

(Nakagawa et al., 1998; Honey et al., 2002; Gommeaux et al., 2009). A recent report also showed that thymic epithelial cells, including cTECs, have high constitutive activity of autophagy, which contributes to MHC class II peptide-mediated T cell repertoire formation and self-tolerance (Nedjic et al., 2008). The unique features of cTECs in protein degradation and self-peptide presentation may govern the positive selection of T cells in both CD4 and CD8 lineages.

The present study reveals that positive selection induced by thymoproteasome-expressing cTECs is essential for the formation of an immunocompetent and self-protective repertoire of CD8<sup>+</sup> T cells. In the absence of thymoproteasome-mediated positive selection, mice exhibited severe defects in the allogeneic responses of CD8<sup>+</sup> T cells and in the survival to influenza virus infection. On the other hand, we detected no signs of autoimmunity in thymoproteasome-deficient mice. Thus, positive selection induced by thymoproteasomes and cTECs specifically governs the installment of diverse repertoire of CD8<sup>+</sup> T cells, which is important for rejecting allogeneic tissues and eradicating virus-infected cells, rather than the establishment of a self-tolerant T cell repertoire, which is separately governed by other mechanisms, including Aire-associated promiscuous gene expression by mTECs (Derbinski et al., 2001; Anderson et al., 2002). Together, this study suggests that positive selection induced by a thymoproteasome-specific repertoire of self-peptides is essential for the installment of an immunocompetent and self-protective repertoire of CD8<sup>+</sup> T cells. We propose that the cTEC expression of thymoproteasomes be considered for the therapeutic reconstitution of T cells by transplantation or regeneration of thymic stromal cells in the clinical setting.

## EXPERIMENTAL PROCEDURES

### Mice

The list of mice used in this study and the procedure for preparation of irradiated bone marrow chimeras are described in Supplemental Experimental Procedures. Animal experiments were performed after obtaining approval from the Animal Experimentation Committee of the University of Tokushima.

### Flow Cytometry Analysis and Cell Sorting

Multicolor flow cytometry analysis and cell sorting were performed with FACS-Calibur and FACS-Vantage (BD Bioscience), as described previously (Ueno et al., 2005). Thymic stromal cells were prepared by digesting thymic fragments with collagenase, dispase, and DNase I (Roche), as described previously (Gray et al., 2006; Hikosaka et al., 2008). To sort thymic epithelial cells, thymic stromal cells were enriched by depleting CD45<sup>+</sup> cells and TER119<sup>+</sup> cells using magnetic-bead-conjugated antibodies specific for CD45 and TER119 (Miltenyi Biotec) prior to FACS cell sorting.

### Reaggregated Thymus Organ Culture

The procedures for fetal thymus organ culture and reaggregated thymus organ culture were described previously (Ueno et al., 2005). DP thymocytes were sorted from adult TCR transgenic mice (purity >99%). Thymic stromal cells were prepared from E15.5 fetal thymus lobes that were cultured for 5 days in the presence of 2-deoxyguanosine. DP thymocytes ( $5 \times 10^5$ ) and thymic stromal cells ( $5 \times 10^4$ ) were reaggregated and organ-cultured for 4 days.

### Analysis of Proteasomal Subunits

Ly51<sup>+</sup> cells were magnetically enriched from adult thymus using biotin-conjugated antibody specific for Ly51 and streptavidin-conjugated magnetic beads. Cells with >90% purity were lysed and subjected to immunoblot analysis.

### Additional Experimental Procedures

See Supplemental Experimental Procedures for additional experimental procedures.

### SUPPLEMENTAL INFORMATION

The Supplemental Information includes five figures and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.immuni.2009.10.009.

### ACKNOWLEDGMENTS

We thank S. Nitta and I. Ohigashi for technical help; T. Hansen, H. Scott, and K. Yasutomo for reagents; R. Germain for discussion and reagents; and A. Singer for reading the manuscript. This study was supported by a MEXT Grant-in-Aid for Scientific Research on Priority Area "Immunological Self" (to Y.T.), Specially Promoted Research (to S.M. and K.T.), Astellas Foundation (to Y.T.), and The Mitsubishi Foundation (to S.M.).

Received: June 16, 2009

Revised: October 3, 2009

Accepted: October 14, 2009

Published online: December 31, 2009

### REFERENCES

- Agenès, F., Dangy, J.P., and Kirberg, J. (2008). T cell receptor contact to restricting MHC molecules is a prerequisite for peripheral interclonal T cell competition. *J. Exp. Med.* 205, 2735–2743.
- Alam, S.M., Travers, P.J., Wung, J.L., Nasholds, W., Redpath, S., Jameson, S.C., and Gascoigne, N.R. (1996). T-cell-receptor affinity and thymocyte positive selection. *Nature* 381, 616–620.
- Allen, P.M. (1994). Peptides in positive and negative selection: a delicate balance. *Cell* 76, 593–596.
- Anderson, M.S., Venanzi, E.S., Klein, L., Chen, Z., Berzins, S.P., Turley, S.J., von Boehmer, H., Bronson, R., Dierich, A., Benoist, C., and Mathis, D. (2002). Projection of an immunological self shadow within the thymus by the aire protein. *Science* 298, 1395–1401.
- Ashton-Rickardt, P.G., Van Kaer, L., Schumacher, T.N., Ploegh, H.L., and Tonegawa, S. (1993). Peptide contributes to the specificity of positive selection of CD8<sup>+</sup> T cells in the thymus. *Cell* 73, 1041–1049.
- Ashton-Rickardt, P.G., Bandeira, A., Delaney, J.R., Van Kaer, L., Pircher, H.P., Zinkernagel, R.M., and Tonegawa, S. (1994). Evidence for a differential avidity model of T cell selection in the thymus. *Cell* 76, 651–663.
- Bender, B.S., Croghan, T., Zhang, L., and Small, P.A., Jr. (1992). Transgenic mice lacking class I major histocompatibility complex-restricted T cells have delayed viral clearance and increased mortality after influenza virus challenge. *J. Exp. Med.* 175, 1143–1145.
- Bevan, M.J. (1977). In a radiation chimera, host H-2 antigens determine immune responsiveness of donor cytotoxic cells. *Nature* 269, 417–418.
- Bouso, P., Bhakta, N.R., Lewis, R.S., and Robey, E. (2002). Dynamics of thymocyte-stromal cell interactions visualized by two-photon microscopy. *Science* 296, 1876–1880.
- Brown, M.G., Driscoll, J., and Monaco, J.J. (1991). Structural and serological similarity of MHC-linked LMP and proteasome (multicatalytic proteinase) complexes. *Nature* 353, 355–357.
- Burgevin, A., Saveanu, L., Kim, Y., Barilleau, E., Kotturi, M., Sette, A., van Endert, P., and Peters, B. (2008). A detailed analysis of the murine TAP transporter substrate specificity. *PLoS ONE* 3, e2402. 10.1371/journal.pone.0002402.
- Choi, E.Y., Jung, K.C., Park, H.J., Chung, D.H., Song, J.S., Yang, S.D., Simpson, E., and Park, S.H. (2005). Thymocyte-thymocyte interaction for efficient positive selection and maturation of CD4 T cells. *Immunity* 23, 387–396.
- Dai, S., Huseby, E.S., Rubtsova, K., Scott-Browne, J., Crawford, F., Macdonald, W.A., Marrack, P., and Kappler, J.W. (2008). Crossreactive T Cells

- spotlight the germline rules for alpha beta T cell-receptor interactions with MHC molecules. *Immunity* 28, 324–334.
- Derbinski, J., Schulte, A., Kyewski, B., and Klein, L. (2001). Promiscuous gene expression in medullary thymic epithelial cells mirrors the peripheral self. *Nat. Immunol.* 2, 1032–1039.
- Doherty, P.C., Topham, D.J., Tripp, R.A., Cardin, R.D., Brooks, J.W., and Stevenson, P.G. (1997). Effector CD4<sup>+</sup> and CD8<sup>+</sup> T-cell mechanisms in the control of respiratory virus infections. *Immunol. Rev.* 159, 105–117.
- Ernst, B., Lee, D.S., Chang, J.M., Sprent, J., and Surh, C.D. (1999). The peptide ligands mediating positive selection in the thymus control T cell survival and homeostatic proliferation in the periphery. *Immunity* 11, 173–181.
- Falk, K., Rötzschke, O., Stevanović, S., Jung, G., and Rammensee, H.G. (1991). Allele-specific motifs revealed by sequencing of self-peptides eluted from MHC molecules. *Nature* 351, 290–296.
- Fehling, H.J., Swat, W., Laplace, C., Kühn, R., Rajewsky, K., Müller, U., and von Boehmer, H. (1994). MHC class I expression in mice lacking the proteasome subunit LMP-7. *Science* 265, 1234–1237.
- Fukui, Y., Ishimoto, T., Utsuyama, M., Gyotoku, T., Koga, T., Nakao, K., Hirokawa, K., Katsuki, M., and Sasazuki, T. (1997). Positive and negative CD4<sup>+</sup> thymocyte selection by a single MHC class II/peptide ligand affected by its expression level in the thymus. *Immunity* 6, 401–410.
- Ge, Q., Bai, A., Jones, B., Eisen, H.N., and Chen, J. (2004). Competition for self-peptide-MHC complexes and cytokines between naive and memory CD8<sup>+</sup> T cells expressing the same or different T cell receptors. *Proc. Natl. Acad. Sci. USA* 101, 3041–3046.
- Gommeaux, J., Grégoire, C., Nguessan, P., Richelme, M., Malissen, M., Guerdier, S., Malissen, B., and Carrier, A. (2009). Thymus-specific serine protease regulates positive selection of a subset of CD4<sup>+</sup> thymocytes. *Eur. J. Immunol.* 39, 956–964.
- Gray, D.H., Seach, N., Ueno, T., Milton, M.K., Liston, A., Lew, A.M., Goodnow, C.C., and Boyd, R.L. (2006). Developmental kinetics, turnover, and stimulatory capacity of thymic epithelial cells. *Blood* 108, 3777–3785.
- Heinemeyer, W., Gruhler, A., Möhrle, V., Mahé, Y., and Wolf, D.H. (1993). PRE2, highly homologous to the human major histocompatibility complex-linked RING10 gene, codes for a yeast proteasome subunit necessary for chymotryptic activity and degradation of ubiquitinated proteins. *J. Biol. Chem.* 268, 5115–5120.
- Hikosaka, Y., Nitta, T., Ohigashi, I., Yano, K., Ishimaru, N., Hayashi, Y., Matsumoto, M., Matsuo, K., Penninger, J.M., Takayanagi, H., et al. (2008). The cytokine RANKL produced by positively selected thymocytes fosters medullary thymic epithelial cells that express autoimmune regulator. *Immunity* 29, 438–450.
- Hogquist, K.A., Jameson, S.C., Heath, W.R., Howard, J.L., Bevan, M.J., and Carbone, F.R. (1994). T cell receptor antagonist peptides induce positive selection. *Cell* 76, 17–27.
- Honey, K., Nakagawa, T., Peters, C., and Rudensky, A. (2002). Cathepsin L regulates CD4<sup>+</sup> T cell selection independently of its effect on invariant chain: a role in the generation of positively selecting peptide ligands. *J. Exp. Med.* 195, 1349–1358.
- Hugo, P., Kappler, J.W., McCormack, J.E., and Marrack, P. (1993). Fibroblasts can induce thymocyte positive selection in vivo. *Proc. Natl. Acad. Sci. USA* 90, 10335–10339.
- Hunt, D.F., Henderson, R.A., Shabanowitz, J., Sakaguchi, K., Michel, H., Sevilir, N., Cox, A.L., Appella, E., and Engelhard, V.H. (1992). Characterization of peptides bound to the class I MHC molecule HLA-A2.1 by mass spectrometry. *Science* 255, 1261–1263.
- Huseby, E.S., White, J., Crawford, F., Vass, T., Becker, D., Pinilla, C., Marrack, P., and Kappler, J.W. (2005). How the T cell repertoire becomes peptide and MHC specific. *Cell* 122, 247–260.
- Huseby, E.S., Kappler, J.W., and Marrack, P. (2008). Thymic selection stifles TCR reactivity with the main chain structure of MHC and forces interactions with the peptide side chains. *Mol. Immunol.* 45, 599–606.
- Ignatowicz, L., Kappler, J., and Marrack, P. (1996). The repertoire of T cells shaped by a single MHC/peptide ligand. *Cell* 84, 521–529.
- Kappler, J.W., Roehm, N., and Marrack, P. (1987). T cell tolerance by clonal elimination in the thymus. *Cell* 49, 273–280.
- Kedzierska, K., Venturi, V., Field, K., Davenport, M.P., Turner, S.J., and Doherty, P.C. (2006). Early establishment of diverse T cell receptor profiles for influenza-specific CD8<sup>(+)</sup>CD62L<sup>(hi)</sup> memory T cells. *Proc. Natl. Acad. Sci. USA* 103, 9184–9189.
- Kirberg, J., Bosco, N., Deloulme, J.C., Ceredig, R., and Agenes, F. (2008). Peripheral T lymphocytes recirculating back into the thymus can mediate thymocyte positive selection. *J. Immunol.* 181, 1207–1214.
- Kisielow, P., Teh, H.S., Blüthmann, H., and von Boehmer, H. (1988). Positive selection of antigen-specific T cells in thymus by restricting MHC molecules. *Nature* 335, 730–733.
- Kurobe, H., Liu, C., Ueno, T., Saito, F., Ohigashi, I., Seach, N., Arakaki, R., Hayashi, Y., Kitagawa, T., Lipp, M., et al. (2006). CCR7-dependent cortex-to-medulla migration of positively selected thymocytes is essential for establishing central tolerance. *Immunity* 24, 165–177.
- Kyewski, B., and Derbinski, J. (2004). Self-representation in the thymus: an extended view. *Nat. Rev. Immunol.* 4, 688–698.
- Li, W., Kim, M.G., Gourley, T.S., McCarthy, B.P., Sant'Angelo, D.B., and Chang, C.H. (2005). An alternate pathway for CD4 T cell development: thymocyte-expressed MHC class II selects a distinct T cell population. *Immunity* 23, 375–386.
- Li, J., Iwanami, N., Hoa, V.Q., Furutani-Seiki, M., and Takahama, Y. (2007). Noninvasive intravital imaging of thymocyte dynamics in medaka. *J. Immunol.* 179, 1605–1615.
- Lie, W.R., Myers, N.B., Connolly, J.M., Gorka, J., Lee, D.R., and Hansen, T.H. (1991). The specific binding of peptide ligand to L<sup>d</sup> class I major histocompatibility complex molecules determines their antigenic structure. *J. Exp. Med.* 173, 449–459.
- Lilić, M., Santori, F.R., Neilson, E.G., Frey, A.B., and Vukmanović, S. (2002). The role of fibroblasts in thymocyte-positive selection. *J. Immunol.* 169, 4945–4950.
- Mareeva, T., Martinez-Hackert, E., and Sykulev, Y. (2008). How a T cell receptor-like antibody recognizes major histocompatibility complex-bound peptide. *J. Biol. Chem.* 283, 29053–29059.
- Marrack, P., and Kappler, J. (1997). Positive selection of thymocytes bearing  $\alpha\beta$  T cell receptors. *Curr. Opin. Immunol.* 9, 250–255.
- Martinic, M.M., Rüllicke, T., Althage, A., Odermatt, B., Höchli, M., Lamarre, A., Dumrese, T., Speiser, D.E., Kyburz, D., Hengartner, H., and Zinkernagel, R.M. (2003). Efficient T cell repertoire selection in tetraparental chimeric mice independent of thymic epithelial MHC. *Proc. Natl. Acad. Sci. USA* 100, 1861–1866.
- Merkenschlager, M., Graf, D., Lovatt, M., Bomhard, U., Zamojska, R., and Fisher, A.G. (1997). How many thymocytes audition for selection? *J. Exp. Med.* 186, 1149–1158.
- Momburg, F., Roelse, J., Howard, J.C., Butcher, G.W., Hämmerling, G.J., and Neeffes, J.J. (1994). Selectivity of MHC-encoded peptide transporters from human, mouse and rat. *Nature* 367, 648–651.
- Mozdzanowska, K., Furchner, M., Maiese, K., and Gerhard, W. (1997). CD4<sup>+</sup> T cells are ineffective in clearing a pulmonary infection with influenza type A virus in the absence of B cells. *Virology* 239, 217–225.
- Murali-Krishna, K., Lau, L.L., Sambhara, S., Lemonnier, F., Altman, J., and Ahmed, R. (1999). Persistence of memory CD8 T cells in MHC class I-deficient mice. *Science* 286, 1377–1381.
- Murata, S., Sasaki, K., Kishimoto, T., Niwa, S., Hayashi, H., Takahama, Y., and Tanaka, K. (2007). Regulation of CD8<sup>+</sup> T cell development by thymus-specific proteasomes. *Science* 316, 1349–1353.
- Nakagawa, T., Roth, W., Wong, P., Nelson, A., Farr, A., Deussing, J., Villadangos, J.A., Ploegh, H., Peters, C., and Rudensky, A.Y. (1998). Cathepsin L: critical role in li degradation and CD4 T cell selection in the thymus. *Science* 280, 450–453.
- Nedjic, J., Aichinger, M., Emmerich, J., Mizushima, N., and Klein, L. (2008). Autophagy in thymic epithelium shapes the T-cell repertoire and is essential for tolerance. *Nature* 455, 396–400.

