{T10A}$ is inactive, the CPs that showed activity must be derived from HP_{EDM4} or HP_{EDM5} that dimerized with $HP_{\beta\Delta 23_{T10A}}$. This was surprising, since HP that form from a mix of EDM4 or 5 and $\beta\Delta 23_{T10A}$ in a 1:1 ratio, failed to dimerize. This difference suggests that the single extended loop by itself can be accommodated in dimerization, but when two extended loop from opposite HPs meet, the dimerization is inhibited. This is consistent with our model that the extended flexible region III can regulate dimerization by populating part of its time outside of the HP, where it can sterically clash with a dimerization partner.

Region I's role in autocatalytic processing

Truncations in the N-terminal region of the propeptide have been shown to affect their incorporation in the HPs. *In vitro* reconstitution assays done using α and β subunits expressed and purified from *E. coli*, have shown that the β subunits with large truncations from the N terminal region accumulated as either assembly intermediates or free

subunits. This indicates that the poorly conserved region I might be important in assembly. It has been noted before that poorly conserved regions can play important roles for the proteasome in studies looking at $\beta 5$ in eukaryotes (Li et al., 2015). To assess a role of Region I in the assembly process of *R.e.* CP, we generated three N-terminal truncation mutants. Further, we combined these truncations with the inactivating T to A mutation in β (Fig. 2.3a). Upon purification and reconstitution of these mutants with α we observed a faster formation of CP in $\Delta 23$ and $\Delta 25$ as compared to wildtype (Fig. 2.3b). We saw the same dimerization and activation behavior upon reconstituting α and β mutants at 4 degrees to form HPs, followed by their dimerization at 30 degrees. (Fig A.2a). This effect was most pronounced in the $\Delta 23$ and $\Delta 25$ truncations where CP formed shortly after HP was detectable. While there was a more rapid rate of dimerization, we surprisingly saw a delayed or reduced proteolytic activity (also see, Fig A.2b). This is particularly interesting as generally the dimerization of HPs appears to be tightly coupled with the activation of CP. For example, despite extensive efforts, preholocomplexes (CP containing all unprocessed propeptides) have not been observed, unless mutants were used that block propeptide processing completely, such as K33A (Kwon et al., 2004) or T1A. We also analyzed assembly over time by monitoring peptidase activity produced upon hydrolysis of LLVY-AMC (Fig 2.3c). Unlike the analysis done on native-PAGE post assembly, all the N-terminal truncation mutants showed severely reduced activity when compared to the reconstitution using β_{wt} .

Figure 2-3 N-terminal truncations in beta subunit affects half proteasome dimerization rate.



(a) N-terminal sequence of N-terminally truncated forms of catalytically active or inactive (TtoA) forms of beta subunits. (b) Equimolar amounts of indicated subunits were first reconstituted at 4 degrees which arrests the assembly process at HP (refer Fig 2.7 for more information). The HPs thus formed were then allowed to dimerize at 30 degrees for indicated time points and analyzed on native gels using LLVY-AMC assay and CBB staining. (Note: LLVY assays of inactive mutants are not shown due to absence of hydrolytic activity of CPs). The mutants are slower and have lower amounts of active CPs at earlier time points w.r.t. β_{wt} , however they do not seem to affect the formation of HPs as observed on CBB stained native-PAGE. (c) Equimolar amounts of α was reconstituted with indicated β subunits and simultaneously assayed for hydrolysis of LLVY-AMC. Accumulation of fluorescent AMC over time is displayed over a period of two hours which indicates the amount of active CP thus formed upon assembly. The average of seven independent experiments are shown with SEM. Activity of mutants reduces significantly as compared to β_{wt} , indicating that these mutants severely affect the assembly process by possibly affecting the dimerization rate.

This faster dimerization of HPs and slow activation of CP suggests that the N-terminal regions play a role in efficient autocatalytic cleavage of the propeptide from β . To know if the reduced or delayed activity in the mutant CPs was due to incomplete or delayed processing of the β propeptide in CPs, we conducted 2D gel analyses (Fig. 2.4a). Here, a first dimension of native PAGE was followed by 2D SDS-page after reconstitution of α and β for 1 hour at 30 degrees. This allowed the determination of the MW of HP and CP components. For wild type α and β , two bands of different MW were visible in the area where HP migrates, namely full-length tagged α (31 kD) and full-length tagged β (32 kD).

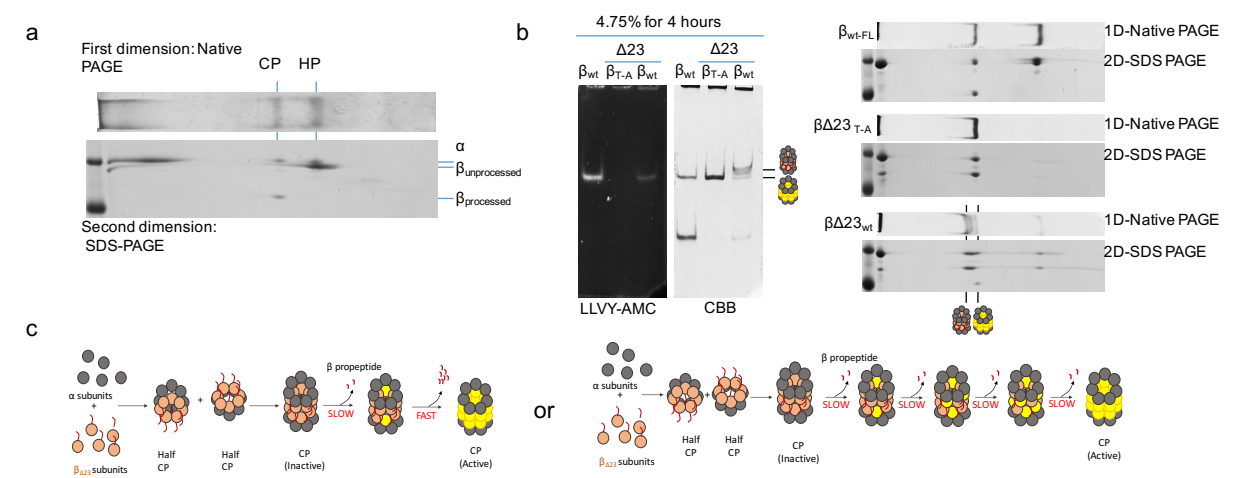
For the CP, we again saw full-length α , but observed a shift in MW of β to ~25 kDa, consistent with the molecular weight of β when the propeptide has been removed. When we used the β_{T10A} mutant in our reconstitutions, both HP and CP were composed of full-length α and β (Fig. A.3). Next, we reconstituted α with $\beta_{\Delta 23}$ and resolved the sample under conditions that we optimized for the best separation of CP bands on the native gel (Fig. 2.4b). To our surprise, the first dimension showed two bands in the CP region of the gel that migrated close together. In-gel LLVY-AMC activity assay showed that only the faster migrating species had proteolytic activity, suggesting we had a population of matured CP and a population of pre-holoenzyme, something that had not been observed in the autocatalysis competent mutant. The 2D gel electrophoresis of these samples shown in the lower right panel of Fig. 2.4b show two distinct spots for β that are distinguishable in migration in vertical as well as horizontal direction. The slower migrating CP species contained a spot of ~28 kDa corresponding to unprocessed β , confirmed by mass spectrometry to be 28,545 Da. The faster active CP species shows a spot of ~24 kDa corresponding to processed β and confirmed by mass spectrometry to be 24,030 Da. α is visible as a single broad spot that encompasses both forms of CP. Thus, truncation of the Region I results in two distinct CP populations, one without proteolytic activity and exclusively composed of β with propeptides, and one with proteolytic active CP where all β had undergone autocatalytic processing.

Cooperativity in maturation

The existence of these two forms of CP is unexpected when considering reports that the maturation of CP from bacteria to archaea and eukaryotes is an autocatalytic process for each active site (Baumeister et al., 1998). If all sites would undergo proteolytic processing independently, then under conditions of slow maturation as we identified here, one would expect to see an array of intermediate species where some β 's are processed while others are not (Fig 2.4c, model on the right). However, we see a sharp delineation with CP populations either containing propeptide or not, suggesting the existence of a cooperative phenomenon during the activation/maturation of CPs (Fig. 2.4c, model on the left). Here, either all active sites transition at the same time to a fast processing mode,

e.g. as a result of simultaneous global conformational changes or, there is a slow initial step that involves the autocatalytic activation of one β subunit. This slow initial step is then followed by a very rapid activation of all other active sites through an unknown mechanism that depends on the presence of one active β subunit.

Figure 2-4 Cooperativity in the activation of CP



(a) α reconstituted with β_{wt} (active) for 1 hour was analyzed on native gel by LLVY-AMC hydrolysis assay followed by separation on a second dimension using SDS-PAGE. This 2D analyses reveals the composition of HPs and CPs in a reconstitution mixture. (b) Native gel analysis of α with β_{wt} , $\beta\Delta 23$ or $\beta\Delta 23_{TtoA}$, reconstituted for 2 hours at 30 degrees (left). The lanes as shown on native gel were then cut and separated on a second dimension to analyze the composition HPs and CPs by CBB staining (right). (c) Model for the mechanism of cooperative activation of core particle (Left). Alternative mechanism for the non-cooperative activation of CP (Right).

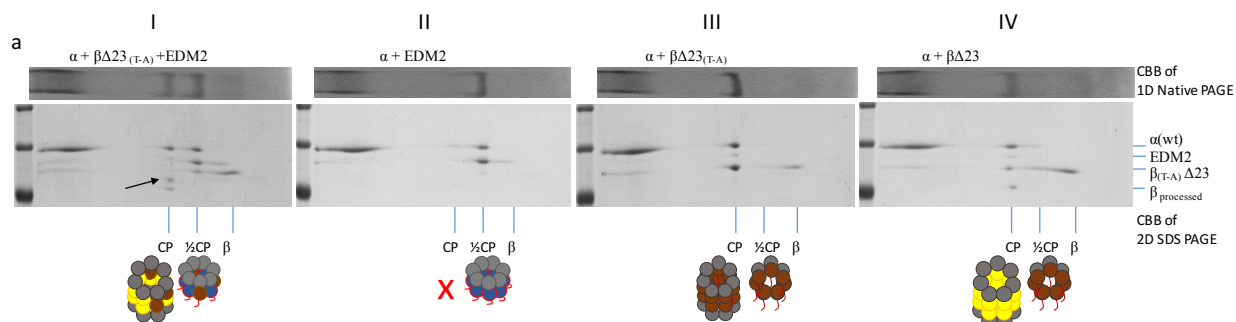
Propeptide trimming by neighboring beta subunits

If a potential mechanism for cooperation involves an initial maturation of one β subunit, we could envision the initial active site trimming of neighboring propeptides prior to additional autocatalysis. This could then remove the constraint responsible for slow autocatalysis. While the N-terminal processing of the precursor β polypeptide is known to be strictly autocatalytic (Chen & Hochstrasser, 1996), it has been shown in eukaryotes that β subunits can cleave neighboring propeptides. This must occur in

eukaryotes, since five β subunits have propeptides, but only three of these β 's have functional active sites (Chen & Hochstrasser, 1996). The close proximity of propeptides to their neighboring active sites (Fig 2.7) and the high concentration of propeptides inside the CP, suggests that it is possible for bacterial β subunits to also cleave their neighboring propeptides. To test cross-processing, we mixed the short loop mutant EDM2 together with an inactive form of beta ($\beta\Delta23_{T-A}$) in a 1:1 molar ratio to get a total of 4 μ M of β subunits and reconstituted this mixture with 4 μ M of α (Fig 2.5a, panel I). We used EDM2 because it failed to form CP when reconstituted with α for 2 hours (Fig 2.5a, Panel II). 2D analysis of the HP thus formed by α and EDM2, showed the presence of two polypeptides: one of MW consistent with α and one of the MW of unprocessed β_{EDM2} . We used $\beta\Delta23_{TtoA}$ because the threonine to alanine substitution makes this subunit inactive and the $\Delta23$ facilitates visualization on SDS-PAGE (Fig 2.5a, panel III) (full length β_{T-A} has molecular weight close to that of α). 2D analysis of CP formed by α and $\beta\Delta23_{TtoA}$ comprised 2 bands neither of which corresponded to a processed β subunit in CP. 2D gel analysis of $\beta\Delta23_{wt}$ reconstituted with 4 μ M of α served as the positive control showing the composition of active CPs and fully processed propeptides (Fig 2.5a, panel IV). As seen in α Fig 2.5a, panel I, mixing the two β mutants (EDM2 and $\beta\Delta23_{TtoA}$), results in CPs containing heterogeneous beta rings comprising both EDM2 and $\beta\Delta23_{TtoA}$. If $\beta\Delta23_{TtoA}$ gets processed, as can be determined by a reduced MW on SDS-PAGE, it must have resulted from processing by a neighboring EDM2 that had undergone autocatalytic processing already. Interestingly, panel I, showed an active CP composed of α , completely processed β , and a novel band which we refer to as trimmed β (Fig 2.5a, arrow). Since EDM2 is a catalytically active mutant and is capable of auto catalytically cleaving its propeptide, these partially processed subunits must represent $\beta\Delta23_{TtoA}$ subunits that have been processed by the EDM2 mutants present in the CP, considering that this novel band remained present even with extended incubation. Likely, the $\beta\Delta23_{TtoA}$ flexible loop would, on occasion, move into the active site of EDM2 resulting in cleavage of the $\beta\Delta23_{TtoA}$ propeptide. However, the neighboring site would be unable to remove the complete propeptide, hence the higher MW band as compared to fully processed β subunits. This processing of $\beta\Delta23_{TtoA}$ by EDM2 shows that a CP activation mechanism can involve cross

processing by neighboring β subunits, providing a potential mechanism for the cooperativity we observed in activation.

Figure 2-5 Active beta subunits can cleave the propeptide of a neighboring beta subunit during CP activation process.



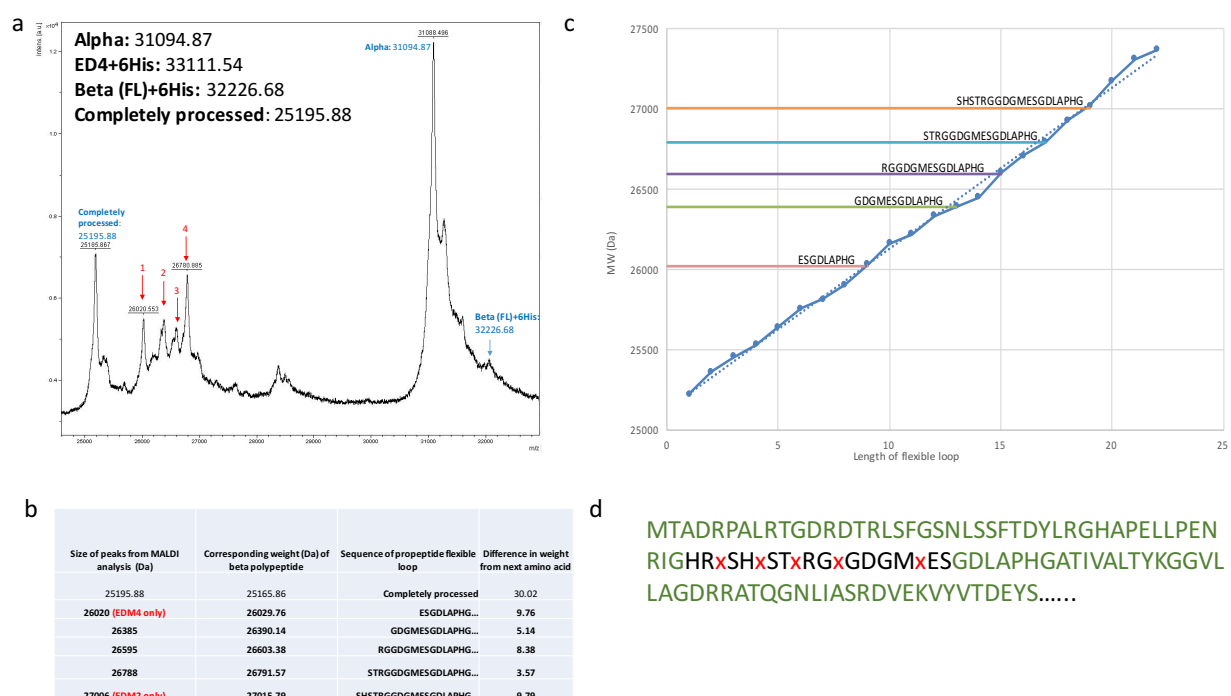
(a) 2D analysis α reconstituted with an equimolar mixture of β mutants as indicated in panel I, II, III and IV. The arrowhead in panel I is pointing at the partially processed band, the size of which is bigger than that fastest migrating-completely processed β band and smaller than any of the β forms used in the assay. (Note: $\beta\Delta23_{TtoA}$ was used for better resolution on SDS-PAGE since full-length α and β_{TtoA} have very little difference in their molecular weight.)

Analysis of the partially processed band formed during CP activation

To accurately determine the exact site where cross β cleavage occurs we estimated the different theoretical molecular weights of the partially processed band and deduced the actual mass of proteins present in the reconstituted samples using MALDI-TOF. To do this, we reconstituted α with an equimolar mixture of EDM2 and $\beta\Delta23_{TtoA}$ before analyzing the samples by MALDI-TOF (Fig 2.6.a). As expected we obtained peaks corresponding to molecular weight of the subunits used for reconstitution and a strong peak corresponding to the molecular weight of β with completely processed propeptide (25,195.88 Da). Instead of obtaining a single peak for the partially processed band, we saw four peaks separated by a molecular weight difference of approximately two amino acids each. Based on the molecular weight of these peaks, we deduced the N-terminal sequence of these partially processed β subunits (Fig 2.6.b). We repeated the reconstitution using EDM4 and β_{TtoA} , and obtained three of the same masses. All cleavage we observed happened in the flexible linker region III (Fig 2.6.c, d). To

understand the propeptide cleavage by neighboring active sites, we also calculated the distance between the active site Thr within beta subunits of a HP and across beta subunits present in two different HPs (Fig 2.7a and 7b). The distances between active site threonine residues within a beta ring are comparable to the distances between active site threonine residues present across two different HPs. This is interesting as it suggests that cross processing of a propeptide between two different beta subunits can also occur across two adjoining HPs.

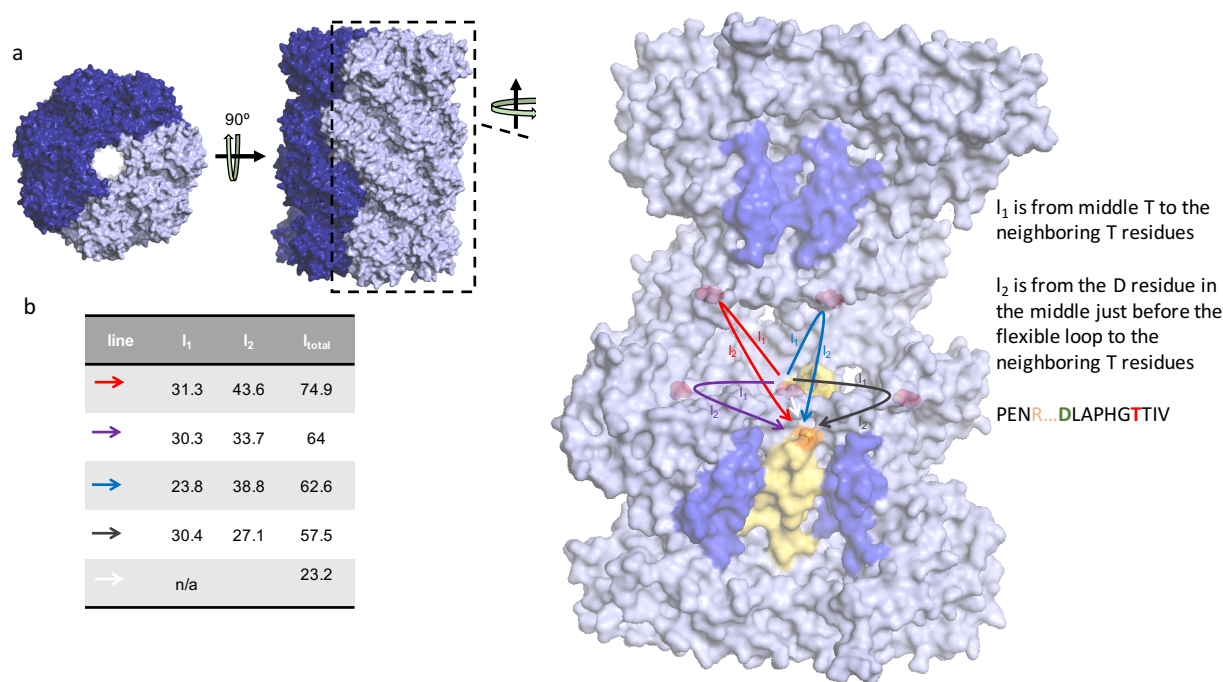
Figure 2-6 Analysis of the partially processed band formed during CP activation



(a) MALDI analysis of the sample obtained by reconstituting 4uM of α with an equimolar mixture containing 2uM of EDM4 and 2uM of β_{ToA} , to estimate the size of partially processed band (pointed by an arrowhead in fig 2.5a). Three out of the four peaks marked by arrowhead here were also obtained upon analysis of reconstitution mixture of α with EDM2 and $\beta_{\Delta 23(T-A)}$ (data not shown). (b) Analysis of MALDI results indicated the existence of 4 major peaks in the molecular weight range between completely processed and un-cleaved β (as seen in (a)). Peaks unique to the reconstitution mixture consisting of either ED4 or ED2 are indicated in the table. By comparing the molecular weight of these peaks with the estimated molecular weight of β polypeptide truncated at different amino acid residues as shown in the figure we estimated the probable sequence of partially processed β polypeptide thus generated. (c) Graphical representation of molecular weight v/s the length of flexible loop ranging from completely processed flexible loop to

varied length partially processed flexible loops. This graph was used to determine the probable sequence of the peaks obtained from MALDI, as indicated on the graph. (d) Partial sequence of β_{ToA} polypeptide with crosses to indicate the cleavage sites in flexible loop that can be accessed by neighboring active site.

Figure 2-7 Calculation of distance between active sites in assembled CP.



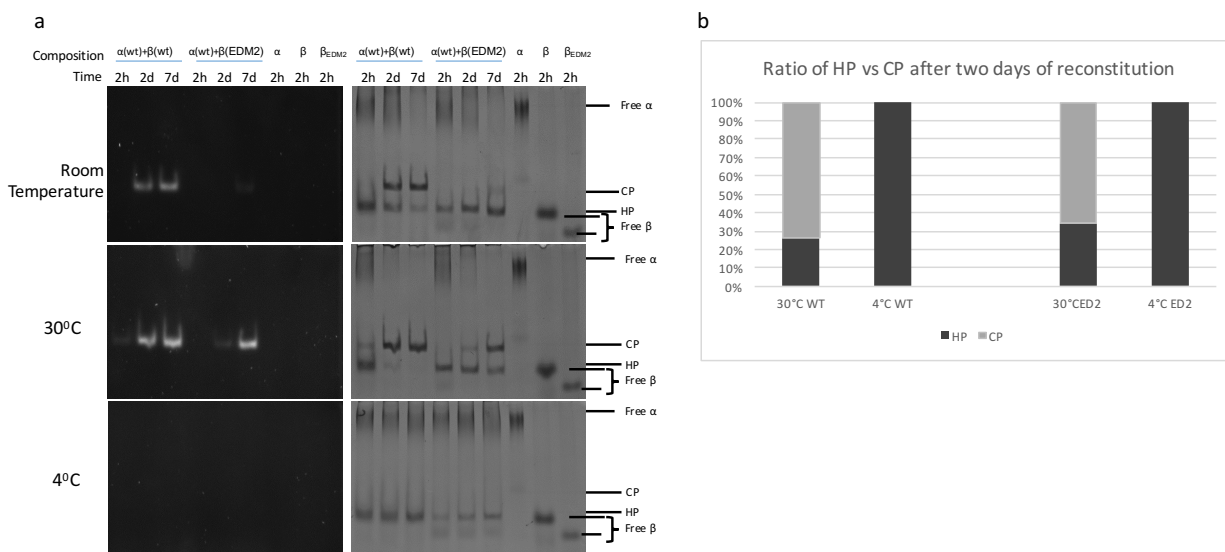
(a) Cross view and Side view of R.e. CP showing the distances of the CA of threonine active sites in blue spheres. (b) Active site (CA) distances within HP, total 7 chains or subunits in the β ring. (c) I_1 is from middle T to the neighboring T residues and I_2 is from the D residue in the middle just before the flexible loop to the neighboring T residues.

Propeptide cleavage across HPs

To be able to test if β can cleave a neighboring propeptide across the dimer interface, we first had to develop an assay where we could form stable HPs and control their dimerization process (Fig 2.8a). We tested the reconstitution of α with either β or EDM2 at different temperatures (30 degrees, room temperature (RT) and 4 degrees) over different time points. Native-PAGE analysis showed that α , β or EDM2 alone do not form HP or CP. As seen in Fig 2.8, free β and EDM2 subunits migrate faster than HP. Upon

reconstitution of α and β at 30 degrees, we observed HPs and active CPs as early as 2-hours followed by a progressive increase in active CP and nearly complete absence of HPs after 2 days. However, EDM2 has a comparatively slower dimerization rate (Fig. 2.2c, lane 2), the reconstitution of α with EDM2 at 30 degrees showed only HP and no CP at 2-hours, with active CPs forming after 2 days. The reconstitution at room temperature showed similar but slower dimerization rates. Interestingly, the reconstitution done at 4 degrees showed no dimerization even after an incubation of seven days (native gels quantified in fig 2.8b). Therefore, we carried out reconstitutions at low temperatures (4 degrees) to form HPs containing specific β mutants and used these HPs to address if cross processing of beta propeptides can occur across two different HPs (Fig.2.9a).

