In the Nick of Time: Understanding the Eviction of an Assembly Chaperone Hsm3 from 26S Proteasomes

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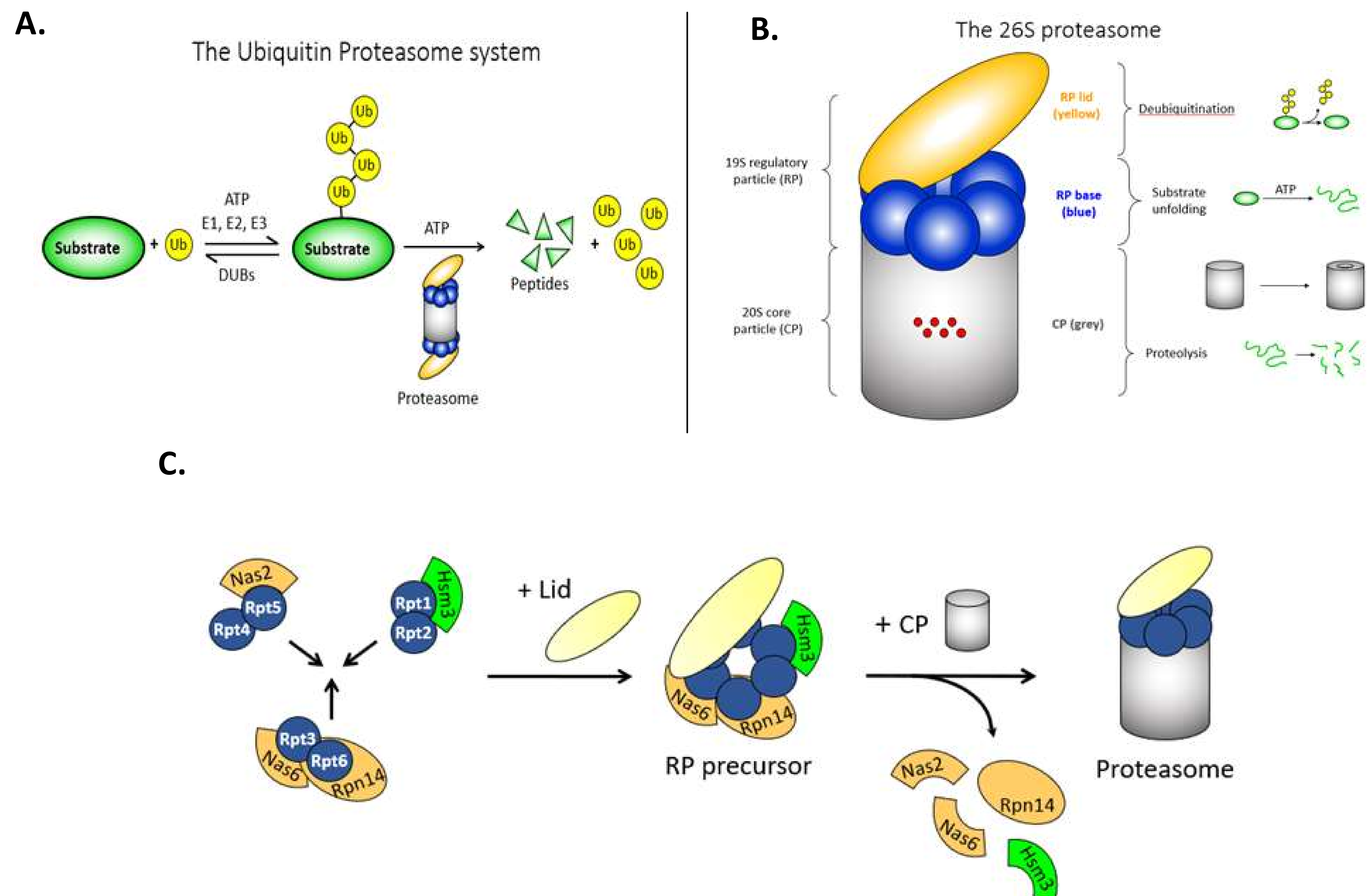
1. Abstract