- Nitta, T., Nitta, S., Lei, Y., Lipp, M., and Takahama, Y. (2009). CCR7-mediated migration of developing thymocytes to the medulla is essential for negative selection to tissue-restricted antigens. *Proc. Natl. Acad. Sci. USA* *106*, 17129–17133.
- Oono, T., Fukui, Y., Masuko, S., Hashimoto, O., Ueno, T., Sanui, T., Inayoshi, A., Noda, M., Sata, M., and Sasazuki, T. (2001). Organ-specific autoimmunity in mice whose T cell repertoire is shaped by a single antigenic peptide. *J. Clin. Invest.* *108*, 1589–1596.
- Palmer, E. (2003). Negative selection—clearing out the bad apples from the T-cell repertoire. *Nat. Rev. Immunol.* *3*, 383–391.
- Park, J.H., Adoro, S., Lucas, P.J., Sarafova, S.D., Alag, A.S., Doan, L.L., Erman, B., Liu, X., Ellmeier, W., Bosselut, R., et al. (2007). 'Coreceptor tuning': cytokine signals transcriptionally tailor CD8 coreceptor expression to the self-specificity of the TCR. *Nat. Immunol.* *8*, 1049–1059.
- Pawlowski, T., Elliott, J.D., Loh, D.Y., and Staerz, U.D. (1993). Positive selection of T lymphocytes on fibroblasts. *Nature* *364*, 642–645.
- Porgador, A., Yewdell, J.W., Deng, Y., Bennink, J.R., and Germain, R.N. (1997). Localization, quantitation, and in situ detection of specific peptide-MHC class I complexes using a monoclonal antibody. *Immunity* *6*, 715–726.
- Reits, E., Griekspoor, A., Neijssen, J., Groothuis, T., Jalink, K., van Veelen, P., Janssen, H., Calafat, J., Drijfhout, J.W., and Neeffjes, J. (2003). Peptide diffusion, protection, and degradation in nuclear and cytoplasmic compartments before antigen presentation by MHC class I. *Immunity* *18*, 97–108.
- Rock, K.L., and Goldberg, A.L. (1999). Degradation of cell proteins and the generation of MHC class I-presented peptides. *Annu. Rev. Immunol.* *17*, 739–779.
- Rock, K.L., Gramm, C., Rothstein, L., Clark, K., Stein, R., Dick, L., Hwang, D., and Goldberg, A.L. (1994). Inhibitors of the proteasome block the degradation of most cell proteins and the generation of peptides presented on MHC class I molecules. *Cell* *78*, 761–771.
- Scollay, R., Wilson, A., D'Amico, A., Kelly, K., Egerton, M., Pearce, M., Wu, L., and Shortman, K. (1988). Developmental status and reconstitution potential of subpopulations of murine thymocytes. *Immunol. Rev.* *104*, 81–120.
- Sebzda, E., Wallace, V.A., Mayer, J., Yeung, R.S., Mak, T.W., and Ohashi, P.S. (1994). Positive and negative thymocyte selection induced by different concentrations of a single peptide. *Science* *263*, 1615–1618.
- Siggs, O.M., Makaroff, L.E., and Liston, A. (2006). The why and how of thymocyte negative selection. *Curr. Opin. Immunol.* *18*, 175–183.
- Singer, A., Mizuochi, T., Munitz, T.I., and Gress, R.E. (1986). Role of self antigen in the selection of the developing T cell repertoire. *Prog. Immunol.* *6*, 60–66.
- Stambas, J., Doherty, P.C., and Turner, S.J. (2007). An *in vivo* cytotoxicity threshold for influenza A virus-specific effector and memory CD8<sup>(+)</sup> T cells. *J. Immunol.* *178*, 1285–1292.
- Starr, T.K., Jameson, S.C., and Hogquist, K.A. (2003). Positive and negative selection of T cells. *Annu. Rev. Immunol.* *21*, 139–176.
- Strasser, A. (2005). The role of BH3-only proteins in the immune system. *Nat. Rev. Immunol.* *5*, 189–200.
- Takahama, Y. (2006). Journey through the thymus: stromal guides for T-cell development and selection. *Nat. Rev. Immunol.* *6*, 127–135.
- Takahama, Y., Suzuki, H., Katz, K.S., Grusby, M.J., and Singer, A. (1994). Positive selection of CD4<sup>+</sup> T cells by TCR ligation without aggregation even in the absence of MHC. *Nature* *371*, 67–70.
- Takahama, Y., Tanaka, K., and Murata, S. (2008). Modest cortex and promiscuous medulla for thymic repertoire formation. *Trends Immunol.* *29*, 251–255.
- Tanchot, C., Lemonnier, F.A., Pérarnau, B., Freitas, A.A., and Rocha, B. (1997). Differential requirements for survival and proliferation of CD8 naïve or memory T cells. *Science* *276*, 2057–2062.
- Uebel, S., Kraas, W., Kienle, S., Wiesmüller, K.H., Jung, G., and Tampé, R. (1997). Recognition principle of the TAP transporter disclosed by combinatorial peptide libraries. *Proc. Natl. Acad. Sci. USA* *94*, 8976–8981.
- Ueno, T., Liu, C., Nitta, T., and Takahama, Y. (2005). Development of T-lymphocytes in mouse fetal thymus organ culture. *Methods Mol. Biol.* *290*, 117–133.
- von Boehmer, H. (1994). Positive selection of lymphocytes. *Cell* *76*, 219–228.
- Yewdell, J.W., Reits, E., and Neeffjes, J. (2003). Making sense of mass destruction: quantitating MHC class I antigen presentation. *Nat. Rev. Immunol.* *3*, 952–961.
- Young, A.C., Nathanson, S.G., and Sacchettini, J.C. (1995). Structural studies of class I major histocompatibility complex proteins: insights into antigen presentation. *FASEB J.* *9*, 26–36.
- Zerrahn, J., Held, W., and Raulet, D.H. (1997). The MHC reactivity of the T cell repertoire prior to positive and negative selection. *Cell* *88*, 627–636.
- Zerrahn, J., Volkman, A., Coles, M.C., Held, W., Lemonnier, F.A., and Raulet, D.H. (1999). Class I MHC molecules on hematopoietic cells can support intrathymic positive selection of T cell receptor transgenic T cells. *Proc. Natl. Acad. Sci. USA* *96*, 11470–11475.
- Zinkernagel, R.M., and Althage, A. (1999). On the role of thymic epithelium vs. bone marrow-derived cells in repertoire selection of T cells. *Proc. Natl. Acad. Sci. USA* *96*, 8092–8097.
- Zinkernagel, R.M., Callahan, G.N., Althage, A., Cooper, S., Klein, P.A., and Klein, J. (1978). On the thymus in the differentiation of "H-2 self-recognition" by T cells: evidence for dual recognition? *J. Exp. Med.* *147*, 882–896.

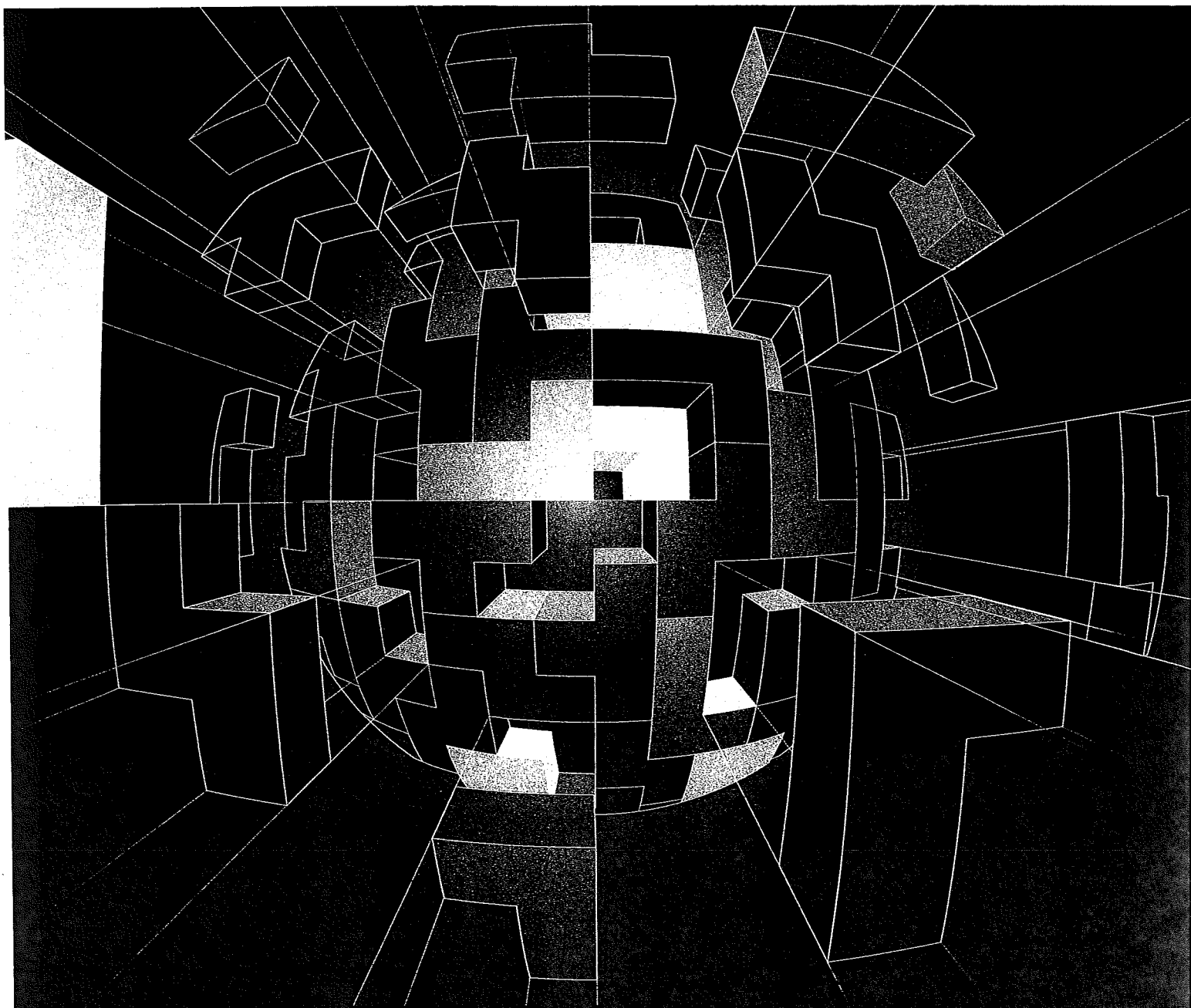
# nature

---

# REVIEWS

february 2009 volume 10 no. 2  
[www.nature.com/reviews](http://www.nature.com/reviews)

## MOLECULAR CELL BIOLOGY



### MICRONA TARGETING

General principles are challenged under *in vivo* conditions

Establishing the body plan

# Molecular mechanisms of proteasome assembly

Shigeo Murata\*, Hideki Yashiroda\* and Keiji Tanaka†

**Abstract** | The 26S proteasome is a highly conserved protein degradation machine that consists of the 20S proteasome and 19S regulatory particles, which include 14 and 19 different polypeptides, respectively. How the proteasome components are assembled is a fundamental question towards understanding the process of protein degradation and its functions in diverse biological processes. Several proteasome-dedicated chaperones are involved in the efficient and correct assembly of the 20S proteasome. These chaperones help the initiation and progression of the assembly process by transiently associating with proteasome precursors. By contrast, little is known about the assembly of the 19S regulatory particles, but several hints have emerged.

## Caspase-like activity

Enzymatic activity that is similar to that of caspases — Cys proteases that have essential roles in apoptosis.  $\beta$ 1 was first reported to cleave after Glu residues, and was thus termed peptidylglutamyl peptide hydrolase. Later, it was found that  $\beta$ 1 also cleaves after Asp acid residues in substrates of caspases.

The 26S proteasome is a eukaryotic ATP-dependent protease that is known to collaborate with the ubiquitin system — the system that tags proteins with polyubiquitin chains as a marker for protein degradation in eukaryotic cells<sup>1,2</sup> (BOX 1). The 26S proteasome is involved in a diverse array of biological processes, including cell-cycle progression, DNA repair, apoptosis, immune response, signal transduction, transcription, metabolism, protein quality control and developmental programmes. It catalyses the precise, rapid and timely degradation and, thus, the irreversible inactivation of targeted proteins. This ensures unidirectional progression of such biological processes.

The 26S proteasome is an unusually large protein complex that consists of two portions: the catalytic 20S proteasome of approximately 700 kDa (also called the 20S core particle (CP)) and the 19S regulatory particle (RP; also called PA700) of approximately 900 kDa, both of which are composed of a set of multiple distinct subunits<sup>1,3</sup> (FIG. 1).

The eukaryotic 20S proteasome is a cylindrical particle that is formed by axial stacking of four heteroheptameric rings — two outer  $\alpha$ -rings and two inner  $\beta$ -rings, each comprising seven structurally similar  $\alpha$ - and  $\beta$ -subunits, respectively.  $\beta$ -rings form a proteolytic chamber and  $\alpha$ -rings serve as a gate for entry into the chamber (FIGS 1,2). Of these 14 subunits,  $\beta$ 1,  $\beta$ 2 and  $\beta$ 5 subunits have hydrolytic activity as Thr proteases for the cleavage of peptide bonds at the carboxyl-terminal side after acidic, basic and hydrophobic residues, respectively. These activities are referred to as caspase-like activity (or peptidylglutamyl-peptide hydrolysing

activity), trypsin-like activity and chymotrypsin-like activity, respectively<sup>4,5</sup>. The active sites of the catalytic  $\beta$ -subunits are located on the inner surface of the proteolytic chamber<sup>6,7</sup>.

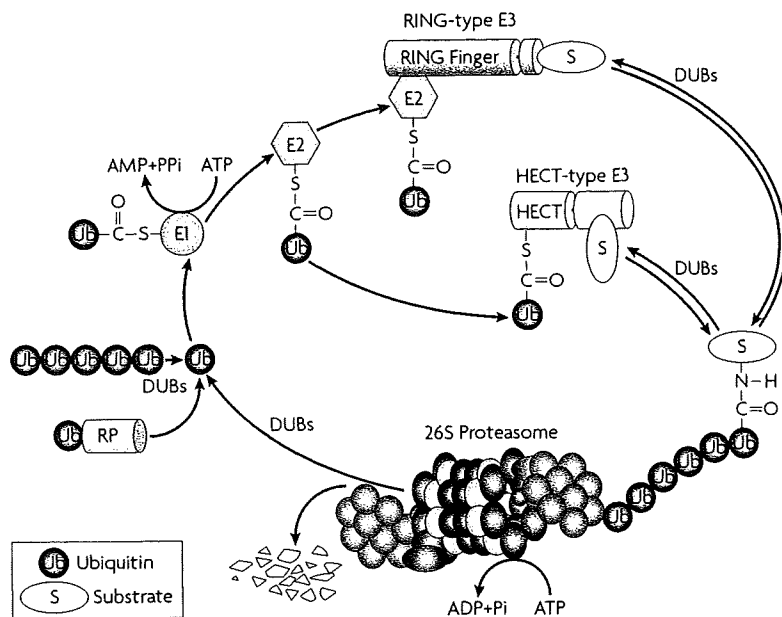
The 19S RP consists of at least 19 different subunits and can be divided into two subcomplexes: the base and the lid<sup>3,8,9</sup> (FIG. 1). The base is composed of six different homologous AAA<sup>+</sup> ATPase subunits, regulatory particle triple-A protein 1 (RPT1)–RPT6, and three non-ATPase subunits, regulatory particle non-ATPase 1 (RPN1), RPN2 and RPN13 (REFS 8,10–14). The ATPase subunits are required for substrate unfolding and  $\alpha$ -ring channel opening, which are prerequisites for threading substrates into the 20S proteasome. RPN1, RPN13, RPT5 and RPN10 capture ubiquitylated proteins either directly or through proteins that contain both UBL (ubiquitin-like) domains and UBA (ubiquitin-associated) domains, such as radiation sensitive 23 (RAD23), dominant suppressor of Kar1 2 (DSK2) and DNA-damage-inducible 1 (DDI1)<sup>3,8,15–20</sup>. RPN10 is assumed to sit at the interface of the lid and base. The lid is composed of nine non-ATPase subunits: RPN3, RPN5–RPN9, RPN11–RPN12 and RPN15 (also called SEM1; DSS1 in mammals)<sup>8,21,22</sup>. The one defined biochemical activity of the lid is the de-ubiquitylation of captured substrates to facilitate their degradation, a process in which the metalloisopeptidase RPN11 has an essential role<sup>23–25</sup>. However, the functions of the other subunits remain unknown. The 19S RP is attached at one or both ends of the 20S proteasome, forming the 26S proteasome. The 26S proteasome acts as an efficient and exhaustive shredder of proteins in living cells. The 19S RP selects proteasomal substrates through

\*Laboratory of Protein Metabolism, Department of Integrated Biology, Graduate School of Pharmaceutical Sciences, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan.

†Laboratory of Frontier Science, Tokyo Metropolitan Institute of Medical Science, 3-18-22 Honkomagome, Bunkyo-ku, Tokyo 113-8613, Japan.