Figure 2-8 Assembly of proteasomes can be regulated by reconstitution at different temperatures.

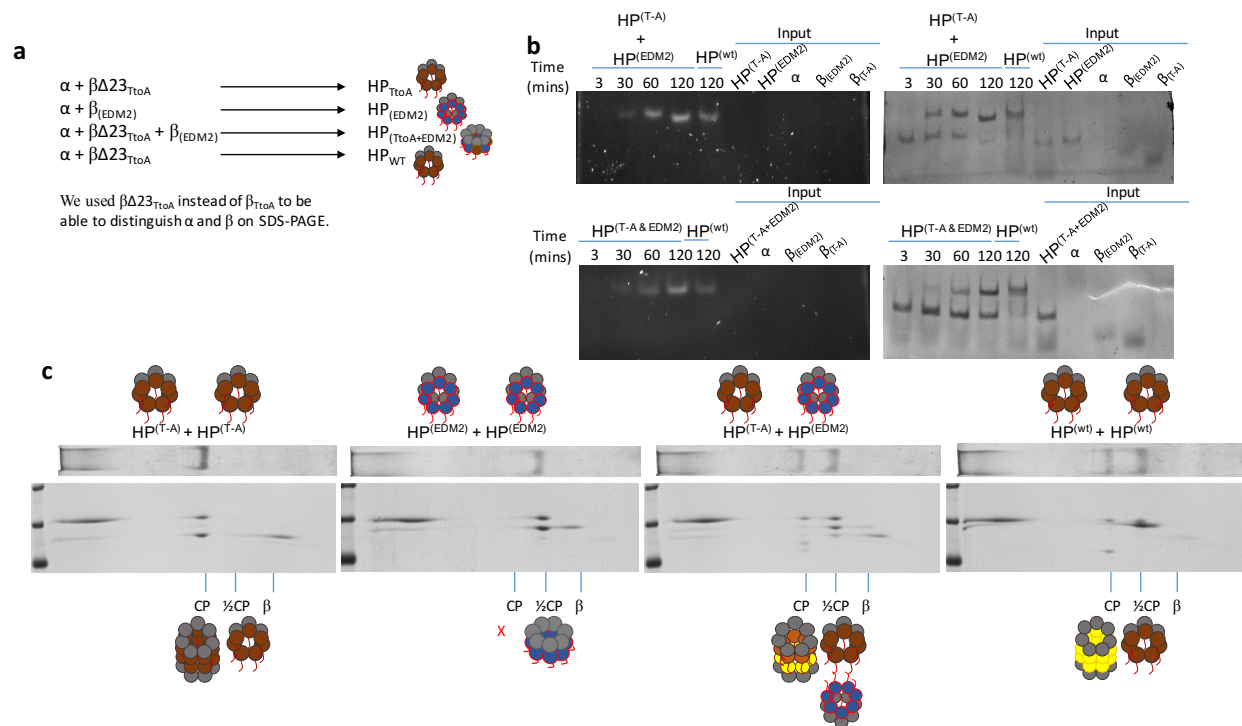


(a) 4 μ M of each subunit were allowed to assemble at indicated temperatures over the course of 7 days. The assembly reaction was then analyzed via Native PAGE activity assay followed by staining with CBB. (b) Quantification of HP vs CPs at 30 degrees v/s 4 degrees. The concentrations of HP (black) and CP (grey) were determined from quantitative analysis of native gel bands using GeneTools [Version 4.03.05.0] from SynGene.

To study the activation of beta propeptides across two HPs, we formed HPs by reconstituting α with different beta subunits at 4 degrees (Fig 2.9a). HP_{EDM2} (HP containing

α and EDM2) was used as it fails to dimerize with another HP_{EDM2}. HP _{Δ 23TtoA} (HP containing α and $\beta\Delta$ 23_{TtoA}) was used as it fails to form active CPs upon dimerization with another HP _{Δ 23TtoA}. Therefore, any active CP formed upon reconstitution of equimolar amounts of HP_{EDM2} with HP _{Δ 23TtoA} must have come from CP with one of each HP_{EDM2} and HP _{Δ 23TtoA}. The reconstitution of HPs at 30 degrees was followed for two hours and showed increased peptidase activity over time (Fig 2.9b). We saw similar results when we dimerized heterogeneous HPs formed by mixing 4 μ M of α with 4 μ M each of EDM2 and $\beta\Delta$ 23_{TtoA} (HP _{Δ 23TtoA+EDM2}) (Fig 2.9c). 2D analysis of the CP formed upon reconstitution of HP_{EDM2} with HP _{Δ 23TtoA} showed the presence of the partially processed β form as we had seen before (Fig 2.5a). MALDI-TOF analyses confirmed the partially processed β of similar MW as previously. In all, our data show that β subunits can be processed across HPs.

Figure 2-9 Active beta subunits can cleave the propeptide of a beta subunit present across dimerizing HP during CP activation process.



Schematic representation of the HPs formed by reconstituting α and respective β subunits at 4 degrees. (Note: The incubation was done overnight to ensure that most of the subunits were

incorporated into HPs and very little no free subunits interfere the reconstitution of HPs). HP formed by only one variant of β is referred as homogenous HP (e.g. $HP^{(T-A)}$), whereas the HPs consisting of two different variant of β is referred as heterogeneous HP (e.g. $HP^{(T-A \& EDM2)}$) (a) The dimerization of HPs was studied by using HPs as reactants in reconstitution over a 2-hour time-course at 30 degrees. The reaction mixtures after the indicated time of incubation were assayed for in gel hydrolytic activity using LLVY and subsequently stained with CBB stain. (b) Two dimensional analysis of a 2-hour reconstitution mixture consisting of indicated HPs was done by first separating on Native gel followed by SDS-PAGE and are stained with CBB.

Discussion

The assembly of CP starts with the formation of HPs. This is followed by two key events, the dimerization of HPs and the processing of propeptides to form active CP. In yeast the dimerization is controlled both positively and negatively by some of the propeptides as well as specific assembly chaperones. Bacteria do not have the same assembly chaperones, but we report here that a flexible loop within the propeptide of *R.e.* β subunit also has a major impact on dimerization. Propeptide processing is not crucial for dimerization, as CP has been formed with mutants incapable of autocatalysis, however, the dimerization always coincides with autocatalytic processing in bacteria when β subunits are competent. Despite substantial research, to our knowledge, no pre-holoenzyme has been observed with wildtype or mutant beta subunits that are capable of autocatalysis. Here, we identified mutants that show rapid dimerization and delayed autocatalytic processing, showing for the first time that these two steps are distinct. We also show that dimerization by itself is not the trigger for autocatalysis. Our data indicate there is a cooperative process that coordinates autocatalysis, potentially involving propeptide trimming of neighboring β subunits by an initially autocatalytically activated β subunit.

Effect on HP dimerization

Here, we have shown that region III of the *R.e.* β -propeptide can negatively regulate the dimerization of HPs. This is a property strikingly similar to an important role that has been demonstrated for eukaryotic assembly chaperones like Ump1 (Ramos et al., 1998), suggesting that in evolution some bacterial propeptide functions have been retained in the eukaryotic chaperones. While some bacterial propeptides have been reported to allow faster rates of dimerization when added in trans to the β -subunit (Zühl et al., 1997), other propeptides are known to have an inhibitory effect on the assembly of proteasomes in other organisms, for example *Mtb* (Li et al., 2010). Our MD simulation data suggests that the residues in region III provide for a highly flexible loop that extends out of the HPs on the dimerization interface. These properties can explain the inability to trace these residues, Gly(-7) to Ile(-23), in crystal structure (Kwon et al., 2004).

Manipulating the length of this loop dramatically reduced the HP dimerization rates when tested *in vitro*, while there was no apparent effect on HP formation. Little to no active CP was detected in most of these mutants even after overnight incubation. This suggests a conservation of optimal size or charge on the flexible disordered loop to allow for proper repositioning of the propeptides during assembly. However, EDM3 was an exception and allowed dimerization of the HPs slower than β_{wt} . A possible explanation for this could be the effect of net charge on the flexible loop in EDM3 which allowed for efficient HP dimerization. The reconstitution experiments done using a mixture of EDM mutants with β_{TtoA} showed that the mutants with shorter flexible loops were fundamentally different from those with extended flexible loops. The smaller flexible loop mutant EDM2, exhibited a severely reduced ability to dimerize by itself, but assembled efficiently when present with β_{TtoA} , that contains a normal length flexible loop. This might indicate a role for specific flexible loop interactions in bringing the dimerization interfaces together, while there is little to no inhibitory effect on activation by the mutant. On the other hand, the extended flexible loops of EDM4 and EDM5, showed little dimerization when mixed in a 1:1 ratio with β_{TtoA} and showed improved CP formation with smaller amounts of the mutant present. Indeed, quantification of the relative amounts of HP versus CP in some of these assays showed that the stalled HP was enriched with the EDM mutants. Thus the extended lengths of the flexible loops seem to inhibit dimerization, likely due to extending out from the HP at the dimerization surface. Interestingly, some recent data suggest the eukaryotic propeptide can also extend out from the HP structure as the yeast $\beta 5$ propeptide can be crosslinked to $\beta 4$ lysine 28, which faces outside the HP. These results thus strengthen our model of conservation of the length of flexible loop in β -subunits and the function of beta propeptides in tuning dimerization.

Competent pre-holocomplexes

Studies in *R.e.* mutants have suggested a mechanism where the assembled proteasomes mature into active proteasomes following a switch mediated by the positioning of S2-S3 loops of β subunits (Witt et al., 2006). Here, the interactions between H3 and H4 helices of the dimerizing HPs drive the positioning of the S2-S3 loops to act as the activation switch by allowing the cleavage of propeptides from beta subunits. When

we introduced N-terminal truncations in the β -subunit propeptide region I, we unexpectedly observed a slower maturation of CP that cannot be readily explained based on our current understanding CP assembly. These mutants allowed us to capture, for the first time, the pre-holoproteasome complex containing β -subunits that are fully competent for autocatalysis. Early studies postulated the autocatalytic cleavage of β to be a rate limiting step (Zühl et al., 1997), which would imply that the pre-holoproteasome is a relatively abundant intermediate. However, this intermediate has always remained elusive (Sharon et al., 2007) and mutations that inhibited autocatalytic processing also interfered with the formation of full CP (Witt et al., 2006), which led to a proposed assembly-dependent activation model. Previous propeptide containing CP structures have all been derived from the reconstitution of α and catalytically dead forms of β -subunits (T1A or K33A) (Kwon et al., 2004; Witt et al., 2006). These forms of CP migrate on native gel similar to matured wildtype CP. However, our competent pre-holoproteasome complex shows a retarded migration on native gel compared to these structures, suggesting it is a distinct form (see Fig.4b) different from previously observed and crystalized structures. These results also highlight a positive role for region I in stimulating the autocatalytic processing of β -propeptide. The molecular mechanism responsible for this is not directly obvious. One possibility is that the mass from region I influences the positioning of the S2-S3 loops. However, based on the crystal structure, where region II crystalizes, region I appears to extend in the direction away from the S2-S3 loop region and towards the gate. A similar direction has been observed for the $\beta 5$ propeptide in yeast, which can be crosslinked to $\alpha 6$, and thus this propeptide seems to be oriented towards the α ring as well. Furthermore, the $\beta 5$ N-terminal propeptide deletion also caused maturation defects (Li et al., 2015). Considering the orientation of this propeptide region, any mechanism that allows the N-terminal portion of the propeptide to stimulate maturation would involve long distance allosteric changes. Long distance allosteric pathways have been proposed for cylindrical proteases like CP's (Shi & Kay, 2013). Alternatively, we could envision the extra mass from the propeptide in the tight cavity of the CP might destabilize propeptide structures that have formed in HP thereby facilitating the proper orientation of the propeptide for autocatalytic processing (Huber et al., 2016).

Cooperativity

The presence of two different populations of CPs (active and inactive forms) suggests the presence of a cooperative phenomenon in the maturation of CP that leads to rapid activation of all β 's in assembled CP. Our mutants show a slow autocatalytic activation and if each site underwent activation independently of others, intermediate forms of CP, e.g. with 50% processed and 50% unprocessed active sites, would be expected (see model Fig. 4c). The absence of any such intermediates, suggests a specific mechanism is responsible for the (almost) simultaneous processing of the propeptides by all β subunits. A possible underlying mechanism that could explain these observations is a two-step process. Here, an initial slow step involves the autocatalytic cleavage of a propeptide from one of the seven β subunits in the ring. This triggers a second step, where the newly created active site initiates hydrolysis of the propeptides of neighboring β subunits. This hydrolysis cannot be at the T_1 site, but would be upstream in the propeptide. This is consistent with our observation that one β can cut the flexible loop of a neighboring β propeptide. Such cross processing of propeptides could stimulate autocatalytic cleavage by that neighboring β if it removed the physical constraints that limit the proper positioning required for autocatalysis of the propeptides (Huber et al., 2016), thus triggering a rapid phase of autocatalysis. This model is consistent with the observation that propeptides provided in trans form mature CP faster (Zühl et al., 1997). In our attempt to map the exact location on the propeptide that was cleaved by a neighboring active site, we observed a few unique cleavage sites in the flexible loop that were separated by 1 or 2 amino acids. Considering that peptides need to be positioned in the active site, with surrounding amino acids contributing to the binding, could indicate two things. First, the different lengths reflect the position of the propeptide from different neighbors. If each flexible loop is positioned differently for the different neighbors it can cause somewhat different lengths or ways, the loop can engage with each neighboring active site. Considering there are four close neighboring sites, this fits well with the 3 to 4 different lengths that were observed (Figure 2.7b). A second possibility is that the different cleavage sites reflect specificity of the active site for certain sequences. Once these preferred sequences are cleaved, the remaining loop fragment is too short or unfavorable

to productively engage with the active site and be further trimmed. For human and yeast proteasomes it has been well established that there are preferred sequences that are recognized by the active sites (Dick et al., 1998; Emmerich et al., 2000; Nussbaum et al., 1998)

Evolutionary perspective

The short length propeptide found in archaea and *Mycobacterium tuberculosis* are not required during assembly. Nevertheless, the longer propeptides found in other bacterial species like *R.e.* used in this paper have been proven indispensable for assembly. Similarly, in eukaryotes, five of the β subunits retain their propeptides. One proposed function of the propeptides is to protect the active site threonine from N-terminal acetylation; however, this does not explain two of the eukaryotic propeptides, as they are found on β subunits that have lost a functional active site. Clearly, the presence of these propeptides in eukaryotes has been conserved in evolution, like the long propeptides found across different bacterial species used for sequence alignment in our study. Here we report a functional conservation between bacterial and eukaryotic CP assembly in regulation of HP dimerization. Our observation that the propeptides influence the transition from pre-holoproteasome to active holoproteasomes is intriguing. This transition is likely to involve a conformational change of the CP, as both species can be separated on native gel. In yeast, there is also a conformational change that triggers maturation and the exchange of immature CP binding from Pba1/2 to RP (Kock et al., 2015; Prashant S Wani et al., 2015). How this switch and maturation are connected remains poorly understood and has been proposed to involve the degradation of Ump1. Our data suggest another potential factor, as are retained might be because they play a role in this conformational switch.

Author contributions

A.S., A.V., F.K. and J.R. performed the experiments and constructed the bacterial plasmids. P.I. and E.J.D., A.B. performed the bioinformatics analysis and molecular dynamics simulations and calculations. Studies were conceived by A.S., E.J.D., and J.R. Manuscript was written by A.S. and J.R. with inputs from all authors.

Chapter 3 - Nucleotide dependent stabilization of RP assembly chaperones on 26S proteasomes by Ecm29

Anjana Suppahia, Faith Mi Ge Kim, Alexander Vontz, Jeroen Roelofs

*Molecular, Cellular, and Developmental Biology Program, Division of Biology, Kansas State University,
338 Ackert Hall, Manhattan, Kansas 66506 USA*

Abstract

Proteasomes in eukaryotes are large multisubunit complexes that consists of 66 subunits assembled to form a 19S regulatory particle (RP) and a 20S core particle (CP). The assembly of RP and CP in all eukaryotes requires the assistance of five unique chaperones. Besides assisting the assembly, these chaperones also prevent pre-mature interactions between the RP and CP sub complexes. For example, RP chaperones Nas2, Hsm3, Rpn14, and Nas6 are known to antagonize the interaction between RP base and CP prior to the completion of RP assembly. Ecm29 is a proteasome interacting protein that possesses multifaceted functions in regulating the activity and stability of the proteasomes. It is described as a proteasome quality control factor that binds faulty proteasomes and causes inhibition of the proteasomal ATPase activity. Here, we describe a novel role of Ecm29 in stabilizing the RP chaperones on 26S proteasomes. To determine if the stabilization of chaperones is dependent on the nucleotide state of specific ATPases, we introduced mutations in the Walker A and Walker B motifs to affect the nucleotide binding and nucleotide hydrolysis respectively, in Rpt1, Rpt3 and Rpt5. Our studies demonstrate that Ecm29 binds and traps the RP base in ATP-bound state, which in turn promotes the stabilization of the RP-CP complexes. Ecm29 is also known to induce a closed confirmation of CP and has also been suggested to cause the dissociation of RP-CP interactions under oxidative stress conditions. Our results suggest a novel role of Ecm29 in stabilizing the proteasomes by preventing the dissociation of RP-CP complexes under oxidative stress conditions.

Introduction

Proteasomes are macromolecular proteases found ubiquitously in all eukaryotic cells. They degrade a vast array of substrates including misfolded proteins, aggregated proteins and several other short lived regulatory proteins that must be degraded in a highly regulated manner to maintain the fast changing proteome of a cell. Proteasomes belong to the AAA-ATPase family of proteases and hydrolyze ATP to generate energy required for unfolding and degrading the substrate proteins. The 26S proteasomes are 2.5 MDa complexes that are composed of two sub-complexes, the 19S regulatory particle (RP) and the 20S core particle (CP). The CP consists of 28 subunits arranged in four hetero-heptameric rings forming a barrel shaped with two outer alpha rings and two inner beta rings. The catalytic activities of proteasome, caspase-like, trypsin-like and chymotrypsin-like, are encoded by β 1, β 2, and β 5 subunits, respectively (Arendt & Hochstrasser, 1997; Heinemeyer et al., 1997). The CP is capped on one or both ends by the RP which opens the CP gates and allows the translocation of substrates into the catalytic chamber. The RP first receives, deubiquitinates and unfolds the substrate before being translocated into the CP. It consists of 19 subunits arranged into two RP sub-complexes called the lid and the base. The RP base contains six AAA-ATPase subunits (Rpt1-6) and three non ATPases (Rpn1, Rpn2, Rpn13) that altogether require five unique chaperones (Nas2, Hsm3, Nas6, Rpn14, Adc17) for proper assembly. The lid however, is known to assemble independently. The ATPase subunits of RP-base undergo extensive inter-subunit interactions and utilize energy generated by the hydrolysis of ATP to unfold the proteasomal substrates (Beckwith et al., 2013). The C-terminal tails of Rpt3, Rpt5 and Rpt2 dock into pockets located on the surface of the alpha rings and induce an open conformation of the gate of CP.

The nucleotide state of the ATPase subunits is known to regulate their interaction with other proteins including RP chaperones and the CP (Rubin et al., 1998). For example, Nas6 chaperone, is known to regulate the interaction of RP base with RP-lid and with CP based on the nucleotide state of the base ATPases (Li et al., 2016). Mutations in the ATP binding or processing capabilities of these ATPases have been reported to severely affect the proteolytic capability of the proteasomes (Kim et al., 2013;

Rubin et al., 1998). It is therefore important to understand the ways nucleotide availability can regulate the activity of the proteasomes, especially under physiological stress conditions.

The ubiquitin proteasome system (UPS) is a major pathway for the degradation of ubiquitinated proteins, yet several stress conditions require reduced proteasomal activity for cell survival. Several factors like Ecm29 and Ubp6 are known to regulate proteasomes by inhibiting the proteolytic activity under physiological stress conditions (Bashore et al., 2016; Lee et al., 2011; Lehmann et al., 2010). Ecm29 has also been reported to disassemble proteasomes under oxidative stress conditions to promote cell survival (Wang et al., 2017). Contrary to this, association of Ecm29 has been shown to stabilize faulty proteasomes by inhibiting the ATPase activity in vivo (De La Mota-peynado et al., 2013). Based on the effects caused by association of Ecm29, it has been speculated to arrest the proteasomes in S1 ground energy configuration, as defined by cryoEM analysis (Finley et al., 2017). On the other hand, deletion of Ecm29 has also been shown beneficial to promote cell survival under conditions that affect proteasome assembly (Park et al., 2011). The precise mechanism by which Ecm29 regulates the activity of proteasomes under cellular stress conditions, still remains to be understood.

In this paper, using a previously described two dimensional (2D) gel approach (Roelofs et al., 2018), we identified novel RP-CP-Ecm29 complexes that were associated with Nas6 and Hsm3 chaperones in *ump1Δ* mutants. Our studies showed the recruitment of chaperones on RP-CP complexes to be dependent on Ecm29. Since nucleotide state of the RP ATPases is known to influence the interaction with various factors including RP chaperones, CP and Ecm29, we investigated the role of nucleotide state of the RP-base in influencing the stability of this novel complex identified. To do this, we used an array of mutants strains each carrying a single mutation in one of the RP ATPases. We introduced mutations that either affected their binding with ATP (Walker A motif mutation) thus generating an ATP-free form of the ATPase or a mutation that affects their ability to hydrolyze ATP (Walker B motif) thus generating an ATP-bound state. We used 2D gel analysis to study the effects of these mutants on the interaction of proteasomes with RP

chaperones in vivo. We also used *in vitro* reconstitution approach to further enhance our understanding of the role of Ecm29 in stabilizing these novel complexes.

Experimental Procedures

Yeast Strains and Plasmids

Information about all the strains used in this study are given in Table 3.1. The primers used for the generation of the strains are also listed in table 3.2.

Rpt1, Rpt3 and Rpt5 mutant strains- We used CEN plasmids to express these mutant forms in the yeast strain. Since the genes encoding RP subunits are essential for the viability of the cells, we made strains that express wildtype RP subunit from a CEN plasmid (sJR1018, sJR1025 and sJR1028) and are deleted for the endogenous copy of the respective RPT gene. These strains were then transformed with plasmids carrying the mutations in Walker-A and Walker-B domains or a wildtype control wildtype form a CEN plasmid with a selection cassette different from the original set (see table 1 genotype for all “pJR” plasmid numbers). The plasmids were swapped by counter-selection against the original plasmids on the on 5-FOA plates.

