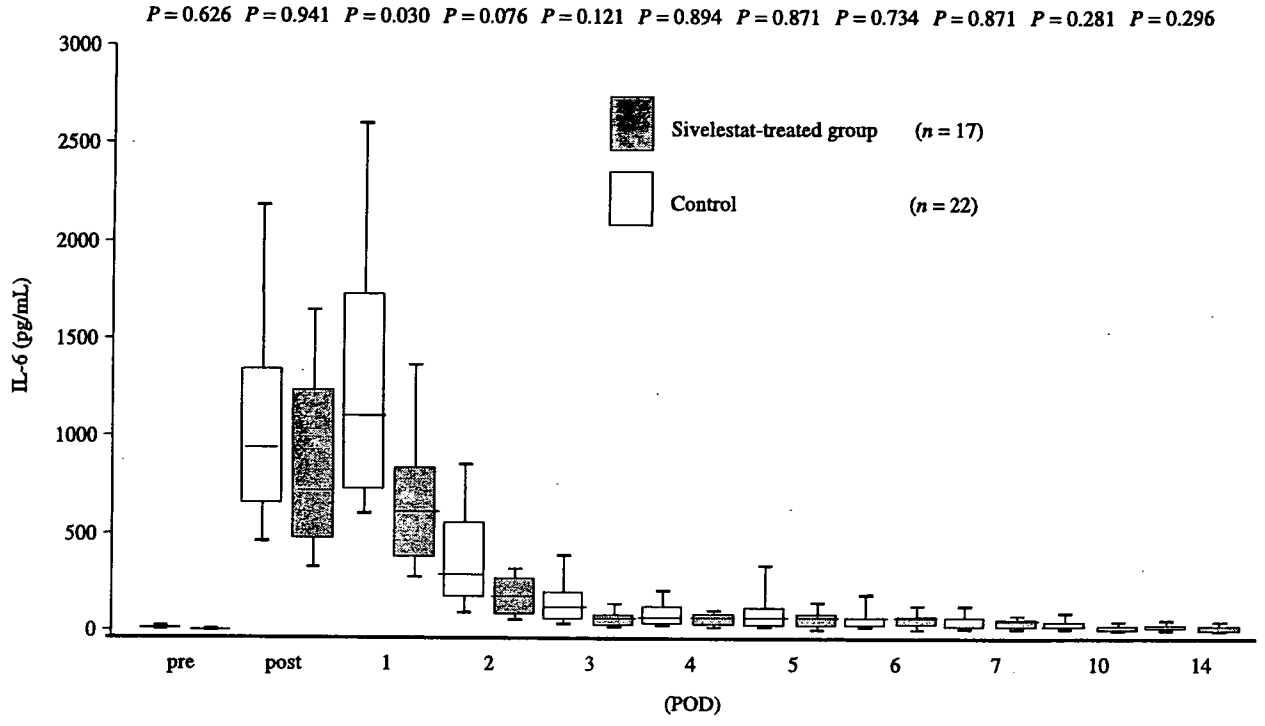

Fig. 3 Pre- and postoperative serum levels of interleukin-6 (IL-6). The median serum IL-6 concentration at each time point is indicated by horizontal bars. The vertical bars indicate the range and the horizontal boundaries of the boxes represent the first and third quartiles.