Correspondence to K.T.  
e-mail: tanaka-kj@igakuken.or.jp  
doi:10.1038/nrm2630

Box 1 | The ubiquitin–proteasome system



Ubiquitin (Ub) is translated as a tandemly fused polyubiquitin or as a fusion protein with the ribosomal protein (RP; see the figure). De-ubiquitylating enzymes (DUBs) hydrolyse the isopeptide bond between Ub molecules or Ub and the RP to produce free Ub. The Ub-conjugation pathway consists of three kinds of enzymes: E1 (Ub-activation enzyme), E2 (Ub-conjugation enzyme) and E3 (Ub ligase). Ub is activated by E1, forming a high-energy thiol ester bond between the carboxy-terminal Gly residue of Ub and the active site Cys of E1 in an ATP-dependent reaction. The activated Ub is transferred to E2, and E3 then attaches Ub to a specific substrate protein. The E3 enzymes are classified into two major classes. RING-type E3s bind to both E2 and a substrate and help E2 to transfer Ub to a substrate, whereas HECT-type E3s form a thiol ester with the activated Ub from E2 before transfer of Ub to a substrate. Ub is covalently attached to Lys residues of a substrate. Polyubiquitylated substrates are recognized and degraded by the 26S proteasome in an ATP-dependent manner. DUBs remove polyubiquitin from proteasome substrates before substrates are translocated into the 20S proteasome and regenerate free Ub from unanchored polyubiquitin chains.

the recognition of polyubiquitin chains, which are then excised coincident with unfolding and translocation into the proteolytic 20S proteasome using energy liberated from ATP hydrolysis<sup>1,9</sup>.

Defining the molecular mechanism that is involved in the assembly of this huge and highly sophisticated protease machinery is important for our understanding of the process of protein degradation, as well as regulation of proteasome activity, and has been a major research challenge. Here, we describe the assembly mechanism of the 20S proteasome, our understanding of which has greatly advanced in the past decade<sup>26–30</sup>. We also discuss the formation of the 19S RP, although the available information on this process is fragmentary and much remains a mystery.

**Assembly of prokaryotic 20S proteasome**

The 20S proteasome was originally isolated from eukaryotes, but subsequent studies documented that this large protease complex is conserved in all three domains of life<sup>31</sup>. The basic understanding of the

assembly of 20S proteasomes came from observations in archaeal 20S proteasomes. Each of the  $\alpha$ - and  $\beta$ -subunits of the 20S proteasome of the archaeobacteria *Thermoplasma acidophilum* is encoded by a single-copy gene and forms a homoheptameric ring<sup>32,33</sup> (BOX 2). Coexpression of *T. acidophilum*  $\alpha$ - and  $\beta$ -subunits in *Escherichia coli* resulted in mature and functional 20S proteasomes. Expression of the  $\alpha$ -subunit alone produced heptameric  $\alpha$ -rings, whereas that of the  $\beta$ -subunit could not assemble and folded incompletely in the absence of  $\alpha$ -subunits<sup>34</sup>. Thus, the assembly of the *T. acidophilum* 20S proteasome is probably initiated by the formation of an  $\alpha$ -ring, to which  $\beta$ -subunits are attached subsequently. The ability to form the  $\alpha$ -ring depends on the amino-terminal H0 helix in the  $\alpha$ -subunit, which contacts the loop preceding the H0 helix of the next  $\alpha$ -subunit<sup>33,34</sup>. This helix does not exist in the  $\beta$ -subunit, the tertiary structure of which otherwise resembles the  $\alpha$ -subunit. The eight residue propeptide of the  $\beta$ -subunit is cleaved off during the assembly of the 20S proteasome, although it is dispensable for the assembly because its deletion did not affect the incorporation of the  $\beta$ -subunit.

In bacteria, the 20S proteasome is found only in actinomycetales. Other bacteria, such as *E. coli*, have a structurally related Thr protease called heat-shock locus gene V (HslV)<sup>35,36</sup>. The 20S proteasome of the actinobacterium *Rhodococcus erythropolis* is composed of two different  $\alpha$ -subunits and two different  $\beta$ -subunits. These are encoded by two operons, each containing a pair of  $\alpha$ - and  $\beta$ -subunits<sup>37</sup> (BOX 2). The two  $\alpha$ -subunits and the two  $\beta$ -subunits are over 80% identical, which suggests a fairly recent duplication of the operon. Therefore, it is not surprising that the expression of any combination of one  $\alpha$ -subunit and one  $\beta$ -subunit (for example,  $\alpha 1\beta 1$ ,  $\alpha 1\beta 2$ ,  $\alpha 2\beta 1$  and  $\alpha 2\beta 2$ ) in *E. coli* and *in vitro* yielded active and mature 20S proteasomes<sup>38</sup>. However, the *R. erythropolis*  $\alpha$ -subunits could not form a ring-like structure by itself, in contrast to *T. acidophilum*  $\alpha$ -subunits, presumably because of a smaller contact region between  $\alpha$ -subunits<sup>38,39</sup>. Heterodimer formation of one  $\alpha$ -subunit and one  $\beta$ -subunit precedes the assembly of *R. erythropolis* half-proteasomes, which are composed of one  $\alpha$ -ring and one  $\beta$ -ring. The crystal structures of the *R. erythropolis* proteasome revealed that the  $\beta$ -propeptide acts as an assembly promoting factor by fitting in the interface of two adjacent  $\alpha$ -subunits to help hold them together<sup>39</sup>.

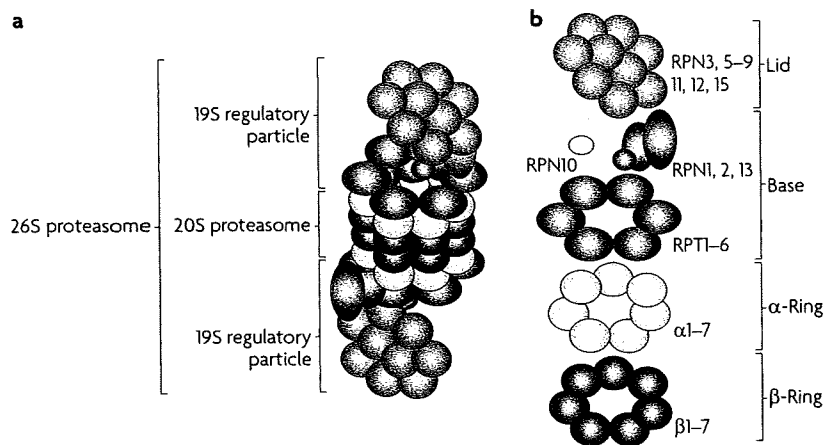
Collectively, archaeobacterial and eubacterial proteasome subunits can assemble autonomously into functional mature proteasomes without the help of any other chaperone proteins.

**Assembly of eukaryotic 20S proteasome**

The eukaryotic 20S proteasome has a more complex subunit composition compared with prokaryotic counterparts. It is composed of seven different  $\alpha$ -subunits and seven different  $\beta$ -subunits, each of which occupies a defined position within the 20S proteasome (FIG. 2; BOX 2).

**Trypsin-like activity**  
 $\beta 2$  cleaves after the basic amino acids Lys and Arg. As this activity resembles that of trypsin, a Ser protease that is secreted in pancreatic juice, it is referred to as 'trypsin-like' activity.

**Chymotrypsin-like activity**  
 $\beta 5$  cleaves after hydrophobic amino acids. This activity resembles that of chymotrypsin, a Ser protease that is secreted in pancreatic juice, and is thus referred to as chymotrypsin-like activity.



**Figure 1 | Schematic diagram of the 26S proteasome.** **a** | The 26S proteasome consists of the catalytic 20S proteasome (a barrel of four stacked rings: two outer  $\alpha$ -rings and two inner  $\beta$ -rings) and the 19S regulatory particle (RP, also known as PA700). **b** | Subunit composition of the 26S proteasome. The regulatory particle is further divided into the base and the lid subcomplexes, which are composed of regulatory particle triple-A (RPT) and regulatory particle non-ATPase (RPN) subunits. RPN10 is coloured yellow because it is supposed to be located at the base–lid interface.

**Assembly of the  $\alpha$ -ring.** The assembly of the eukaryotic 20S proteasome is assumed to start with  $\alpha$ -ring formation. This assumption is based on observations that the expression of *Trypanosoma brucei*  $\alpha 5$  subunit or human  $\alpha 7$  in *E. coli* results in the formation of four stacked or double homoheptameric rings, respectively<sup>40,41</sup>. However, neither homoheptameric rings nor stacked  $\alpha$ -rings occur *in vivo*. Moreover, the  $\alpha$ -ring is usually composed of seven different  $\alpha$ -subunits that occupy defined positions in the  $\alpha$ -ring, which suggests the existence of mechanisms that correctly arrange the seven  $\alpha$ -subunits into a heteroheptamer and prevent oligomerization of  $\alpha$ -rings. Recent studies identified two dimeric complexes that are dedicated to proteasome assembly both in human cells (proteasome assembling chaperone 1 (PAC1)–PAC2 and PAC3–PAC4)<sup>42,43</sup> and in yeast (proteasome biogenesis-associated 1 (Pba1; also known as Poc1), Pba2 (also known as Add66 and Poc2), Pba3 (also known as Poc3, Dmp2 and Irc25) and Pba4 (also known as Poc4 and Dmp1), which are orthologues of human PAC1, PAC2, PAC3 and PAC4, respectively)<sup>44–49</sup> (TABLE 1). This demonstrates a chaperone-assisted mechanism for the efficient assembly of eukaryotic 20S proteasomes.

The PAC1–PAC2 heterodimer was originally identified in a search for proteasome-associated proteins in human cells<sup>42</sup>. PAC1–PAC2 was found to be mainly associated with a proteasome assembly intermediate with a major peak of  $\alpha$ -subunits in glycerol gradient centrifugation analysis. Purification of this intermediate revealed that this complex includes all seven  $\alpha$ -subunits and PAC1–PAC2 but no  $\beta$ -subunits, and has a size of approximately 230 kDa. This is most likely to be the size of the  $\alpha$ -ring<sup>42</sup>. The PAC1–PAC2 complex could bind directly to  $\alpha 5$  and  $\alpha 7$  *in vitro*, and indeed it is associated *in vivo* with a subset of  $\alpha$ -subunits, including  $\alpha 5$  and  $\alpha 7$ , that is presumably an intermediate on the way to the  $\alpha$ -ring<sup>42</sup>.

Knockdown of PAC1 or PAC2 by short interfering RNA (siRNA) decreases normal  $\alpha$ -ring assembly and results in the accumulation of off-pathway products — presumably  $\alpha$ -ring dimers<sup>42</sup>. Interestingly, knockdown of PAC1 results in loss of PAC2, and vice versa, indicating that PAC1 and PAC2 are stable only when they form a heterodimer. These findings show that PAC1–PAC2 does not only assist  $\alpha$ -ring formation but also prevents aberrant dimerization of  $\alpha$ -rings<sup>42</sup>. PAC1–PAC2 sticks to proteasome precursors until the 20S proteasome is completely formed. It is then degraded by the newly formed 20S proteasome and is therefore short-lived, with a half-life of approximately 30–40 minutes, which is consistent with the estimated maturation period of the mammalian 20S proteasome from 30 minutes to 2 hours<sup>42,50</sup> (FIG. 3a).

In the budding yeast, Pba1–Pba2 is probably the counterpart of PAC1–PAC2 because Pba1 and Pba2 have weak sequence similarities to PAC1 and PAC2, respectively, and form a heterodimer that binds to proteasome precursors. Similar to PAC1–PAC2, this heterodimer is degraded by newly formed 20S proteasomes<sup>44,47,48</sup>. However, yeast strains that lack Pba1–Pba2 grow normally and show only modest defects in proteasome assembly with slightly increased assembly intermediates, in contrast to the phenotypes that are observed in human cells with deleted PAC1–PAC2 (REFS 42, 48).

However, several lines of evidence suggest that compromised proteasome function occurs in cells that lack Pba1 and Pba2. The loss of Pba2 causes accumulation of polyubiquitylated proteins in cells, stabilizes certain endoplasmic reticulum-associated degradation (ERAD) substrates and exhibits synthetic phenotypes when combined with the loss of inositol-requiring enzyme 1 (Ire1), a transducer of the unfolded protein response (UPR)<sup>44,47,48,51</sup>. Furthermore, deletion of Pba1 or Pba2 changes the phenotypes of mutants for proteasome-related genes;  $\Delta pba1$  or  $\Delta pba2$  mutants partially suppress the growth defect of ubiquitin-mediated proteolysis 1 ( $\Delta ump1$ ) mutants, another proteasome-dedicated chaperone (see below), whereas they worsen the phenotypes of cells that lack  $\alpha 3$  (the only non-essential subunit of the yeast 20S proteasome) and Rpn4 (a transcription factor that positively regulates proteasome-related genes)<sup>48</sup>. The first observation suggests that slower proteasome formation owing to a lack of Pba1–Pba2 reduces the influence of  $\Delta ump1$ , which causes premature dimerization of incomplete half-proteasomes<sup>48,52</sup> (see below). The genetic interaction with  $\Delta rpn4$  is often observed in mutants that have potentially damaged proteasome activities.

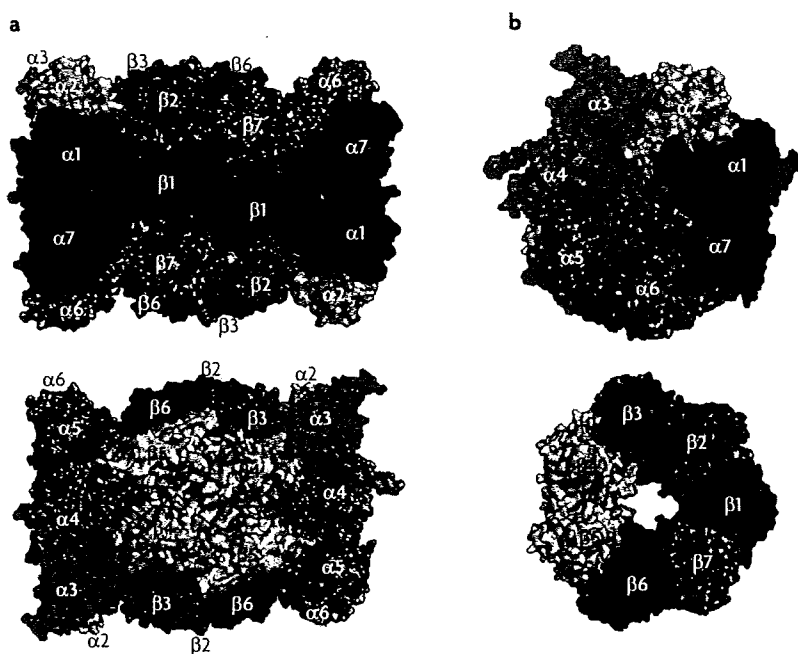
PAC3 was identified as a 14 kDa protein co-purified with  $\alpha$ -rings and PAC1–PAC2 in human cells<sup>43</sup>. As with PAC1–PAC2, most PAC3 is associated with  $\alpha$ -rings in cells. However, in contrast to PAC1–PAC2, PAC3 is a long-lived protein and is released before the completion of 20S proteasome formation. Knockdown of PAC3 causes a reduction in  $\alpha$ -rings and 20S proteasomes, an effect that is similar to PAC1–PAC2 knockdown<sup>43</sup>. However, ectopic expression of PAC3 does not compensate for the loss of PAC1–PAC2, suggesting that PAC1–PAC2 and PAC3 have roles in proteasome assembly at different steps<sup>43</sup>. In support of this conclusion, PAC3 and

**AAA<sup>+</sup> ATPase**  
(ATPase associated with diverse cellular activities). ATP-hydrolysing enzyme that contains one or two conserved ATP-binding domains, which are in turn comprised of conserved A and B motifs. AAA<sup>+</sup> ATPases assemble into oligomeric assemblies (often hexamers) that form a ring-shaped structure with a central pore.

**UBL**  
(Ubiquitin-like). A protein domain with motifs that have significant sequence and structural similarity to ubiquitin. Type 1 UBLs are comprised solely of this motif and generally work as modifiers that are covalently attached to target proteins, in a fashion similar to the ubiquitin. Type 2 UBLs are larger proteins that contain this motif as a part of the protein.

**UBA**  
(Ubiquitin-association). A domain of ~ 45 amino acids that adopts a structure that comprises a three  $\alpha$ -helix bundle. The UBA domain binds to ubiquitin through a conserved hydrophobic surface patch.





**Figure 2 | Molecular structures of eukaryotic 20S proteasomes.** **a** | Side views of the bovine 20S proteasome. The overall shape of the bovine 20S proteasome is an elongated cylinder with large central cavities and narrow constrictions. The approximate length and diameter are 150 Å and 115 Å, respectively. The  $\alpha$ -subunits are located at the ends, whereas the  $\beta$ -subunits form the two inner rings. The carboxy-terminal extension of  $\beta 2$  embraces adjacent  $\beta 3$ , and the C-terminal extension of  $\beta 7$  intercalates between  $\beta 1$  and  $\beta 2$  in the opposing  $\beta$ -ring. **b** | Top views of the  $\alpha$ -ring (upper panel) and the  $\beta$ -ring (lower panel). The C-terminal helices of the  $\alpha 3$  and  $\alpha 4$  subunits protrude from the core with high flexibility. Whereas the  $\beta$ -ring has an open space in its centre, the  $\alpha$ -ring is closed by the amino-terminal extensions of the  $\alpha$ -subunits.

PAC1 knockdown have additive effects on proteasome assembly; simultaneous depletion of PAC1 and PAC3 causes a severe reduction in  $\alpha$ -rings and the 20S proteasome compared with depletion of PAC1 or PAC3 alone. Interestingly, whereas the knockdown of only PAC3 is not associated with the accumulation of off-pathway products, such as  $\alpha$ -ring dimers that are observed in PAC1-depleted cells, simultaneous knockdown of PAC1 and PAC3 causes the accumulation of half-proteasomes that lack  $\beta 5$  (REF. 43). These findings suggest that PAC1–PAC2 and PAC3 work differently but cooperate in the assembly of  $\alpha$ -rings and the correct formation of half-proteasomes.