Table 3-1 Strains

Strain	Genotype	Figure
sJR1018	MAT α ecm29::pGPD-His6-Ecm29 (ClonNAT) rpn11::rpn11-TEV-ProA (HIS3) rpt1::HIS3 pEL36 (ProA-TEV-Rpt1 TRP Cen plasmid)	
sJR1025	ecm29::pGPD-ECM29 (G418) rpn11:RPN11-Tev-ProA(HIS3) rpt3::HIS3 Dp75(Rpt3K129R)	
sJR1028	ecm29::pGPD-ECM29 (G418) rpn11:RPN11-Tev-ProA(HIS3) rpt5::HIS3 Sp12(Rpt5K228R)	
sJR1029	MAT α ecm29::Pgpd-His6-Ecm29 (ClonNAT) rpn11::rpn11-TEV-ProA (HIS3) rpt1::HIS3 pJR751(Rpt1 URA Cen plasmid)	4a; 5 and S3
sJR1050	MAT α ecm29::Pgpd-His6-Ecm29 (ClonNAT) rpn11::rpn11-TEV-ProA (HIS3) rpt1::HIS3 pJR752 (Rpt1 TRP Cen plasmid)	4a; 5 and S3
sJR1030	MAT α ecm29::Pgpd-His6-Ecm29 (ClonNAT) rpn11::rpn11-TEV-ProA (HIS3) rpt1::HIS3 pJR754(Rpt1KtoS URA Cen plasmid)	4a; 5 and S3
sJR1048	MAT α ecm29::Pgpd-ECM29 (G418) rpn11:RPN11-Tev-ProA(HIS3) rpt3::HIS3 pJR768 (Rpt3wt, URA, CEN)	4b; 5 and S3
sJR1049	MAT α ecm29::Pgpd-ECM29 (G418) rpn11:RPN11-Tev-ProA(HIS3) rpt3::HIS3 pJR768 (Rpt3EtoQ, URA, CEN)	4b; 5 and S3

sJR1065	ecm29::Pgpd-ECM29 (G418) rpn11::RPN11-Tev-ProA(HIS3) rpt3::HIS3 pJR768 (Rpt3 KtoS, TRP, CEN)	4b; 5 and S3
sJR1056	ecm29::Pgpd-ECM29 (G418) rpn11::RPN11-Tev-ProA(HIS3) rpt5::HIS3 pJR753(Rpt5 wt, URA CEN)	4c; 5 and S3
sJR1083	ecm29::Pgpd-ECM29 (G418) rpn11::RPN11-Tev-ProA(HIS3) rpt5::HIS3 pJR793(Rpt5EtoQ ; URA CEN)	4c; 5 and S3
sJR1068	ecm29::Pgpd-ECM29 (G418) rpn11::RPN11-Tev-ProA(HIS3) rpt5::HIS3 pJR793(Rpt5KtoS ; URA CEN)	4c; 5 and S3
sJR1413	Ump1::HYGRO Ecm29::G418 rpn11::rpn1-GFP (HIS3) in Sub61 (lys2-801 leu2-3, 2-112 ura3-52 his3-Δ 200 trp1-1)	1a, b and S1a, b
sJR1414	Ump1::HYGRO α1::α1-GFP (HIS) in Sub61 (lys2-801 leu2-3, 2-112 ura3- 52 his3-Δ 200 trp1-1)	1a, b and S1a, b
sJR1415	MAT α Ump1::HYGRO rpn11::rpn1-GFP (HIS3) in Sub61	1a, b and S1a, b
sJR944	Ecm29Δ ump1Δ	2
sJR945	Ump1Δ	2
sJR736	MAT A rpn11::RPN11-TEVProA (HIS3) ecm29::(NAT) pGDP-HIS7- ECM29	2, 3 and 6a, b
sDL133	MAT α rpn11::RPN11-TEVProA (HIS3)	2, S2
sJR211	MAT α ecm29::TRP rpn11::RPN11-TEVProA (HIS3)	6a, b
sJR861	MAT α Ump1::HYGRO rpn11::rpn1-GFP (HIS3) in Sub61	7a, b and S4
sJR798	Ecm29::G418 rpn11::rpn1-GFP (HIS3) in Sub61 (lys2-801 leu2-3, 2-112 ura3-52 his3-Δ 200 trp1-1)	7a, b and S4
sJR1136	rpn11::RPN1-GFP (HIS) Ecm29::PADH-G418-ECM29	7a, b and S4
sJR1135	rpn11::RPN1-GFP (HIS) ecm29::PGPD-ClonNAT-ECM29	7a, b and S4
sJR1290	α1::α1-LoxP-3xHA-GFP-TADH1-HphMX-LoxP-3xT7-RFP	8a,b,c
sJR1292	ecm29::ecm29-LoxP-3xHA-GFP-TADH1-HphMX-LoxP-3xT7-RFP	8a,b,c

Table 3-2 Primers

Primer	Genotype	Template	Sequence (5'-3')
pRL465	Rpt1 + Flanks (Amp)	SC gDNA	ggatccACATATACCCGCCATTGTTGCACTATAAGG
pRL466	Rpt1 + Flanks (Amp)	SC gDNA	ggatccAAAAACCTCCAGCATATGCCCTATTAGG
pRL467	Rpt1::Rpt1KtoS (Amp)	pJR685	CTGGTACTGGTTCTACATTATGTGCTCGTGCTGTTG

pRL468	Rpt1::Rpt1KtoS (Amp)	pJR685	GTGGCCCATATAATAAGATACCCTTTGGTG
pRL469	Rpt1::Rpt1EtoQ (Amp)	pJR685	TATTCTTCGATCAAATCGACGCTGTTGGTGGTGC
pRL470	Rpt1::Rpt1EtoQ (Amp)	pJR685	TAATGCAAGCCTTTTTGGTACGAG
pRL471	Rpt3 + Flanks (Amp)	SC gDNA	ggatccTGACGCTCCCCTAGTCTTTGACACC
pRL472	Rpt3 + Flanks (Amp)	SC gDNA	ggatccACACAAATCGAATAGCAAATCTACACTGGGATGAAG
pRL473	Rpt3::Rpt3KtoS (Amp)	pJR686	ACCAGGTACCGGTTCTACGATGCTTGTTAAAGCCGTCGC
pRL474	Rpt3::Rpt3KtoS (Amp)	pJR686	GGACCATATAATAGAACACCCCTAGGAGGGTCAATACCG
pRL475	Rpt3::Rpt3EtoQ (Amp)	pJR686	TTCATAGATCAAGTAGATTCCATTGCCACAAAGCG
pRL476	Rpt3::Rpt3EtoQ (Amp)	pJR686	TATAATGGATGGAGCATTTTCCCTGGCGAG
pRL477	Rpt5 + Flanks (Amp)	SC gDNA	ggatccTAAGCATGCTATATATCAGTAGTATGTATTTTGCAGC
pRL478	Rpt5 + Flanks (Amp)	SC gDNA	ggatccATACTCAAAGCACCCGTAGCCGTCG
pRL479	Rpt5::Rpt5KtoS (Amp)	pJR687	GGTACAGGTTCTACTTTATTAGCTCGTGCTTGCGCG
pRL480	Rpt5::Rpt5KtoS (Amp)	pJR687	CGGAGGCCCGTACATTAGGGC
pRL481	Rpt5::Rpt5EtoQ (Amp)	pJR687	TCATTGACCAATTGGATGCCATTGGTACTAAGAG
pRL482	Rpt3::Rpt5EtoQ (Amp)	pJR687	AAATAATAGTTGGTGCCTTTTCCTTGGCCAG
pRL669	$\alpha 1$:: $\alpha 1$ -LoxP- 3xHA-GFP- TADH1-HphMX- LoxP-3xT7-RFP	pKV016	GAACATCGAAGAAAGGCTAGTAGCAATTGCTGAACAAGAT GGT GGA TCT GGT GGA TCT
pRL671	$\alpha 1$:: $\alpha 1$ -LoxP- 3xHA-GFP-	pKV016	CGCGTGTGATTTACATTATGTTGTGGCAGGAAGTATG TCAGGCGCCGGTGGAGTGGCG

	TADH1-HphMX- LoxP-3xT7-RFP		
pRL677	ecm29::ecm29- LoxP-3xHA-GFP- TADH1-HphMX- LoxP-3xT7-RFP	pKV016	AACGAGCAGAATTGAGTTGGAATTGAAAAATATTGGTCTA GGT GGA TCT GGT GGA TCT
pRL679	ecm29::ecm29- LoxP-3xHA-GFP- TADH1-HphMX- LoxP-3xT7-RFP	pKV016	TAAGTGTCTATTTATCAATATGAAAAAGTTTCGGAGT <u>TCAGGCGCCGGTGGAGTGGCG</u>

Note: SC gDNA= on *Saccharomyces cerevisiae* genomic DNA.

pJR685 was made by integrating blunt cloning vector to the product of pRL465 and pRL466 using SC gDNA. Similarly, pRL471 and pRL472 resulted in pJR686 and pRL477 and pRL478 resulted in pJR687.

pKV016: see reference (Terweij et al., 2013)

Antibodies

The polyclonal antibodies to recognize Ecm29, Hsm3, and Nas6 were generously provided by Dr. Dan Finley (Harvard Medical School, Boston, MA). Pgk1 antibody (Invitrogen, catalog no.459250), Blm10 antibody (Enzo Life Sciences) were purchased. The polyclonal antibody for β 5-propeptide was provided as a gift from the lab of Dr. John Hanna (Harvard Medical School, Boston, MA).

Yeast growth conditions

Yeast cultures were grown in YPD or YPR at 30 degrees for indicated times. YPD medium contains 0.17% yeast nitrogen base, 0.5% $(\text{NH}_4)_2 \text{SO}_4$, 2% dextrose/raffinose. For glucose starvation, cultures grown overnight were used to inoculate fresh YPD/YPR at A_{600} of 0.5 and allowed to grow for 4 hours or until an A_{600} of 1.5 was reached. The cells were collected and washed with sterile H_2O and re-suspended in minimal media lacking glucose. Culture was then indicated at 30 degrees under constant shaking. At indicated time points, cells were collected, centrifuged, and the pellets were re-suspended

in remaining liquid after aspiration and frozen as droplets in liquid nitrogen and stored thereafter at -80 degrees.

Yeast cell lysis by cryo grinding and Native gels and Two-Dimensional (2D) gels using purified proteasomes

Done as previously described (Roelofs et al., 2018).

Imaging

The Native gel, CBB stained gels, western blot images were acquired using G-Box imaging system (Syngene) using GeneSnap software. The GFP and RFP images of native gels were captured by Typhoon 9410 imager. For GFP images excitation 488, 526 SP filter were used and for RFP images excitation 633, 580 SP filters were used.

Fluorescence Microscopy

Yeast cells expressing tagged versions of proteasome subunits were analyzed by growing in the required medium, collecting cells at specific time-points and washing them with sterile water before transferring them on 1% agarose solution solidified (not dried) on slides. The cells are covered with coverslip before capturing images at room temperature on a Nikon Eclipse TE 2000-S microscope at 600X magnification using a Plan Apo X 60/1.40 objective and the 490/528 nm (GFP images) and 633/580 nm (RFP images). The images were collected using Metamorph (Molecular Devices) and processed using Fiji (ImageJ).

Proteasome purification

The proteasomes were purified by including modifications in protocols described previously (Leggett et al., 2005). The yeast strains containing ProA affinity tag on Rpn11 were grown overnight in 1.5 to 3 liter YPD media. The cells were collected and washed with sterile water and re-suspended in lysis buffer (50mM Tris [pH8.0], 5mM MgCl₂, 1mM EDTA, and 1mM ATP). The suspended cells were lysed using French Press at 1200 psi

and the total cell lysates (TLs) were centrifuged at 10,000 g for 30 minutes at 4 degrees to get clear lysates (CLs). The CLs were filtered through cheese cloth and incubated with equilibrated IgG resin at 4 degrees for 1 hour under constant rotation. The IgG resin was collected by centrifugation at 500g for 2minutes at 4 degrees and washed with 50 bed volumes of ice-cold wash buffer (50 mM Tris (pH 7.5), 5 mM MgCl₂, 50 mM NaCl, 1 mM EDTA, 1 mM ATP). The resin was then washed with 20 bed volumes of elution buffer (50 mM Tris (pH 7.5), 5 mM MgCl₂, 1 mM DTT, and 1 mM ATP). The proteasomes were eluted from the IgG resin by incubating with GST-Tev protease at 4 degrees overnight. Next, the eluted sample was incubated with glutathione resin (GoldBio) at 4 degrees for 30 mins under constant rotation, to remove the GST-Tev protease. The purified material was analyzed using native-PAGE, SDS-PAGE and 2D PAGE assay. When nucleotides other than ATP were used, ATP was replaced with 0.5mM ATPyS, or 1mM ADP.

Recombinant chaperone purification

For expression of recombinant proteins, plasmid carrying the gene of interest was transformed into competent Rossetta cells. GST-Hsm3, GST-Nas6, and GST-Rpn15 were expressed and purified from *E. coli* by inducing the overexpression by IPTG at 30 degrees for 3 h. The cells were collected and the pellet was re-suspended in lysis buffer (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 1% Triton X-100, and protease inhibitors). The cells were lysed by French press at 900 psi and cleared by centrifugation. The CLs were filtered through cheese cloth and incubated with glutathione resin at 4 degrees for 1 hour under constant rotation. The resin was then washed with 50 bed volumes of wash buffer (PBS, 0.1% TritonX-100, 0.02% azide). The GST-tagged proteins were eluted by re-suspending the resin in 20 bed volumes of elution buffer (50 mM Tris (pH7.5), 100 mM NaCl, 1 mM EDTA, 0.25 mM DTT) containing 60 mM reduced glutathione. The elution step was subsequently repeated four times with changing concentration of reduced glutathione to 120mM, 250mM, 400mM and 500mM respectively. The elutes containing highest concentration of the protein of interest were pooled and dialyzed to separate reduced glutathione from the purified protein using dialysis buffer (50 mM Tris(pH7.5), 100 mM NaCl, 1 mM EDTA, 0.25 mM DTT).

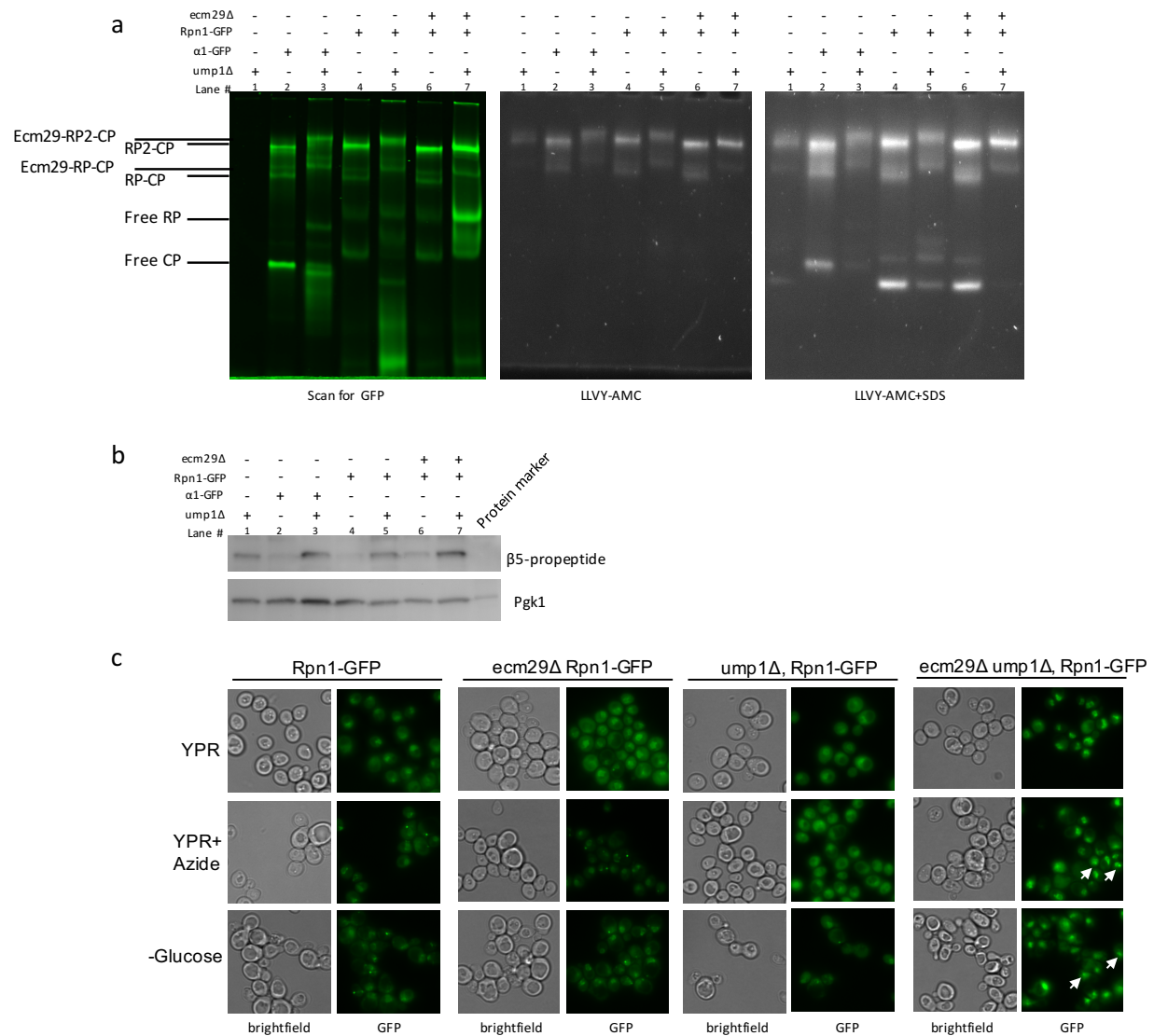
Results

Ecm29 stabilizes interactions of the chaperones Hsm3 and Nas6 with RP-CP complexes.

It is known that the deletion of UMP1, a CP assembly chaperone, causes defects in assembly of RP base and leads to the accumulation of RP base intermediates and free lid (Kusmierczyk et al., 2008). Deletion of ECM29 in the *ump1Δ* strain was later shown to rescue base assembly defects and as a result, cause elevation in the amount of free RP (Park et al., 2011) (Figure 3.1a). These results suggest that the impairment in RP assembly observed in *ump1Δ* mutants is mediated by Ecm29. We analyzed the total lysates of strains where either alpha1 or Rpn1 subunit of the proteasome were tagged with GFP. Consistent with what was shown before, *ump1Δ* mutants showed an overall reduction in the proteasome levels and the amounts of free CP (Figure 3.1a, lane 1, 3, 5, and 7). Furthermore, we saw an increase in the amounts of fast migrating, Rpn1 containing-RP assembly intermediates (lane 5 and 7). The accumulation of these RP intermediates was abolished upon deletion of Ecm29 (lane 7) with a concurrent increase in the amount of free RP.

We also saw an increased association of Blm10 (PA200 in humans) with CP in *ump1Δ* mutants (Figure B.1a). Blm10, like RP, is a proteasome activator and has been shown to regulate CP activity by docking in $\alpha 5$ - $\alpha 6$ pocket on the outer ring of CP (Savulescu & Glickman, 2011). This $\alpha 5$ - $\alpha 6$ pocket is also the binding site for Pba1-Pba2 dimer chaperones that assist CP maturation (Li et al., 2007). Studies have suggested that the degradation of *ump1* chaperone during the maturation of CP, causes conformational changes in alpha-ring that leads to reduction in the affinity of Pba1-Pba2 dimer towards CP (Kock et al., 2015; Wani et al., 2015). Consistent with this model, the *ump1Δ* mutants used in our study also suggest a reduced affinity for Pba1-Pba2 dimer which would increase the availability of $\alpha 5$ - $\alpha 6$ pocket for binding Blm10 and thus provide an explanation for the enrichment of Blm10 complexes in the lysates.

Figure 3-1 Ecm29 dependent rescue of RP base assembly defects.



(a) Strains expressing indicated tagged-GFP proteasome subunits were analyzed on native PAGE to determine the impact of UMP1 and ECM29 deletion on proteasome assembly. Total lysates obtained by cryo grinding were separated on non-denaturing native-PAGE and scanned on a typhoon imager to visualize the distribution of GFP tagged subunits. Next, we used fluorogenic proteasome substrate LLVY-AMC to determine the localization of active proteasome complexes. Addition of 0.02 % SDS to the activity assay allows for the visualization of the proteasomal activity by physically interfering with CP gating. The lysates for cryo grinding were obtained as described previously (Roelofs et al., 2018). (b) Immunoblot of samples loaded in (a) for β5-propeptide shows the accumulation in ump1Δ strains of non-autocatalytically processed β5, the Pgk1 (phosphoglycerate kinase) immunoblot blot served as the loading control. (c)

Indicated strains were visualized by fluorescence microscopy to visualize the localization of Rpn1-GFP tagged subunits in logarithmically growing cells and after 24 hours of treatment with azide and glucose starvation conditions.

Glucose starvation or induction of oxidative stress by adding sodium azide to the growth medium induces granule formation by the proteasomes (Marshall & Vierstra, 2018) (Figure 3.1c). We tested if granule formation under stress conditions was affected in *ump1Δ* mutant, because the *ump1Δ* mutants have defects in assembly of RP base as well as CP. While the wildtype strain and *ecm29Δ* showed granule formation upon carbon starvation and under oxidative stress conditions, *ump1Δ* mutants failed to form granules in the presence of azide and showed severely reduced levels of granules upon glucose starvation (Figure 3.1c). Next, we tested the effect of knocking out ECM29 in *ump1Δ* strain, since it is known to rescue the RP base assembly defects. We expected the *ecm29Δ ump1Δ* double knockout strain to show recovery in the ability to form granules. We indeed observed only a slight increase in the formation of granules in *ecm29Δ ump1Δ* strain (marked by arrows in Figure 3.1c, *ecm29Δ ump1Δ* strain data). Additionally, we observed an increase in the intensity of signal for Rpn1-GFP around the nuclear pore complex in *ecm29Δ ump1Δ* strain as opposed to the more diffused nuclear localization in *ump1Δ* strain. Assembly defects in RP and CP were also observed by analyzing the total cell lysates from α 1-GFP or Rpn1-GFP tagged strains used in Figure 3.1, on Native gels (Figure B.1b). Here, *ecm29Δ ump1Δ* strain showed an increase in the amount of 26S and free RP but not free CP. Suggesting that the partial rescue of granule formation in *ecm29Δ ump1Δ* strain, was due to the rescue of RP assembly and no substantial effect on CP assembly. In sum, we observed defects in granule formation in *ump1Δ* strain which was rescued partially upon deletion of ECM29, supporting our aforementioned idea of Ecm29 dependent regulation of RP assembly in *ump1Δ* strain.

In a wildtype background, pba1-pba2 protects RP and presumably RP-base assembly intermediates from interacting with the CP, which might allow for the proper assembly of both CP and RP before they engage in the next assembly steps. We hypothesized that the mutant in CP assembly, *ump1Δ*, lacks the pba1/pba2 and allows RP base intermediates that have a weak affinity to CP to associate and form unstable base intermediates-CP complexes. Ecm29, a proteasome quality control factor, could

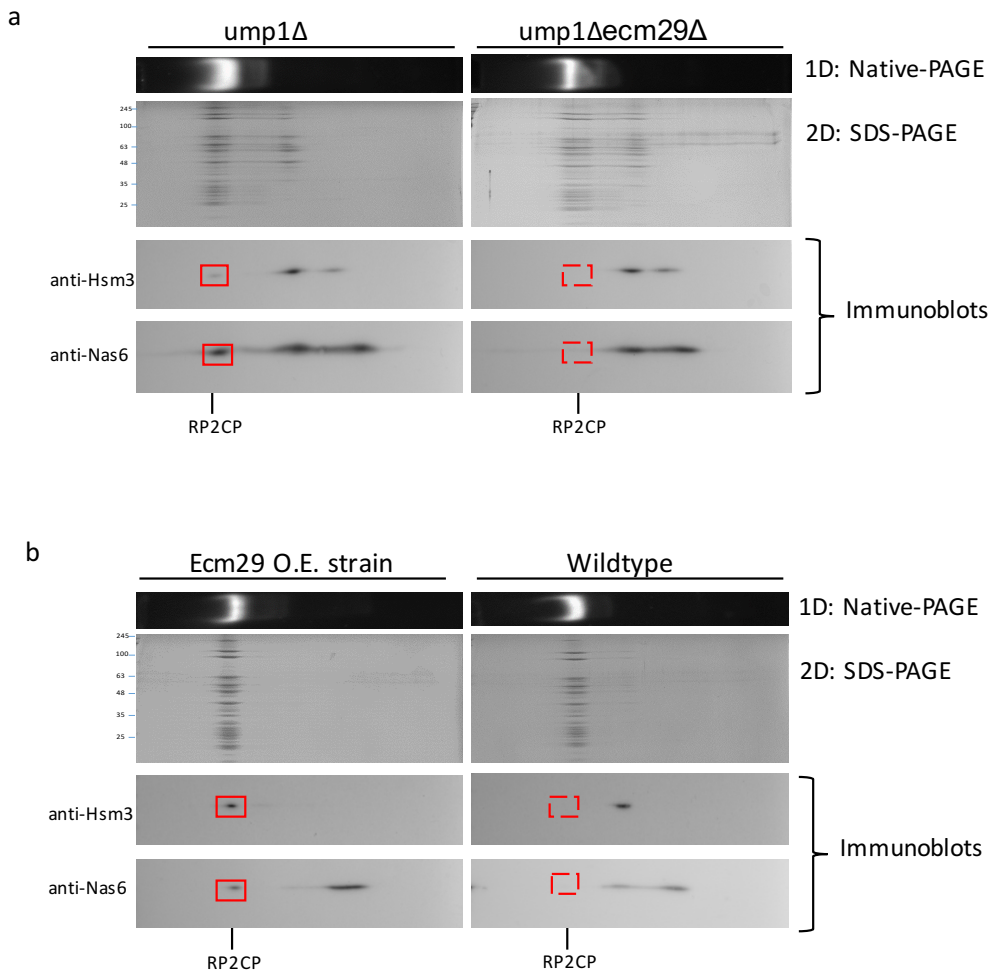
recognize and stabilize such complexes through its ability to bind both CP and RP base (De La Mota-peynado et al., 2013; Prashant S Wani et al., 2015). We therefore tested to see if *Ump1Δ* strains contained any RP-base intermediates-CP complexes stabilized by Ecm29. To test this, we followed a 2D gel approach as described previously (Roelofs et al., 2018). We first separated purified proteasomes on native gel (the first dimension) and then on SDS-PAGE (the second dimension). Native gel separates the different proteasome complexes in their native state as present in the purified preparations. Whereas the second dimension SDS-PAGE leads to the denaturation of native complexes and separates the individual proteins present in native complexes on the basis of their molecular size. Immunoblotting 2D gel of the proteasome prep from *ump1Δ* strain showed the presence of RP chaperones Hsm3 and Nas6 on 26S proteasomes (Figure 3.2a). We excluded analyzing Nas2 as it is present only on early RP assembly intermediate and is expelled early on during the incorporation of Rpt1 in RP base (Tomko et al., 2010). Unlike Hsm3 and Nas6, Rpn14 was not observed on 26S complexes (data not shown). The presence of any RP chaperone on 26S complexes was unusual as they are usually only found on RP-base assembly intermediates or on free RP complexes, but not RP-CP complexes (Park et al., 2013; Roelofs et al., 2009; Satoh et al., 2014; Sokolova et al., 2015). Instead, these chaperones are known to prevent RP-CP interaction and must be expelled out of the RP modules upon AAA-ATPase base ring formation (Park et al., 2009; Roelofs et al., 2009)

This expulsion is critical as the chaperones interact with the C-termini tail of ATPases which is also required to open the CP gates by docking into the alpha pockets of CP (Gillette et al., 2008; Smith et al., 2008). Next, we tested if the recruitment of chaperones on 26S might be dependent on Ecm29 (Lehmann et al., 2010; Park et al., 2011). As expected, 2D analysis of the proteasome prep from an *Ecm29Δ ump1Δ* double knockout indeed showed an absence of Hsm3 and Nas6 chaperones from 26S (Figure 3.2a).

To further support the model that the recruitment of chaperones was indeed dependent on Ecm29, we overexpressed Ecm29 (Ecm29 O.E.) in wildtype strain and compared it with a wildtype strain to look for differences in the localization of chaperones on 26S

(Figure 3.2b). We did not observe detectable amounts of chaperones on 26S in wildtype strain, whereas the Ecm29 O.E. strain showed a strong increase in the amount of chaperones on 26S (Figure 3.2b). Thus, Ecm29 stabilize the interaction of RP chaperones on 26S complexes.

