論文の内容の要旨

論文題目　Analysis of the turnover mechanism of the proteasome

(プロテアソームのターンオーバー機構の解析)

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The 26S proteasome is an enzymatic complex that mainly degrades ubiquitinated proteins. This large protein complex is composed of a central 20S core particle (CP) capped at either one or both ends by 19S regulatory particles (RP). The 20S CP is responsible for its proteolytic activity. The 19S RP plays an important role in the capture of ubiquitinated proteins. In addition to the constitutive proteasome subunits, proteasome interacting proteins (PIPs) that transiently associate with the proteasome and play auxiliary roles have been identified. While a number of PIPs are well-characterized, there still remain many PIPs whose functions are unknown. Thus, the proteasome activity is regulated by numerous proteins, but there is still room for further investigation.

It is known that the proteasome plays pivotal roles in various cellular events such as cell cycle, transcription, and immune response. In addition, it has been reported that a couple of diseases such as cancer and neurodegenerative diseases are associated with aberrant proteasome activity. Therefore, regulation of proteasome activity is important for understanding pathogenesis of human diseases.

Indeed, numerous researches on the regulation of the proteasome have been conducted. For instance, identification of proteasome-interacting proteins, assembly mechanisms of the proteasome complex, and development of proteasome activators and inhibitors have been studied.

Here, I propose that the turnover of the proteasome itself be engaged in the regulation of proteasome activities. The proteasome is known to be modulated by a number of post-translational modifications, and previous reports suggest that some of those modifications decreased enzymatic activities of the proteasome. In addition, the proteasome

contains three catalytically active subunits, but these are replaced by immunoproteasome subunits when cells are exposed to inflammatory stimuli such as γ-type interferon (IFN-γ). Thus, it is expected that damaged or unnecessary proteasomes have to be removed and that the regulation of the turnover of the proteasome is important for maintaining cellular homeostasis. Indeed, since the proteasome is a protein complex that has a measurable half-life, its turnover must be under control. As there are few studies on alterations of the proteasome after its biogenesis, this study is aimed at clarifying the turnover mechanism of the proteasome, especially with regard to characterization of aged proteasomes and degradation of the proteasome.

To address this issue, this study was conducted by two strategies. The first was to characterize the old proteasome by biochemical approaches.

To purify the old proteasome, I made use of the Rpn11-Flag/EGFP tag-exchangeable knock-in mice (Fig A). Rpn11 is one of the subunits of 19S RP. In the knock-in allele, a sequence encoding Flag epitope was fused to the 3' end of the Rpn11 coding sequence in the exon 12. The modified exon 12 was flanked by two loxP sequences. EGFP tag was also incorporated with the exon 12 of Rpn11. Accordingly, the knock-in allele expresses Flag-tagged Rpn11. Once it is subjected to Cre-recombinase, the region between the two loxP sequences is excised, switching to express EGFP-tagged Rpn11 and stopping expression of Rpn11-Flag. Using this tool, we can purify old proteasomes by Flag tag after expression of Cre-recombinase in the knock-in cells (Fig B). After a certain period of time from the expression of Cre-recombinase, proteasomes that incorporate Flag-tagged Rpn11 are considered as the old proteasome, while incorporation of EGFP-tagged Rpn11 is a marker of the new proteasome.