Pba3 and Pba4, the budding yeast orthologues of human PAC3 and PAC4, were identified independently through bioinformatics searches<sup>45</sup> and genetic screens of mutations that suppress lethal DNA damage<sup>44</sup>, confer sensitivity to amino acid analogues<sup>46</sup> or stabilize ornithine decarboxylase protein<sup>49</sup>. Pba3 and Pba4 form a heterodimer complex, and this complex can be co-purified with proteasome precursors that consist of all seven  $\alpha$ -subunits and an unprocessed  $\beta 2$  subunit *in vivo*<sup>46</sup>. Pba3–Pba4 directly interacts with  $\alpha 5$  *in vitro*, and the affinity-purified complex with tagged Pba3–Pba4 includes a large amount of  $\alpha 4$ ,  $\alpha 5$ ,  $\alpha 6$  and  $\alpha 7$  compared with other  $\alpha$ -subunits<sup>45,46</sup>. These observations suggest that Pba3–Pba4 has a role at an early stage

of  $\alpha$ -ring assembly, starting with the formation of a Pba3–Pba4– $\alpha 5$  tertiary complex, which in turn recruits neighbouring  $\alpha$ -subunits, with Pba3–Pba4 remaining bound until the incorporation of  $\beta 2$ .

Deletion of Pba3 or Pba4 in cells markedly decreases 20S proteasomes and causes the accumulation of assembly intermediates. These cells show accumulation of dead-end complexes that comprise  $\beta 2$  and all  $\alpha$ -subunits except  $\alpha 4$  (REF. 46). However,  $\Delta pba4$  cells produce an alternative proteasome that is found in  $\Delta \alpha 3$  cells; 20–50% of the assembled 20S proteasomes in  $\Delta pba4$  cells contained a second copy of  $\alpha 4$  in place of  $\alpha 3$  (REF. 45). Such alternative proteasomes confer resistance to heavy metal stress on  $\Delta pba3$ – $pba4$  cells as well as  $\Delta \alpha 3$  cells, suggesting that proteasome-dedicated chaperones provide structural flexibility of the proteasome depending on the cellular environment<sup>45</sup>. Whether such alternative proteasomes would be observed in the absence of PAC3–PAC4 in mammalian cells remains to be elucidated. Taken together, these studies show that Pba3–Pba4 catalyses correct subunit orientation of the  $\alpha$ -ring, presumably by facilitating the recruitment of  $\alpha 4$  to  $\alpha 5$ , which might prevent incorporation of a second copy of  $\alpha 4$  in the position that is normally occupied by  $\alpha 3$  in the  $\alpha$ -ring.

**$\beta$ -ring formation on the  $\alpha$ -ring.** The  $\alpha$ -ring serves as a scaffold for the assembly of  $\beta$ -subunits. Catalytic  $\beta$ -subunits and non-catalytic  $\beta 6$  and  $\beta 7$  are synthesized with N-terminal propeptides, which are removed at the final step of assembly to expose the catalytic Thr residues of  $\beta 1$ ,  $\beta 2$  and  $\beta 5$ . The N-terminal active sites of  $\beta$  subunits are on the inner surface of the  $\beta$ -rings, whereas the C termini of  $\beta$  subunits are on the outer surface of the 20S proteasome. During the assembly pathway from the  $\alpha$ -ring through the half-proteasome, each  $\beta$  subunit assembles on the  $\alpha$ -ring. An intermediate called the 13S complex, which is composed of one  $\alpha$ -ring and unprocessed  $\beta 2$ ,  $\beta 3$  and  $\beta 4$ , has been identified both in yeast and mammals, indicating the rank order of  $\beta$ -subunit positioning onto the  $\alpha$ -ring<sup>48,53,54</sup>.

Recent studies in yeast showed that the addition of other  $\beta$  subunits, except  $\beta 7$ , form another intermediate that is referred to as the half-mer ( $-\beta 7$ ) precursor complex<sup>48,55</sup>. siRNA-mediated silencing of each  $\beta$ -subunit in mammalian cells caused an accumulation of a specific ‘assembly-arrested’ intermediate just before incorporation of the knocked-down  $\beta$ -subunit<sup>56</sup>. This approach suggests the defined order of  $\beta$ -subunit assembly on the  $\alpha$ -ring: assembly begins with  $\beta 2$ , followed by  $\beta 3$ ,  $\beta 4$ ,  $\beta 5$ ,  $\beta 6$ ,  $\beta 1$  and finally  $\beta 7$  (REF. 56) (FIG. 3a). During  $\beta$ -ring assembly, release of PAC3 is coupled to  $\beta 3$  incorporation in human cells<sup>43,56</sup> (FIG. 3a). This is consistent with the observation that Pba3–Pba4 is selectively co-purified with  $\beta 2$  but not with other  $\beta$ -subunits in yeast<sup>44,46</sup>, which indicates a conserved mechanism in the proteasome assembly between PAC3–PAC4 and Pba3–Pba4. As PAC3 directly binds to  $\beta 3$  *in vitro*,  $\beta 3$  might be recruited to the assembly intermediate by transient interaction with PAC3, whose release is an obligatory step in the incorporation of  $\beta 3$  (REFS 43,56).

#### Operon

A unit of genes in prokaryotes that is expressed as a single messenger RNA.

#### ERAD

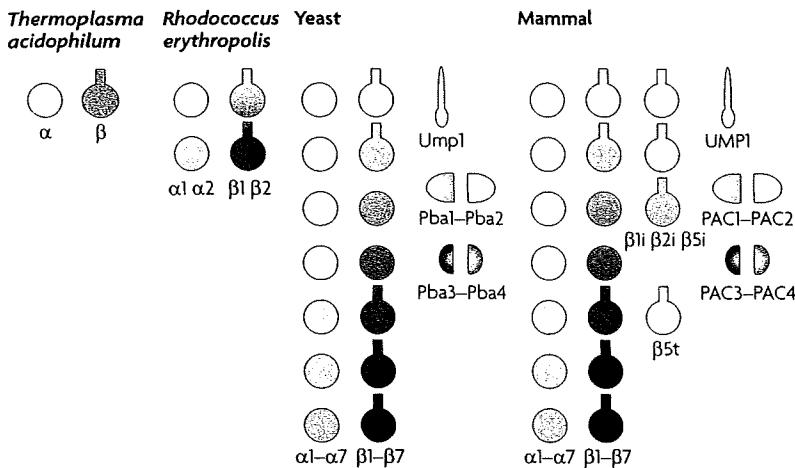
(Endoplasmic reticulum-associated degradation). A system that ubiquitylates misfolded proteins in the endoplasmic reticulum for degradation by the 26S proteasome.

#### Unfolded protein response

A cellular stress response that is induced by the accumulation of unfolded proteins in the endoplasmic reticulum.



Box 2 | Evolution of proteasome assembly in different organisms



Most archaea and actinobacteria have one  $\alpha$ - and one  $\beta$ -type subunit (see the figure), but their composition varies from one  $\alpha$ -type and two  $\beta$ -type, two  $\alpha$ -type and two  $\beta$ -type, and two  $\alpha$ -type and one  $\beta$ -type subunits. Among the species that synthesize two  $\beta$ -type subunits, one of two  $\beta$ -type subunits is thought to be inactive in the *Sulfolobus*, *Pyrobaculum* and *Aeropyrum* species. Contrary to the diversity of the composition observed in prokaryotes, all eukaryotic 20S proteasomes consist of seven different  $\alpha$ - and seven different  $\beta$ -type subunits. Of the seven  $\beta$ -type subunits, only three  $\beta$ -subunits,  $\beta 1$ ,  $\beta 2$  and  $\beta 5$ , have hydrolytic activities. Primitive eukaryotes, such as yeast, have a single kind of 20S proteasome composed of seven distinct  $\alpha$ - and seven distinct  $\beta$ -type subunits. However, many higher eukaryotes have paralogous genes. For example, all but  $\alpha 7$ ,  $\beta 1$ ,  $\beta 6$  and  $\beta 7$  are duplicated in *Arabidopsis thaliana* and the paralogous  $\alpha 3$ ,  $\alpha 4$ ,  $\alpha 6$ ,  $\beta 2$ ,  $\beta 4$  and  $\beta 5$  genes in *Drosophila melanogaster* are expressed in a male-specific manner, although the functions of these duplicated genes are unknown. In jawed vertebrates including mammals, the constitutive catalytic subunits  $\beta 1$ ,  $\beta 2$  and  $\beta 5$  are replaced with  $\beta 1i$ ,  $\beta 2i$  and  $\beta 5i$  by interferon (IFN) $\gamma$  stimulation, which results in formation of the immunoproteasome. The immunoproteasomes, which have higher trypsin-like and chymotrypsin-like activities than standard proteasomes, are thought to have a role in better presentation of antigenic peptides on major histocompatibility complex (MHC) class I molecules. In the thymus, another catalytic subunit,  $\beta 5t$ , is incorporated in the 20S proteasomes instead of  $\beta 5$  or  $\beta 5i$  along with  $\beta 1i$  and  $\beta 2i$ . This thymus-specific proteasome is called thymoproteasome and is thought to have an essential role in positive selection of MHC class I restricted T cells, a process that increases useful T cell repertoire. Eukaryotes have acquired proteasome-dedicated extrinsic chaperones, namely ubiquitin-mediated proteolysis 1 (Ump1) or UMP1 and proteasome biogenesis-associated 1 (Pba1)–4 or proteasome assembling chaperone 1 (PAC1)–4, which coincides with the acquisition of subunit complexity.

**Insight from the Pba3–Pba4– $\alpha 5$  complex structure.**

Crystal structure analysis has revealed that the tertiary structure of the Pba3–Pba4 heterodimer closely resembles that of the PAC3 homodimer, despite low sequence similarities<sup>46</sup> (FIG. 4a). However, it is uncertain whether the PAC3 homodimers exist *in vivo*. Intriguingly, Pba3–Pba4 and PAC3 have a  $\beta$ -sandwich structure that is formed by two six-stranded  $\beta$ -sheets surrounded by two helices on each side. This structure resembles those of  $\alpha$ - and  $\beta$ -subunits, although the two  $\beta$ -sheets in  $\alpha$ - and  $\beta$ -subunits are made up of five  $\beta$ -strands<sup>46</sup> (FIG. 4b). Analysis of the Pba3–Pba4– $\alpha 5$  complex revealed that Pba3–Pba4 is attached to the surface of the  $\alpha$ -ring where  $\beta$ -subunits are assembled (FIG. 4c). However, the binding mode of Pba3–Pba4 is different from that of  $\beta$ -subunits; Pba3–Pba4 is located at the more inner space of the  $\alpha$ -ring, which enables Pba3–Pba4 to interact with

three different  $\alpha$ -subunits ( $\alpha 4$ ,  $\alpha 5$  and  $\alpha 6$ ), compared with  $\beta$ -subunits that interact with two neighbouring  $\alpha$ -subunits (FIG. 4c). This feature of Pba3–Pba4 might be useful in initiating  $\alpha$ -ring assembly. This location of Pba3–Pba4 is consistent with biochemical analyses that indicate that Pba3–Pba4 or PAC3 detach from  $\alpha$ -rings during  $\beta$ -ring formation (FIG. 3a).

Structural analyses of PAC1–PAC2 and Pba1–Pba2 are not available, but the observation that the binding of PAC1–PAC2 or proteasome activator 28 kDa (PA28; also known as PSME1) to assembly intermediates is mutually exclusive suggests that the binding surface of PAC1–PAC2 on the  $\alpha$ -ring is opposite to that of PAC3 (REF. 56). PA28 is an activator complex that is known to bind to the outer surface of the  $\alpha$ -ring in place of 19S RP and is involved in major histocompatibility complex (MHC) class I antigen presentation<sup>57,58</sup>.

**Dual role of Ump1 in  $\beta$ -ring formation and dimerization.**

Before the discovery of Ump1, the 20S proteasome was presumed to assemble autonomously. In yeast, *ump1* mutants are defective in ubiquitin-mediated proteolysis, and Ump1 was the first identified extrinsic assembly factor for 20S proteasomes<sup>52</sup>. Ump1 is not essential for cell viability, but  $\Delta$ *ump1* cells exhibit significant growth defects and hypersensitivity to various stresses. Ump1 is specifically associated with assembly intermediates of 20S proteasomes and seems to enter the assembly pathway after association of  $\beta 2$ ,  $\beta 3$  and  $\beta 4$  in yeast<sup>48</sup>. Following dimerization of half-proteasomes, Ump1 is encapsulated and degraded within the newly formed 20S proteasome<sup>52</sup>. Loss of Ump1 causes the accumulation of assembly intermediates and 20S proteasomes that contain unprocessed  $\beta$ -subunits, which indicates that Ump1 coordinates the processing of  $\beta$ -subunits and dimerization of half-proteasomes.

The human orthologue of Ump1 was identified by a database search and an amino acid sequence analysis of a small precursor proteasome protein and was named UMP1 (REF. 59), proteasembilin<sup>60</sup> or proteasome maturation protein (POMP)<sup>61</sup>. UMP1 is included in precursor proteasomes with unprocessed  $\beta$ -subunits and is degraded on the completion of proteasome assembly with a similar half-life to PAC1–PAC2 (REF. 42). However, knockdown of UMP1 does not cause the accumulation of assembly intermediates that contain unprocessed  $\beta$ -subunits, which contrasts with  $\Delta$ *ump1* cells, which accumulate such intermediates<sup>42,43,52,56</sup>. Furthermore, knockdown of UMP1 impairs  $\beta 5$  recruitment<sup>62</sup>, and UMP1 can bind to the  $\alpha$ -ring in the absence of any  $\beta$ -subunits *in vitro*<sup>63</sup>, suggesting that it is incorporated into proteasome precursors earlier than yeast Ump1 and that UMP1 is required for  $\beta$ -ring formation.

Subsequent studies showed that UMP1 knockdown results in the accumulation of  $\alpha$ -rings that do not contain  $\beta$ -subunits, and that incorporation of UMP1 is coupled with  $\beta 2$  incorporation *in vivo*: UMP1 is required for  $\beta 2$  incorporation, and vice versa<sup>56</sup> (FIG. 3a). Therefore, UMP1 is required for the initiation of  $\beta$ -ring formation, a role that has not been observed for yeast Ump1. Furthermore, UMP1 also regulates

Table 1 | Proteasome assembling chaperones in humans and yeast

Humans				Yeast				
Name	Alias	HUGO	Length (aa)	Name	Alias	Systematic gene name	Length (aa)	Sequence identity
<i>Proteasome assembling chaperones</i>								
PAC1	DSCR2	PSMG1	288	Pba1	Poc1	YLR199C	276	<10%
PAC2	HCCA3	PSMG2	264	Pba2	Add66 and Poc2	YKL206C	267	19%
PAC3	MGC10911	PSMG3	122	Pba3	Poc3, Dmp2 and lrc25	YLR021W	179	<10%
PAC4	C6orf86	PSMG4	123	Pba4	Poc4 and Dmp1	YPL144W	148	<10%
UMP1	Proteasemblin	POMP	141	Ump1	Rns2	YBR173C	148	24%
<i><math>\alpha</math>-subunit</i>								
$\alpha$ 5	Zeta	PSMA5	241	$\alpha$ 5	Pup2 and Doa5	YGR253C	260	60%

For comparison, we provide information on the  $\alpha$ 5 subunit. aa, amino acids; Doa5, degradation of  $\alpha$ 2; DSCR2, down syndrome critical region gene 2; HCCA3, hepatocellular carcinoma associated gene 3; MGC10911, mammalian gene collection 10911; PAC, proteasome assembling chaperone; Pba, proteasome biogenesis-associated; POMP, proteasome maturation protein; Rns, RNase hypersensitive; UMP1, ubiquitin-mediated proteolysis 1.

the recruitment of precursor proteasome at the endoplasmic reticulum, which seems to be the main location of proteasome assembly in mammalian cells<sup>63</sup>.