Figure 3-2 Ecm29 upregulation or overexpression recruit chaperones on 26S.



(a) The total cell lysates from *ump1Δ* and *ump1Δecm29Δ* strains were resolved on native gels and visualized by LLVY-AMC assay. Next, lanes from native gels were excised and loaded on second dimension SDS-PAGE. The 2D SDS-PAGE were then transferred on PVDF membranes and immunoblotted for indicated chaperones Hsm3 and Nas6. The boxes indicate location where chaperones bound to 26S are anticipated to be detected. Solid boxes indicate chaperone presence was detected by immune blot, while dashed boxes indicate no detectable levels of chaperones were observed in *ump1Δecm29Δ* cells. (b) Similar to (a) only now comparing 2D

analysis of total lysates from cells over expressing Ecm29 under GPD promotor and the wildtype cells. Note: since the proteasomes were purified using Rpn11-ProA tag, our preps were enriched with RP sub-complexes base and lid and also with free RP. These complexes when not bound to CP are known to stay associated with RP chaperones, as seen on Hsm3 and Nas6 immunoblots.

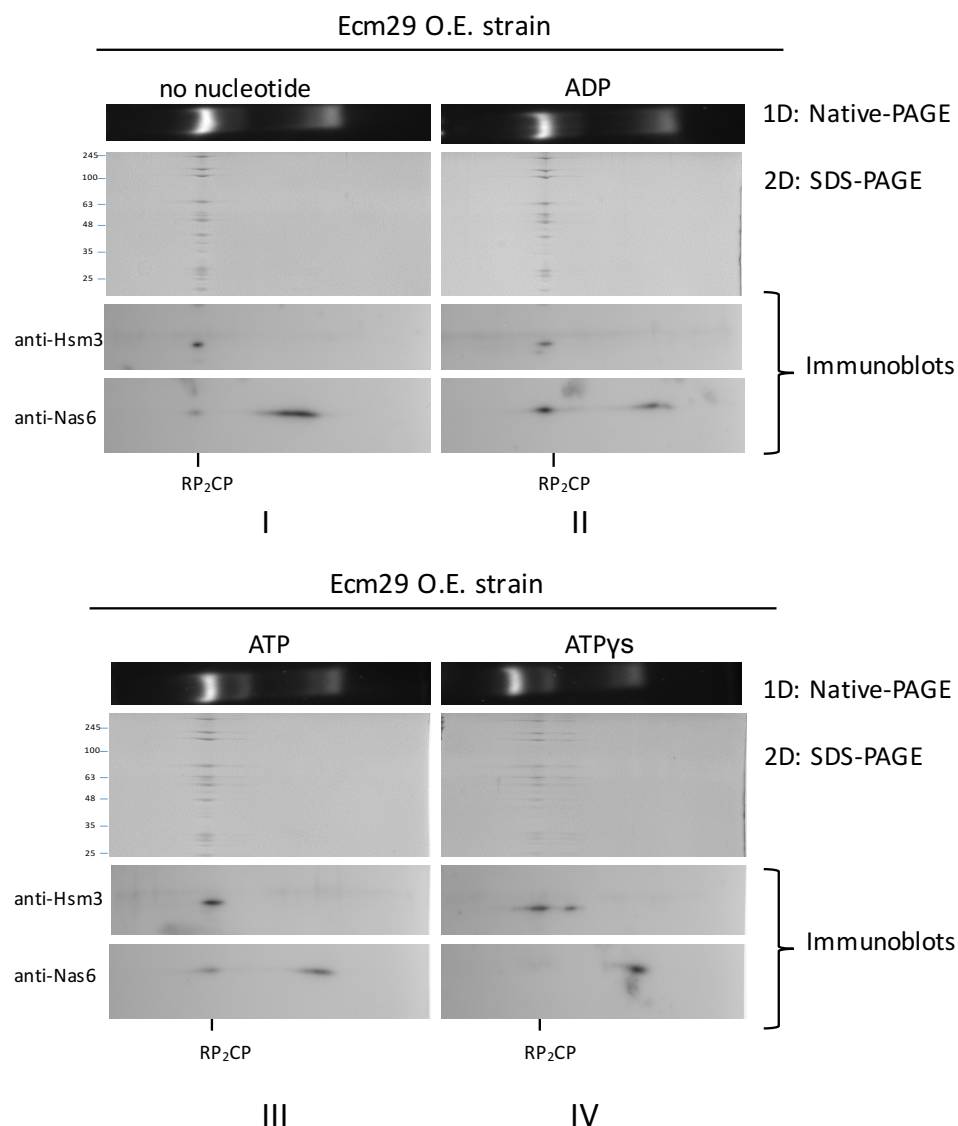
Nucleotide state of RP ATPases has no significant effect on the interaction of chaperones with Ecm29-RP-CP complexes

The nucleotide state of the RP ATPase subunits plays an important role in governing the interactions with various factors including Ecm29 and the chaperones (Li et al., 2016; Verma et al., 2000). Depending on the nucleotide state of the RP ATPases, three different states of proteasomes have been defined by cryoEM studies, the ground state with inhibited proteasome activity named S1, intermediate state named S2 and a high energy ATP bound state named S3 (Schweitzer et al., 2014). Since the affinity of Ecm29 to these three energy states is suggested to vary, we tested if changing the nucleotide availability to the 26S proteasomes in vitro can affect the recruitment of chaperones to 26S complexes bound to Ecm29 (Prashant S Wani et al., 2016). To mimic the different states of RP base we purified proteasomes from Ecm29 O.E. mutant strain using buffers containing either ATP (1 mM), ADP (1 mM), ATP γ S (0.5 mM), or in the complete absence of any nucleotide and performed the aforementioned 2D analysis of the purified proteasomes (Figure 3.3). The proteolytic activity assay done after the separation on native gel showed no significant differences in the LLVY-AMC activity of the 26S proteasomes. Despite the absence of nucleotides in purification process, the RP-CP complexes were stabilized by the binding of Ecm29 (Figure 3.3, lane I). This was consistent with the finding that Ecm29 can stabilize RP-CP interactions independent of the nucleotide state of RP base subunits (Kleijnen et al., 2007). The immunoblot analysis showed no difference in the binding of chaperones to 26S proteasomes in all four preps, except Nas6 blot in panel IV (Figure 3.3, all panels). Nas6 is known to influence the association of RP base with RP-lid and the CP depending on the nucleotide state of the RP-base (F. Li et al., 2016). Binding of ATP γ S mimics nucleotide bound-active state of the RP base, where Nas6 does not interfere in RP-base and CP association (F. Li et al., 2016). Consistent with this, we saw a reduced presence of Nas6 in preps done in the

presence of ATP γ S (Figure 3.3, panel IV). We similarly analyzed proteasomes purified from wildtype strain using buffers containing ATP, ADP, ATP γ S or in the complete absence of any nucleotide. As expected, wildtype strain showed no chaperones bound to 26S proteasomes under any conditions (Figure B.2).

The RP ATPases bind and hydrolyze ATP stochastically to execute the unfoldase activity. Proteasomes purified in the absence of ATP have been reported to retain ATP molecules tightly bound to certain ATPase subunits in RP (Kleijnen et al., 2007). Thus, our results using proteasomes purified in the presence of different nucleotides, represent only the net effect of the perhaps different nucleotide state of the ATPases. In order to dissect if the nucleotide state of specific ATPases can influence the stabilization of chaperones on RP-CP complexes by Ecm29, we introduced mutations in Walker A and Walker B sequence of individual ATPase subunits that mimicked a permanently ATP-free or ATP-bound states *in vivo*, respectively (Beckwith et al., 2013).

Figure 3-3 Ecm29 stabilizes chaperones on 26S independent of the nucleotide form available during purification process.



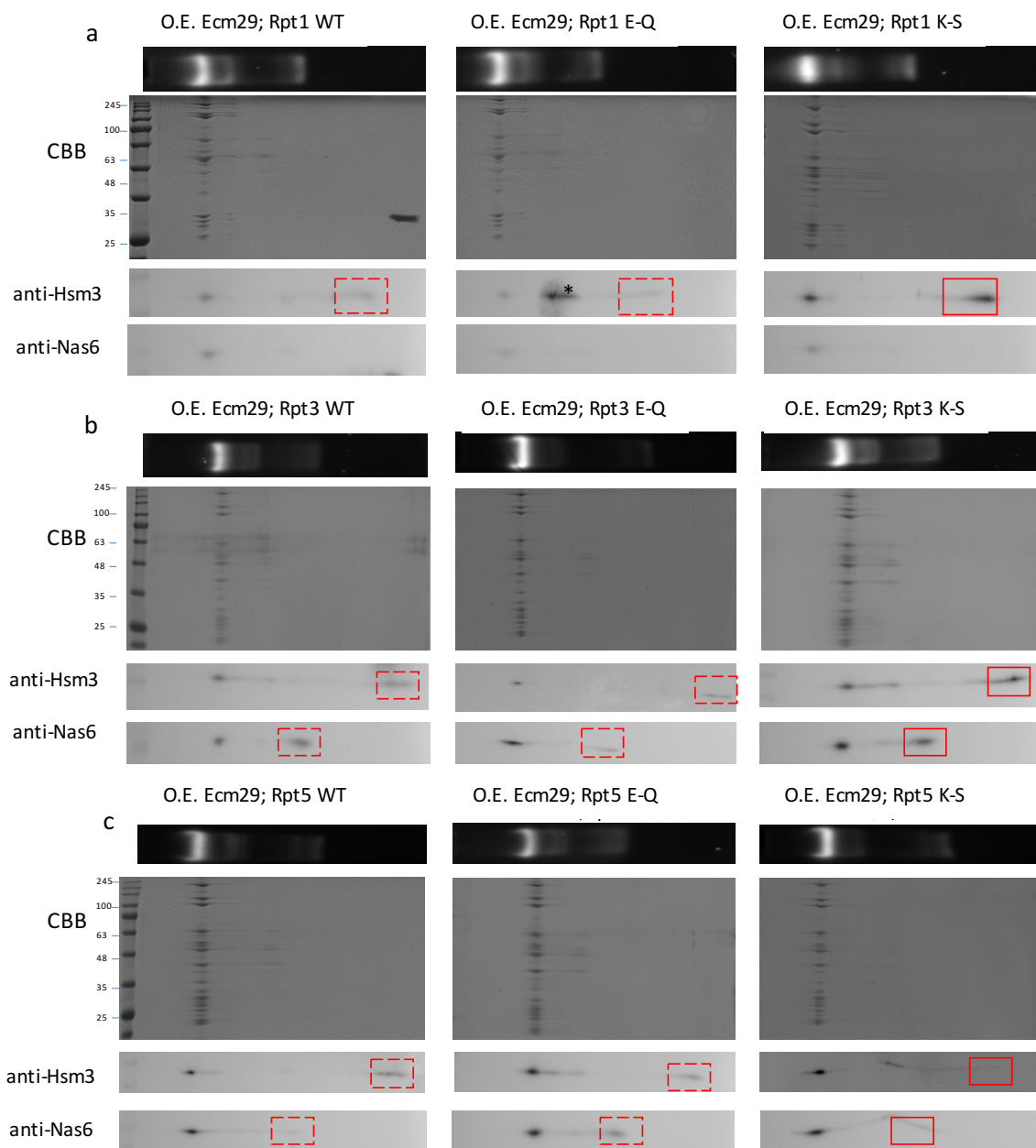
Proteasomes were purified from yeast strain over expressing Ecm29 by replacing the endogenous promotor with a strong GPD promotor. The purifications were carried out in the presence of ADP, ATP or ATPYs or in the absence of any nucleotide using affinity tag ProA on Rpn11 (Rpn11-ProA tag). The purified proteasomes were analyzed by previously described 2D approach and the presence of Hsm3 and Nas6 chaperones in purified material was analyzed by immunoblotting.

Mutation affecting nucleotide hydrolysis by RP ATPases has no effect on Ecm29 dependent recruitment of chaperones *in vivo*

To examine the nucleotide dependent binding of chaperones to individual RP ATPase subunits, we introduced glutamate to glutamine (EtoQ) mutation in the Walker B motif of Rpt1, Rpt3 and Rpt5 *in vivo*. This mutation of conserved glutamate is known to interfere with ATP hydrolyzing activity of the ATPases and causes them to mimic a constitutively ATP-bound (active) state (Beckwith et al., 2013). We introduced the E to Q mutation in only one out of six ATPase subunits in a mutant strain and tested for the effect of nucleotide state of the mutant ATPase subunit on the stabilization of chaperones on 26S bound with Ecm29. We followed a previously described 2D approach to analyze the proteasomes containing E to Q mutants of Rpt1, Rpt3 and Rpt5 subunits and compare them with the wildtype strains (Figure 3.4). As seen for Ecm29 O.E. strain, immunoblot analysis of E to Q mutants also contained chaperones bound to 26S complexes. We saw no major difference in the binding of chaperones to 26S proteasomes. E to Q mutation although renders the mutated ATPase unable to hydrolyze ATP, these mutant subunits do not interfere with the RP base assembly or the RP base-CP interaction (Beckwith et al., 2013). Proteasomes bound with non-hydrolysable ATPγS show increased stability as compared to ATP or ADP bound forms. Since E-Q mutation also mimics ATP bound state, we saw an increased proteasome stability and a consequently less amounts of free CP on native gels (Figure B.3).

We also analyzed the physiological effect of E to Q mutations *in vivo* by analyzing the phenotype of strains under heat stress (Figure 3.5). Heat stress induces protein misfolding *in vivo* which requires enhanced proteasomal activity and thus several proteasome mutants have been reported to be sensitive to heat stress, when grown at 37 degrees. Moreover, heat stress has been suggested to increase chaperone mediated RP ring assembly (Hanssum et al., 2014). The mutant strains carrying E to Q mutation in any of the three RP ATPases did not exhibit sensitivity at 37 degrees. This suggest that mutating the ATP hydrolysis ability of one out of six RP ATPases, does not cause any significant functional defects in the RP or the 26S proteasome.

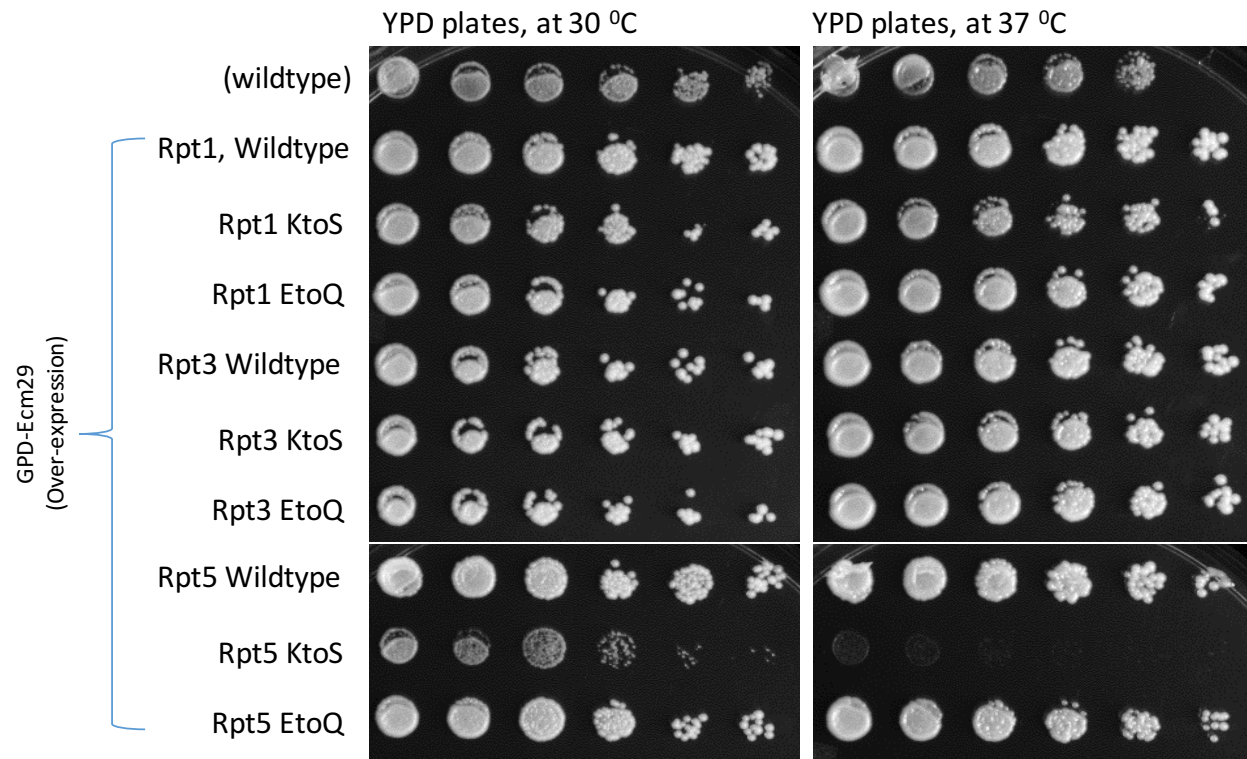
Figure 3-4 ATP-bound state of ATPases promotes stabilization of chaperones.



2D analysis of Ecm29 over expressing strain with base variants consisting of single-base ATPase mutant. (a) 2D analysis were used to assess their effect on the stability of chaperones by the E to Q mutation in the Walker-B motif of the Rpt1 subunit, which mimics the nucleotide occupied-state, or the K to S mutation in the walker A motif, which mimics the empty-state. Presence of free chaperones in purified preps was used as an indication of their reduced affinity towards 26S proteasomes. Chaperones when not bound to the ATPases, are faster migrating species on native gels, and were captured on immunoblots of 2D gels and are marked by solid or dashed

boxes indicating the presence or absence of free chaperones, respectively. * represents background signal (b) 2D analysis of occupied-state Walker-B E to Q mutation or empty-state Walker-A K to S mutation in Rpt3 subunit to assess their effect on the stability of chaperones on 26S. (c) 2D analysis of occupied-state Walker-B E to Q mutation or empty-state Walker-A K to S mutation in Rpt5 subunit to assess their effect on the stability of chaperones on 26S.

Figure 3-5 Nucleotide state of Rpt5 is critical for the cellular physiology under heat stress.



Cells equivalent to A_{600} of 1.0 were collected and used for fivefold serial dilutions before spotting on YPD plates. The plates were incubated at 30 degrees and 37 degrees for 2 days. The dilution assay was performed in replicates of three, all showing similar growth patterns. The growth of all the strains remains unaffected at 30 degrees and 37 degrees except for the Rpt5 K to S mutant. The slow growth at 30 degrees and severely affected growth at 37 degrees suggests indicates the importance of nucleotide state of Rpt5 in RP base.

Mutation affecting nucleotide binding of RP ATPases destabilizes Ecm29 dependent recruitment of chaperones *in vivo*

As the mutations that mimic the nucleotide bound state of the Rpt subunits showed little difference with wildtype conditions, we next introduced mutations that cause the Rpt subunits to instead remain in ATP free state due to strongly reduced ability to bind nucleotide. We introduced the lysine to serine (K to S) mutation in Walker A motif of Rpt1, Rpt3 and Rpt5 to analyze the effect of ATP-free state of the ATPases on the stabilization of chaperones on RP-CP complexes. Rpt2 is highly sensitive to these mutations and a mutation of conserved Lys has been reported lethal for the cells (Rubin et al., 1998). Similarly, mutation in Rpt4 and Rpt6 are also known to be sensitive where the mutant strains exhibited severe growth defects (Rubin et al., 1998). The mutation of conserved Lys residue results in the inhibition of ATP binding to the ATPase (Rubin et al., 1998). By preventing nucleotide binding to ATPases, we introduced an ATP-free state in only one out of six ATPase subunits present in the RP-base. Considering the stochastic nature of ATP hydrolysis by different ATPase subunits present in RP-base ring, the mutant strains carrying K to S mutation in one of the three aforementioned ATPases allowed us to analyze the effect of ATP-free state of specific ATPase on the stabilization of chaperones on RP-CP complexes (Figure 3.4). Similar to the E to Q mutants, we observed chaperones on 26S complexes in K to S mutant strains as well. However, we also noticed an increase in the amounts of free chaperones upon immunoblot analysis of the 2D gels (Figure 3.4, see K to S mutants). As mentioned previously, these proteasomes were purified using a C-terminal Pro-A tag present on Rpn11 subunit of the RP-lid. Presence of Hsm3 and Nas6 chaperones in the purified material would represent the chaperones bound either to free RP or the ones preset on 26S proteasomes. High resolution 2D gels and their western blot analysis helped in assigning the complexes containing these chaperones. Comparison immunoblots probed for Hsm3 showed an increased free Hsm3 in Rpt1_{KtoS} mutants as compared to the proteasomes carrying wildtype Rpt1 (Figure 3.4). This suggests a decreased affinity of Hsm3 for ATP-free Rpt1 in Ecm29 bound RP-CP complexes. The binding of Nas6 remains unchanged in this mutant as Nas6 binds to Rpt3 which is unchanged in Rpt1_{KtoS} mutant. Similarly, 2D analysis of Rpt3_{KtoS} mutants showed

an increase in free Nas6 chaperone as compared to the wildtype proteasome (Figure 3.4).

On the other hand, Rpt5_{KtoS} mutant proteasomes exhibited no difference in their binding with Hsm3 and Nas6. Since Ecm29 binds to RP through Rpt5, our results suggest that the K-S mutation does not affect interaction of Ecm29 with Rpt5 (Lee et al., 2011). However, the phenotypic growth assay showed that the Rpt5 K to S mutant was heat sensitive and exhibited growth defects at 37 degrees (Figure 3.5). This suggests that the nucleotide state of Rpt5 plays an important role in regulating the proteasomal activity. The analysis of proteasome preps on native gel does not show any significant effect on the stability of proteasomes, however the K to S mutation causes Rpt5 to stay in a constitutively ATP free state which causes severe growth defects under heat stress (Figure B.3).

A K to S mutation in the walker A motif causes the C-terminal tail to be buried in the RP base such that it is unavailable for docking into CP (Beckwith et al., 2013). This confirmation of tail can also potentially affect its interaction with chaperone. However, co purification of chaperones with 26S suggests that the mutation in Walker A motif does not abrogate the interaction of ATPases with chaperones. The presence of free chaperones in purified material suggests that the binding of Ecm29 causes a conformational change that causes the C-terminal tail of mutated ATPase to be partially available for binding with chaperones. However, the chaperones fall off these complexes and run as free proteins on Native gels due a reduction in their affinity towards the ATP-free ATPase.

Since the K to S caused a reduction in the binding affinity of chaperones, we hypothesized the E-Q mutants to mimic the nucleotide state of an “Ecm29 trapped” ATPase. Consistent with our hypothesis, our E to Q mutants exhibited high similarities in the binding pattern of chaperones as seen in wildtype proteasomes bound to Ecm29 (Figure 3.4). We saw insignificant changes in the binding affinity of Hsm3 or Nas6 in Rpt1_{EtoQ}, Rpt3_{EtoQ} and Rpt5_{EtoQ}. These results support the idea of Ecm29 trapping the RP-base in ATP bound state, which was further validated using *in vitro* approach.

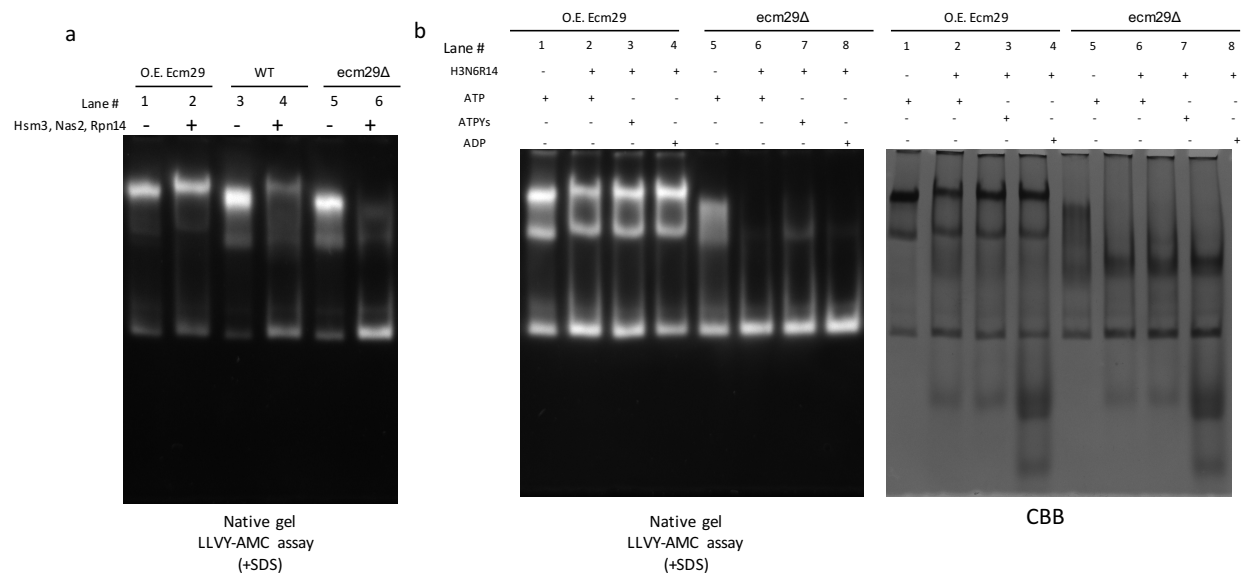
***In vitro* analysis confirms the role of Ecm29 in stabilizing chaperones on RP-CP complexes**

In order to understand the role of Ecm29 in stabilizing the RP chaperones on 26S, we conducted *in vitro* experiments by reconstituting purified proteasomes from yeast strains, with ten times (10x) excessive amounts of recombinant RP chaperones, Hsm3, Nas6 and Rpn14 expressed and purified from *E. coli*. The proteasomes were purified from three different yeast strains, Ecm29 O.E. mutant strain, wildtype strain and an Ecm29 knockout (*ecm29Δ*) strain using ProteinA tagged Rpn11 subunit of the RP-lid of the proteasomes. The proteasomes and chaperones were reconstituted for 1 hour at 30 degrees and then analyzed on native gel (Figure 3.6a). Binding of Ecm29 to RP₂CP and RP-CP complexes leads to a shift in their mobility on native gels (Figure 3.6a, compare lane 1, 3, and 5). Binding of 10x chaperones to proteasomes also causes a shift in their mobility (see lane 2 and 4). Addition of 10x chaperones to proteasomes purified from Ecm29Δ mutant strain leads to a severe dissociation of RP₂CP and RP-CP complexes (see lane 6) and causes an accumulation of free CP. On the other hand, proteasomes purified from Ecm29 O.E. strain were not affected by the presence of chaperones (see lane 2). Since most of the proteasomes from Ecm29 O.E. are bound and stabilized by Ecm29, addition of chaperones does not lead to the dissociation of 26S or accumulation of free CP. As discussed before, binding of chaperones to 26S also creates a shift and the band now appears higher than Ecm29 bound 26S (compare lane 1 and 2). Meanwhile, the proteasome purified from the Rpn11-tagged wildtype strain looked like an intermediate of the other two strains. Upon addition of chaperones, the proteasomes stabilized by Ecm29 were prevented from dissociating, where as the ones not bound to Ecm29 fell apart leading to a moderate increase in the amount of free CP observed (lane 3 and 4).

