論文の内容の要旨

論文題目　A novel ubiquitin-interacting protein that counteracts aggregate formation of ubiquitinated proteins
(新規ユビキチン会合分子によるユビキチン化タンパク質凝集化の抑制)

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The ubiquitin-proteasome system (UPS) is the principal pathway for protein degradation in eukaryotic cells. In the UPS, proteins that are to be eliminated are tagged with ubiquitin chains and degraded by the 26S proteasome. The UPS takes important roles in various biological events, and therefore dysfunction of it leads to diseases such as cancer and neurodegeneration. Recent reports revealed that various intracellular molecules were involved in the UPS. Moreover, because of molecular diversity of these proteins, molecules involved in the UPS and complexity of the UPS is increased in higher eukaryotes. Hence, it is challenging to examine how the UPS is strictly regulated in multicellular organisms.

To identify new genes that modify proteasome function, I designed a genetic screen using *Drosophila* by which I sought genes that suppress visible phenotypes caused by inverted repeat (IR)-induced RNAi of a proteasome-related gene. I found that *pnr-GAL4*-driven dorsal midline-specific knockdown of fly *TXNL1* (*CG5495*), a proteasome-associating reductase, induced abnormal pigmentation. Since the role of TXNL1 on the UPS is unclear, I examined it by fly models. *Sca-GAL4*-driven proneural cluster-specific knockdown of *Rpt6* (*CG1489*), one of the subunits of 26S proteasomes, caused loss of macrochaetae in the scutellum, suggesting that dysfunction of the UPS causes loss of bristles. Upregulation of TXNL1 partially restored the loss of bristles, suggesting that TXNL1 upregulate the UPS function in flies. Collectively, melanization induced by TXNL1 knockdown might reflect dysfunction of the UPS and should be a good marker for the designed screen. To seek suppressors of the melanization, I generated *pnr>TXNL1-IR* flies and crossed them with the Gene Search (GS) lines. I examined about 13,000 progenies and found eight strains and three genes suppressing the melanization. I focused on one gene (Modifier A) that completely suppressed loss of TXNL1-induced melanization.

Modifier A is widely conserved, and human also has its ortholog. Modifier A has a putative ubiquitin-associated (UBA) domain. To examine the interaction of human Modifier A with ubiquitin, I performed glutathione S-transferase (GST) pull-down assays. This approach showed that Modifier A was associated with ubiquitin conjugates, which was not observed in mutants lacking the putative UBA domain. These suggest that human Modifier A has bona fide UBA domain. To clarify further interacting partners of Modifier A, Flag-tagged Modifier A was expressed in HEK293T cells, and its complexes were analyzed by

tandem mass spectrometry. This revealed that Modifier A was associated with heat shock 70 kDa protein 1A/1B (Hsp70) and its family proteins (Hsc70 and Hsp105). Collectively, I renamed Modifier A HUIP (*h*eat shock proteins- and *u*biquitin-*i*nteracting *p*rotein).

To investigate whether HUIP is involved in the UPS, I generated HUIP-knockout (KO) cells. In steady state, the amount of ubiquitin conjugates in HUIP-KO HCT116 cells were almost the same as that in parental wild type (WT) cells. However, after treatment with bortezomib, a reversible inhibitor of the proteasome, more detergent-insoluble ubiquitinated proteins were accumulated in HUIP-KO cells than in WT cells. In addition, immunostaining showed that loss of HUIP increased the number of cells with ubiquitin-positive aggregates. By contrast, overexpression of full-length HUIP decreased the number of cells with ubiquitin-positive aggregates generated upon proteasome inhibition, which is completely failed by overexpression of HUIP lacking the UBA domain. Moreover, the full-length HUIP effect was canceled by knockdown of Hsp70. These data demonstrated that HUIP inhibits aggregation of ubiquitinated proteins and that interaction of HUIP with ubiquitin and activities of Hsp70 were required for the inhibition.

Immunostaining analysis suggested two possibilities for HUIP function: preventing ubiquitinated proteins from aggregating, promoting disaggregation of them, or both. To verify the latter possibility, I introduced Tet-On system to express HUIP after generating ubiquitin-positive aggregates and traced clearance of the aggregates in the inhibitor-free medium. This revealed no significant differences in clearance of ubiquitin aggregates between the presence and absence of induced HUIP, suggesting that HUIP inhibits aggregate formation of ubiquitinated proteins rather than disaggregates them.

I sought specific substrates of HUIP. The C-terminal fragment of TAR DNA-binding protein (TDP-43) and mutants of superoxide dismutase 1 (SOD1) have been linked to familial ALS and reported to frequently form ubiquitin-positive aggregates in patient tissues. Cytological analysis revealed that HUIP colocalized with cytosolic aggregates of the C-terminal fragment of TDP-43 (C-TDP-43) and mutant SOD1 in cultured cells. Additionally, immunoprecipitation analysis showed that mutant SOD1 but not WT SOD1 was associated with HUIP, suggesting that HUIP recognizes ubiquitinated state of SOD1. When mutants were expressed in HUIP-KO 293T cells, both mutants were more accumulated in insoluble fractions in KO cells compared to WT cells, which was more significant upon proteasome inhibition. Similar to endogenous ubiquitinated proteins, aggregation of C-TDP-43 was enhanced by HUIP knockout, while suppressed by HUIP overexpression, suggesting that HUIP inhibits aggregation of ALS-linked mutants. I next investigated whether HUIP affects degradation of these mutants. Cycloheximide chase experiments revealed that HUIP accelerated degradation of mutant SOD1, which was attenuated by knockdown of Hsp70. Overexpression of HUIP decreased steady state levels of insoluble C-TDP-43 and soluble mutant SOD1, which was abolished by treatment with bortezomib but not with lysosome inhibitor chloroquine. These data suggest that HUIP promotes proteasomal degradation of ALS-linked mutants in Hsp70-dependent fashion.

Finally I investigated the physiological importance of HUIP. Knockdown of HUIP in HeLa cells increased sensitivity to bortezomib. Knockdown of fly HUIP (dHUIP) in fly whole bodies exhibited larval lethality. Moreover, *GMR-GAL4*-driven eye-specific knockdown of dHUIP caused a dose-dependent degeneration and accumulation of insoluble ubiquitinated proteins. These data indicate that exacerbated aggregation of ubiquitinated proteins by depletion of HUIP augments toxicity to cell viability. To test the genetic interaction between dHUIP and ALS-linked TDP-43, I used flies expressing mutant TDP-43 (M337V). Ectopic expression of mutant TDP-43 in the eye caused age-dependent degeneration, which was exacerbated by dHUIP knockdown while alleviated by dHUIP upregulation without affecting mRNA levels of TDP-43. These results indicate that toxicity of TDP-43 is regulated by the levels of dHUIP. To examine the effect of dHUIP on degradation of mutant TDP-43, fly heads were subjected to immunoblotting. dHUIP knockdown increased insoluble high-molecular-weight (HMW) bands of TDP-43 that are observed in patient tissues with TDP-43 proteinopathy. On the other hand, upregulation of dHUIP decreased the protein amount of monomeric TDP-43 and insoluble HMW bands at day one and 12 posteclosion, respectively. These indicate that dHUIP protects from TDP-43 toxicity by inhibiting aggregation and promoting degradation of TDP-43 in vivo.

On the whole, the current model is that HUIP counteracts aggregation of ubiquitinated and aggregation-prone proteins in concert with Hsp70/Hsc70, which leads to promotion of proteasomal degradation of them. This study demonstrates a new mechanism for protein degradation via the UPS and a new insight into protection against ALS disease.