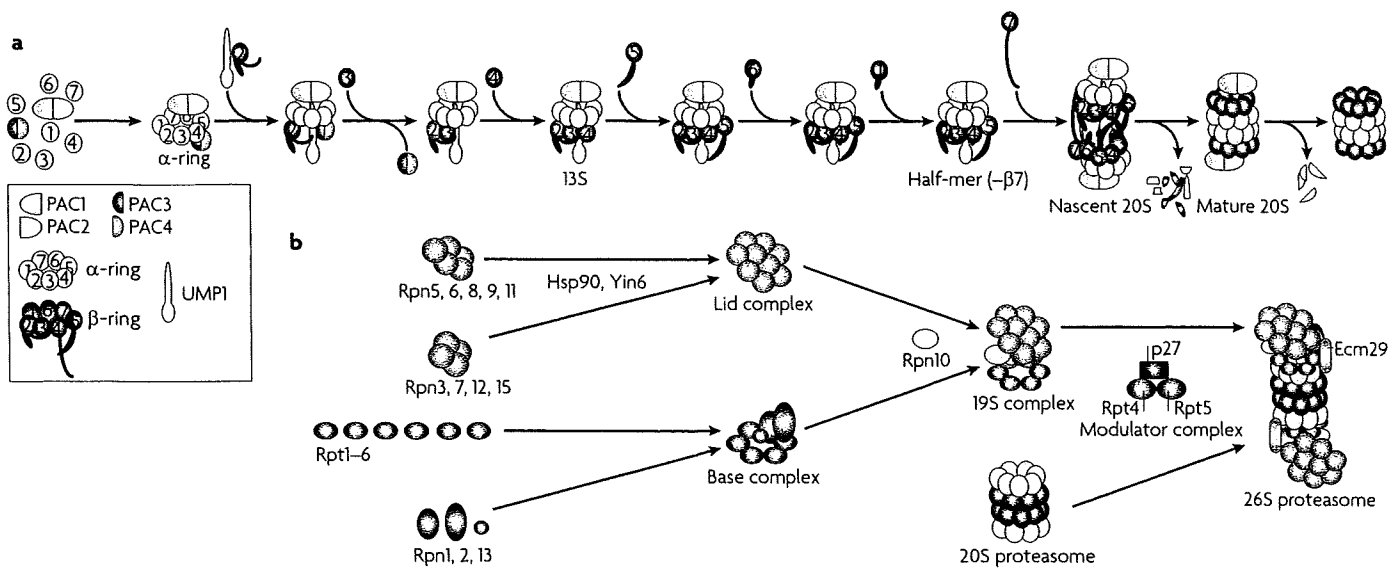
**Intramolecular chaperones promote proteasome assembly.** Propeptides of some proteases are known to facilitate their own folding or molecular assembly, working as intramolecular chaperones<sup>64</sup>. The propeptides and tails of 20S proteasome  $\beta$ -subunits have such roles. Bacterial *R. erythropolis*  $\beta$ -subunit propeptides promote subunit folding as well as proteasome assembly<sup>38,39</sup>. The roles of N-terminal propeptides and C-terminal tails of  $\beta$ -subunits have been studied in yeast and humans and these C-terminal tails have been shown to have important roles in proteasome assembly by providing specific interactions with *cis*- and *trans*- $\beta$ -rings. The propeptide of  $\beta$ 5 facilitates its incorporation into the 20S proteasome and is essential for yeast viability<sup>65</sup>. In human cells,  $\beta$ 5 propeptide is not required for its own incorporation but rather for  $\beta$ 6 recruitment<sup>56</sup>. The propeptides of  $\beta$ 1 and  $\beta$ 2 are dispensable for cell viability but are known to protect the N-terminal catalytic Thr residue of  $\beta$ 1 and  $\beta$ 2 against N<sup>ε</sup>-acetylation, and mutants lacking these two propeptides display modest defects in proteasome biogenesis<sup>66</sup>. In human cells, loss of  $\beta$ 2 propeptide leads to failure of  $\beta$ 3 recruitment and is therefore fatal<sup>56</sup>. The C-terminal tail of  $\beta$ 2, which wraps around  $\beta$ 3 in the same  $\beta$ -ring, is also essential for proteasome biogenesis both in yeast and human cells<sup>6,7,56,67</sup> (FIG. 2). The C-terminal tail of  $\beta$ 7, which is inserted into a groove between  $\beta$ 1 and  $\beta$ 2 in the opposite ring, also has an important role in the dimerization of half-proteasomes<sup>6,7,55,56,67</sup> (FIG. 2) as well as stabilization of the active conformation of  $\beta$ 1 (REF. 67).

Interestingly, the propeptides of human  $\beta$ -subunits are different from those of yeast counterparts, compared with the mature  $\beta$ -subunits, which are well conserved between yeast and humans (Supplementary information S1 (figure)). Such differences are also noted in the proteasome-dedicated chaperones (TABLE 1). It might be assumed that non-essential propeptides and chaperones do not need to be highly conserved during evolution,

whereas their basic functions and tertiary structures are maintained: PAC3–PAC4 and Pba3–Pba4 are good examples (see above). However, the C-terminal extension of  $\beta$ 2, which has an essential role by directly associating with  $\beta$ 3, is highly conserved (Supplementary information S1 (figure)). In this sense, it is curious that the essential  $\beta$ 5 propeptide is poorly conserved (Supplementary information S1 (figure)). As the  $\beta$ 5 propeptide associates both physically and functionally with Ump1 or UMP1 (see below), the  $\beta$ 5 propeptide might have evolved to work cooperatively with Ump1 and its orthologues, the sequences of which have significantly changed during evolution.

**Dimerization of the half-proteasome.** Incorporation of  $\beta$ 7 into half-mers ( $-\beta$ 7) and intercalation of its C-terminal tail between  $\beta$ 1 and  $\beta$ 2 of the opposing  $\beta$ -ring triggers dimerization of the half-proteasomes in yeast and human<sup>48,55,56,67</sup>. Overexpression of  $\beta$ 7 suppresses the lethality of a  $\beta$ 5 mutant that lacks its propeptide ( $\beta$ 5 $\Delta$ pro), which suggests that  $\beta$ 5 propeptide has a partially redundant role for half-mer dimerization. Intriguingly,  $\Delta$ ump1 and  $\beta$ 6 $\Delta$ pro also rescued the lethality of  $\beta$ 5 $\Delta$ pro, and  $\Delta$ ump1 rescued the lethality of  $\beta$ 6 $\Delta$ NTE (NTE stands for N-terminal extension; a unique N-terminal nine residue sequence in mature  $\beta$ 6). These observations suggest that  $\beta$ 7,  $\beta$ 5 propeptide and  $\beta$ 6 NTE promote proteasome assembly, whereas Ump1 and  $\beta$ 6 propeptide have an inhibitory role on dimerization of half-mers. Ump1 could be an assembly checkpoint factor that inhibits this dimerization until a full set of  $\beta$ -subunits are recruited on to the  $\alpha$ -ring, and thus mediates productive proteasome assembly<sup>48</sup>.

Bleomycin-sensitive 10 (Blm10) was identified as a component of proteasome precursors that have been purified with Ump1 in yeast<sup>68</sup>.  $\Delta$ blm10 cells grow apparently normally under normal conditions but show mild temperature sensitivity. The turnover of Ump1 and the processing of  $\beta$ 5 are accelerated in  $\Delta$ blm10 cells, which suggests a role for Blm10 in preventing the premature formation of 20S proteasomes<sup>68</sup>.



**Figure 3 | Model for proteasome assembly. a** | The assembly of human 20S proteasomes. Proteasome assembling chaperone 1 (PAC1)–PAC2 heterodimers and PAC3–PAC4 complexes assist  $\alpha$ -ring formation. PAC1–PAC2 suppresses off-pathway aggregation of  $\alpha$ -rings. Sequential incorporation of  $\beta$ -subunits starts from the association of  $\beta$ 2 and ubiquitin-mediated proteolysis 1 (UMP1) on the  $\alpha$ -ring. UMP1 is required for association of  $\beta$ 2 in the early assembly intermediates. PAC3–PAC4 is released following the association of  $\beta$ 3 and is recycled. Subsequent orderly incorporation of other  $\beta$ -subunits is assisted by intramolecular chaperones such as the propeptides of  $\beta$ 2 and  $\beta$ 5 and the carboxy-terminal tail of  $\beta$ 2. Dimerization of half-proteasomes without  $\beta$ 7, also called half-mers ( $-\beta$ 7), is triggered by incorporation of  $\beta$ 7 and intercalation of its C-terminal tail into the groove between  $\beta$ 1 and  $\beta$ 2 of the opposing  $\beta$ -ring. This is followed by removal of  $\beta$ -subunit propeptides ( $\beta$ 1,  $\beta$ 2,  $\beta$ 5,  $\beta$ 6 and  $\beta$ 7) and UMP1 degradation. PAC1–PAC2 is subsequently degraded by the newly formed active 20S proteasomes. **b** | The assembly of 19S regulatory particle (RP) is largely unknown, but it is suggested that the base and the lid are assembled independently. In yeast, Yin6 and heat-shock protein 90 (Hsp90) seem to be involved in the assembly of the lid, which is thought to be formed from two different subclusters. Regulatory particle non-ATPase 10 (Rpn10) stabilizes the connection between the lid and the base. In yeast, extracellular matrix 29 (Ecm29) has a role in tethering the 20S to the RP. In mammals, the so-called modulator complex facilitates the association of the RP with the 20S by an unknown mechanism. Rpt, regulatory particle triple-A.

However, the combination of Blm10 deletion and  $\beta$ 7 C-terminal truncation results in severe impairment of proteasome activity and  $\beta$ 2 processing, indicating that Blm10 promotes proteasome maturation, presumably by stabilizing nascent 20S proteasomes<sup>55</sup>. A single model that can account for these two conflicting roles of Blm10 remains elusive at this stage. Additional functions beyond 20S maturation have been reported for Blm10 and its mammalian homologue PA200 (also known as PSME4). Blm10 and PA200 bind to the ends of mature 20S proteasomes and open the axial channel, thus enhancing peptide hydrolysis, most notably after acidic residues, which is responsible for a role for Blm10 and PA200 in maintaining genomic stability<sup>69–74</sup>.

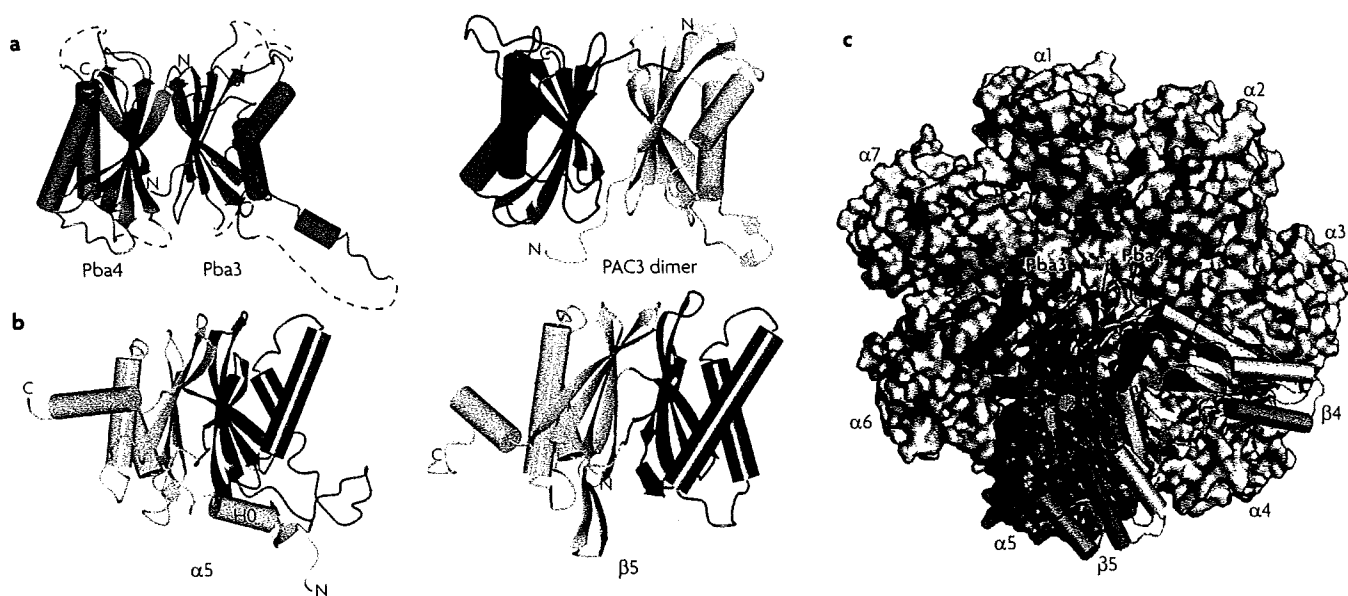
The 19S RP might also participate in stabilizing nascent 20S proteasomes, because the RP associates with an Ump1-containing complex in the absence of Blm10, and an *rpn2* mutant that is defective in the 19S RP–20S interaction shows a large accumulation of precursor complexes that contain unprocessed  $\beta$ 2 when combined with  $\Delta$ *blm10* (REF. 55).

The correct dimerization of half-proteasomes is followed by the removal of  $\beta$ -propeptides and degradation of Ump1 coincides with the completion of proteasome maturation, followed by degradation of PAC1–PAC2 (REFS 42,52) (FIG. 3a).

### Tissue specific proteasomes

Vertebrates encode four additional catalytic  $\beta$ -subunits: three interferon (IFN) $\gamma$ -inducible  $\beta$ 1i,  $\beta$ 2i,  $\beta$ 5i immunosubunits and one thymus-specific  $\beta$ 5t subunit, which are incorporated in the place of their most closely related  $\beta$ -subunits, thus forming distinct subtypes of proteasomes with altered catalytic activities. These are called immunoproteasomes and thymoproteasomes<sup>75–77</sup>. These alternative proteasomes have key roles in acquired immunity by altering antigen processing. The immunoproteasome has increased chymotrypsin-like and trypsin-like activities, which are favourable for the production of antigenic peptides that bind to the groove of MHC class I molecules<sup>76,78</sup>. The thymoproteasome has reduced chymotrypsin-like activity, which is thought to be important for the production of a unique peptide repertoire in the thymus<sup>77,79,80</sup>. By comparison, the 20S proteasome, including constitutively expressed catalytic subunits  $\beta$ 1,  $\beta$ 2 and  $\beta$ 5, is often called the standard or constitutive proteasome.

In *Drosophila melanogaster*, approximately one-third of the proteasome subunits are found to have testis-specific isoforms<sup>81</sup>. One of these, proteasome subunit  $\alpha$ 6 testis-specific (PROSA6T), is required for spermatogenesis<sup>82</sup>. However, whether there are specific mechanisms for the assembly of such testis-specific subtypes has not been explored.



**Figure 4 | Structural basis of Pba3-Pba4 and PAC3.** **a** | Ribbon diagrams of the proteasome biogenesis-associated 3 (Pba3)-Pba4 heterodimer (in yeast) and the proteasome assembling chaperone 3 (PAC3) homodimer (in mammals). Both complexes have  $\beta$ -sandwich structures that are formed by two six-stranded  $\beta$ -sheets, and these sandwich structures are surrounded by four helices.  $\alpha$ -helices and  $\beta$ -strands are shown by cylinders and arrows, respectively. **b** | Ribbon diagrams of the  $\alpha 5$  and  $\beta 5$  subunits. All  $\alpha$ - and  $\beta$ -subunits have the same basic structures, except the helical structure of H0 at the amino terminus of the  $\alpha$ -subunits. **c** | Model of the Pba3-Pba4- $\alpha$ -ring- $\beta 4$ - $\beta 5$  complex. Pba3-Pba4,  $\beta 4$  and  $\beta 5$  are shown in ribbon presentations. Pba3-Pba4 is located deeper in the  $\alpha$ -ring compared with  $\beta$ -subunits and thus interacts with three  $\alpha$ -subunits,  $\alpha 4$ ,  $\alpha 5$  and  $\alpha 6$ , whereas  $\beta 5$  interacts with only two  $\alpha$ -subunits,  $\alpha 4$  and  $\alpha 5$ . This model also shows that the presence of Pba3-Pba4 on the  $\alpha$ -ring causes steric hindrance with  $\beta 4$ .

**The immunoproteasome.** The mechanism of immunoproteasome assembly has been well studied. The propeptides of the immunosubunits and UMP1 have key roles in this process. Similar to the standard proteasome, the immunoproteasome is assembled in a stepwise manner, but one remarkable difference is that  $\beta 1i$  enters the assembly pathway of the immunoproteasome earlier than in standard proteasome assembly, forming an assembly intermediate containing an  $\alpha$ -ring,  $\beta 1i$ ,  $\beta 2i$ ,  $\beta 3$  and  $\beta 4$  (REF. 54). In this intermediate, the incorporation of  $\beta 2i$  depends on  $\beta 1i$ , and the incorporation of  $\beta 1i$  is in turn facilitated by  $\beta 2i$ , although  $\beta 1i$  can be incorporated in the absence of  $\beta 2i$ <sup>83-85</sup>.  $\beta 5i$  is incorporated preferentially over  $\beta 5$  into the intermediates that contain  $\beta 1i$  and  $\beta 2i$  and is required for the processing of  $\beta 1i$  and  $\beta 2i$ , which is dependent on the  $\beta 5i$  propeptide but not on  $\beta 5i$  catalytic activity<sup>84,85</sup>. Such interdependency provides homogenous formation of immunoproteasomes that contain all three inducible subunits.  $\beta 5i$  can also be incorporated into the standard proteasome. Consistent with these observations,  $\beta 2i$  processing and incorporation is severely impaired in  $\beta 1i$ -deficient cells, and  $\beta 1i$  incorporation is partially impaired in  $\beta 2i$ -deficient cells, whereas  $\beta 5i$  incorporation is not affected in both cells<sup>86,87</sup>.  $\beta 5i$ -deficient cells that have been treated with concanavalin-A, a lectin that is known to activate lymphocytes, exhibit significant retardation of proteasome assembly and accumulation of proteasome precursors containing unprocessed  $\beta 1i$  and  $\beta 2i$ <sup>86</sup>.

Intriguingly, IFN $\gamma$  stimulation increased the transcription of *UMP1* mRNA along with that of immunosubunits<sup>59-61</sup>, but it decreased UMP1 protein levels, thereby accelerating its turnover by approximately fourfold<sup>62</sup>. This rapid turnover was coupled with the maturation of active immunoproteasomes, which indicates that the generation of immunoproteasomes is fourfold faster than that of standard proteasomes<sup>62</sup>. The higher affinity of UMP1 to  $\beta 5i$  compared with  $\beta 5$  is likely to contribute to the rapid maturation of immunoproteasomes<sup>62</sup>. Through these mechanisms, immunoproteasomes are preferentially assembled over standard proteasomes, despite the coexistence of both immunosubunits and standard subunits.

**The thymoproteasome.** Another vertebrate-specific 20S proteasome is the thymoproteasome, which is exclusively expressed in cortical thymic epithelial cells (cTECs). cTECs are responsible for a process that is referred to as positive selection, which enriches the useful repertoire of developing T cells in the thymus<sup>77</sup>. The catalytic subunits of the thymoproteasome are composed of  $\beta 1i$ ,  $\beta 2i$  and  $\beta 5t$ . Ectopically expressed  $\beta 5t$  in a human cell line that does not express immunosubunits can be readily processed and incorporated into the proteasome<sup>77</sup>, suggesting that  $\beta 5t$  is preferentially incorporated over  $\beta 5$  and that  $\beta 1i$  and  $\beta 2i$  are not prerequisites for its incorporation. As most proteasomes in cTECs are thymoproteasomes, it is presumed that  $\beta 5i$  is also expressed in cTECs and that  $\beta 5t$  is preferentially incorporated over  $\beta 5i$ .