Ecm29 is a large protein that contacts both RP and CP and is speculated to affect the ATPase activity by trapping it in the S1 energy state (Finley et al., 2017). In order to understand the mechanism behind regulation of ATPase activity by Ecm29, we carried out previously described *in vitro* reconstitution experiments in the presence of different ATP analogs and compared the stability of 26S proteasomes from Ecm29 O.E. mutant

strain and *ecm29Δ* strain (Figure 3.6b). As expected, the proteasomes purified from Ecm29 O.E. mutant strain did not undergo any dissociation upon the addition of chaperones and we saw a shift in their mobility upon the binding to chaperones (see lanes 1-4). On the other hand, the reconstitution done with *ecm29Δ* strain showed expected disruption of RP-CP complexes in the presence of ATP and ADP nucleotides. However, they exhibited an increased stability in the presence of ATPγS, a non-hydrolysable analog of ATP (Figure 3.6b, see lane 7 on native gel and CBB stained gel). These stable RP-CP complexes showed a shift in mobility on native gel, suggesting the presence of chaperones on the RP-base. The stabilization of RP-CP complexes in the presence of ATPγS mimics the stabilization of proteasomes by Ecm29 and supports our aforementioned hypothesis that Ecm29 traps the RP-base in ATP bound state. Contrary to the popular idea that Ecm29 traps proteasomes in S1 energy state, our results hints towards Ecm29 dependent regulation of base confirmation to an active and ATP-bound S3 state (Finley et al., 2017).

Figure 3-6 In vitro reconstitution suggests the stabilization of proteasomes by Ecm29 in ATP bound state.



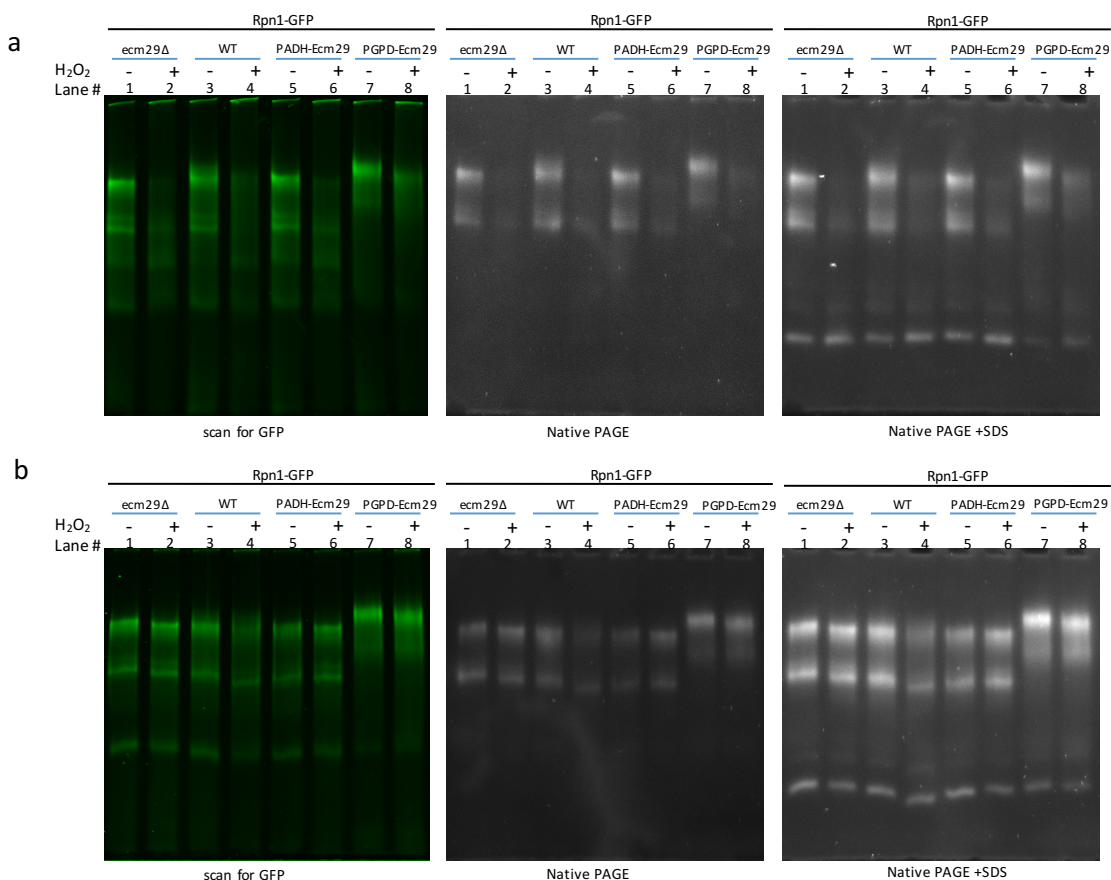
(a) 0.125 μ M of purified proteasomes from indicated strains were reconstituted with 1.25 μ M (10x more) of Hsm3, Nas6, and Rpn14 for 30 mins at 30 degrees. The reconstituted samples were separated on native-PAGE and analyzed for peptidase activity using the LLVY-AMC hydrolysis assay. (b) testing the effect of nucleotide state of the RP base, we first reconstituted 0.125 μ M of proteasome preps with ATP (1 mM), ATP γ S (0.5 mM) or ADP (1 mM). The tubes containing ADP were also supplied with hexokinase (0.2 units/ul) and 2-deoxy D-glucose (20mM) to convert any potential ATP bound to RP base to ADP. Next, we added 1.25 μ M each of Hsm3, Nas6 and Rpn14 chaperones and incubating the mixture for 30 mins at 30 degrees. The reconstituted samples were then analyzed on native-PAGE for the activity of proteasomes by performing LLVY-AMC assay and for stability by performing coomassie brilliant blue (CBB) staining.

Ecm29 causes stabilization of proteasomes under oxidative stress conditions

Biochemical and mass spectrometry studies in yeast and mammals have suggested a role of Ecm29 in mediating the structural changes in proteasomes during oxidative stress (Wang et al., 2011). It was shown that Ecm29 exhibits increased association with RP and causes the dissociation of proteasomes. As we saw in *ump1 Δ* strains, deletion of ECM29 also causes a rescue in amounts of total assembled 26S complexes in oxidative stressed cells. Since our results suggested a role of Ecm29 in stabilizing proteasomes and assisting in their assembly, we decided to study the effect of Ecm29 over expression on the stability of proteasomes under oxidative stress. We analyzed the total cell lysates collected 2 hours and 24 hours post induction of oxidative stress from different strains by separating on native PAGE. For analysis we used Rpn1-GFP tagged strains expressing wildtype levels of Ecm29 (labelled as WT) and compared them to Ecm29 knock out strain (*ecm29 Δ*), strain expressing Ecm29 under a weak promotor (ADH-Ecm29) and strain over expressing Ecm29 (GPD-Ecm29) (Figure 3.7a). We induced oxidative stress in logarithmically growing cells by adding 10mM hydrogen peroxide (H₂O₂). Consistent with previous studies, we saw dissociation of proteasomes and appearance of free CP (see Native PAGE) and RP (see typhoon scan of GFP), 2 hours following the induction of oxidative stress in wildtype strain (Figure 3.7a, lane 3 and 4). However, we saw similar destabilization of 26S in *ecm29 Δ* and ADH-Ecm29 strains (lane 1 and 2) as well, as opposed to previous findings that suggested increased proteasome stabilization upon deleting ECM29. Interestingly, Ecm29 over expressing strain, GDP-Ecm29, exhibited increased stabilization of 26S proteasomes. Immunoblot analysis confirmed that the stable 26S complexes were bound to Ecm29, supporting the idea that the stabilization of

proteasomes in WT and GPD-Ecm29 strain was mediated by Ecm29 (Figure B.4). After 24 hours, we saw greatly reduced effect of oxidative stress which resulted in minimal dissociation of RP-CP complexes in WT strain and no dissociation at all in all other strains (Figure 3.7b).

Figure 3-7 Ecm29 stabilizes proteasomes under oxidative stress.



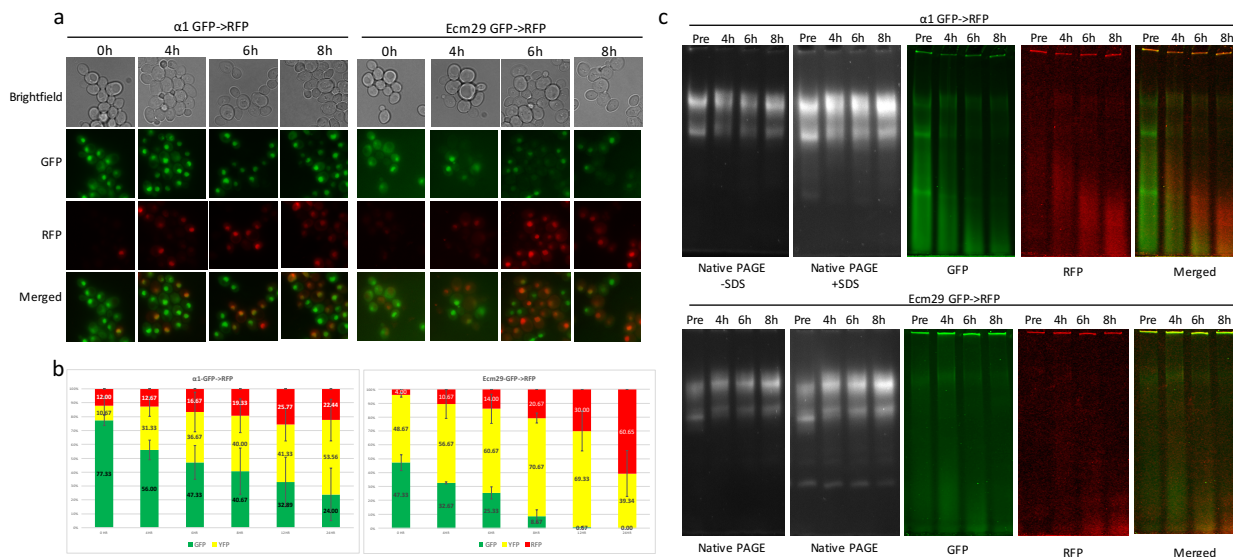
(a) Indicated strains were logarithmically grown in YPD to an A_{600} of 1.5, divided into 2 sets where one sample was incubated with 10 mM H₂O₂. After 2 hours, cells were collected, lysed by cryogrinding, and the cleared total cell lysates were analyzed on native gels. Proteasome integrity was analyzed by scanning for fluorescent signal of the GFP-tagged proteasome subunits Rpn1. The stability and activity of proteasomes was analyzed by using LLVY-AMC assay. (b) Experimental conditions identical to (a), only lysates were generated 24 hrs after treatment.

As seen on native gel in Figure 3.7b, Ecm29 eventually dissociates from 26S complexes after 24 hours of H₂O₂ treatment. To understand this further, we studied the stabilization of Ecm29 as compared to the 26S proteasomes in a cell, under normal conditions. We

used recombination-induced tag exchange (RITE) approach to study the dynamic behavior of Ecm29 with respect to the proteasomes. RITE approach allows for a time controlled permanent tag switch on the C-terminal of a protein of interest which allows to study the dynamics of old and new proteins in vivo (Verzijlbergen et al., 2010). We created strains by integrating RITE cassette that can switch from GFP to RFP tag, downstream of $\alpha 1$ ($\alpha 1$ -GFP->RFP) and Ecm29 (Ecm29-GFP->RFP) (Figure B.5). Prior to the induction of switch, the strains expressed on the GFP tagged version of $\alpha 1$ and Ecm29 ($\alpha 1$ -GFP and Ecm29-GFP, respectively) (Figure 3.8a, 0h). We scored cells (n=100) representative of the yeast culture at each time point for the total GFP and RFP signals and used the ratio of GFP v/s RFP to determine the dynamics of the expression and stability of the tagged protein. We scored the cells as GFP positive (represented by green bars on the graph) if they contained “old” protein only, RFP positive (red bars on graph) if the cells had switched and completely degraded all the “old” proteins or Both (yellow bars on graph) if they contained substantial amounts of both “new” and “old” protein (Figure 3.8b). At 0hr, 77.33% of the $\alpha 1$ -GFP->RFP and 47.33% of Ecm29-GFP->RFP tagged cells were GFP positive. We detected RFP expression at 0h, prior to the induction of switch in the media. To understand the dynamics of the tagged proteins, we compared the intensity of signals between progressing time-points. The tag switch was then initiated by inducing Cre-lox dependent genetic recombination that caused removal of GFP tag situated between loxP sites. As a result, the “old” GFP tag is genetically exchanged with “new” RFP tag and the strains now expressed RFP tagged version of $\alpha 1$ and Ecm29 post-switch ($\alpha 1$ -RFP and Ecm29-RFP, respectively) (Figure 3.8a, 4h). With increasing time points post switch, we saw a gradual reduction of “old” GFP-tagged proteins and an increase in RFP-tagged “new” proteins. The persistence of $\alpha 1$ -GFP and Ecm29-GFP signals post switch (green and yellow bars), indicated the stability of the respective proteins. The quantification of GFP and RFP data showed that $\alpha 1$ subunit was more stable than Ecm29 (Figure 3.8b). We also analyzed the total cell lysates collected at the aforementioned time-points on Native-PAGE to study the stabilization of Ecm29 on proteasomes (Figure 3.8c). As seen in figure 3.8a and 8b, $\alpha 1$ -GFP->RFP lysates collected before switch (pre) showed only GFP signal and post switch, they exhibited a gradual reduction of GFP signal

and an increase in RFP signal. We saw similar pattern in Ecm29-GFP->RFP strain, despite weaker signal as compared to the $\alpha 1$ -tagged strain.

Figure 3-8 Analysis of old and new proteasomes and Ecm29 by RITE method.



(a) Fluorescent microscopy images representing yeast strains with RITE cassette integrated at the C-terminal of $\alpha 1$ ($\alpha 1$ -GFP->RFP) or Ecm29 (Ecm29-GFP->RFP). The induction and activation of Cre recombinase with beta-estradiol, allows for the genetic excision of the initial GFP tag, resulting in the appearance of an in frame RFP tag. The cells were collected for microscopy before inducing tag switch (pre) and at the indicated time-points post induction. (b) To quantify the results in (a) 100 cells were scored for their green and red fluorescence. The ratio of total GFP and RFP signals in the nucleus was measured and used to classify cells as only GFP signal positive (GFP), only RFP positive (RFP), or positive for both GFP and RFP signals (BOTH) and is depicted as percentage of total cells in the sample. The average of three independent experiments is shown with S.E.M. (c) The cells collected at indicated time-points were also lysed by cryogrinding and the total lysates were analyzed on Native PAGE by LLVY-AMC assay and scanned for GFP and RFP signals.

Discussion

The core structure of proteasome regulatory particle base consists of the six AAA-ATPase subunits that are crucial for energy dependent degradation as they unfold substrates. These ATPase subunits, Rpt 1 to 6, vary in function and form a non-planar, stair case like arrangement when not bound to a substrate (Beckwith et al., 2014). Upon binding substrate, they form a more planar and ring like structure of ATPases that utilizes energy derived from ATP hydrolysis to unfold substrates. While base assembly is facilitated by five unique assembly chaperones, we provide evidences supporting a role of Ecm29 in mediating the assembly process. Studies done in past few years have suggested pleiotropic roles of Ecm29 in regulating the stability and activity of proteasomes. Since Ecm29 binds both RP and CP, it is intuitive to think of Ecm29 as a mediator of allosteric interactions between the two proteasomal sub-complexes. In this study, we use UMP1 Δ mutants to study the role of Ecm29 in mediating such interactions in proteasomes. Ecm29 is known to causes base assembly defects in strains lacking a CP chaperone, Ump1. Our study provides mechanistic insights into the role of Ecm29 in regulating the RP-base assembly.

Ecm29 has been described as a proteasome quality control factor, our results provide evidence in support this functional role of Ecm29. We show that Ecm29 can bind and allow for the stabilization of RP chaperones on 26S complexes. The nucleotide state of the RP base is known to affect the interaction with other factors, we show that they also play a pivotal role in the stabilization of chaperones by Ecm29.

RP chaperones and the CP are known to be mutually exclusive in their interaction with RP base subunits. Using *ump1* Δ strains and the strain over expressing Ecm29, we show that Ecm29 stabilizes chaperones on RP-CP complexes. In order to understand if Ecm29 binding affects the nucleotide state of the ATPases, we introduced mutations in ATPases that permanently arrest them in ATP bound or ATP-free state. We compared the purified proteasomes from mutant strains with the wildtype ATPase bearing strain to see if we can mimic the effect of Ecm29 in stabilizing the interactions of chaperones with RP-CP complexes. We also tested if any of the mutant forms could counteract the role of Ecm29

and destabilize the interaction of chaperones with RP-CP complexes. Since not all ATPases in the RP base stay in the same nucleotide state, we expected the mutations of each of the subunits to have different effects on the binding of chaperones to RP-CP complexes (Kleijnen et al., 2007). Rpt5_{KtoS} mutation causing the ATPase to stay in a nucleotide free state, in particular, had the most severe effect on the physiology of cells under heat stress condition. K to S mutation in the Walker A motif of Rpt1 and Rpt5, also had effects on their binding with RP assembly chaperones. The Walker B mutants carrying E to Q mutation in Rpt1, Rpt3, and Rpt5 subunits showed no detectable differences in the stabilization of chaperones, when compared to the strain carrying wildtype Walker B domains and over-expressing Ecm29. This led to propose that the stabilization of chaperones on RP-CP complexes is favored by the ATP bound state of the ATPases. The *in vitro* experiments done in this study support this idea by showing that ATPγS can stabilize RP-CP complexes in the absence of Ecm29. Binding of ATPγS in the *in vitro* experiments would also create the same conformational difference as the E to Q mutation would *in vivo*, since ATPγS is a non-hydrolysable analog of ATP. Since the ATPases bind nucleotide in tandem, and are not uniform in their nucleotide state, more experiments are needed to confirm the state of Rpt2, Rpt4 and Rpt6 upon binding of RP base to Ecm29 (Kleijnen et al., 2007).

Glucose starvation is known to induce the proteasomes to form granules. Knocking out UMP1 showed severe reduction in granule formation which was partially rescued upon deletion of ECM29. However, since UMP1 knockouts have an overall reduced levels of proteasomes, it was unclear if the rescue in granule formation was caused by lower levels of proteasomes in general or if it was mediated by Ecm29. Therefore, we analyzed a double knockout lacking both Ump1 and Ecm29 and saw increase in the amount of 26S proteasome and increased in signal intensity for Rpn1-GFP representing RP population, but saw no increase in the amount of free CP. Overall, our results suggest that *ump1Δ* strain failed to form granules under stress conditions due to the reduced levels of assembled proteasomes. Knocking out ECM29 partially rescues the granule formation by rescuing RP formation and enhancing the total 26S complexes in the strain.

Ecm29 is known to regulate the activity of proteasomes by inhibiting the activity of the ATPases *in vivo* (De La Mota-peynado et al., 2013). On the other hand, it is also known to cause dissociation of proteasomes under oxidative stress conditions. Altogether, Ecm29 possesses ambivalent functions in regulating the proteasomes under stress conditions. Our results show a role of Ecm29 in stabilizing the proteasomes under oxidative stress conditions. Since Ecm29 can inhibit the ATPase activity of the proteasomes, Ecm29 may play an essential role in coping with oxidative stress conditions. Oxidative stress is one of the most common physiological stress condition prevalent in a number of disorders in human body, including neurological disorders and cancer. It is therefore important to study the role of Ecm29 in regulating the activity or assembly of essential complex, proteasomes.

In summary, we have showed an additional role of Ecm29 in regulating the assembly of proteasomes. We have identified novel complexes composed of Ecm29-RP chaperones-RP-CP. Our ATPase mutants revealed that this regulatory activity of Ecm29 was dependent upon the nucleotide state of the ATPases. We also showed that the ATPase activity of Rpt5 subunits is critical for the survival of cells under stress conditions. Comparison of the proteasome preps from Ump1 Δ strain and Ecm29 over expressing strain showed that the novel complexes identified were not caused by the CP assembly defects and that Ecm29 overexpression can alone cause the recruitment of chaperones to 26S complexes. We show that Ecm29 upon binding the proteasomes can trap the ATPases in RP base in nucleotide bound state. The physiological importance of binding and stabilizing proteasomes was also shown under oxidative stress conditions. Prolonged stress conditions that leads to the inhibition of proteasomes can cause ubiquitination and degradation of proteasomes through lysosome dependent autophagy (selective degradation of complex, also termed as proteaphagy). Our results suggest that Ecm29 prevents autophagy by stabilizing RP base intermediates on CP and promoting their assembly by stabilizing their weak interaction.

Author contributions

A.S., A.V., F.K. and J.R performed the experiments and constructed the yeast strains. Studies were conceived by A.S.and J.R. Manuscript was written by A.S. and J.R. with input from all authors.

Chapter 4 - Discussion

The biomolecular organization of cells consists of numerous essential multisubunit complexes. Elaborately programmed mechanisms regulate efficient assembly, activation, and degradation of these complexes. Cross-talk between these regulatory processes is essential to enable a cell to respond to environmental flux. Regulated degradation of proteins is an important cellular process that plays essential roles in the maintenance of homeostasis of an organism. Proteasomes are non-lysosomal proteases found in the cytosol and the nucleus that carry out the degradation of proteins that have been specifically marked for degradation. Proteasomes are intricate multisubunit protease complexes found in all three domains of life. Although ubiquitous, they vary substantially in terms of their composition, structure, catalytic activity, accessory interacting factors and targeted substrates.

While proteasomes are essential for the viability and survival of eukaryotic cells, the dispensability of proteasomes varies among different bacterial strains. Eukaryotic 26S proteasome consists of a 20S core particle (CP) and a 19S regulatory particle (RP). The functional complex requires the proper assembly of a total of 66 subunits, including some structurally homologous but functionally different subunits. Whereas the prokaryotic proteasomes have a simpler organization consisting of only a CP composed of one (or sometimes two) isoforms of alpha and beta subunits. Several ATPases and non-ATPases (as discussed in Chapter 1) are known in prokaryotes to unfold and channel protein substrates to the catalytic core. Eukaryotic proteasomes selectively bind substrates targeted for degradation by tagged poly-ubiquitin chains. Ubiquitin like molecules have also been identified in prokaryotes that help in targeting substrates for degradation. SAMPylation and PUPylation are two most commonly known post-translational modifications that target substrates to archaeal and bacterial proteasomes, respectively (Darwin, 2009; Pearce et al., 2009). In eukaryotes, proteasomal activity influences numerous biological processes ranging from housekeeping functions to cell cycle progression. Similarly, proteasomes found in bacterial strains have been known to regulate a number of processes that affect bacterial physiology. For example,

proteasomal activity in *Mycobacterium tuberculosis* provides resistance to the bacteria against copper and nitric oxide (NO) produced by the phagocytic cells of the infected host (Darwin & Ehrt, 2003; Festa et al., 2011). *M. tuberculosis* is a highly infectious, multidrug resistant bacteria causing tuberculosis in humans. Understanding of the roles of proteasomes in facilitating survival of *M. tuberculosis* could open new drug developmental opportunities against one of the leading causes of death globally. Regardless of the significant differences in the overall complexity of proteasomes across the three domains, the structure of the core particle is conserved from bacteria to humans. It consists of two outer alpha rings that form gated entries to the proteolytic chamber formed by two inner beta rings which house the active site. The CP assembly process culminates by autocatalytic removal of propeptide chains from beta subunits. This universal autocatalytic mechanism involves a nucleophilic attack by the active site threonine residue (Thr1) on the preceding peptide bond with glycine (Gly-1), causing release of propeptide sequence and exposing the active site in the catalytic chamber (Ditzel et al., 1998).

