Dissecting the Localization of 26S Proteasome Subunits During De Novo Assembly in living cells

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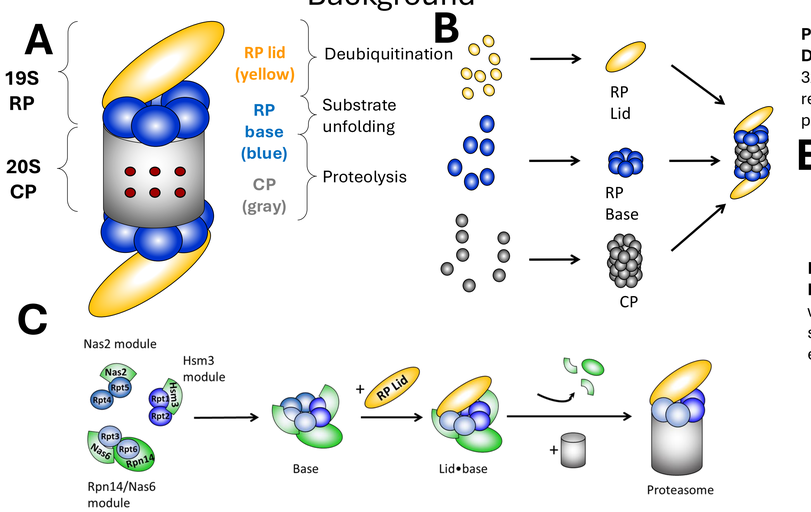
Abstract

The 26S Proteasome is responsible for the vast majority of regulated protein degradation and is vital for maintaining cellular homeostasis. The proteasome is composed of ~ 66 individual subunits and although the general mechanism for assembly has been identified the localization for assembly is largely unknown. In this work we are attempting to address these limitations by developing a non-radioactive pulse–chase system in *Saccharomyces cerevisiae* (budding yeast), and combined this with immunofluorescence and live-cell fluorescence imaging approaches to investigate the subcellular localization of proteasome assembly. Our preliminary results support the hypothesis that early localization and assembly steps occur in the cytosol with entry into the nucleus occurring after formation of a metastable intermediate, and final assembly steps occurring in the nucleus. Proteasome dysfunction has been documented in neurological cases, specifically neurodegenerative diseases like Alzheimer's Disease, which is characterized by a lack of activity and abundance of 26S proteasomes. Understanding changes in localization during proteasome assembly may provide strategies for potential therapeutic development.

Background

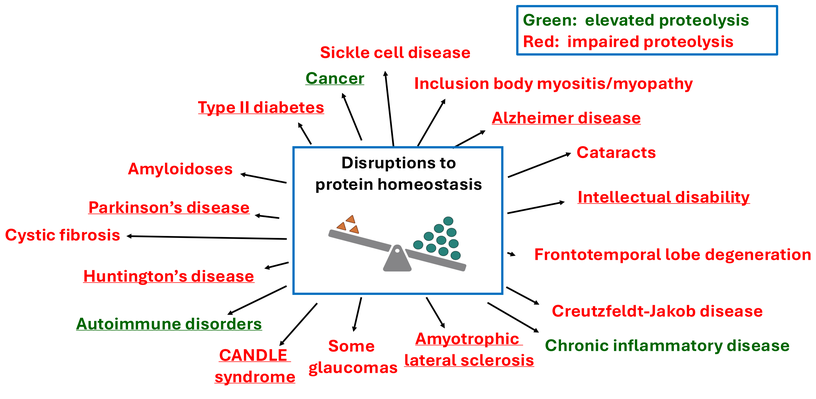


A. The 26S Proteasome is a multi-subunit complex made up of multiple subunits, including the lid that recognizes polyubiquitinated proteins, the base that helps unfold the protein and translocate it into the core particle, which contains the catalytic sites to degrade the protein.