The proteasome is a complex cellular machine that is responsible for destroying unneeded or unwanted proteins. The proteasome consists of three subcomplexes: the lid, base, and core particle. Each subcomplex can assemble on its own before combining to form mature proteasomes. Under normal conditions, the assembly of the base is controlled by four chaperone proteins. However, how the chaperones are evicted prior to assembly of mature proteasomes is not well understood. We established a transition metal Förster resonance energy transfer (TM-FRET) assay to monitor the eviction of one of these chaperone proteins, Hsm3 from its binding partner in the base, Rpt1. We first produced a single-cysteine version of Hsm3 for labeling at a defined site with the small molecule fluorophore TAMRA. We next engineered two histidines into an α-helix of the base subunit Rpt1 near the Hsm3 binding site to create a Ni2+ chelating site. We performed growth assays on these mutants to confirm that they fully complemented deletion strains, indicating the mutations were innocuous. We will use this TM-FRET system to understand how Hsm3 is evicted during proteasome biogenesis. Proteasome dysfunction has been implicated in several diseases, and is a validated drug target in treating multiple myeloma. Understanding proteasome assembly, and specifically chaperone eviction, can be exploited in developing novel drugs.

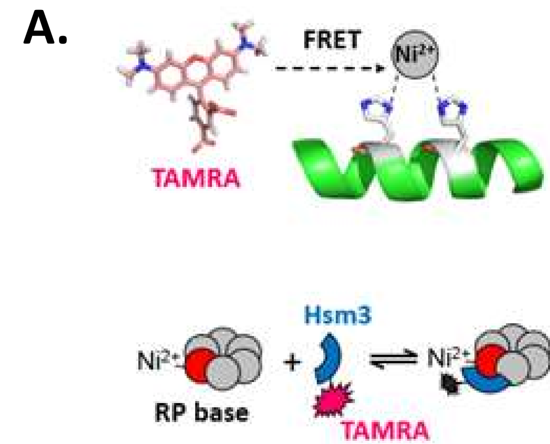
2. Introduction to the 26S proteasome



A. The ubiquitin proteasome is a highly regulated and controlled system that involves tagging unwanted proteins with ubiquitin followed by degradation by the proteasome. **B.** The 26S proteasome, a complex cellular machine affectionately known as the "recycling center" of the cell, consists of three subcomplexes: the lid, base, and core particle (CP). The lid removes the polyubiquitin targeting signal via the lone intrinsic deubiquitinating enzyme, the heterohexameric ring of ATPases utilize energy from ATP hydrolysis to mechanically unfold and translocate the substrate into the CP, which cleaves it into short peptides. **C.** The base subcomplex can assemble on its own with the help of 4 evolutionarily conserved chaperone proteins (shown in orange and green). In order to form mature proteasomes, the chaperones are evicted via a poorly defined mechanism.

4. Transition Metal Förster Resonance Energy Transfer

A.