The propeptide or exceptionally extended C-terminal tail (51 amino acids) of  $\beta 5t$  might be involved in the assembly of thymoproteasomes, but there is no evidence in support of this.

### Assembly of the 19S RP

The 19S RP is found only in eukaryotes. Compared with the 20S proteasome, the mechanism involved in the assembly of the 19S RP is mostly unknown. In an N-terminally truncated mutant of Rpn2, a base subunit, 26S proteasomes were almost completely abolished at the restrictive temperature, but the complete lid complex was observed in yeast<sup>88</sup>. Conversely, the base complex was found in the *rpn5-1* lid mutant<sup>88</sup>. These findings suggest that the base and the lid are assembled independently, after which these two subcomplexes are joined (FIG. 3b). However, accumulation of Rpt5-containing particles of various sizes was detected in cells with impaired 20S proteasome formation ( $\Delta ump1$ ,  $\Delta pba3$  and  $\Delta pba4$ ) or  $\Delta \alpha 3$  cells<sup>45</sup>. This indicates erroneous RP formation in the presence of defective 20S proteasomes, raising the possibility that the 20S proteasome functions as an assembly factor for the RP<sup>45</sup>.

**Putting together the base.** The base is composed of six related AAA<sup>+</sup> ATPase and three non-ATPase subunits. The six ATPase subunits are thought to form a ring-like structure by analogy to other AAA<sup>+</sup> ATPase complexes, such as cell division cycle 48 (Cdc48) and proteasome-activating nucleotidase, which are successfully assembled into homo-hexamers when expressed in *E. coli*<sup>34,89</sup>. However, coexpression of the six proteasome ATPases in *E. coli* failed to yield a heterohexameric complex<sup>90</sup>. Rather, coexpression of Rpt1 and Rpt2 or solo expression of Rpt4 yielded high molecular weight complexes<sup>90</sup>. These results suggest the existence of chaperones that discriminate and arrange the six homologous ATPase subunits in a defined order, as is observed in the assembly of 20S  $\alpha$ -ring. Recently, a model in which the RPN1–RPN2 complex is located as the central unit of the base, around which the six ATPase subunits wrap to complete the base, has been presented. This might suggest a role of RPN1–RPN2 as a platform for base assembly<sup>91</sup>.

Several extrinsic factors of unknown functions that associate with certain base subunits have been identified: p27 forms a complex with RPT4 and RPT5 (REF. 92), p28 (also known as gankyrin) with RPT3 (REFS 93–95), and subunit 5b (S5b) with RPT1, RPT2 and RPN1 (REF. 19). These subcomplexes might be assembly intermediates of the base complex, and these non-proteasomal factors might act as chaperones to help assemble the base complex correctly.

**Assembling the lid.** Based on the detection of partially assembled lid subcomplexes in yeast lid mutants<sup>96</sup> and results of mass spectrometry analysis of intact lid<sup>97</sup>, two clusters in the lid complex have been proposed: one is composed of Rpn5, Rpn6, Rpn8, Rpn9 and Rpn11 and the other of Rpn3, Rpn7, Rpn12 and Rpn15, in which the interaction of Rpn3 and Rpn5 connects the two clusters (FIG. 3b). These observations are consistent with the reported subunit interaction maps that are based on yeast

two-hybrid analysis<sup>98,99</sup>. However, whether these clusters are authentic assembly intermediates and, if so, how these clusters are assembled, remains elusive.

Hsp90, the most abundant molecular chaperone known to assist in protein folding and cell signalling<sup>100</sup>, is thought to have a role in both the assembly and maintenance of the lid in yeast<sup>101</sup>. Inactivation of Hsp90 in yeast led to disassembly of the lid complex, which was then reassembled into the 26S proteasome by reactivation of Hsp90 *in vivo* or by adding normal Hsp90 and ATP *in vitro*<sup>101</sup>. These findings suggest that the ATP-dependent chaperone activity of Hsp90 contributes to the assembly of the lid as well as the 26S proteasome. However, the molecular role of Hsp90 in the 26S proteasome assembly remains to be identified.

*Schizosaccharomyces pombe* Yin6 is the homologue of the mammalian oncoprotein INT6. It has a proteasome–COP9–initiation factor domain (PCI domain) and is known to interact with the 26S proteasome through Rpn5 (REF. 102). The lid itself also contains four PCI domain proteins (Rpn3, Rpn5, Rpn6 and Rpn9) and the PCI domain is suggested to function in protein–protein interaction. In  $\Delta yin6$  cells, Rpn5 does not efficiently incorporate into 26S proteasomes owing to mislocalization of Rpn5, which results in defective 26S proteasome formation<sup>102</sup>. Overexpression of Rpn7 rescues proteasome mislocalization or disassembly in  $\Delta yin6$  cells, suggesting further functional interaction between Yin6 and the 26S proteasome<sup>103</sup>. However, there is no evidence for mammalian INT6 being involved in the assembly of 26S proteasomes. Furthermore, INT6 binds not only to the lid but also to the COP9 signalosome and eukaryotic translation initiation factor 3 (eIF3)<sup>104</sup>, and therefore the role of INT6 and Yin6 might not necessarily reflect a role in proteasome function.

### Regulation of 20S–RP interactions

The eukaryotic 20S proteasome is a latent protease on its own because the centre of the  $\alpha$ -ring, through which unfolded polypeptides enter the chamber of the 20S proteasome, is gated by the N termini of the  $\alpha$ -subunits<sup>6,105</sup>. Association of the C termini of RPT2 and RPT5 of the base is proposed to open the gate to the chamber of the 20S and enables the 20S proteasome to degrade proteins by sticking the conserved C-terminal residues of RPT2 and RPT5 into pockets between  $\alpha$ -subunits<sup>106–108</sup>. ATP binding to the ATPase subunits is required for this interaction<sup>107–109</sup>. Thus, regulation of 20S–RP interactions is a crucial step in proteasome function.

Intriguingly, in a yeast lid mutant (*rpn5-1*) that exhibits defective lid assembly but normal base and 20S assembly, the base and the 20S proteasome could not form a stable complex, suggesting that the association of the lid with the base induces a conformational change of the base that is necessary for the stable association of the RP with the 20S proteasome<sup>88</sup>. Inhibition of proteasome active sites also stabilized 26S proteasomes, suggesting that the interface between the RP and the 20S proteasome dynamically changes depending on the activities of the 20S proteasome<sup>110</sup>. However, it has been reported that the disassembly of the 26S proteasome

#### Cdc48

A chaperone-like AAA<sup>+</sup> ATPase that is required for various cellular processes, such as cell cycle progression, homotypic membrane fusion and ERAD. The human counterpart of Cdc48 is valosin-containing protein.

#### Proteasome-activating nucleotidase

An AAA<sup>+</sup> ATPase ring complex and archaeal regulatory particle triple-A homologue that opens the gate of 20S proteasomes and activates protein degradation.

#### PCI domain

The homology domain of unclear function that is present in several components of the proteasome, the COP9 signalosome and eukaryotic translation initiation factor 3.

#### COP9 signalosome

An eight-subunit protein complex that regulates protein ubiquitylation and turnover in various developmental and physiological contexts. Extensively characterized in plants but fundamental to all eukaryotes, this complex post-translationally modifies the cullin subunit of E3 ubiquitin ligases by cleaving the covalently coupled polypeptide Nedd8.

and even dissociation of the RP into subcomplexes or subunits are induced following ATP-dependent degradation of a substrate protein in yeast<sup>111</sup>. By contrast, mammalian 26S proteasomes can degrade substrate proteins without any release of subunits or subcomplexes<sup>112</sup>, and therefore this 'chew and spew' model observed in yeast is still controversial<sup>112-114</sup>.

Some auxiliary factors have been shown to regulate RP association with the 20S proteasome. Extracellular matrix 29 (Ecm29) is a protein of approximately 200 kDa and can bind to both the RP and the 20S proteasome in yeast<sup>115</sup>. Purified 26S proteasomes from  $\Delta$ ecm29 cells tend to dissociate into RPs and 20S proteasomes. Together with the findings of electron micrographs of Ecm29-20S proteasome complexes, it is likely that Ecm29 stabilizes 26S proteasomes by tethering the 20S proteasome to the RP<sup>115</sup>, a function that can overcome even the absence of ATP<sup>110</sup>. The so-called modulator complex, which is a complex of p27, RPT4 and RPT5 and is found in mammalian cells, enhanced the proteolytic activity of the proteasome by promoting the association of RPs with 20S proteasomes<sup>116,117</sup> (FIG. 3b). However, the mechanism of this action remains unknown. Nob1, which was identified as a binding partner of Rpn12 and Pno1, was reported to interact with the RP-half-proteasome precursor that contains Ump1, and is required for this precursor maturation, resulting in both 20S and 26S proteasome formation in yeast<sup>118</sup>. However, Nob1 was also reported as an endonuclease for processing of the 20S pre-rRNA to the mature 18S rRNA, and its role in proteasome biogenesis has been challenged<sup>5,119</sup>. Discrepancies between these results remain to be reconciled.

### Concluding remarks

The regulation of proteasome activity in tumour cells is clinically important<sup>120</sup>. Based on the successful clinical use of bortezomib, an inhibitor of proteasomal catalytic activity, in the treatment of multiple myeloma, attempts

to broaden its application for other types of malignant neoplasms as well as development of new proteasome inhibitors are currently underway<sup>120-122</sup>. In contrast to increased proteasome activities in tumour cells, decreased proteasome activities have been suggested in neurodegenerative diseases and ageing<sup>123-126</sup>. In addition, it has been reported that a single nucleotide polymorphism in the *PSMA6* gene that increases the expression of the  $\alpha 1$  subunit confers susceptibility to myocardial infarction<sup>127</sup>, although the precise mechanism is unclear. Therefore, elucidating proteasome regulation at various steps, such as transcription, assembly, catalytic activity and localization, could provide clues to treatment of these disorders.

Among these potential regulatory processes, the assembly mechanism of the 20S proteasome is well studied. 20S proteasome assembly in yeast and human cells have been proven to closely resemble each other, sharing common assembly chaperones as well as intramolecular chaperones, although some of their roles are slightly different between yeast and humans. As the crystal structure of the mammalian 20S proteasome is known, it might be feasible to design drugs that inhibit contact surfaces that are important for the assembly between subunits or subunits and assembly chaperones. Furthermore, the mechanism of RP assembly should be studied more extensively. At present, we do not even know the initial events or the requirements for certain assembly chaperones in the RP assembly. Extensive genetic screening has been done to identify genes that modulate the ubiquitin-proteasome pathway, and numerous proteasome mutants and mutants that affect the 20S proteasome assembly have been identified. However, the exact molecules that are essential for RP assembly remain undiscovered at present. This could also suggest that the assembly of the 19S RP is autonomous, these molecules have been simply overlooked, or a novel and alternative screening approach is needed to identify such molecules.

- Coux, O., Tanaka, K. & Goldberg, A. L. Structure and functions of the 20S and 26S proteasomes. *Annu. Rev. Biochem.* **65**, 801-847 (1996).
- Baumeister, W., Watz, J., Zuhl, F. & Seemuller, E. The proteasome: paradigm of a self-compartmentalizing protease. *Cell* **92**, 367-380 (1998).
- Schmidt, M., Hanna, J., Elsasser, S. & Finley, D. Proteasome-associated proteins: regulation of a proteolytic machine. *Biol. Chem.* **386**, 725-737 (2005).
- Arendt, C. S. & Hochstrasser, M. Identification of the yeast 20S proteasome catalytic centers and subunit interactions required for active-site formation. *Proc. Natl Acad. Sci. USA* **94**, 7156-7161 (1997).
- Heinemeyer, W., Ramos, P. C. & Dohmen, R. J. The ultimate nanoscale mincer: assembly, structure and active sites of the 20S proteasome core. *Cell. Mol. Life Sci.* **61**, 1562-1578 (2004).
- Groll, M. *et al.* Structure of 20S proteasome from yeast at 2.4 Å resolution. *Nature* **386**, 463-471 (1997).
- Unno, M. *et al.* The structure of the mammalian 20S proteasome at 2.75 Å resolution. *Structure* **10**, 609-618 (2002).
- Glickman, M. H. *et al.* A subcomplex of the proteasome regulatory particle required for ubiquitin-conjugate degradation and related to the COP9-signalosome and eIF3. *Cell* **94**, 615-623 (1998).
- Glickman, M. H. & Ciechanover, A. The ubiquitin-proteasome proteolytic pathway: destruction for the sake of construction. *Physiol. Rev.* **82**, 373-428 (2002).
- Finley, D. *et al.* Unified nomenclature for subunits of the *Saccharomyces cerevisiae* proteasome regulatory particle. *Trends Biochem. Sci.* **23**, 244-245 (1998).
- Yao, T. *et al.* Proteasome recruitment and activation of the Uch37 deubiquitinating enzyme by Adrm1. *Nature Cell Biol.* **8**, 994-1002 (2006).
- Jorgensen, J. P. *et al.* Adrm1, a putative cell adhesion regulating protein, is a novel proteasome-associated factor. *J. Mol. Biol.* **360**, 1043-1052 (2006).
- Hamazaki, J. *et al.* A novel proteasome interacting protein recruits the deubiquitinating enzyme UCH37 to 26S proteasomes. *EMBO J.* **25**, 4524-4536 (2006).
- Qiu, X. B. *et al.* hRpn13/ADRM1/GP110 is a novel proteasome subunit that binds the deubiquitinating enzyme, UCH37. *EMBO J.* **25**, 5742-5753 (2006).
- Elsasser, S. *et al.* Proteasome subunit Rpn1 binds ubiquitin-like protein domains. *Nature Cell Biol.* **4**, 725-730 (2002).
- Saeki, Y., Sone, T., Toh-e, A. & Yokosawa, H. Identification of ubiquitin-like protein-binding subunits of the 26S proteasome. *Biochem. Biophys. Res. Commun.* **296**, 813-819 (2002).
- Schreiner, P. *et al.* Ubiquitin docking at the proteasome through a novel pleckstrin-homology domain interaction. *Nature* **453**, 548-552 (2008).
- Husnjak, K. *et al.* Proteasome subunit Rpn13 is a novel ubiquitin receptor. *Nature* **453**, 481-488 (2008).
- Deveraux, Q., Jensen, C. & Rechsteiner, M. Molecular cloning and expression of a 26 S protease subunit enriched in dileucine repeats. *J. Biol. Chem.* **270**, 23726-23729 (1995).
- Lam, Y. A., Lawson, T. G., Velayutham, M., Zweier, J. L. & Pickart, C. M. A proteasomal ATPase subunit recognizes the polyubiquitin degradation signal. *Nature* **416**, 763-767 (2002).
- Funakoshi, M., Li, X., Velichutina, I., Hochstrasser, M. & Kobayashi, H. Sem1, the yeast ortholog of a human BRCA2-binding protein, is a component of the proteasome regulatory particle that enhances proteasome stability. *J. Cell Sci.* **117**, 6447-6454 (2004).
- Sone, T., Saeki, Y., Toh-e, A. & Yokosawa, H. Sem1p is a novel subunit of the 26 S proteasome from *Saccharomyces cerevisiae*. *J. Biol. Chem.* **279**, 28807-28816 (2004).
- Verma, R. *et al.* Role of Rpn11 metalloprotease in deubiquitination and degradation by the 26S proteasome. *Science* **298**, 611-615 (2002).
- Yao, T. & Cohen, R. E. A cryptic protease couples deubiquitination and degradation by the proteasome. *Nature* **419**, 403-407 (2002).
- Cope, G. A. *et al.* Role of predicted metalloprotease motif of Jab1/Csn5 in cleavage of Nedd8 from Cul1. *Science* **298**, 608-611 (2002).
- Ramos, P. C. & Dohmen, R. J. PAComakers of proteasome core particle assembly. *Structure* **16**, 1296-1304 (2008).