B. Each subcomplex can assemble independently of the others.

C. Proposed assembly of the base subcomplex from proteasome-specific chaperone-bound intermediates, followed by association with the lid to form the lid*base subcomplex, and final docking onto the CP to form a mature proteasome

Motivation

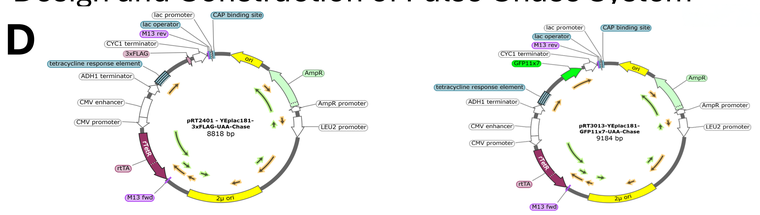


Understanding proteasome localization changes during assembly can greatly inform how many diseases develop, including neurodegenerative disorders and cancers, where proteolytic activity is unregulated and has been disregulated. Specifically, pharmaceuticals are commonly used to treat multiple myeloma, and our findings on localization changes during proteasome assembly could lead to more targeted or effective therapies.

Split GFP System

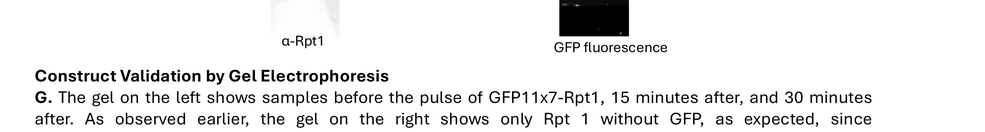
The split GFP system is comprised of two main parts: a large fragment of GFP, 1-10 (beta strands 1-10), and a small fragment of GFP, 11 (beta strand 11). Individually, these fragments do not fluoresce, but when they come together, they start to fluoresce. This allows us to observe the localization of newly tagged subunits.

Design and Construction of Pulse Chase System



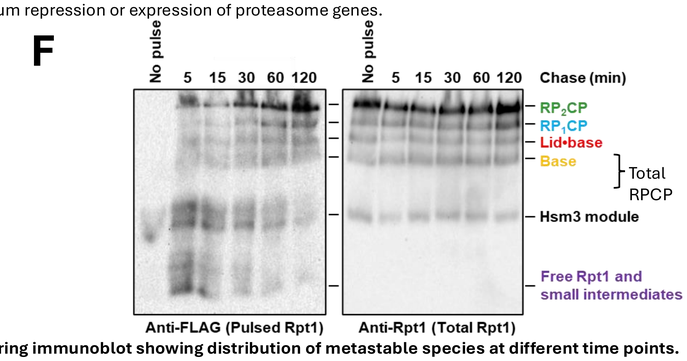
Plasmid Design and Assembly

D. Plasmids were constructed using two plasmids: one serving as the backbone containing the Rpt subunit and a 3x Flag tag, and the other containing GFP repeated 11 times. The image on the left shows GFP being cut out with restriction enzymes and inserted into the other plasmid after it was cut with the same enzymes. Since the programs have modular capabilities, this allows us to insert different subunits to study localization independently.



Inhibition of protein synthesis using doxycycline and O-methyltyrosine.

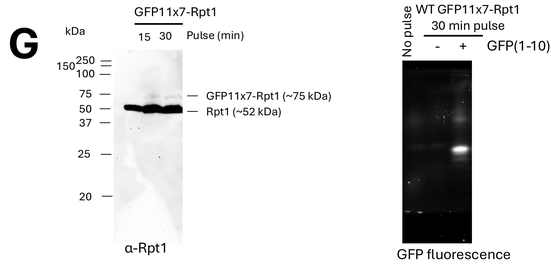
E. Pulse-Chase of proteasome subunits. Kinetics were studied using a pulse-chase method. Yeast was treated with O-methyl-tyrosine to activate proteasome synthesis for specific periods and to label newly produced subunits during the pulse phase. The yeast was then exposed to two antibiotics, tetracycline and doxycycline, to ensure maximum repression or expression of proteasome genes.



Non-denaturing immunoblot showing distribution of metastable species at different time points.

F. The gel on the left shows the formation of smaller subunits early in the maturation cycle, compared to later when they are incorporated into a higher-order 26S proteasome subcomplex. Meanwhile, the gel on the right indicates that the cell's distribution of proteasomal species remains unaffected by the pulse chase system.