Proteasomes are present in a limited number of bacterial species from orders actinomycete and nitrospirales but not in all gram positive bacteria. This suggests that, evolutionarily, their presence can be attributed to lateral gene transfer events. Since eukaryotes require multiple assembly chaperons during CP assembly, it is interesting to note that fewer homologs of CP assembly chaperones have been identified in prokaryotes and that, in their absence, assembly proceeds efficiently in vitro. Although bacteria encode only one (or two) isoforms of CP subunits, it is an intriguing how bacteria manage to assemble functional proteasomes in the absence of chaperones. Despite recent advancements in our understanding of proteasomal activity in bacteria, we still only partly understand the assembly and activation of bacterial CP. The research presented in Chapter 2 suggests the propeptide sequence present on all bacterial beta subunits potentially function as assembly chaperones.

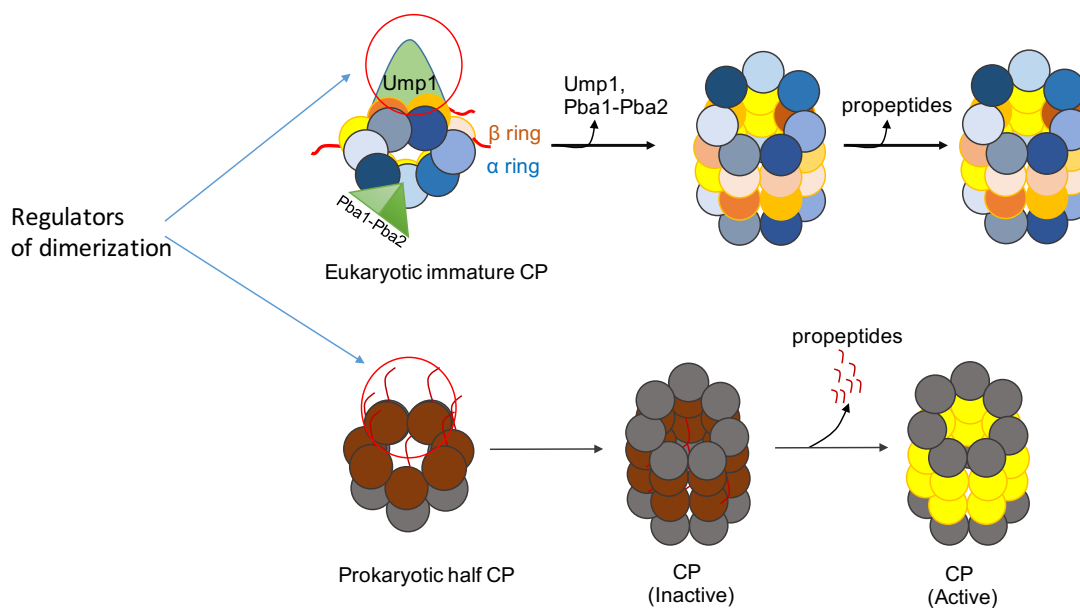
Role of bacterial propeptides in the maturation

Within eukaryotic proteasomes, five out of seven beta subunits possess propeptides: $\beta 1$, $\beta 2$, $\beta 5$, $\beta 6$ and $\beta 7$. Out of these, only three possess a functional active site: $\beta 1$, $\beta 2$ and $\beta 5$. While active site bearing β subunits can undergo autocatalytic removal of their propeptide sequence, inter molecular processing is involved in the removal of the propeptide sequences from $\beta 6$ and $\beta 7$ (Groll et al., 1999). On the other hand, homo-heptameric β ring found in bacterial CP has seven β subunits that possess both a propeptide sequence and active site. Our studies show strong evidence for cross processing of beta propeptides in bacterial CP, thus supporting an evolutionary link between bacterial and eukaryotic CP. Despite all beta subunits possessing an active site, we propose bacteria utilize cross processing of propeptides to allow for a faster and more efficient CP maturation process. In eukaryotes, incorporation of $\beta 7$ triggers the HP dimerization process which is tightly coupled with the autocatalysis process (Marques et al., 2007). Similarly, our studies show that bacterial dimerization is regulated by the flexible loop of propeptide, but further studies are required to determine what triggers autocatalysis in bacterial CP.

The role of propeptides as intrinsic chaperones that facilitate folding of beta subunits has been studied in both eukaryotes and prokaryotes (Chen & Hochstrasser, 1996; Zühl et al., 1997). Eukaryotic beta sequences possess propeptides of different lengths and are each known to have subunit specific function. The propeptide found on yeast $\beta 5$ subunit and its mammalian ortholog LMP7 is essential for efficient incorporation into CP (Braun et al., 1999; Chen & Hochstrasser, 1996). Additionally, $\beta 5$ propeptide is also known to mediate the dimerization of half-proteasomes in eukaryotes (Chen & Hochstrasser, 1996). In bacteria, beta propeptides have been shown to assist in the formation of alpha rings. In addition to the multifaceted roles played by propeptide sequences in eukaryotes, our study revealed functional significance of the different regions of bacterial propeptide sequences. The presence of a glycine rich region (referred as Region III) seen upon multiple sequence alignment prompted us to test its influence on the confirmation and function of beta subunits. Indeed, the function of Region III of propeptides had functions parallel to the role of eukaryotic chaperone Ump1 in CP

maturation. Our molecular dynamics simulations, confirmed by biochemical studies, showed that mutations in Region III that affected its overall length and %glycine residue, can inhibit HP dimerization. Our results show an important role of the glycine rich region in the assembly process and provide evidence for an evolutionary pressure to conserve the overall flexibility in this region.

Figure 4-1 Role of propeptides in regulation of HP dimerization in prokaryotes is orthologous to the role of Ump1 in eukaryotes.



The role of propeptides in CP assembly is similar to the role played by well-studied eukaryotic CP chaperone Ump1, where it is known to inhibit the premature dimerization of HPs (Figure 4.1). Ump1 binds immature CPs before beta ring formation starts and facilitates the incorporation of beta subunits during CP maturation (Ramos et al., 1998). Maturation of eukaryotic CP involves degradation of Ump1 trapped in the catalytic chamber. This is accompanied by a structural change in CP, which causes the release of assembly chaperone Pba1/Pba2, and promotes association of regulatory particle to form a functional 26S proteasome (Wani et al., 2015). The degradation of Ump1 by mature active CP is also similar to the degradation of propeptide sequence upon maturation and activation of bacterial CP.

Whether or not the maturation of pre-holoenzyme into holoenzyme as seen in bacteria also involves a structure based activation switch still needs to be determined. Our data shows the presence of two distinct CP populations comprising of pre-holoenzyme and holoenzyme, respectively, and supports the idea of an activation switch like mechanism in bacteria. Further structural analysis would help understand if this

activation switch also involves a eukaryotic like structural change during CP activation. We can envision a mechanism where the presence of propeptide could cause the beta ring to be present in an open confirmation where the central chamber accommodates the flexible loop bearing propeptide sequence. Following the cooperative activation process and the catalytic removal of propeptide sequence, a conformational change in beta ring can take place which in turn may affect the overall CP structure. Structural analysis of bacterial CP would allow us to identify any structural changes that might occur during CP maturation and would also assist in identifying novel allosteric interactions that would allow for differential association of mature CP with proteasomal activators.

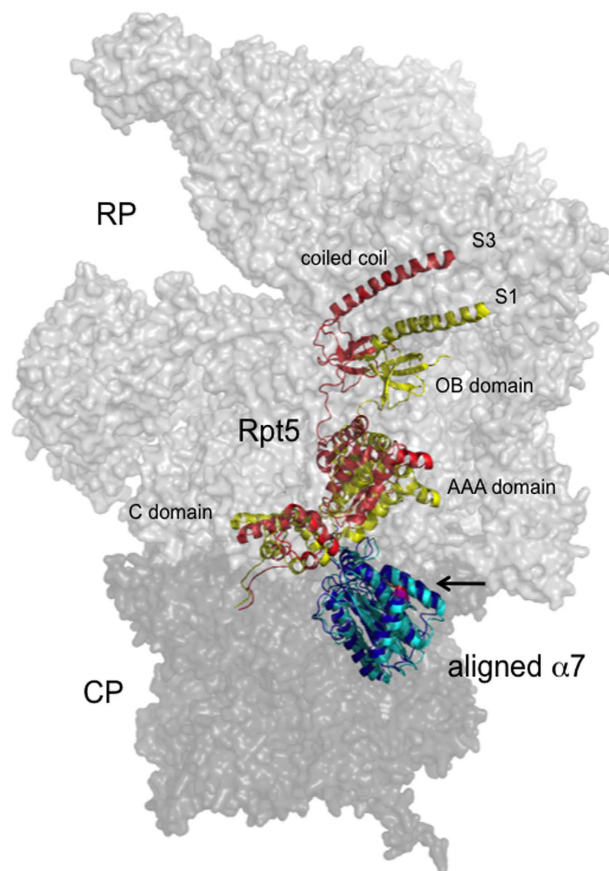
Ecm29 mediated interaction between RP and CP

The assembly of a functional eukaryotic proteasome holoenzyme requires both RP and CP. This RP-CP interaction is tightly regulated by a number of cellular conditions including salt concentrations, pH, metabolic state, and accumulation of degradation products within the CP (Bajorek et al., 2003; Leggett et al., 2005). In addition to this, other factors affecting RP-CP stability include the availability of nucleotides, post translational modifications of proteasome subunits, RP and CP assembly chaperones, CP interacting proteins like Blm10 and PA28, RP interacting proteins like PAAF-1 and proteasome holoenzyme interacting factors like Ecm29 (Kleijnen et al., 2007; Lehmann et al., 2010; Satoh et al., 2001; Smith et al., 2006; Wani et al., 2016). Studies on RP-CP interactions have revealed allosteric interactions between the RP and active sites within CP (Kleijnen et al., 2007). Chemical inhibitors that bind CP active sites also stabilize of RP-CP interactions providing evidence for the flow of information from the active sites to the RP interaction surface. It has also been hypothesized that CP can function as a template for the assembly of RP base. This idea was put forth when studies showed accumulation of RP base intermediates upon CP assembly defects or under conditions that affects the docking of Rpt subunits C terminal tails in CP (Kusmierczyk et al., 2008; Park et al., 2009). It was later shown that the RP base assembly defects could be rescued upon the deletion of Ecm29 in strains lacking Ump1 (Park et al., 2011).

Chapter 3 in this dissertation provides an additional layer to the role of Ecm29 in regulating RP-CP association. The precise role of Ecm29 in proteasome stress response has remained elusive. Ecm29 has been shown to stabilize proteasomes under nucleotide deficit conditions, however, it has also been shown to cause destabilization of RP-CP complexes under oxidative stress conditions (De La Mota-peynado et al., 2013; Haratake et al., 2016). Our studies in chapter 3 show a role of Ecm29 in stabilizing proteasomes under stress conditions. As opposed to the disassembly of RP-CP complexes as seen under oxidative stress, our results show stabilization of 26S in the presence of Ecm29. This apparent contradiction in results likely indicates there are some important physiological or cellular factors that regulate Ecm29, but remain unknown. Although our results contradict the studies showing Ecm29 dependent disassembly of RP-CP complexes, the idea of Ecm29 dependent regulation to survive under stress still remains valid (Wang et al., 2017). It will be interesting to see if the level of Ecm29 is regulated upon oxidative stress for better survival.

Studies from our lab have shown that the binding of Ecm29 to CP depends on the phosphorylation of C-terminal tail of $\alpha 7$ (P.S. Wani et al., 2016). The selectivity of Ecm29 binding to proteasomes was previously suggested to depend on the nucleotide status of the RP. The nucleotide state of the base subunits causes structural changes in mature proteasomes that were defined by cryo-EM studies as s1, s2 and s3 conformations (Schweitzer et al., 2014). Since the relative orientation of Rpt5 and $\alpha 7$ changes under these conformations as shown in figure 4.2, it can be envisioned that Ecm29 favors binding proteasomes in certain conformations over others (P.S. Wani et al., 2016).

Figure 4-2 Structural changes in the 26 S proteasome at the Ecm29 binding region.



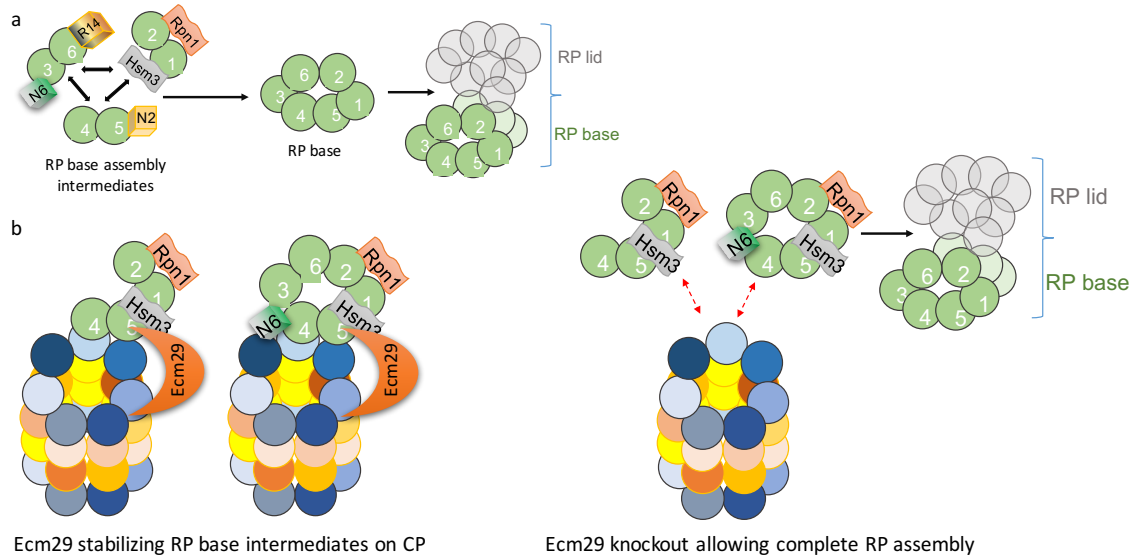
Alignment of $\alpha 7$ domain of 26S proteasomes in S1 (ground, 4CR2) and S3 (active, ATPyS-bound, 4CR4) states and the change in relative orientation of coiled coil domain of Rpt5 subunit. Only the $\alpha 7$ and Rpt5 subunits are depicted in color. Picture adapted from Wani et al., 2016.

Our results from chapter 3 provide evidence in support of specificity of Ecm29 towards proteasomes under specific conformations. The analysis of yeast strains bearing EtoQ mutant Rpt ATPases incapable of ATP hydrolysis suggested association of Ecm29 to proteasomes in an active ATP bound state. *In vitro* studies using proteasomes purified from Ecm29 knockout and over expressing strains also supported Ecm29 dependent stabilization of proteasomes in an active state. Whether Ecm29 binds the proteasomes already present in ATP bound state or if it induces a change in the nucleotide state of the Rpt ATPases upon binding, still needs to be determined. Our results unveiled a role of Ecm29 in stabilizing RP chaperones, Nas6 and Hsm3, on RP-CP complexes. We envision a mechanism where RP intermediates, accumulated in an Ump1 knockout strain, can interact with CP and form unstable RP intermediates-CP complexes. Ump1 knockout

causes defects in the activation of CP by affecting the autocatalysis of propeptides; this can cause structural changes in CP and, thus, affect CP-RP interactions (Li et al., 2015). Ecm29, as a proteasome quality control factor, recognizes and binds these non-native complexes. However, the fate of these complexes still needs to be determined. We observed dramatic reduction in the formation of RP or CP granules under different conditions in an Ump1 knockout strain. These results refute a model in which Ecm29 binds and sequesters these complexes to target them for degradation through autophagy. An alternative mechanism by which Ecm29 can regulate RP-CP interactions is by binding and stabilizing RP intermediates-CP complexes and allowing for the completion of RP base assembly process. Mass spec analysis of these complexes obtained on native gels can help identify if these complexes contain RP intermediates bound to CP or if they contain complete RP along with the chaperones bound to CP. The localization of the complexes on native gel suggests that the complex contains chaperones bound to 26S proteasomes. Although, it is also possible that our native gel analysis failed to capture RP intermediates-CP complexes since native gel does not separate native complexes based on their size.

Finally, figure 4.3 summarizes our model for the role of Ecm29 in stabilizing chaperones on RP-CP complexes. Ecm29 has been shown to function ambiguously in mediating the proteasome stress response by both stabilizing or mediating the disassembly of proteasomes under specific cellular conditions. We identified novel complexes stabilized by Ecm29 that contained RP chaperones bound to 26S proteasomes. Based on our model, Ecm29 stabilizes RP base intermediates on CP and allows for the completion of RP base assembly. Knocking out Ecm29 allows the disassembly of unstable RP intermediates-CP complexes, and leads to the accumulation of free RP complexes.

Figure 4-3 Rescue of RP-base assembly by Ecm29.s



(a) Summary for the assembly of RP. (b) Model summarizing the role of Ecm29 in stabilizing different RP intermediates on CP that causes accumulation of free lid. As shown on right, deletion of Ecm29 allows for the unstable complexes to fall apart and allows for the association of base and lid to form 19S RP.

References- Chapter 1 to 4

- 1996 Intramolecular processing of beta .pdf. (n.d.).
- Arendt, C., & Hochstrasser, M. (1997). Identification of the yeast 20S proteasome catalytic centers and subunit interactions required for active-site formation, *94*(July), 7156–7161.
- Arendt, C. S., & Hochstrasser, M. (1999). Eukaryotic 20S proteasome catalytic subunit propeptides prevent active site inactivation by N-terminal acetylation and promote particle assembly. *EMBO Journal*, *18*(13), 3575–3585. <https://doi.org/10.1093/emboj/18.13.3575>
- Bajorek, M., Finley, D., & Glickman, M. H. (2003). Proteasome Disassembly and Downregulation Is Correlated with Viability during Stationary Phase, *13*, 1140–1144. <https://doi.org/10.1016/S>
- Barraault, M., Richet, N., Godard, C., Murciano, B., Le, B., & Rousseau, E. (2012). Dual functions of the Hsm3 protein in chaperoning and scaffolding regulatory particle subunits during the proteasome assembly, *109*(17), 6368–6369. <https://doi.org/10.1073/pnas.1116538109>
- Bashore, C., Dambacher, C. M., Goodall, E. A., Matyskiela, M. E., Lander, G. C., & Martin, A. (2016). Ubp6 deubiquitinase controls conformational dynamics and substrate degradation of the 26S proteasome, *22*(9), 712–719. <https://doi.org/10.1038/nsmb.3075.Ubp6>
- Baumeister, W., Walz, J., Zühl, F., & Seemüller, E. (1998). The proteasome: Paradigm of a self-compartmentalizing protease. *Cell*, *92*(3), 367–380. [https://doi.org/10.1016/S0092-8674\(00\)80929-0](https://doi.org/10.1016/S0092-8674(00)80929-0)
- Becker, S. H., & Darwin, K. H. (2017). Bacterial Proteasomes: Mechanistic and Functional Insights. *Microbiology and Molecular Biology Reviews : MMBR*, *81*(1), e00036-16. <https://doi.org/10.1128/MMBR.00036-16>
- Beckwith, R., Estrin, E., Worden, E. J., & Martin, A. (2013). Reconstitution of the 26S proteasome reveals functional asymmetries in its AAA + unfoldase. *Nature Publishing Group*, *20*(10), 1164–1172. <https://doi.org/10.1038/nsmb.2659>
- Beckwith, R., Estrin, E., Worden, E. J., & Martin, A. (2014). Reconstitution of the 26S proteasome reveals functional asymmetries in its AAA+ unfoldase, *20*(10), 1164–1172. <https://doi.org/10.1038/nsmb.2659.Reconstitution>
- Bochtler, M., Ditzel, L., Groll, M., & Huber, R. (1999). The proteasome, 295–317.
- Bochtler, M., Hartmann, C., Song, H. K., Bourenkov, G. P., Bartunik, H. D., & Huber, R. (2000). The structures of HslU and the ATP- dependent protease HslU – HslV, *403*(February), 13091–13096.
- Braun, B. C., Glickman, M., Kraft, R., Dahlmann, B., Kloetzel, P., Finley, D., & Schmidt, M. (1999). The base of the proteasome regulatory particle exhibits chaperone-like activity,

1(August), 221–226.

- Budenholzer, L., Cheng, C. L., Li, Y., & Hochstrasser, M. (2017). Proteasome Structure and Assembly. *Journal of Molecular Biology*, 429(22), 3500–3524. <https://doi.org/10.1016/j.jmb.2017.05.027>
- Burns, K. E., Pearce, M. J., & Darwin, K. H. (2010). Prokaryotic Ubiquitin-Like Protein Provides a Two-Part Degron to Mycobacterium Proteasome Substrates, 192(11), 2933–2935. <https://doi.org/10.1128/JB.01639-09>
- Butler, S. M., Festa, R. A., Pearce, M. J., & Darwin, K. H. (2006). Self-compartmentalized bacterial proteases and pathogenesis, 60, 553–562. <https://doi.org/10.1111/j.1365-2958.2006.05128.x>
- Chen, P., & Hochstrasser, M. (1996). Autocatalytic subunit processing couples active site formation in the 20S proteasome to completion of assembly. *Cell*, 86(6), 961–972. [https://doi.org/10.1016/S0092-8674\(00\)80171-3](https://doi.org/10.1016/S0092-8674(00)80171-3)
- Coux, O., & Goldberg, A. L. (1996). STRUCTURE AND FUNCTIONS OF THE 20s AND 26s.
- Darwin, K. H. (2009). Prokaryotic ubiquitin-like protein (Pup), proteasomes and pathogenesis. *Nature Publishing Group*, 7(7), 485–491. <https://doi.org/10.1038/nrmicro2148>
- Darwin, K. H., & Ehrt, S. (2003). The Proteasome of Mycobacterium tuberculosis Is Required for Resistance to Nitric Oxide, 302(December), 1963–1967.
- De La Mota-peynado, A., Lee, S. Y., Pierce, B. M., Wani, P., Singh, C. R., & Roelofs, J. (2013). The Proteasome-associated Protein Ecm29 Inhibits Proteasomal ATPase Activity and in Vivo Protein Degradation by the Proteasome *, 288(41), 29467–29481. <https://doi.org/10.1074/jbc.M113.491662>
- Dick, T. P., Nussbaum, A. K., Deeg, M., Heinemeyer, W., Groll, M., Schirle, M., ... Tu, D.-. (1998). Contribution of Proteasomal B-Subunits to the Cleavage of Peptide Substrates Analyzed with Yeast Mutants *, 273(40), 25637–25646.
- Ditzel, L., Huber, R., Mann, K., Heinemeyer, W., Wolf, D. H., & Groll, M. (1998). Conformational Constraints for Protein Self-cleavage in the Proteasome, 1187–1191.
- Djuranovic, S., Hartmann, M. D., Habeck, M., Ursinus, A., Zwickl, P., Lupas, A. N., & Zeth, K. (2009). Structure and Activity of the N-Terminal Substrate Recognition Domains in Proteasomal ATPases, 580–590. <https://doi.org/10.1016/j.molcel.2009.04.030>
- Ehlinger, A., & Walters, K. J. (2014). Structural Insights into Proteasome Activation by the 19S Regulatory Particle, 52(21). <https://doi.org/10.1021/bi400417a.Structural>
- Elsasser, S., Gali, R. R., Schwickart, M., Larsen, C. N., Leggett, D. S., Müller, B., ... Cell, M. (2003). Proteasome subunit Rpn1 binds ubiquitin-like protein domains, 4(September 2002). <https://doi.org/10.1038/ncb845>

- Elsasser, S., Schmidt, M., & Finley, D. (2005). Characterization of the proteasome using native gel electrophoresis. *Methods in Enzymology*, 398(05), 353–363. [https://doi.org/10.1016/S0076-6879\(05\)98029-4](https://doi.org/10.1016/S0076-6879(05)98029-4)
- Emmerich, N. P. N., Nussbaum, A. K., Stevanovic, S., Priemer, M., & Tu, D.-. (2000). The Human 26 S and 20 S Proteasomes Generate Overlapping but Different Sets of Peptide Fragments from a Model Protein Substrate *, 275(28), 21140–21148. <https://doi.org/10.1074/jbc.M000740200>
- Festa, R. A., Jones, M. B., Butler-wu, S., Sinsimer, D., Gerads, R., Bishai, W. R., ... Darwin, K. H. (2011). A novel copper-responsive regulon in Mycobacterium tuberculosis, 79(October 2010), 133–148. <https://doi.org/10.1111/j.1365-2958.2010.07431.x>
- Finley, D. (2009). Recognition and Processing of Ubiquitin-Protein Conjugates by the Proteasome. <https://doi.org/10.1146/annurev.biochem.78.081507.101607>
- Finley, D., Chen, X., Walters, K. J., & Section, P. (2017). Gates, channels, and switches: elements of the proteasome machine, 41(1), 77–93. <https://doi.org/10.1016/j.tibs.2015.10.009.Gates>
- Fukunaga, K., Kudo, T., Toh-e, A., Tanaka, K., & Saeki, Y. (2010). Dissection of the assembly pathway of the proteasome lid in Saccharomyces cerevisiae. *Biochemical and Biophysical Research Communications*, 396(4), 1048–1053. <https://doi.org/10.1016/j.bbrc.2010.05.061>
- Funakoshi, M., Jr, R. J. T., Kobayashi, H., & Hochstrasser, M. (2009). Multiple Assembly Chaperones Govern Biogenesis of the Proteasome Regulatory Particle Base. *Cell*, 137(5), 887–899. <https://doi.org/10.1016/j.cell.2009.04.061>
- Gaczynska, M., Rockt, K. L., & Goldberg, A. L. (1993). γ · Interferon and expression of MHC genes regulate peptide hydrolysis by proteasomes, 365(September), 16–19.
- Gandotra, S., Schnappinger, D., Monteleone, M., Hillen, W., & Ehrt, S. (2011). In vivo gene silencing identifies the Mycobacterium tuberculosis proteasome as essential for persistence in mice, 13(12), 1515–1520. <https://doi.org/10.1038/nm1683.In>
- Gille, C., Goede, A., Schlo, C., Preißner, R., Kloetzel, P., & Go, U. B. (2003). A Comprehensive View on Proteasomal Sequences : Implications for the Evolution of the Proteasome, 2836(02), 1437–1448. [https://doi.org/10.1016/S0022-2836\(02\)01470-5](https://doi.org/10.1016/S0022-2836(02)01470-5)
- Gillette, T. G., Kumar, B., Thompson, D., Slaughter, C. A., & Demartino, G. N. (2008). Differential Roles of the COOH Termini of AAA Subunits of PA700 (19 S Regulator) in Asymmetric Assembly and Activation of the 26 S Proteasome * □, 283(46), 31813–31822. <https://doi.org/10.1074/jbc.M805935200>
- Glickman, M. H., Rubin, D. M., Fried, V. A., & Finley, D. (1998). The Regulatory Particle of the Saccharomyces cerevisiae Proteasome, 18(6), 3149–3162.
- Groll, M., Ditzel, L., Lowe, J., Stock, D., Bochtler, M., Bartunikt, H. D., & Huber, R. (1997).