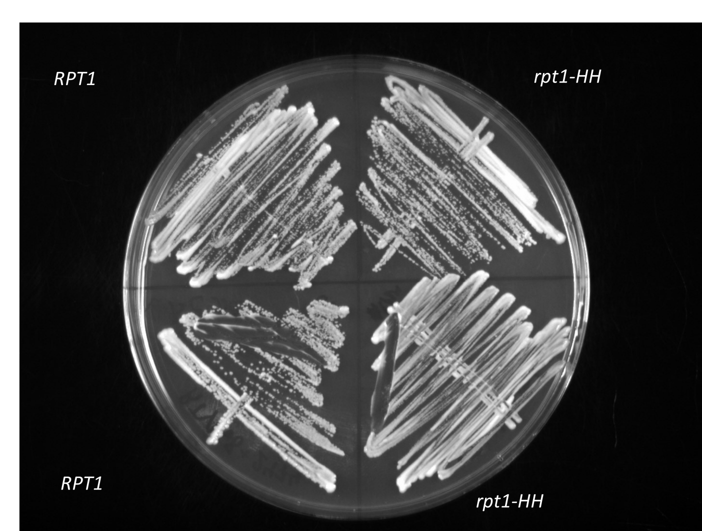
We have established a transition metal Förster resonance energy transfer (TM-FRET) system based on TAMRA quenching by a chelated Ni2+ ion to monitor interaction of Hsm3 with proteasomes or assembly intermediates. This assay exploits the binding of Hsm3 to an α-helical small domain of its cognate ATPase subunit Rpt1. We first produced a single-cysteine version of Hsm3 for labeling at a defined site with the small molecule fluorophore TAMRA. We next engineered two histidines into an α-helix of Rpt1 (*rpt1-HH*) near the Hsm3 binding site to create a Ni2+-chelating site.

5. Cloning of *HSM3* and *RPT1* plasmids

The *HSM3* gene was cloned into the yeast expression plasmid YCplac22 using standard molecular cloning techniques. A 2022 bp BamHI SalI fragment containing the promoter, ORF, and terminator of *HSM3* was subcloned into YCplac22. The resultant plasmid was verified by restriction enzyme digest with BamHI and SalI. To clone YCplac22-*hsm3ΔC*, an approximately 5 kb fragment was amplified from the above plasmid and gap repaired into a BamHI and XhoI fragment containing hsm3ΔC. The resultant plasmid was verified by DNA sequencing. Plasmids with single cysteine hsm3 mutations were constructed using site-directed mutagenesis using YCplac22-*hsm3ΔC* as a template. The resultant plasmids were verified by DNA sequencing. Plasmids containing the histidine mutations in *RPT1* were constructed using site-directed mutagenesis using YCplac22-*RPT1* as a template. The resultant plasmids were verified by DNA sequencing.