# REVIEWS

27. Kusmierczyk, A. R. & Hochstrasser, M. Some assembly required: dedicated chaperones in eukaryotic proteasome biogenesis. *Biol. Chem.* **389**, 1143–1151 (2008).
28. Rosenzweig, R. & Glickman, M. H. Chaperone-driven proteasome assembly. *Biochem. Soc. Trans.* **36**, 807–812 (2008).
29. Ellis, R. J. Molecular chaperones: assisting assembly in addition to folding. *Trends Biochem. Sci.* **31**, 395–401 (2006).
30. Williamson, J. R. Cooperativity in macromolecular assembly. *Nature Chem. Biol.* **4**, 458–465 (2008).
31. Volker, C. & Lupas, A. N. Molecular evolution of proteasomes. *Curr. Top. Microbiol. Immunol.* **268**, 1–22 (2002).
32. Zwickl, P. *et al.* Primary structure of the Thermoplasma proteasome and its implications for the structure, function, and evolution of the multicatalytic proteinase. *Biochemistry* **31**, 964–972 (1992).
33. Lowe, J. *et al.* Crystal structure of the 20S proteasome from the archaeon *T. acidophilum* at 3.4 Å resolution. *Science* **268**, 533–539 (1995).
34. Zwickl, P., Ng, D., Woo, K. M., Klenk, H. P. & Goldberg, A. L. An archaeobacterial ATPase, homologous to ATPases in the eukaryotic 26 S proteasome, activates protein breakdown by 20 S proteasomes. *J. Biol. Chem.* **274**, 26008–26014 (1999).
35. Yoo, S. J. *et al.* Purification and characterization of the heat shock proteins HslV and HslU that form a new ATP-dependent protease in *Escherichia coli*. *J. Biol. Chem.* **271**, 14035–14040 (1996).
36. Rohrwild, M. *et al.* HslV–HslU: a novel ATP-dependent protease complex in *Escherichia coli* related to the eukaryotic proteasome. *Proc. Natl Acad. Sci. USA* **93**, 5808–5813 (1996).
37. Tamura, T. *et al.* The first characterization of a eubacterial proteasome: the 20S complex of *Rhodococcus*. *Curr. Biol.* **5**, 766–774 (1995).
38. Zuhl, F., Seemuller, E., Colbik, R. & Baumeister, W. Dissecting the assembly pathway of the 20S proteasome. *FEBS Lett.* **418**, 189–194 (1997).
39. Kwon, Y. D., Nagy, I., Adams, P. D., Baumeister, W. & Jap, B. K. Crystal structures of the *Rhodococcus* proteasome with and without its pro-peptides: implications for the role of the pro-peptide in proteasome assembly. *J. Mol. Biol.* **335**, 233–245 (2004).
40. Yao, Y. *et al.*  $\alpha 5$  subunit in *Trypanosoma brucei* proteasome can self-assemble to form a cylinder of four stacked heptamer rings. *Biochem. J.* **344**, 349–358 (1999).
41. Gerards, W. L. *et al.* The human  $\alpha$ -type proteasomal subunit Hsc8 forms a double ringlike structure, but does not assemble into proteasome-like particles with the  $\beta$ -type subunits HsDelta or HsBPROS26. *J. Biol. Chem.* **272**, 10080–10086 (1997).
42. Hirano, Y. *et al.* A heterodimeric complex that promotes the assembly of mammalian 20S proteasomes. *Nature* **437**, 1381–1385 (2005). **Discovery of the PAC1–PAC2 heterodimer as a proteasome assembly-dedicated chaperone.**
43. Hirano, Y. *et al.* Cooperation of multiple chaperones required for the assembly of mammalian 20S proteasomes. *Mol. Cell* **24**, 977–984 (2006). **Discovery of PAC3 as a proteasome-assembly-dedicated chaperone.**
44. Le Tallec, B. *et al.* 20S proteasome assembly is orchestrated by two distinct pairs of chaperones in yeast and in mammals. *Mol. Cell* **27**, 660–674 (2007). **References 44–46 and 49 show that Pba3–Pba4 helps to assemble the yeast 20S proteasome. In reference 44, PAC4 was identified by a homology search of Pba4.**
45. Kusmierczyk, A. R., Kunjappu, M. J., Funakoshi, M. & Hochstrasser, M. A multimeric assembly factor controls the formation of alternative 20S proteasomes. *Nature Struct. Biol.* **15**, 237–244 (2008).
46. Yashiroda, H. *et al.* Crystal structure of a chaperone complex that contributes to the assembly of yeast 20S proteasomes. *Nature Struct. Biol.* **15**, 228–236 (2008). **Shows the crystal structure of Pba3–Pba4– $\alpha 5$ .**
47. Scott, C. M. *et al.* ADD66, a gene involved in the endoplasmic reticulum-associated degradation of  $\alpha 1$ -antitrypsin-Z in yeast, facilitates proteasome activity and assembly. *Mol. Biol. Cell* **18**, 3776–3787 (2007).
48. Li, X., Kusmierczyk, A. R., Wong, P., Emili, A. & Hochstrasser, M.  $\beta$ -subunit appendages promote 20S proteasome assembly by overcoming an Ump1-dependent checkpoint. *EMBO J.* **26**, 2339–2349 (2007). **References 48 and 55 provide evidence that  $\beta 7$  incorporation is a late limiting step in dimerization of precursor complexes. Reference 55 also shows that Blm10 and the 19S RP have a redundant function in the 20S proteasome maturation.**
49. Hoyt, M. A. *et al.* A genetic screen for *Saccharomyces cerevisiae* mutants affecting proteasome function, using a ubiquitin-independent substrate. *Yeast* **25**, 199–217 (2008).
50. Yang, Y., Fruh, K., Ahn, K. & Peterson, P. A. *In vivo* assembly of the proteasomal complexes, implications for antigen processing. *J. Biol. Chem.* **270**, 27687–27694 (1995).
51. Palmer, E. A. *et al.* Differential requirements of novel A1Piz degradation deficient (ADD) genes in ER-associated protein degradation. *J. Cell Sci.* **116**, 2361–2373 (2003).
52. Ramos, P. C., Hockendorff, J., Johnson, E. S., Varshavsky, A. & Dohmen, R. J. Ump1p is required for proper maturation of the 20S proteasome and becomes its substrate upon completion of the assembly. *Cell* **92**, 489–499 (1998). **The identification of the first proteasome-dedicated chaperone for the efficient assembly and maturation of the 20S proteasome.**
53. Frenzel, S., Pesold-Hurt, B., Seelig, A. & Kloetzel, P. M. 20S proteasomes are assembled via distinct precursor complexes. Processing of LMP2 and LMP7 propeptides takes place in 13–16 S preproteasome complexes. *J. Mol. Biol.* **236**, 975–981 (1994).
54. Nandi, D., Woodward, E., Ginsburg, D. B. & Monaco, J. J. Intermediates in the formation of mouse 20S proteasomes: implications for the assembly of precursor  $\beta$  subunits. *EMBO J.* **16**, 5363–5375 (1997).
55. Marques, A. J., Gianemann, C., Ramos, P. C. & Dohmen, R. J. The C-terminal extension of the  $\beta 7$  subunit and activator complexes stabilize nascent 20S proteasomes and promote their maturation. *J. Biol. Chem.* **282**, 34869–34876 (2007).
56. Hirano, Y. *et al.* Dissecting  $\beta$ -ring assembly pathway of the mammalian 20S proteasome. *EMBO J.* **27**, 2204–2213 (2008). **Shows the order of association of  $\beta$  subunits and UMP1 on  $\alpha$ -ring.**
57. Whitby, F. C. *et al.* Structural basis for the activation of 20S proteasomes by 11S regulators. *Nature* **408**, 115–120 (2000).
58. Strehl, B. *et al.* Interferon- $\gamma$ , the functional plasticity of the ubiquitin–proteasome system, and MHC class I antigen processing. *Immunol. Rev.* **207**, 19–30 (2005).
59. Burri, L. *et al.* Identification and characterization of a mammalian protein interacting with 20S proteasome precursors. *Proc. Natl Acad. Sci. USA* **97**, 10348–10353 (2000).
60. Griffin, T. A., Slack, J. P., McCluskey, T. S., Monaco, J. J. & Colbert, R. A. Identification of proteasomblin, a mammalian homologue of the yeast protein, Ump1p, that is required for normal proteasome assembly. *Mol. Cell Biol. Res. Commun.* **3**, 212–217 (2000).
61. Witt, E. *et al.* Characterisation of the newly identified human Ump1 homologue POMP and analysis of LMP7( $\beta 5$ ) incorporation into 20 S proteasomes. *J. Mol. Biol.* **301**, 1–9 (2000).
62. Heink, S., Ludwig, D., Kloetzel, P. M. & Kruger, E. IFN- $\gamma$ -induced immune adaptation of the proteasome system is an accelerated and transient response. *Proc. Natl Acad. Sci. USA* **102**, 9241–9246 (2005).
63. Fricke, B., Heink, S., Steffen, J., Kloetzel, P. M. & Kruger, E. The proteasome maturation protein POMP facilitates major steps of 20S proteasome formation at the endoplasmic reticulum. *EMBO Rep.* **8**, 1170–1175 (2007).
64. Shinde, U. & Inouye, M. Intramolecular chaperones: polypeptide extensions that modulate protein folding. *Semin. Cell Dev. Biol.* **11**, 35–44 (2000).
65. Chen, P. & Hochstrasser, M. Autocatalytic subunit processing couples active site formation in the 20S proteasome to completion of assembly. *Cell* **86**, 961–972 (1996).
66. Arendt, C. S. & Hochstrasser, M. Eukaryotic 20S proteasome catalytic subunit propeptides prevent active site inactivation by N-terminal acetylation and promote particle assembly. *EMBO J.* **18**, 3575–3585 (1999).
67. Ramos, P. C., Marques, A. J., London, M. K. & Dohmen, R. J. Role of C-terminal extensions of subunits  $\beta 2$  and  $\beta 7$  in assembly and activity of eukaryotic proteasomes. *J. Biol. Chem.* **279**, 14323–14330 (2004).
68. Fehlker, M., Wendler, P., Lehmann, A. & Erenkel, C. Blm3 is part of nascent proteasomes and is involved in a late stage of nuclear proteasome assembly. *EMBO Rep.* **4**, 959–963 (2003).
69. Ustrell, V., Hoffman, L., Pratt, G. & Rechsteiner, M. PA200, a nuclear proteasome activator involved in DNA repair. *EMBO J.* **21**, 3516–3525 (2002).
70. Kajava, A. V., Gorbea, C., Ortega, J., Rechsteiner, M. & Steven, A. C. New HEAT-like repeat motifs in proteins regulating proteasome structure and function. *J. Struct. Biol.* **146**, 425–430 (2004).
71. Ortega, J. *et al.* The axial channel of the 20S proteasome opens upon binding of the PA200 activator. *J. Mol. Biol.* **346**, 1221–1227 (2005).
72. Schmidt, M. *et al.* The HEAT repeat protein Blm10 regulates the yeast proteasome by capping the core particle. *Nature Struct. Biol.* **12**, 294–303 (2005).
73. Iwanczyk, J. *et al.* Structure of the Blm10–20 S proteasome complex by cryo-electron microscopy. Insights into the mechanism of activation of mature yeast proteasomes. *J. Mol. Biol.* **363**, 648–659 (2006).
74. Blickwedehl, J. *et al.* Role for proteasome activator PA200 and postglutamyl proteasome activity in genomic stability. *Proc. Natl Acad. Sci. USA* **105**, 16165–16170 (2008).
75. Tanaka, K. & Kasahara, M. The MHC class I ligand-generating system: roles of immunoproteasomes and the interferon- $\gamma$ -inducible proteasome activator PA28. *Immunol. Rev.* **163**, 161–176 (1998).
76. Kloetzel, P. M. Generation of major histocompatibility complex class I antigens: functional interplay between proteasomes and TPII. *Nature Immunol.* **5**, 661–669 (2004).
77. Murata, S. *et al.* Regulation of CD8<sup>+</sup> T cell development by thymus-specific proteasomes. *Science* **316**, 1349–1353 (2007).
78. Rock, K. L., York, I. A. & Goldberg, A. L. Post-proteasomal antigen processing for major histocompatibility complex class I presentation. *Nature Immunol.* **5**, 670–677 (2004).
79. Murata, S., Takahama, Y. & Tanaka, K. Thymoproteasome: probable role in generating positively selecting peptides. *Curr. Opin. Immunol.* **20**, 192–196 (2008).
80. Takahama, Y., Tanaka, K. & Murata, S. Modest cortex and promiscuous medulla for thymic repertoire formation. *Trends Immunol.* **29**, 251–255 (2008).
81. Yuan, X., Miller, M. & Belote, J. M. Duplicated proteasome subunit genes in *Drosophila melanogaster* encoding testes-specific isoforms. *Genetics* **144**, 147–157 (1996).
82. Zhong, L. & Belote, J. M. The testis-specific proteasome subunit Prosa6T of *D. melanogaster* is required for individualization and nuclear maturation during spermatogenesis. *Development* **134**, 3517–3525 (2007).
83. Groettrup, M., Standera, S., Stohwasser, R. & Kloetzel, P. M. The subunits MECL1 and LMP2 are mutually required for incorporation into the 20S proteasome. *Proc. Natl Acad. Sci. USA* **94**, 8970–8975 (1997).
84. Griffin, T. A. *et al.* Immunoproteasome assembly: cooperative incorporation of interferon  $\gamma$  (IFN- $\gamma$ )-inducible subunits. *J. Exp. Med.* **187**, 97–104 (1998). **Shows that interdependence of  $\beta 11$ ,  $\beta 2i$  and  $\beta 5i$  for their incorporation into the 20S proteasome.**
85. Kingsbury, D. J., Griffin, T. A. & Colbert, R. A. Novel propeptide function in 20 S proteasome assembly influences  $\beta$  subunit composition. *J. Biol. Chem.* **275**, 24156–24162 (2000).
86. De, M. *et al.*  $\beta 2$  subunit propeptides influence cooperative proteasome assembly. *J. Biol. Chem.* **278**, 6153–6159 (2003).
87. Basler, M., Moeblus, J., Elenich, L., Groettrup, M. & Monaco, J. J. An altered T cell repertoire in MECL1-deficient mice. *J. Immunol.* **176**, 6665–6672 (2006).
88. Isono, E. *et al.* The assembly pathway of the 19S regulatory particle of the yeast 26S proteasome. *Mol. Biol. Cell* **18**, 569–580 (2007). **Shows that the 20S proteasome, the base and the lid can be formed and imported into the nucleus independently.**



89. DeLaBarre, B. & Brunger, A. T. Complete structure of p97/valosin-containing protein reveals communication between nucleotide domains. *Nature Struct. Biol.* **10**, 856–863 (2003).
90. Takeuchi, J. & Tamura, T. Recombinant ATPases of the yeast 26S proteasome activate protein degradation by the 20S proteasome. *FEBS Lett.* **565**, 39–42 (2004).
91. Rosenzweig, R., Osmulski, P. A., Gaczynska, M. & Glickman, M. H. The central unit within the 19S regulatory particle of the proteasome. *Nature Struct. Biol.* **15**, 573–580 (2008).
92. DeMartino, G. N. *et al.* Identification, purification, and characterization of a PA700-dependent activator of the proteasome. *J. Biol. Chem.* **271**, 3112–3118 (1996).
93. Hori, T. *et al.* cDNA cloning and functional analysis of p28 (Nas6p) and p40.5 (Nas7p), two novel regulatory subunits of the 26S proteasome. *Gene* **216**, 113–122 (1998).
94. Dawson, S. *et al.* Gankyrin is an ankyrin-repeat oncoprotein that interacts with CDK4 kinase and the S6 ATPase of the 26 S proteasome. *J. Biol. Chem.* **277**, 10893–10902 (2002).
95. Nakamura, Y. *et al.* Structure of the oncoprotein gankyrin in complex with S6 ATPase of the 26S proteasome. *Structure* **15**, 179–189 (2007).
96. Isono, E., Saito, N., Kamata, N., Saeki, Y. & Toh, E. A. Functional analysis of Rpn6p, a lid component of the 26 S proteasome, using temperature-sensitive *rpn6* mutants of the yeast *Saccharomyces cerevisiae*. *J. Biol. Chem.* **280**, 6537–6547 (2005).
97. Sharon, M., Taverner, T., Ambroggio, X. I., Deshaies, R. J. & Robinson, C. V. Structural organization of the 19S proteasome lid: insights from MS of intact complexes. *PLoS Biol.* **4**, e267 (2006). Together with reference 96, this paper provides evidence that the lid consists of two subclusters.
98. Fu, H., Reis, N., Lee, Y., Glickman, M. H. & Vierstra, R. D. Subunit interaction maps for the regulatory particle of the 26S proteasome and the COP9 signalosome. *EMBO J.* **20**, 7096–7107 (2001).
99. Davy, A. *et al.* A protein–protein interaction map of the *Caenorhabditis elegans* 26S proteasome. *EMBO Rep.* **2**, 821–828 (2001).
100. Whitesell, L. & Lindquist, S. L. HSP90 and the chaperoning of cancer. *Nature Rev. Cancer* **5**, 761–772 (2005).
101. Imai, J., Maruya, M., Yashiroda, H., Yahara, I. & Tanaka, K. The molecular chaperone Hsp90 plays a role in the assembly and maintenance of the 26S proteasome. *EMBO J.* **22**, 3557–3567 (2003).
102. Yen, H. C., Gordon, C. & Chang, E. C. *Schizosaccharomyces pombe* Int6 and Ras homologs regulate cell division and mitotic fidelity via the proteasome. *Cell* **112**, 207–217 (2003).
103. Sha, Z. *et al.* Isolation of the *Schizosaccharomyces pombe* proteasome subunit Rpn7 and a structure–function study of the proteasome–COP9–initiation factor domain. *J. Biol. Chem.* **282**, 32414–32423 (2007).
104. Hoareau Alves, K., Bochar, V., Rety, S. & Jalinet, P. Association of the mammalian proto-oncoprotein Int-6 with the three protein complexes eIF3, COP9 signalosome and 26S proteasome. *FEBS Lett.* **527**, 15–21 (2002).
105. Groll, M. *et al.* A gated channel into the proteasome core particle. *Nature Struct. Biol.* **7**, 1062–1067 (2000).
106. Gillette, T. G., Kumar, B., Thompson, D., Slaughter, C. A. & Demartino, G. N. Differential roles of the COOH termini of AAA subunits of PA700 (19S regulator) in asymmetric assembly and activation of the 26S proteasome. *J. Biol. Chem.* **283**, 31813–31822 (2008).
107. Smith, D. M. *et al.* Docking of the proteasomal ATPases' carboxyl termini in the 20S proteasome's a ring opens the gate for substrate entry. *Mol. Cell* **27**, 731–744 (2007).
108. Rabl, J. *et al.* Mechanism of gate opening in the 20S proteasome by the proteasomal ATPases. *Mol. Cell* **30**, 360–368 (2008).
109. Liu, C. W. *et al.* ATP binding and ATP hydrolysis play distinct roles in the function of 26S proteasome. *Mol. Cell* **24**, 39–50 (2006).
110. Kleijnen, M. F. *et al.* Stability of the proteasome can be regulated allosterically through engagement of its proteolytic active sites. *Nature Struct. Biol.* **14**, 1180–1188 (2007).
111. Babbitt, S. E. *et al.* ATP hydrolysis-dependent disassembly of the 26S proteasome is part of the catalytic cycle. *Cell* **121**, 553–565 (2005).
112. Kriegenburg, F. *et al.* Mammalian 26S proteasomes remain intact during protein degradation. *Cell* **135**, 355–365 (2008).
113. Cascio, P., Hilton, C., Kisselev, A. F., Rock, K. L. & Goldberg, A. L. 26S proteasomes and immunoproteasomes produce mainly N-extended versions of an antigenic peptide. *EMBO J.* **20**, 2357–2366 (2001).
114. Hendil, K. B., Hartmann-Petersen, R. & Tanaka, K. 26 S proteasomes function as stable entities. *J. Mol. Biol.* **315**, 627–636 (2002).
115. Leggett, D. S. *et al.* Multiple associated proteins regulate proteasome structure and function. *Mol. Cell* **10**, 495–507 (2002).
116. Adams, G. M., Crotchet, B., Slaughter, C. A., DeMartino, G. N. & Gogol, E. P. Formation of proteasome–PA700 complexes directly correlates with activation of peptidase activity. *Biochemistry* **37**, 12927–12932 (1998).
117. Adams, G. M. *et al.* Structural and functional effects of PA700 and modulator protein on proteasomes. *J. Mol. Biol.* **273**, 646–657 (1997).
118. Tone, Y. & Toh, E. A. Nob1p is required for biogenesis of the 26S proteasome and degraded upon its maturation in *Saccharomyces cerevisiae*. *Genes Dev.* **16**, 3142–3157 (2002).
119. Fatica, A., Oeffinger, M., Dlakic, M. & Tollervey, D. Nob1p is required for cleavage of the 3' end of 18S rRNA. *Mol. Cell Biol.* **23**, 1798–1807 (2003).
120. Adams, J. The proteasome: a suitable antineoplastic target. *Nature Rev. Cancer* **4**, 349–360 (2004).
121. Nickeleit, I. *et al.* Argyrin A reveals a critical role for the tumor suppressor protein p27(kip1) in mediating antitumor activities in response to proteasome inhibition. *Cancer Cell* **14**, 23–35 (2008).
122. Schwartz, A. L. & Ciechanover, A. Targeting proteins for destruction by the ubiquitin system: implications for human pathobiology. *Annu. Rev. Pharmacol. Toxicol.* **3 Oct 2008** (doi:10.1146/annurev.pharmtox.051208.165340).
123. Rubinsztein, D. C. The roles of intracellular protein-degradation pathways in neurodegeneration. *Nature* **443**, 780–786 (2006).
124. Breusing, N. & Grune, T. Regulation of proteasome-mediated protein degradation during oxidative stress and aging. *Biol. Chem.* **389**, 203–209 (2008).
125. Goldberg, A. L. On prions, proteasomes, and mad cows. *N. Engl. J. Med.* **357**, 1150–1152 (2007).
126. Kristiansen, M. *et al.* Disease-associated prion protein oligomers inhibit the 26S proteasome. *Mol. Cell* **26**, 175–188 (2007).
127. Ozaki, K. *et al.* A functional SNP in PSMA6 confers risk of myocardial infarction in the Japanese population. *Nature Genet.* **38**, 921–925 (2006).