- Structure of 20S proteasome from yeast at 2.4Å resolution, *38613*(April).
- Groll, M., Heinemeyer, W., Ger, Si. J., Ullrich, T., Bochtler, M., Wolf, D., & Huber, A. (1999). The catalytic sites of 20S proteasomes and their role in subunit maturation : A mutational and crystallographic study, *96*(September), 10976–10983.
- Hanssum, A., Zhong, Z., Rousseau, A., Krzyzosiak, A., Sigurdardottir, A., & Bertolotti, A. (2014). An Inducible Chaperone Adapts Proteasome Assembly to Stress. *Molecular Cell*, *55*(4), 566–577. <https://doi.org/10.1016/j.molcel.2014.06.017>
- Haratake, K., Sato, A., & Chiba, T. (2016). KIAA0368 -deficiency affects disassembly of 26S proteasome under oxidative stress condition, *159*(6), 609–618. <https://doi.org/10.1093/jb/mvw006>
- He, J., Kulkarni, K., Fonseca, P. C. A., Krutauz, D., Glickman, M. H., Barford, D., & Morris, E. P. (2012). The Structure of the 26S Proteasome Subunit Rpn2 Reveals Its PC Repeat Domain as a Closed Toroid of Two Concentric α -Helical Rings. *Structure/Folding and Design*, *20*(3), 513–521. <https://doi.org/10.1016/j.str.2011.12.015>
- Heinemeyer, W., Fischer, M., Krimmer, T., Stachon, U., & Wolf, D. H. (1997). The Active Sites of the Eukaryotic 20 S Proteasome and Their Involvement in Subunit Precursor Processing *, *272*(40), 25200–25209.
- Hirano, Y., Kaneko, T., Okamoto, K., Bai, M., Yashiroda, H., Furuyama, K., ... Murata, S. (2008). Dissecting b -ring assembly pathway of the mammalian 20S proteasome, *27*(16), 2204–2213. <https://doi.org/10.1038/emboj.2008.148>
- Howell, L. A., Tomko, R. J., & Kusmierczyk, A. R. (2017). Putting it all together : intrinsic and extrinsic mechanisms governing proteasome biogenesis. <https://doi.org/10.1007/s11515-017-1439-1>
- Hu, G., Lin, G., Wang, M., Dick, L., Xu, R. M., Nathan, C., & Li, H. (2006). Structure of the Mycobacterium tuberculosis proteasome and mechanism of inhibition by a peptidyl boronate. *Molecular Microbiology*, *59*(5), 1417–1428. <https://doi.org/10.1111/j.1365-2958.2005.05036.x>
- Huber, E. M., Heinemeyer, W., Li, X., Arendt, C. S., Hochstrasser, M., & Groll, M. (2016). A unified mechanism for proteolysis and autocatalytic activation in the 20S proteasome. *Nature Communications*, *7*, 1–10. <https://doi.org/10.1038/ncomms10900>
- Imai, K., & Mitaku, S. (2005). Mechanisms of secondary structure breakers in soluble proteins, *1*, 55–65. <https://doi.org/10.2142/biophysics.1.55>
- Imkamp, F., Rosenberger, T., Striebel, F., Keller, P. M., Amstutz, B., Sander, P., & Weber-ban, E. (2010). Deletion of dop in Mycobacterium smegmatis abolishes pupylation of protein substrates in vivo, *75*(January), 744–754. <https://doi.org/10.1111/j.1365-2958.2009.07013.x>
- Isono, E., Nishihara, K., Saeki, Y., Yashiroda, H., Kamata, N., Ge, L., ... Toh-e, A. (2007). The

- Assembly Pathway of the 19S Regulatory Particle of the Yeast 26S Proteasome □, 18(February), 569–580. <https://doi.org/10.1091/mbc.E06>
- Jastrab, J. B., & Darwin, H. K. (2015). Bacterial Proteasomes. *Annual Review of Microbiology*, 18, 109–127. <https://doi.org/10.1146/annurev-micro-091014-104201>. Bacterial
- Kaczowka, S. J., & Maupin-Furlow, J. A. (2003). Subunit topology of two 20S proteasomes from *Haloferax volcanii*. *Journal of Bacteriology*, 185(1), 165–174. <https://doi.org/10.1128/JB.185.1.165-174.2003>
- Kaneko, T., Hamazaki, J., Iemura, S., Sasaki, K., Furuyama, K., & Natsume, T. (2009). Assembly Pathway of the Mammalian Proteasome Base Subcomplex Is Mediated by Multiple Specific Chaperones. *Cell*, 137(5), 914–925. <https://doi.org/10.1016/j.cell.2009.05.008>
- Kim, Y., Li, X., Thompson, D., & Demartino, G. N. (2013). ATP Binding by Proteasomal ATPases Regulates Cellular Assembly and Substrate-induced Functions of the 26 S, 288(5), 3334–3345. <https://doi.org/10.1074/jbc.M112.424788>
- Kleijnen, M. F., Roelofs, J., Park, S., Hathaway, N. A., Glickman, M., King, R. W., & Finley, D. (2007). Stability of the proteasome can be regulated allosterically through engagement of its proteolytic active sites, 14(12). <https://doi.org/10.1038/nsmb1335>
- Kloetzel, P. M. (2004). Generation of major histocompatibility complex class I antigens : functional interplay between proteasomes and TPPII, 5(7), 661–669. <https://doi.org/10.1038/ni1090>
- Kock, M., Nunes, M. M., Hemann, M., Kube, S., Dohmen, R. J., Herzog, F., ... Wendler, P. (2015). Proteasome assembly from 15S precursors involves major conformational changes and recycling of the Pba1–Pba2 chaperone. <https://doi.org/10.1038/ncomms7123>
- Kunjappu, M. J., & Hochstrasser, M. (2014). Assembly of the 20S proteasome. *Biochimica et Biophysica Acta - Molecular Cell Research*, 1843(1), 2–12. <https://doi.org/10.1016/j.bbamcr.2013.03.008>
- Kusmierczyk, A. R., Kunjappu, M. J., Funakoshi, M., & Hochstrasser, M. (2008). A multimeric assembly factor controls the formation of alternative 20S proteasomes. *Nature Structural and Molecular Biology*, 15(3), 237–244. <https://doi.org/10.1038/nsmb.1389>
- Kusmierczyk, A. R., Kunjappu, M. J., Kim, R. Y., & Hochstrasse, M. (2011). terminal HbYX Motif for Proteasomal Precursor Binding, 18(5), 622–629. <https://doi.org/10.1038/nsmb.2027.A>
- Kusmierczyk, A. R., Kunjappu, M. J., Kim, R. Y., & Hochstrasser, M. (2011). A conserved 20S proteasome assembly factor requires a C-terminal HbYX motif for proteasomal precursor binding. *Nature Publishing Group*, 18(5), 622–629. <https://doi.org/10.1038/nsmb.2027>
- Kwon, Y. Do, Nagy, I., Adams, P. D., Baumeister, W., & Jap, B. K. (2004). Crystal structures of

- the Rhodococcus proteasome with and without its pro-peptides: Implications for the role of the pro-peptide in proteasome assembly. *Journal of Molecular Biology*, 335(1), 233–245. <https://doi.org/10.1016/j.jmb.2003.08.029>
- Lee, S. Y., Mota-peynado, A. D. La, & Roelofs, J. (2011). Loss of Rpt5 Protein Interactions with the Core Particle and Nas2 Protein Causes the Formation of Faulty Proteasomes That Are Inhibited by Ecm29 Protein * □, 286(42), 36641–36651. <https://doi.org/10.1074/jbc.M111.280875>
- Leggett, D. S., Glickman, M. H., & Finley, D. (2005). Purification of Proteasomes, Proteasome Subcomplexes, and Proteasome-Associated Proteins From Budding Yeast, 301(3).
- Lehmann, A., Niewianda, A., Jechow, K., Janek, K., & Enenkel, C. (2010). Ecm29 Fulfills Quality Control Functions in Proteasome Assembly. *Molecular Cell*, 38(6), 879–888. <https://doi.org/10.1016/j.molcel.2010.06.016>
- Levitt, M. (1978). Conformational Preferences of Amino Acids in Globular Proteins. <https://doi.org/10.1021/bi00613a026>
- Li, Xia, Li, Yanjie, Arendt, C. S., & Hochstrasser, and M. (2015). Distinct Elements in the Proteasomal beta-5 Subunit Propeptide Required for Autocatalytic Processing and Proteasome Assembly. *Journal of Biological Chemistry*. <https://doi.org/10.1074/jbc.M115.677047>
- Li, D., Li, H., Wang, T., Pan, H., Lin, G., & Li, H. (2010). Structural basis for the assembly and gate closure mechanisms of the Mycobacterium tuberculosis 20S proteasome. *EMBO Journal*, 29(12), 2037–2047. <https://doi.org/10.1038/emboj.2010.95>
- Li, F., Tian, G., Langager, D., Sokolova, V., Finley, D., & Park, S. (2016). Nucleotide-dependent switch in proteasome assembly mediated by the Nas6 chaperone. <https://doi.org/10.1073/pnas.1612922114>
- Li, X., Kusmierczyk, A. R., Wong, P., Emili, A., & Hochstrasser, M. (2007). β-Subunit appendages promote 20S proteasome assembly by overcoming an Ump1-dependent checkpoint. *EMBO Journal*, 26(9), 2339–2349. <https://doi.org/10.1038/sj.emboj.7601681>
- Lin, G., Hu, G., Tsu, C., Kunes, Y. Z., Li, H., Dick, L., ... Nathan, C. (2006). Mycobacterium tuberculosis prcBA genes encode a gated proteasome with broad oligopeptide specificity. *Molecular Microbiology*, 59(5), 1405–1416. <https://doi.org/10.1111/j.1365-2958.2005.05035.x>
- Luan, B., Huang, X., Wu, J., Mei, Z., Wang, Y., Xue, X., ... Wang, F. (2016). Structure of an endogenous yeast 26S proteasome reveals two major conformational states, 113(10), 1–6. <https://doi.org/10.1073/pnas.1601561113>
- Lupas, A., Frank, Z., Tamura, T., Wolf, S., & Baumeister, W. (1997). Eubacterial proteasomes, 125–131.

- Marques, A. nio J., Glanemann, C., Ramos, P. C., & Dohmen, R. J. rgen. (2007). The C-terminal Extension of the α_7 Subunit and Activator Complexes Stabilize Nascent 20 S Proteasomes and Promote Their Maturation *, 282(48), 34869–34876. <https://doi.org/10.1074/jbc.M705836200>
- Marshall, R. S., & Vierstra, R. D. (2018). Proteasome Storage Granules Protect Proteasomes from Autophagic Degradation upon Carbon Starvation.
- Matyskiela, M. E., Lander, G. C., & Martin, A. (2014). Conformational switching of the 26S proteasome enables substrate degradation, 20(7), 781–788. <https://doi.org/10.1038/nsmb.2616.Conformational>
- Maupin-Furlow, J. A. (2014). Prokaryotic Ubiquitin-Like Protein Modification. *Annual Review of Microbiology*, 68(1), 155–175. <https://doi.org/10.1146/annurev-micro-091313-103447>
- Maupin-Furlow, J. A., Kaczowka, S. J., Reuter, C. J., Zuobi-Hasona, K., & Gil, M. A. (2003). Archaeal proteasomes: Potential in metabolic engineering. *Metabolic Engineering*, 5(3), 151–163. [https://doi.org/10.1016/S1096-7176\(03\)00030-2](https://doi.org/10.1016/S1096-7176(03)00030-2)
- Maupin-Furlow, J. A., Humbard, M. A., Kirkland, P. A., Li, W., Reuter, C. J., Wright, A. J., & Zhou, G. B. T.-C. T. in D. B. (2006). Proteasomes from Structure to Function: Perspectives from Archaea (Vol. 75, pp. 125–169). Academic Press. [https://doi.org/https://doi.org/10.1016/S0070-2153\(06\)75005-0](https://doi.org/https://doi.org/10.1016/S0070-2153(06)75005-0)
- Mc Cormack, T., Baumeister, W., Grenier, L., Moomaw, C., Plamondon, L., Pramanik, B., ... Dick, L. (1997). Active site-directed inhibitors of Rhodococcus 20 S proteasome. Kinetics and mechanism. *Journal of Biological Chemistry*, 272(42), 26103–26109. <https://doi.org/10.1074/jbc.272.42.26103>
- Murata, S., Yashiroda, H., & Tanaka, K. (2009). Molecular mechanisms of proteasome assembly, 10(fEbRuARy). <https://doi.org/10.1038/nrm2630>
- Nussbaum, A. K., Dick, T. P., Keilholz, W., Ietz, K. L. D., Einemeyer, W. O. H., Roll, M. I. G., ... Uber, R. O. H. (1998). Cleavage motifs of the yeast 20S proteasome B subunits deduced from digests of enolase 1, 95(October), 12504–12509.
- Panfair, D., Ramamurthy, A., & Kusmierczyk, A. R. (2015). Alpha-ring Independent Assembly of the 20S Proteasome. *Scientific Reports*, 5, 1–14. <https://doi.org/10.1038/srep13130>
- Park, S., Kim, W., Tian, G., Gygi, S. P., & Finley, D. (2011). Structural Defects in the Regulatory Particle-Core Particle Interface of the Proteasome Induce a Novel Proteasome Stress Response * □, 286(42), 36652–36666. <https://doi.org/10.1074/jbc.M111.285924>
- Park, S., Li, X., Kim, H. M., Singh, C. R., Tian, G., Hoyt, M. A., ... Finley, D. (2013). Reconfiguration of the proteasome during chaperone-mediated assembly, 497(7450), 512–516. <https://doi.org/10.1038/nature12123.Reconfiguration>
- Park, S., Roelofs, J., Kim, W., Robert, J., Schmidt, M., Gygi, S. P., & Finley, D. (2009).

- Hexameric assembly of the proteasomal ATPases is templated through their C termini. *Nature*, 459(7248), 866–870. <https://doi.org/10.1038/nature08065>
- Pearce, M. J., Mintseris, J., Ferreyra, J., Gygi, S. P., & Heran, K. (2009). Ubiquitin-Like Protein Involved in the Proteasome Pathway of *Mycobacterium tuberculosis*, 322(5904), 1104–1107. <https://doi.org/10.1126/science.1163885>. Ubiquitin-Like
- Ramnath, G., Nagy, I., Sled, P., Steyaert, J., Förster, F., Bracher, A., & Baumeister, W. (2014). Crystal structure of the proteasomal deubiquitylation module Rpn8-Rpn11, 111(8), 2984–2989. <https://doi.org/10.1073/pnas.1400546111>
- Ramos, P. C., Höckendorff, J., Johnson, E. S., Varshavsky, A., & Dohmen, R. J. (1998). Ump1p is required for proper maturation of the 20S proteasome and becomes its substrate upon completion of the assembly. *Cell*, 92(4), 489–499. [https://doi.org/10.1016/S0092-8674\(00\)80942-3](https://doi.org/10.1016/S0092-8674(00)80942-3)
- Roelofs, J., Park, S., Haas, W., Tian, G., McAllister, F. E., Huo, Y., ... Finley, D. (2009). Chaperone-mediated pathway of proteasome regulatory particle assembly. *Nature*, 459(7248), 861–865. <https://doi.org/10.1038/nature08063>
- Roelofs, J., Supphiah, A., Waite, K. A., & Park, S. (2018). Native Gel Approaches in Studying Proteasome Assembly and Chaperones, 1844, 237–260.
- Rubin, D. M., Glickman, M. H., Larsen, C. N., Dhruvakumar, S., & Finley, D. (1998). Active site mutants in the six regulatory particle ATPases reveal multiple roles for ATP in the proteasome, 17(17), 4909–4919.
- Saeki, Y., Toh-e, A., Kudo, T., Kawamura, H., & Tanaka, K. (2009). Multiple Proteasome-Interacting Proteins Assist the Assembly of the Yeast 19S Regulatory Particle. *Cell*, 137(5), 900–913. <https://doi.org/10.1016/j.cell.2009.05.005>
- Satoh, K., Sasajima, H., Nyomura, K., Yokosawa, H., & Sawada, H. (2001). Assembly of the 26S Proteasome Is Regulated by Phosphorylation of the p45 / Rpt6 ATPase Subunit, 314–319. <https://doi.org/10.1021/bi001815n>
- Satoh, T., Saeki, Y., Hiromoto, T., Wang, Y., Uekusa, Y., Yagi, H., ... Kato, K. (2014). Structural Basis for Proteasome Formation Controlled by an Assembly Chaperone Nas2. *Structure/Folding and Design*, 22(5), 731–743. <https://doi.org/10.1016/j.str.2014.02.014>
- Savulescu, A. F., & Glickman, M. H. (2011). Proteasome Activator 200 : The HEAT is on, 3–10. <https://doi.org/10.1074/mcp.M110.006890>
- Schweitzer, A., Pfeifer, G., Plitzko, J. M., Unverdorben, P., Beck, F., Sled, P., ... Förster, F. (2014). Deep classification of a large cryo-EM dataset defines the conformational landscape of the 26S proteasome z, 111(15), 1–6. <https://doi.org/10.1073/pnas.1403409111>
- Sharon, M., Witt, S., Glasmacher, E., Baumeister, W., & Robinson, C. V. (2007). Mass spectrometry reveals the missing links in the assembly pathway of the bacterial 20 S

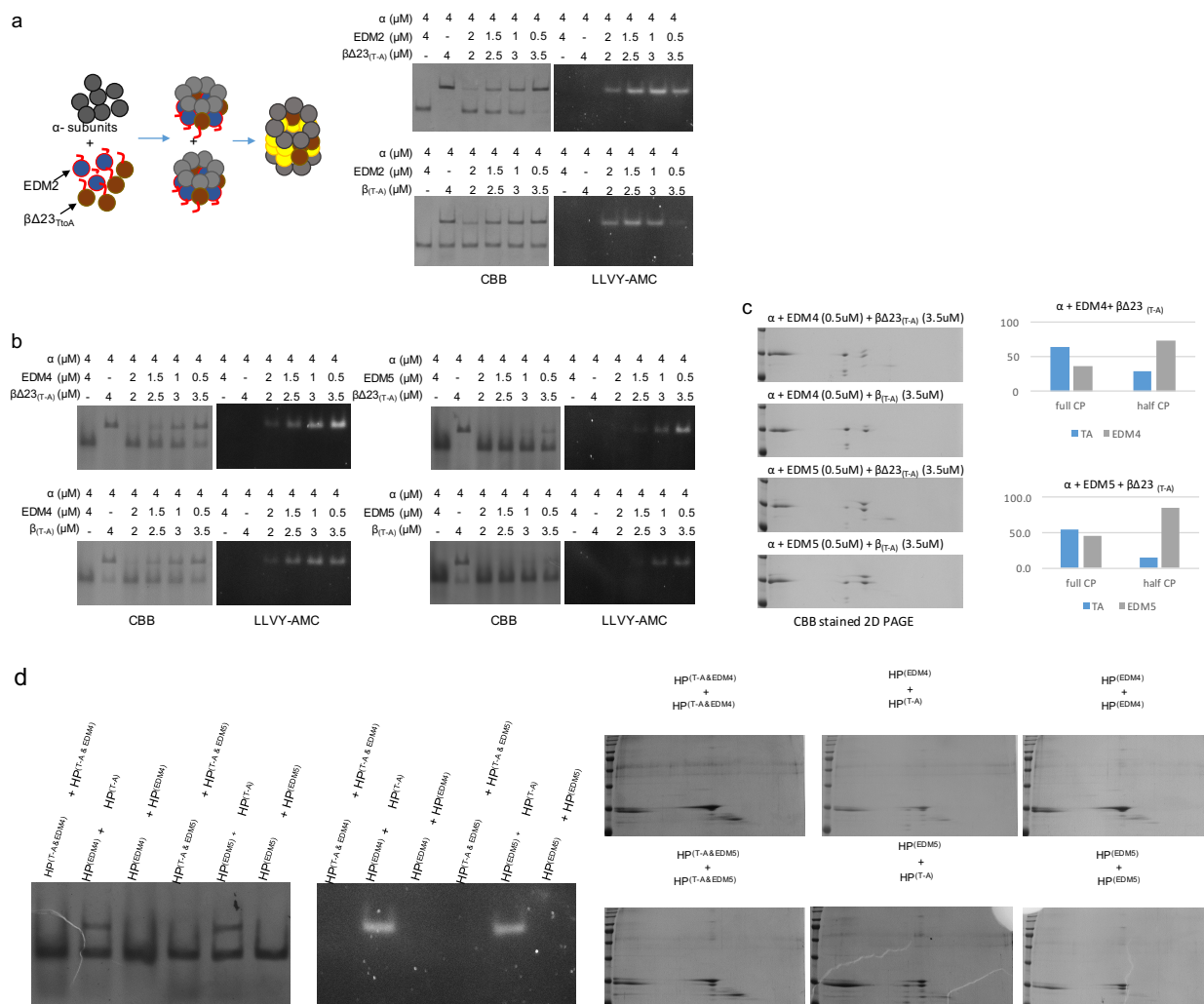
- proteasome. *Journal of Biological Chemistry*, 282(25), 18448–18457.
<https://doi.org/10.1074/jbc.M701534200>
- Shi, L., & Kay, L. E. (2013). Tracing an allosteric pathway regulating the activity of the HslV protease. <https://doi.org/10.1073/pnas.1318476111>
- Sikdar, A., Satoh, T., Kawasaki, M., & Kato, K. (2014). Biochemical and Biophysical Research Communications Crystal structure of archaeal homolog of proteasome-assembly chaperone PbaA. *Biochemical and Biophysical Research Communications*, 453(3), 493–497.
<https://doi.org/10.1016/j.bbrc.2014.09.114>
- Singh, C. R., Mehzaheen, N., Chowdhury, W. Q., Eric, S., & Battaile, P. (2014). 1.15 Å resolution structure of the proteasome- assembly chaperone Nas2 PDZ domain, 500, 418–423. <https://doi.org/10.1107/S2053230X14003884>
- Smith, D. M., Benaroudj, N., & Goldberg, A. (2006). Proteasomes and their associated ATPases : A destructive combination, 156, 72–83. <https://doi.org/10.1016/j.jsb.2006.04.012>
- Smith, D. M., Chang, S., Park, S., Finley, D., Cheng, Y., & Goldberg, A. (2008). Docking of the Proteasomal ATPases' C-termini in the 20S Proteasomes alpha Ring Opens the Gate for Substrate Entry David, 27(5), 731–744.
- Sokolova, V., Li, F., Polovin, G., & Park, S. (2015). Proteasome Activation is Mediated via a Functional Switch of the Rpt6 C-terminal Tail Following Chaperone-dependent Assembly. *Nature Publishing Group*, (July), 1–15. <https://doi.org/10.1038/srep14909>
- Soppa, J. (2010). Protein acetylation in archaea, bacteria, and eukaryotes. *Archaea*, 2010. <https://doi.org/10.1155/2010/820681>
- Stadtmueller, B. M., & Hill, C. P. (2012). NIH Public Access, 41(1), 8–19.
<https://doi.org/10.1016/j.molcel.2010.12.020> Proteasome
- Streich, F. C., & Lima, C. D. (2014a). Structural and Functional Insights to Ubiquitin-Like Protein Conjugation. <https://doi.org/10.1146/annurev-biophys-051013-022958>
- Streich, F. C., & Lima, C. D. (2014b). Structural and Functional Insights to Ubiquitin-Like Protein Conjugation. <https://doi.org/10.1146/annurev-biophys-051013-022958>
- Striebel, F., Imkamp, F., Sutter, M., Steiner, M., Mamedov, A., & Weber-ban, E. (2009). Bacterial ubiquitin-like modifier Pup is deamidated and conjugated to substrates by distinct but homologous enzymes, 16(6), 647–652. <https://doi.org/10.1038/nsmb.1597>
- Talleg, B. Le, Barrault, M.-B. 'né dicte, Courbeyrette, R. 'gis, Guérois, R. 'l, & Marsolier-Kergoat, Marie-Claude Peyroche, A. (2007). Article 20S Proteasome Assembly Is Orchestrated by Two Distinct Pairs of Chaperones in Yeast and in Mammals, 660–674.
<https://doi.org/10.1016/j.molcel.2007.06.025>
- Tamura, T., Nagy, I., Lupas, A., Lottspeich, F., Cejka, Z., Schoofs, G., ... Baumeister, W.