6. Transformation of plasmids into yeast strains

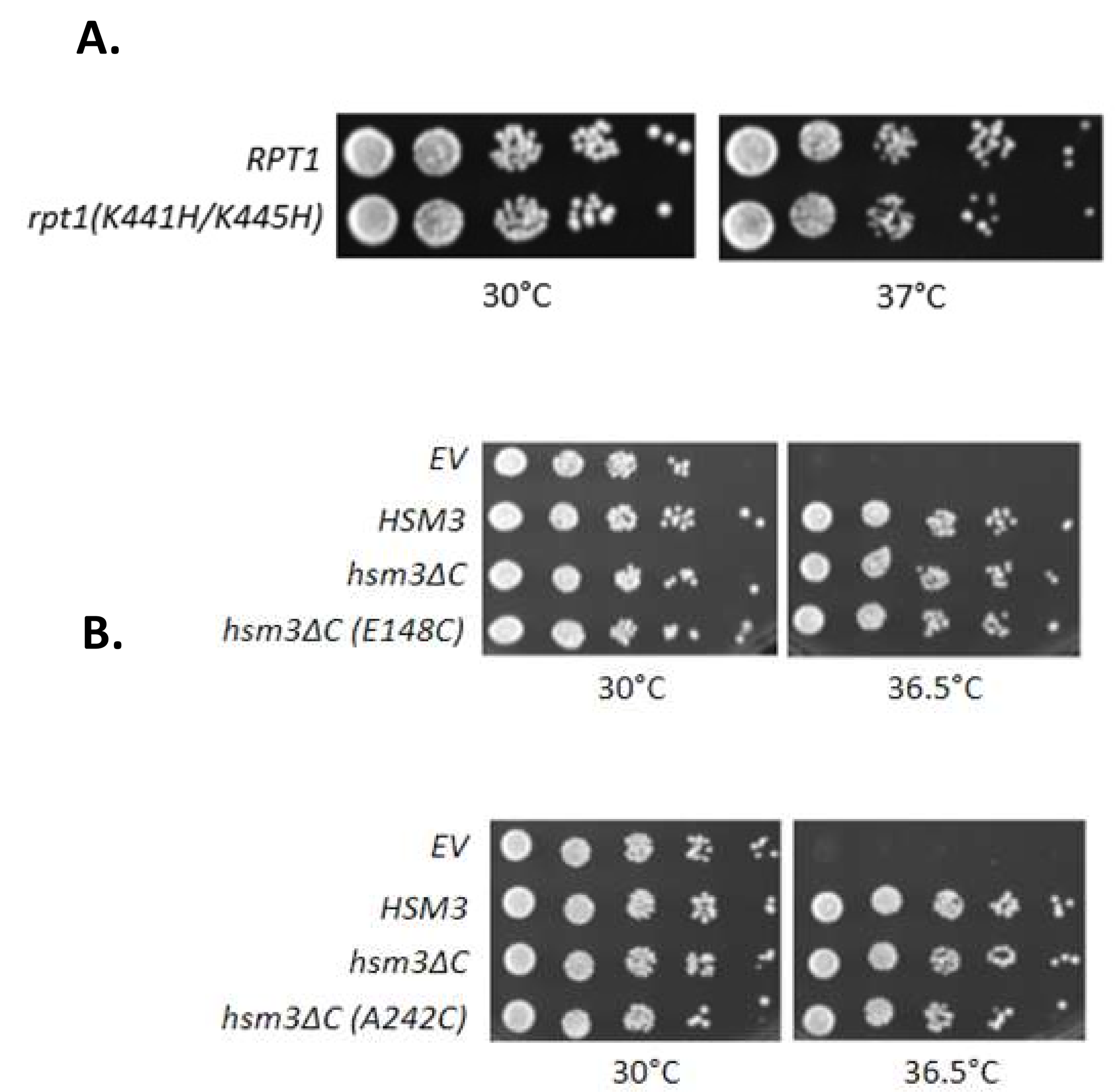
A.



WT HSM3 and single cysteine plasmids were introduced into either *WT* or *hsm3Δ nas2Δ* yeast strains by standard yeast transformation and plated on sc-Trp plates. Yeast strains were grown overnight to saturation and 500 µl of yeast cells were used for the transformation.

WT RPT1 and *rpt1(K441H K445H) (rpt1-HH)* plasmids were introduced into a *rpt1Δ* shuffle strain as described above and plated on sc-Trp plates. **A.** The cover plasmid was evicted by streaking single colonies on plates containing 5-fluoroorotic acid (FOA).

7. *rpt1* and *hsm3* mutants fully complement deletion strains



For growth assays, the indicated strains were diluted to OD600 = 0.1 with sterile water and spotted as six-fold serial dilutions onto the indicated media. The plates were incubated for 2 days at the indicated temperatures. **A.** The *hsm3Δ* and single cysteine mutants rescue the *hsm3Δ nas2Δ* growth defect. **B.** There is no growth defect in the *rpt1(K441H/K445H)* mutant.

8. Conclusion

- Mutating the endogenous cystines to alanine or serine doesn't affect the function of Hsm3.
- The single cysteine Hsm3 proteins fully complement the *nas2Δ hsm3Δ* double deletion yeast strain.
- The engineered Rpt1 histidine mutants fully complemented the *rpt1Δ* yeast strain.

9. Future Directions

- Confirm that the single cysteine Hsm3 protein has similar folding to WT via circular dichroism.
- Test that there is quenching of Hsm3 fluorescence in a manner that is depending on nickel and the engineered histidines in Rpt1.

10. References

- Howell, L. A., Tomko, R. J., & Kusmierczyk, A. R. (2017, March 2). Putting it all together: Intrinsic and extrinsic mechanisms governing proteasome biogenesis - frontiers in biology. SpringerLink. Retrieved September 7, 2021, from https://link.springer.com/article/10.1007/s11515-017-1439-1