#### Acknowledgements

We are grateful to T. Mizushima, Nagoya City University, Nagoya, Japan, for preparing figures of crystal structures. This work was supported by grants to S.M., H.Y. and K.T. from the Ministry of Education, Science and Culture of Japan (MEXT) and the Target Protein Project of MEXT (to K.T.).

#### DATABASES

Entrez Gene: <http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=gene>

*ump1*

UniProtKB: <http://www.uniprot.org>

DSS1 | PAC1 | PAC2 | PAC3 | PAC4 | Pba1 | Pba2 | Pba3 | Pba4 | RPN1 | RPN2 | RPN3 | RPN5 | RPN9 | RPN10 | RPN11 | RPN12 | RPN13 | RPT1 | RPT5 | RPT6 |

#### FURTHER INFORMATION

Shigeo Murata's homepage:

<http://www.f.u-tokyo.ac.jp/~tanpaku/index-e.htm>

Hideki Yashiroda's homepage:

<http://www.f.u-tokyo.ac.jp/~tanpaku/index-e.htm>


Keiji Tanaka's homepage:

<http://www.rinshoken.or.jp/englishsite/indexen.html>

#### SUPPLEMENTARY INFORMATION

See online article: S1 (figure)

ALL LINKS ARE ACTIVE IN THE ONLINE PDF

© 2009  nature publishing group

To order reprints, please contact:

*In the Americas:*

Tel +1 212 726 9631; Fax 212 679 0843; [reprints@natureny.com](mailto:reprints@natureny.com)

*Europe/UK/ROW:*

Tel +44 (0)20 7843 4967; Fax + 44 (0)20 7843 4839; [reprints@nature.com](mailto:reprints@nature.com)

*Japan & Korea:*

Tel +81 3 3267 8751; Fax +81 3 3267 8746; [reprints@naturejpn.com](mailto:reprints@naturejpn.com)

January 2009

Vol.85

No.1

pp. 12-36

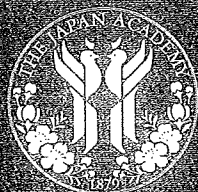
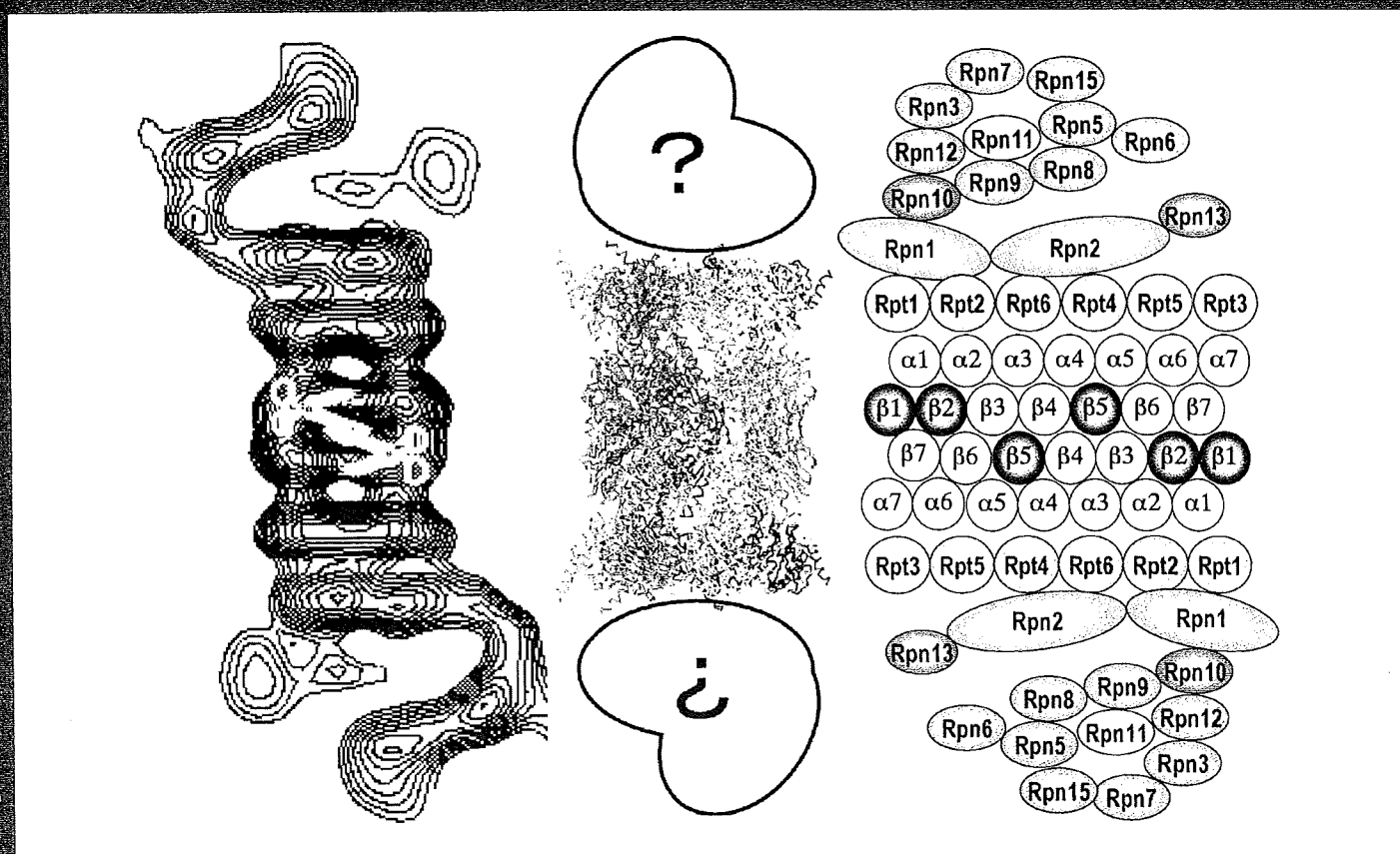
PJABDW  
ISSN 0386-2208

Now on  
**MEDLINE**

<http://www.japan-acad.go.jp/pjab>

# PROCEEDINGS OF THE JAPAN ACADEMY SERIES B

## PHYSICAL AND BIOLOGICAL SCIENCES



*Molecular organization of the 26S proteasome*

# THE JAPAN ACADEMY

PRESIDENT: Masaaki KUBO

VICE PRESIDENT: Takashi SUGIMURA

CHAIRPERSON OF SECTION I: Chie NAKANE

CHAIRPERSON OF SECTION II: Yoshihide KOZAI

## PUBLICATION COMMITTEE

CHAIRMAN: Takashi SUGIMURA

## EDITORIAL BOARD FOR PROCEEDINGS OF THE JAPAN ACADEMY, SERIES B

EDITOR-IN-CHIEF: Tamio YAMAKAWA

EXECUTIVE EDITOR: Masanori OTSUKA

## EDITORIAL MEMBERS

Teruhiko BEPPU (*Applied Microbiology*)

Leo ESAKI (*Physics*)

Osamu HAYAISHI (*Biochemistry, Medical Chemistry*)

Heisuke HIRONAKA (*Mathematics*)

Kenichi HONDA (*Applied Chemistry*)

Hiroo IMURA (*Internal Medicine*)

Hiroo INOKUCHI (*Physical Chemistry*)

Masaki KASHIWARA (*Mathematics*)

Yoshihide KOZAI (*Astronomy*)

Taroh MATSUNO (*Meteorology, Geophysics*)

Shigefumi MORI (*Mathematics*)

Jun-ichi NISHIZAWA (*Electronics, Communication*)

Ryoji NOYORI (*Organic Chemistry*)

Fumio OOSAWA (*Biophysics*)

Masanori OTSUKA (*Pharmacology*)

Takao SEKIYA (*Pharmaceutical Science, Nucleic Acid Chemistry*)

Hideki SHIRAKAWA (*Material Science*)

Takashi SUGIMURA (*Biochemistry, Oncology*)

Kunihiko SUZUKI (*Neurochemistry, Neurology, Genetic Neurological Disorder*)

Koichi TANAKA (*Mass Spectrometry*)

Kumao TOYOSHIMA (*Virology, Oncology*)

Yasuyuki YAMADA (*Plant Molecular and Cell Biology*)

Tamio YAMAKAWA (*Biochemistry*)

Toshimitsu YAMAZAKI (*Nuclear Physics*)

---

ADVISORY MEMBERS: Kiyoshi KUROKAWA (*Medical Science*)

---

*Cover Illustration: The structure and subunits of the 26S proteasome consisting of the 20S proteasome (the 20S core particle) and the 19S regulatory particle.*

The 26S proteasome (left panel, its averaged image based on electron micrographs) is made up of two subcomplexes, the 20S core complex (CP) and the 19S regulatory particle (RP). Much attention is now focused on the structure and functions of the 26S proteasome because it functions as a protein-destruction machine in ubiquitin-dependent proteolysis, which plays essential roles in almost all cellular events such as cell cycle progression, signal transduction, cell death, immune responses, metabolism, protein quality control and development. The 20S CP (also known as the 20S proteasome) is classified as a threonine protease that contains two pairs of three different catalytic sites (see the left panel, the two scissors shown in yellow). The middle panel shows its overall tertiary structure, whereas the structures of the terminal 19S RP have not yet been determined and are depicted by question marks. The 26S proteasome consists of many different subunits (right panel). The central 20S CP forms an  $\alpha_1\text{-}\beta_1\text{-}\beta_1\text{-}\beta_1\text{-}\alpha_1\text{-}\beta_1\text{-}\beta_1\text{-}\alpha_1\text{-}\beta_1\text{-}\beta_1\text{-}\alpha_1\text{-}\beta_1\text{-}\beta_1\text{-}\alpha_1$  structure with axial stacking of two outer  $\alpha$  and two inner  $\beta$  rings, which are made up of seven similar  $\alpha$  and  $\beta$  subunits, respectively:  $\beta_1$ ,  $\beta_2$  and  $\beta_5$  contain catalytic sites with caspase-like, trypsin-like and chymotrypsin-like activities, respectively. The 19S RP comprises approximately 20 different subunits that can be classified into Rpt and Rpn ones and comprises two subcomplexes, the lid and the base: The former subcomplex is composed of at least nine Rpn subunits (Rpn3, Rpn5, Rpn6, Rpn7, Rpn8, Rpn9, Rpn11, Rpn12 and Rpn15), while the latter is composed of six Rpt (Rpt1-Rpt6) and four Rpn (Rpn1, Rpn2, Rpn10 and Rpn13) subunits. Rpn10 and Rpn13 function as ubiquitin receptors that trap polyubiquitylated proteins, Rpn11 functions as a metalloprotease that cleaves the polyubiquitin chains tagged to client proteins at a proximal site and a hexameric ring containing Rpt1-Rpt6 promotes unfolding of client proteins and the gate opening of the 20S CP. There are several subtypes of the vertebrate proteasomes: The immunoproteasome and the thymoproteasome, which have different subunit structures and substrate specificities from those of the standard (or constitutive) proteasome, play important roles in antigen processing and positive selection of developing thymocytes, respectively. In addition, various proteasome inhibitors have been developed and one of the inhibitors has recently been approved for the treatment of multiple myeloma.

Hideyoshi Yokosawa  
RIKEN Brain Research Institute

© The Japan Academy 2009

## Review

### The proteasome: Overview of structure and functions

By Keiji TANAKA<sup>\*1,†</sup>

(Communicated by Takao SEKIYA, M.J.A.)

**Abstract:** The proteasome is a highly sophisticated protease complex designed to carry out selective, efficient and processive hydrolysis of client proteins. It is known to collaborate with ubiquitin, which polymerizes to form a marker for regulated proteolysis in eukaryotic cells. The highly organized proteasome plays a prominent role in the control of a diverse array of basic cellular activities by rapidly and unidirectionally catalyzing biological reactions. Studies of the proteasome during the past quarter of a century have provided profound insights into its structure and functions, which has appreciably contributed to our understanding of cellular life. Many questions, however, remain to be elucidated.

**Keywords:** proteasome, ubiquitin, intracellular proteolysis, multisubunit complex, molecular chaperone

#### Introduction

The proteasome is a large protein complex responsible for degradation of intracellular proteins, a process that requires metabolic energy. Polymerization of ubiquitin, a key molecule known to work in concert with the proteasome, serves as a degradation signal for numerous target proteins; the destruction of a protein is initiated by covalent attachment of a chain consisting of several copies of ubiquitin (more than four ubiquitin molecules), through the concerted actions of a network of proteins, including the E1 (ubiquitin-activating), E2 (ubiquitin-conjugating) and E3 (ubiquitin-ligating) enzymes.<sup>1,2)</sup> The polymerized ubiquitin chain acts as a signal that shuttles the target proteins to the proteasome, where the substrate is proteolytically broken down. For accurate selection of the proteins, numerous enzymes (e.g., 2 E1 proteins, approximately 30 E2 proteins and more than 500 different species of E3 in humans) are mobilized with this cascade system. The set of E3 proteins is highly diverse, because each E3 enzyme usually

selectively recognizes a protein substrate for ubiquitylation. Furthermore, it should be noted that ubiquitylation is a reversible reaction, because many cysteine-protease and metalloprotease deubiquitylating enzymes (DUBs) are present in the cell. Interestingly, the human genome encodes approximately 95 putative DUBs.<sup>3)</sup> Certain DUBs are responsible for the maturation of ubiquitin from its precursor proteins and products of genes that encode polyubiquitin or ubiquitin fused with ribosomal proteins. Other DUBs function at the initial stage during the breakdown of ubiquitin-tagged proteins to allow ubiquitins to be recycled. The ubiquitin-proteasome system (UPS) controls almost all basic cellular processes—such as progression through the cell cycle, signal transduction, cell death, immune responses, metabolism, protein quality control and development—by degrading short-lived regulatory or structurally aberrant proteins.<sup>4)–6)</sup> The divergent roles of the UPS have been reported in detail and reviewed comprehensively.<sup>1)–6)</sup> In this review, I provide an overview of the structure and functions of uniquely specified proteasomes. Due to space limitations, I have primarily cited review articles with the exception of particularly important or recently published papers.

#### 1. 26S and 30S Proteasomes

The proteasome is made up of two subcom-

<sup>\*1</sup> Laboratory of Frontier Science, Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan.

<sup>†</sup> Correspondence should be addressed: K. Tanaka, Laboratory of Frontier Science, Tokyo Metropolitan Institute of Medical Science, 3-18-22, Honkomagome, Bunkyo-ku, Tokyo 113-8613, Japan (e-mail: tanaka-kj@igakuken.or.jp).