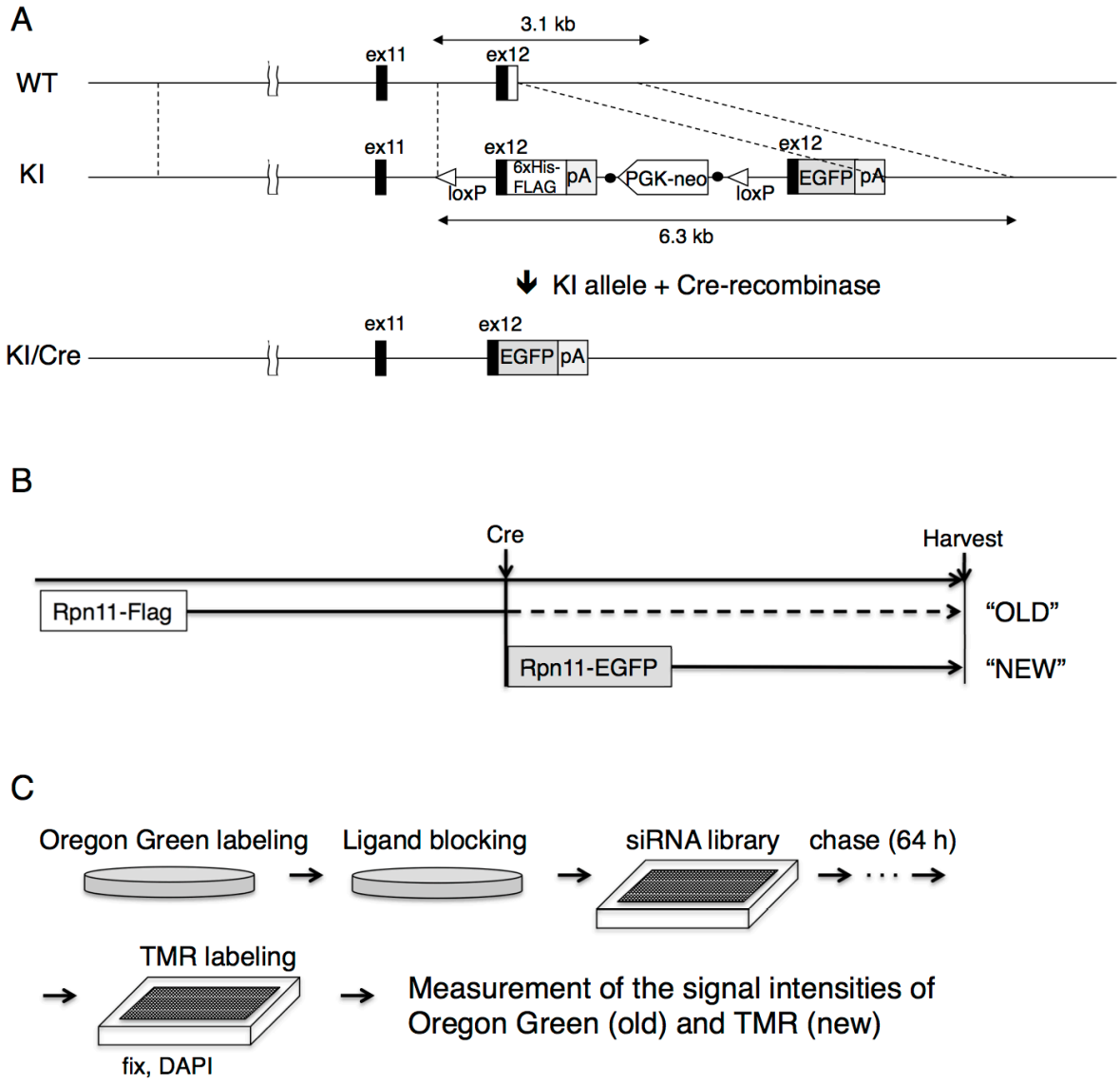
Thus, Cre-recombinase was expressed by virus transmission into mouse embryonic fibroblasts (MEFs) derived from Rpn11-Flag knock-in mice, and the old proteasome was selectively purified using Flag-tag three days after the virus transmission. This showed that the old proteasome did not show alterations in the composition of 20S CP and 19S RP. However, a couple of PIPs showed enhanced interactions to the old proteasome. In addition, the old proteasome was oxidized and one of the subunits in the old proteasome was phosphorylated. The phosphorylation site was identified by mass spectrometry analysis. For the confirmation and further studies of the phosphorylation site of the old proteasome, antibodies specific for phosphorylated and unphosphorylated forms of the subunit were generated. This antibody specifically recognized the old proteasome, demonstrating that the old proteasome was certainly phosphorylated.

Since the old proteasome showed alterations in protein-protein interactions and also in oxidation and phosphorylation states, I next examined whether these modifications influenced the enzymatic activities and the localization of the old proteasome. As a result, the activities of the old proteasome were consistent with its modifications. In addition, immunofluorescence and fractionation analysis revealed that the localization of the proteasome might be dependent of its age.

Although the old proteasome was biochemically characterized, the process of the turnover, especially of its degradation, was not clarified from these results. Hence, a human whole-genome knockdown screen was performed to investigate the turnover mechanism of the proteasome as a second strategy in this study. This experiment was based on HaloTag technology, which is a protein fusion tag that can be labeled with fluorescent chemical ligands after HaloTag is expressed in cells. To monitor the turnover of the proteasome, knock-in HeLa cells in which Rpn11 is tagged with HaloTag (Rpn11-Halo) have been generated by CRISPR/Cas9 system. Oregon Green-labeled Rpn11-Halo knock-in HeLa cells were subsequently subjected to the blocking ligand, and seeded onto 384-well siRNA library plates. After incubating for 64 hours, the cells were then labeled with TMR, followed by cell fixation and nuclear staining. The number of the cells and the fluorescence intensities of Oregon Green and TMR per cells, which indicated the old and the new Rpn11-Halo, respectively, were quantified by high-throughput image analyzer (Fig C).

In this screening, approximately 18 thousand targets were narrowed down to 10 possible genes that might be engaged in the turnover of the proteasome. Surprisingly, knockdown of two genes among the final 10 qualifiers affected a post-translational modification of the old proteasome, which was observed in the first strategy. These data suggest that this post-translational modification is involved in the turnover of the aged proteasome.

Figure



(A) Construct of Rpn11-Flag/EGFP tag-exchangeable knock-in mice. WT, KI, ex11, ex12, pA, PGK, and neo denote wild-type, knock-in, exon 11, exon 12, polyadenylation signal, PGK promoter sequence, and neomycin-resistant cassette, respectively.

(B) Concept of the old and the new proteasome.

(C) Schematic view of the primary screen using Rpn11-Halo knock-in HeLa cells.