- (1995). The first characterization of a eubacterial proteasome: the 20S complex of *Rhodococcus*. *Current Biology*, 5(7), 766–774. [https://doi.org/10.1016/S0960-9822\(95\)00153-9](https://doi.org/10.1016/S0960-9822(95)00153-9)
- Terweij, M., Welsem, T. Van, Deventer, S. Van, Verzijlbergen, K. F., Menendez-benito, V., Ontoso, D., ... Neefjes, J. (2013). Recombination-Induced Tag Exchange (RITE) Cassette Series to Monitor Protein Dynamics in *Saccharomyces cerevisiae*, 3(August), 1261–1272. <https://doi.org/10.1534/g3.113.006213>
- Thrower, J. S., Hoffman, L., Rechsteiner, M., & Pickart, C. M. (2000). Recognition of the polyubiquitin proteolytic signal, 19(1), 94–102.
- Tomko, R. J., Funakoshi, M., Schneider, K., Wang, J., & Hochstrasser, M. (2010). Heterohexameric Ring Arrangement of the Eukaryotic Proteasomal ATPases : Implications for Proteasome Structure and Assembly. *Molecular Cell*, 38(3), 393–403. <https://doi.org/10.1016/j.molcel.2010.02.035>
- Tomko, R. J., & Hochstrasser, M. (2013). Molecular Architecture and Assembly of the Eukaryotic Proteasome. <https://doi.org/10.1146/annurev-biochem-060410-150257>
- Tomko, R. J., Taylor, D. W., Chen, Z. A., Wang, H., Hochstrasser, M., Tomko, R. J., ... Rappsilber, J. (2015). A Single a Helix Drives Extensive Remodeling of the Proteasome Lid and Completion of Regulatory Particle Assembly. *Cell*, 163(2), 432–444. <https://doi.org/10.1016/j.cell.2015.09.022>
- Varshavsky, A. (2012). The Ubiquitin System , an Immense Realm. <https://doi.org/10.1146/annurev-biochem-051910-094049>
- Verma, R., Chen, S., Feldman, R., Schieltz, D., Dohmen, J., & Deshaies, R. J. (2000). Proteasomal Proteomics : Identification of Nucleotide- sensitive Proteasome-interacting Proteins by Mass Spectrometric Analysis of Affinity-purified Proteasomes, 11(October), 3425–3439.
- Verzijlbergen, K. F., Menendez-benito, V., Welsem, T. Van, Deventer, S. J. Van, & Lindstrom, D. L. (2010). Recombination-induced tag exchange to track old and new proteins, 107(1). <https://doi.org/10.1073/pnas.0911164107>
- Wang, X., Chemmama, I. E., Yu, C., Huszagh, A., Xu, Y., Viner, R., ... Huang, L. (2017). *The proteasome-interacting Ecm29 protein disassembles the 26S proteasome in response to oxidative stress* (Vol. 292). <https://doi.org/10.1074/jbc.M117.803619>
- Wang, X., Yen, J., Kaiser, P., & Huang, L. (2011). Regulation of the 26S Proteasome Complex During Oxidative Stress, 3(151), 1–17. <https://doi.org/10.1126/scisignal.2001232.Regulation>
- Wani, P. S., Rowland, M. A., Ondracek, A., Deeds, E. J., & Roelofs, J. (2015). Maturation of the proteasome core particle induces an affinity switch that controls regulatory particle

- association. *Nature Communications*, 6, 1–11. <https://doi.org/10.1038/ncomms7384>
- Wani, P. S., Suppahia, A., Capalla, X., Ondracek, A., & Roelofs, J. (2016). Phosphorylation of the C-terminal tail of proteasome subunit α 7 is required for binding of the proteasome quality control factor Ecm29. *Nature Publishing Group*, (June), 1–14. <https://doi.org/10.1038/srep27873>
- Wani, P. S., Suppahia, A., Capalla, X., Ondracek, A., & Roelofs, J. (2016). Phosphorylation of the C-terminal tail of proteasome subunit α 7 is required for binding of the proteasome quality control factor Ecm29. *Scientific Reports*, 6. <https://doi.org/10.1038/srep27873>
- Witt, S., Kwon, Y. Do, Sharon, M., Felderer, K., Beuttler, M., Robinson, C. V., ... Jap, B. K. (2006). Proteasome Assembly Triggers a Switch Required for Active-Site Maturation. *Structure*, 14(7), 1179–1188. <https://doi.org/10.1016/j.str.2006.05.019>
- Yu, Z., Livnat-levanon, N., Kleifeld, O., Mansour, W., Nakasone, M. A., Carlos, A., ... Glickman, M. H. (2015). Base-CP proteasome can serve as a platform for stepwise lid formation, 1–14. <https://doi.org/10.1042/BSR20140173>
- Zühl, F., Seemüller, E., Golbik, R., & Baumeister, W. (1997). Dissecting the assembly pathway of the 20S proteasome. *FEBS Letters*, 418(1–2), 189–194. [https://doi.org/10.1016/S0014-5793\(97\)01370-7](https://doi.org/10.1016/S0014-5793(97)01370-7)
- Zühl, F., Tamura, T., Dolenc, I., Cejka, Z., Nagy, I., De Mot, R., & Baumeister, W. (1997). Subunit topology of the *Rhodococcus* proteasome. *FEBS Letters*, 400(1), 83–90. [https://doi.org/10.1016/S0014-5793\(96\)01403-2](https://doi.org/10.1016/S0014-5793(96)01403-2)
- Zwickl, P., Klein, J., & Baumeister, W. (1994). Critical elements in proteasome assembly. *Nature Structural Biology*, 1(11), 765–770. <https://doi.org/10.1038/nsb1194-765>

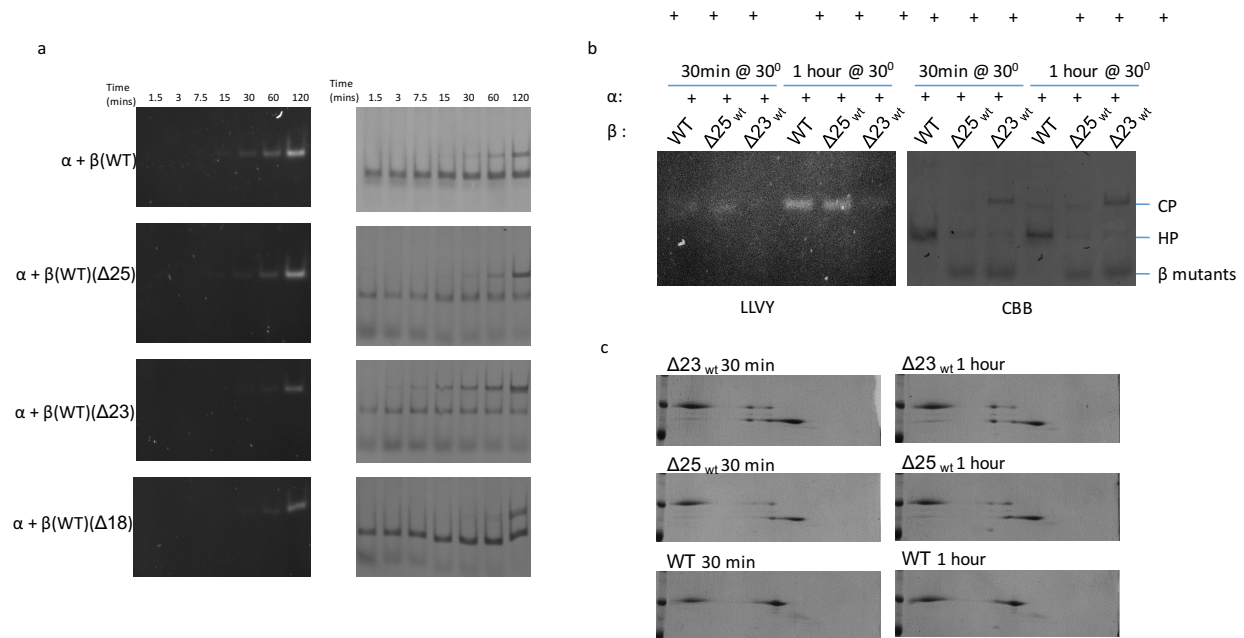
Appendix A - Supplementary information for chapter 1

Fig A.1: Incorporation of EDMs into mature CP upon mixing with β containing complete propeptide.



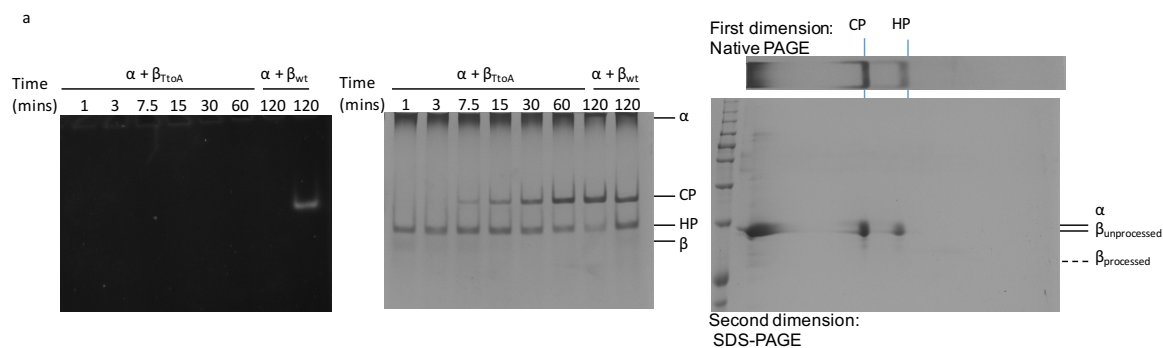
(a) Graphical representation of the assay (left), LLVY-AMC hydrolytic assay and CBB stain of gel containing samples obtained from reconstitution of α with ratios β_{EDM2} and β_{T10A} combined at indicated molar ratios for two hours at 30 degrees (right). (b) Reconstitution of EDM4/5 with $\beta\Delta 23$ (T-A/WT) at different molar ratios, analyzed by LLVY-AMC assay and CBB stain. (c) 2D analysis of respectively labelled samples were reconstituted at 30 degrees for 2 hours. (d) Quantification of the gels from (c). (e) 2D gel analysis of full CPs formed by mixing indicated HPs (HP (EDM4 or 5) or HP (T-A $\Delta 23$)), see figure 8 for reconstitution temperature used for HP dimerization.

Figure A.2: Reconstitution of 20S R.e. proteasome from α and β subunits.



(a) Equimolar amounts of wildtype α was mixed with specified β mutants for different time points in vitro as described previously. The reaction was then analyzed on Native-PAGE by LLVY assay and CBB staining. As seen in fig 4b, the truncated mutants dimerize faster and activate slower. (b) 4 μ M of wildtype α subunits were reconstituted with 4 μ M of specific β mutants as indicated. After reconstituting the mixture for 30 mins or 1 hour as indicated, the samples were analyzed for activity and dimerization using LLVY-AMC assay and CBB staining, respectively. (c) 2D analysis of a reconstitution mixture containing α and the indicated beta mutant subunits, reconstituted for 30 mins and 1 hour (see fig 3 c for 1D native of these gels).

Figure A.3: 2-D gel analysis of mixture of α and β_{TtoA} .

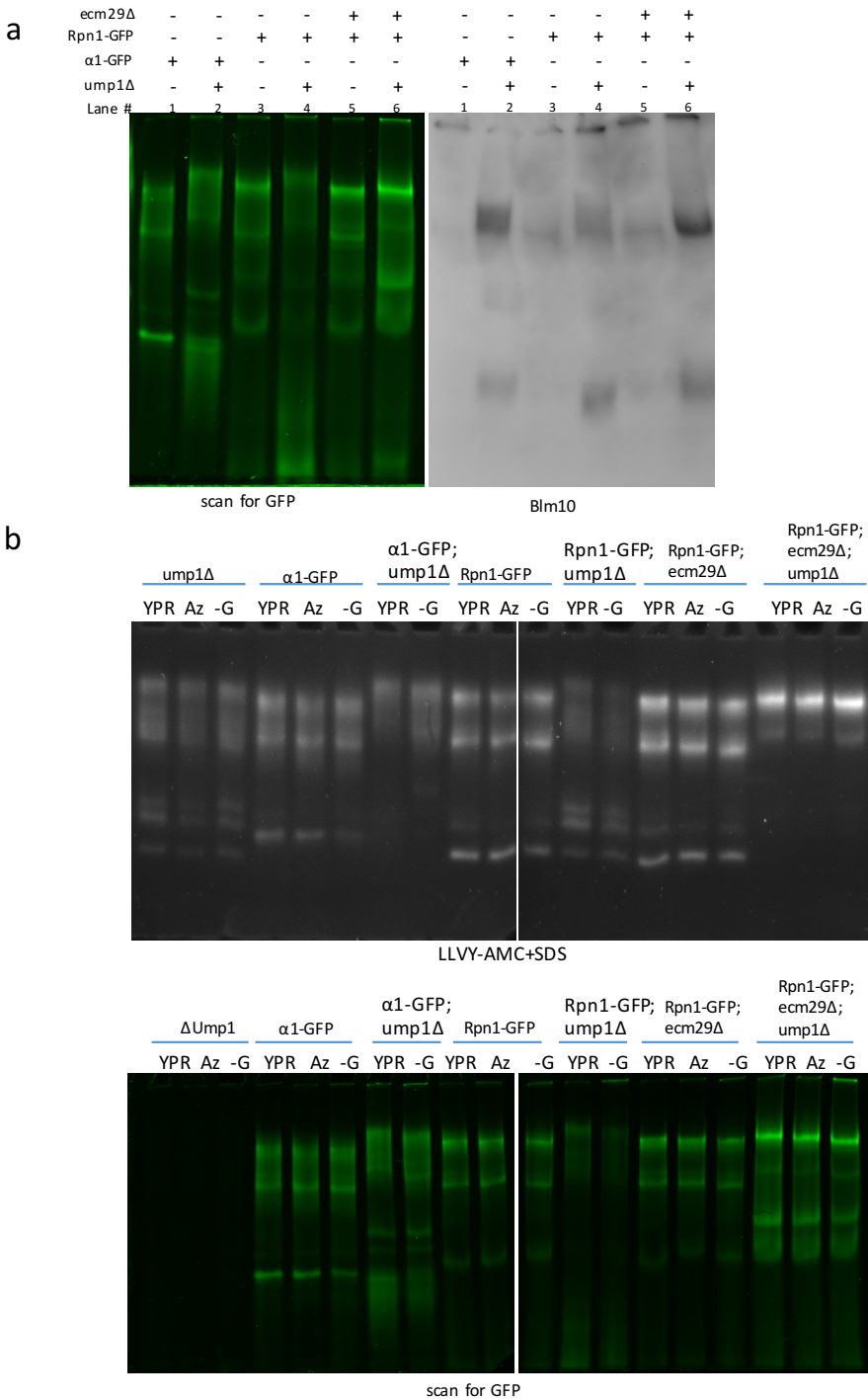


(a) Time-course analysis to monitor the assembly of α and β_{TtoA} reconstitution mixture on 1D native gel. Sample from 1-hour reconstitution was also separated on a second

dimension SDS-PAGE, as described before. Since β_{TtoA} is an inactive form of β , we do not see any band representing $\beta_{processed}$ in CP.

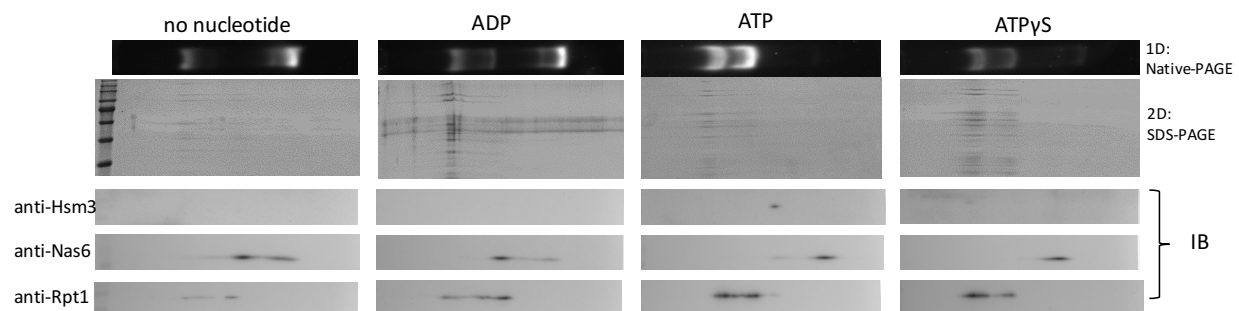
Appendix B - Supplementary information for chapter 3

Figure B.1. Ecm29 dependent rescue of RP base assembly defects.



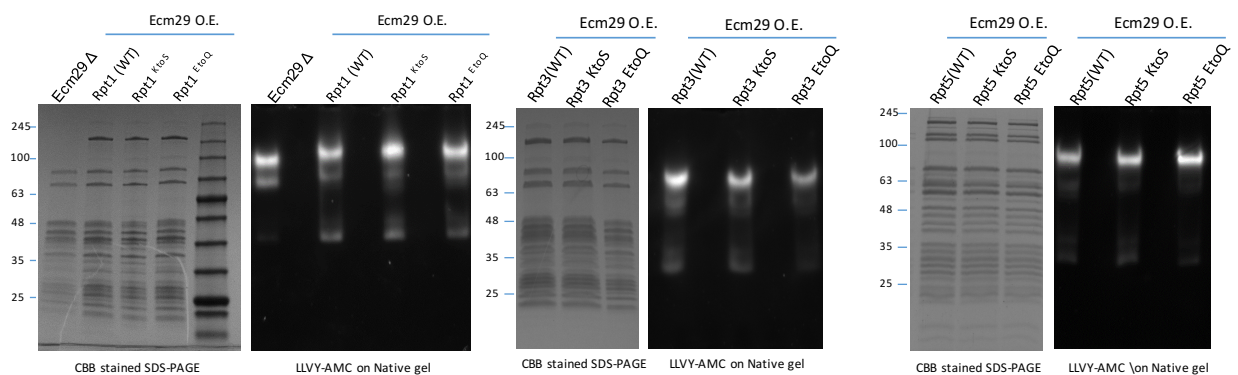
(a) Total cell lysates were resolved on native PAGE and scanned for GFP signal and transferred on PVDF membrane for immunoblot analysis. The immunoblot of the total lysates show enrichment of Blm10 on Ump1 Δ strains. Knocking out Ecm29 further increased the amounts of blm10-RP-CP complexes, lane 6. (b) The amounts and activity of 26S proteasomes from indicated strains was analyzed by native PAGE analysis of the lysates of cells collected after 24 hours of growth in yeast extract peptone raffinose (YPR), YPR+ sodium azide (Az) or under glucose starvation conditions (-glucose). Lysates from α 1-GFP and α 1-GFP Ump1 Δ strains were also included to see the stability of CP upon deleting Ump1.

Figure B.2. No chaperones were seen on 26S in wildtype strain independent of the nucleotide available during purification process.



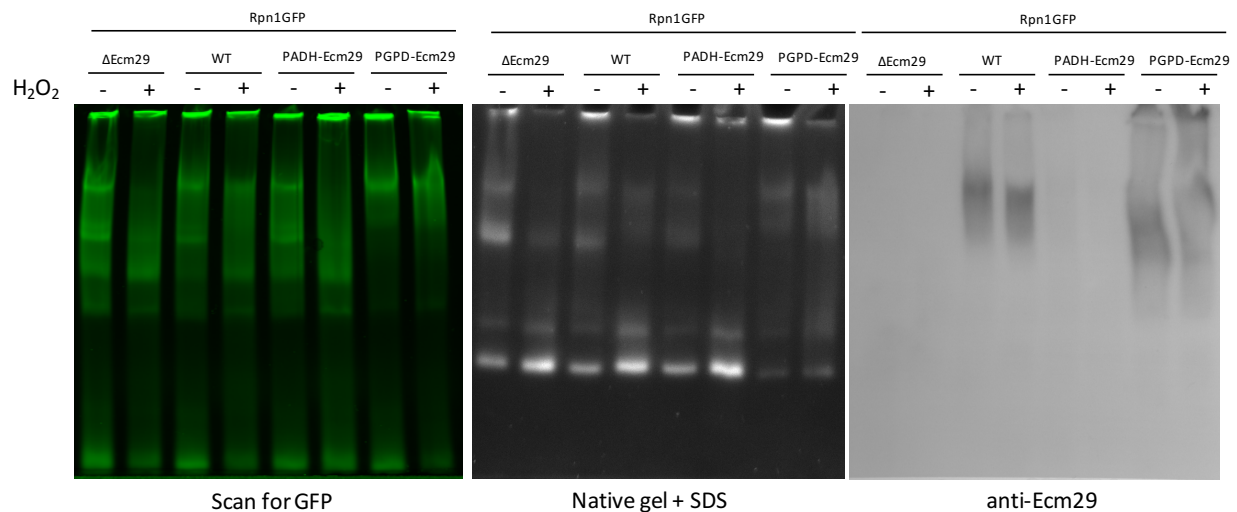
Proteasomes were purified from wildtype yeast strain using affinity tag ProA on Rpn11 (Rpn11-ProA tag). The purifications were carried out in the presence of ADP, ATP or ATPγS or in the absence of any nucleotide. The purified proteasomes were analyzed by previously described 2D approach and the presence of Hsm3 and Nas6 chaperones in purified material was analyzed by immunoblotting.

Figure B.3. Single-base ATPase mutation does not affect the overall activity and stability of 26S proteasomes.



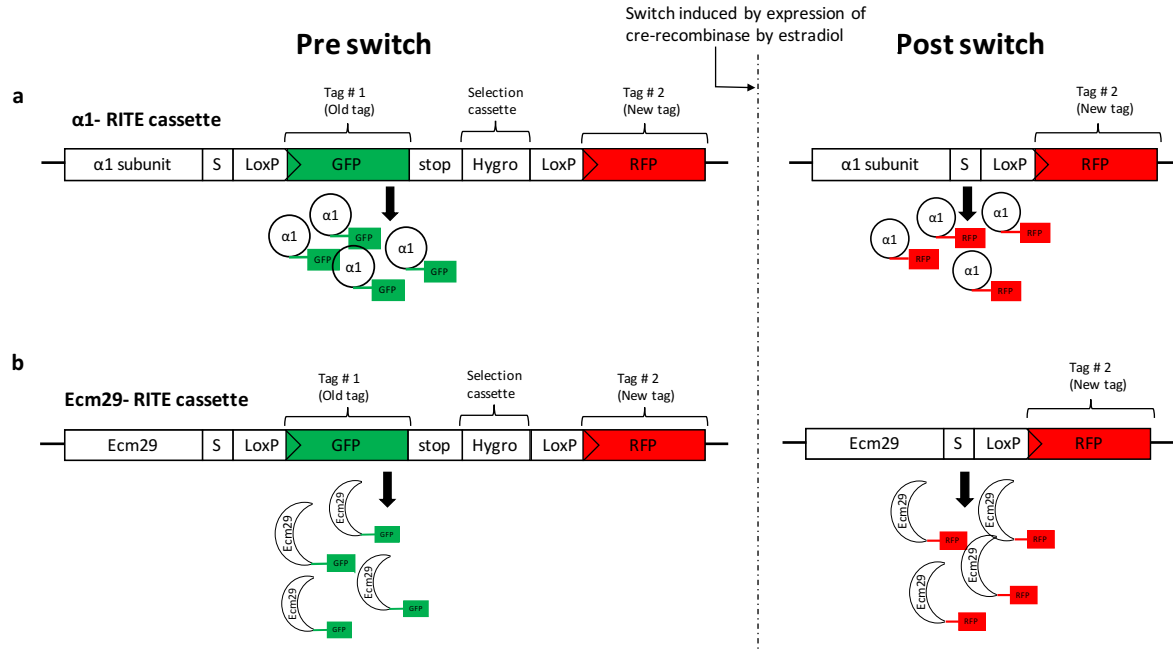
1D SDS- PAGE and 1D native page analysis of proteasome preps purified from indicated strains. SDS-PAGE was stained with CBB stain to visualize the proteasomal subunits purified. The Native gels were visualized by adding fluorogenic proteasome substrate LLVY-AMC in the presence of SDS to visualize free CP as well. Lane 1 contains proteasomes purified from *Ecm29Δ* strain and thus do not show a shift in migration of RP2-CP or RP-CP complexes as seen on all other strains over expressing *Ecm29*.

Figure B.4. Ecm29 stabilizes proteasomes under oxidative stress.



Indicated strains were logarithmically grown in YPD to an A₆₀₀ of 1.5, divided into 2 sets before adding 10mM H₂O₂ in one of the culture flasks. After 2 hours, cells were collected for cryogrinding and the total cell lysates were analyzed by resolving on native gels, scanned for GFP signal. The stability and activity of proteasomes was analyzed by using LLVY-AMC assay. Immunoblot analysis of the native gel was done using polyclonal *Ecm29* antibody to test if the stabilized proteasomes were bound to *Ecm29*.

Figure B.5: Schematic representation of recombination-induced tag exchange (RITE) method.



(a) RITE cassette integrated at the C-terminal of the $\alpha 1$ subunit. Following the ORF is present a spacer region (S), loxP site, followed by GFP-tag ("old" tag), STOP codon, a selection cassette for hygromycin (hygro), followed by another loxP site and the RFP tag ("new" tag). Prior to switch, the cells are resistant to hygro and synthesize $\alpha 1$ -GFP. After the induction of Cre recombinase, the loxP sites undergo recombination which leads to the removal of GFP tag and hygro selection cassette. The cells get genetically modified and synthesize $\alpha 1$ with the new tag $\alpha 1$ -RFP. At a given time, a cell may contain both old and new tagged protein depending upon the stability of the protein. (b) RITE cassette integrated at the C-terminal of Ecm29. Prior to switch, the cells are resistant to hygro and synthesize Ecm29-GFP. After the induction of Cre recombinase, genetically modified cells synthesize Ecm29 with the new tag Ecm29-RFP.