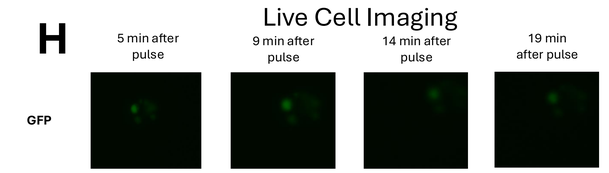
Validation of pulse split GFP pulse chase system



Construct Validation by Gel Electrophoresis

G. The gel on the left shows samples before the pulse of GFP11x7-Rpt1, 15 minutes after, and 30 minutes after. As observed earlier, the gel on the right shows only Rpt 1 without GFP, as expected, since Omethyltyrosine hasn't been used to express GFP. After the pulse, the expected sizes of GFP and Rpt1 are visible at 15 and 30 minutes, respectively. The gel on the right shows cell lysates separated by non-denaturing PAGE. GFP1-10 was added to the cell lysates and incubated for 30 minutes. Fluorescence confirms complementation with pulsed subunits; GFP fluoresces only when it contacts GFP 1-10 and GFP 11. The middle column shows a 30-minute pulse without GFP 11, and the far-left column shows no pulse.

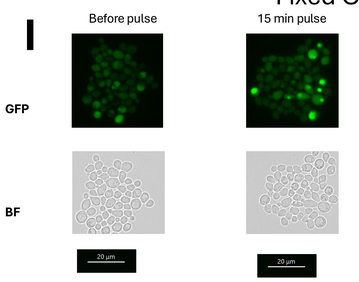
Live Cell Imaging



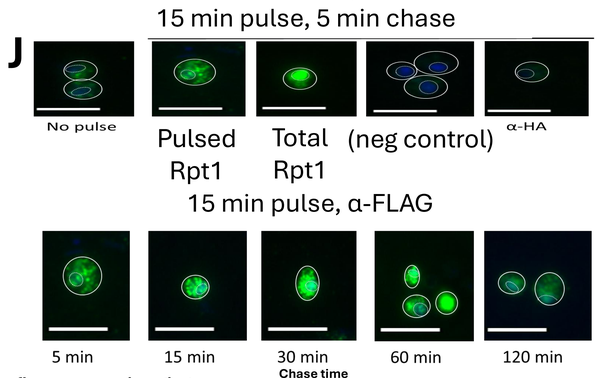
Live Cell Imaging (Preliminary)

H. Live cell imaging was more challenging to obtain than fixed cell images, as there was no apparent change in the GFP signal over the pulse time. This approach may have been limited by the maturation of the chromophore, since it can take upwards of 10 minutes or insufficient transformation efficiency.

Fixed Cell Imaging



I. Fixed cell imaging using paraformaldehyde showed promising results, with noticeable differences in localization and signal strength. After a 15-minute pulse with fixed cells, we detected fluorescence, suggesting that the split GFP system is functioning. Additionally, the cells remained static in shape and movement, with a 30% increase in fluorescence over the 15-minute period. We are still working to improve transformation efficiency, as we suspect some cells did not receive the plasmid, leaving them visually dark when checked for fluorescence.



J. Immunofluorescence imaging.

Immunofluorescence, where fixed cells were incubated with an antibody recognizing the tag and a secondary antibody containing the fluorophore (Alexa 488), showed more fluorescence than the negative control. The images at the bottom indicate that, early after the pulse, subunit localization occurred mostly in the cytosol, and over time they further integrated into the nucleus. During this period, you can observe the localization of the 26S proteasome in the nucleus (indicated by the dotted lines), with separate subunits assembling in the cytoplasm and then being transported into the nucleus.

Conclusions

- Immunofluorescence suggests that newly synthesized subunits of the proteasome are formed in the cytosol, and over time, these subunits move into the nucleus to be incorporated and assembled into the 26S proteasome.
- The observations support the hypothesis that biogenesis involves heavily regulated localization of newly synthesized subunits
- Pulse-Chase enables observation of subunit trafficking during early stages of assembly.

Future directions

Given that these experiments were conducted during the log-rate phase of yeast growth, which shows healthy maturation and proteasome activity, this experiment could be performed during the post-log-rate phase to model and demonstrate the function of the proteasome in neurodegenerative disease. This is because yeast activity in this phase closely resembles that in cells affected by these diseases.

References

- Betancourt et al., 2023 – Biomolecules
- Davidson & Pickering, 2023 – Frontiers in Cell and Developmental Biology
- Smoyer et al., 2016 – Journal of Cell Biology
- Stelter & Hurt, 2014 – Methods in Cell Biology