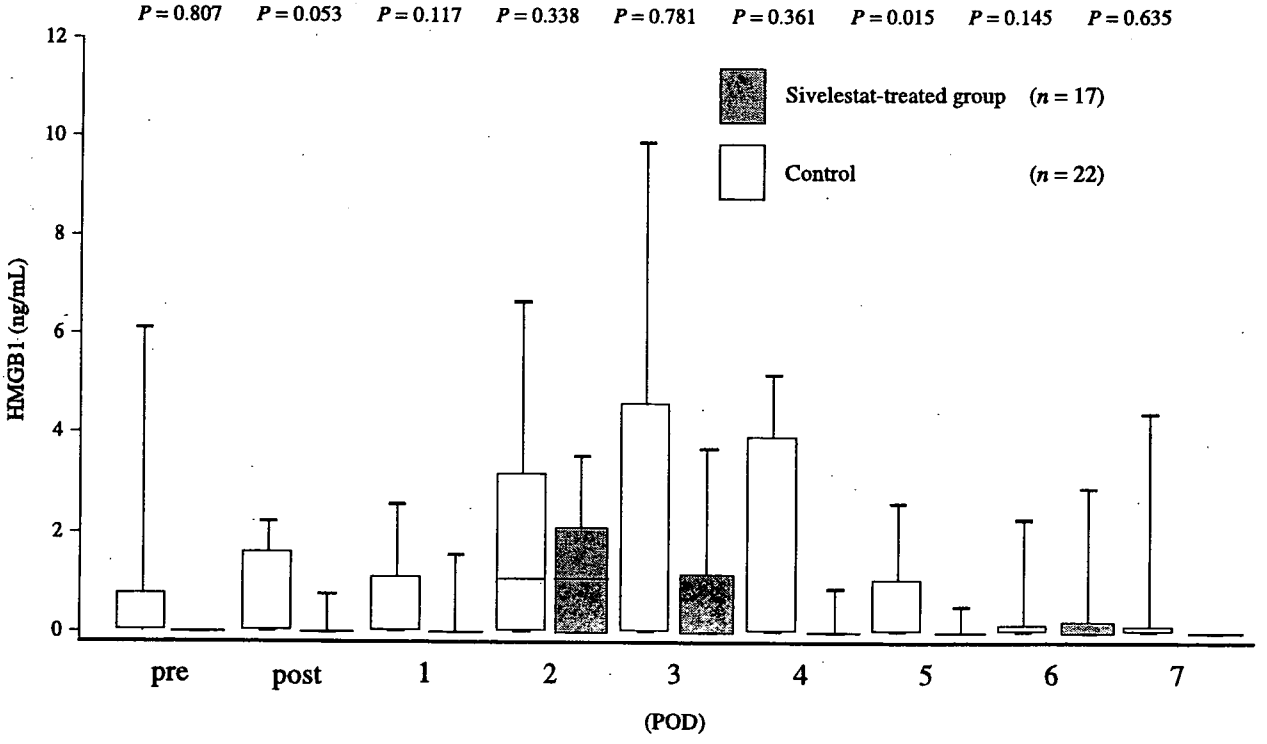

Fig. 4 Pre- and postoperative serum levels of high mobility group box chromosomal protein 1 (HMGB1). The median serum HMGB-1 concentration at each time point is indicated by horizontal bars. The vertical bars indicate the range and the horizontal boundaries of the boxes represent the first and third quartiles.

so short that the present study lacked information on long-term complications after esophagectomy and the late effect of the drug. Second, the treatment significantly suppressed NE activities and concentrations of IL-1β, IL-6 and HMGB1 in serum and improved the SIRS condition.

As reported by Zeiher *et al.*, Sivelestat has been effective in multiple animal models of ALI.[21,31] A

phase III study conducted in Japan demonstrated that Sivelestat improved investigator assessment of pulmonary function improvement and significantly reduced the duration of the patient's ICU stay.[21,25] Sivelestat has been approved and is commercially available in Japan as a treatment for ALI associated with SIRS.[21] All the patients in this study met the standards of SIRS in both groups, at least from immediately after surgery to POD 1, and all but two patients in the control group met those of ALI at POD 1. Therefore, administration of Sivelestat immediately after transthoracic esophagectomy was warranted. In the Sivelestat-treated group, PaO_2/FiO_2 ratios on POD 1 were significantly improved compared to the control group, and this was considered to be related to the significantly shorter duration of mechanical ventilation and ICU stay.

Although a STRIVE study, another multicenter prospective trial, showed that continuously infused Sivelestat had no effect on the primary end points of 28-day all-cause mortality or ventilator-free days in a heterogeneous ALI patient population managed with low-tidal volume mechanical ventilation, Sivelestat was effective, but without statistical significance, for ALI patients who had a relatively high PaO_2/FiO_2 ratio (100–300) and non-infectious etiology, according to the analyses of the subgroups.[21] Sivelestat can thus be effective when administered as early as any insults that may induce ALI. Because transthoracic esophagectomy is non-infectious, the PaO_2/FiO_2 ratio of the patients is relatively high (median of the lowest PaO_2/FiO_2 ratio of the patients without complications in the control group [PaO_2/FiO_2 ratio at POD 1]; 250 [233–286]), and moreover, initiation of the insult, that is, at the start of the operation, is clear. Transthoracic esophagectomy is considered to be a good indication for Sivelestat treatment, even in patients before they develop complications.

In the present study, serum NE activities were significantly suppressed in the Sivelestat-treated group while Sivelestat was being administered. This result meant that the dosage, (0.2 mg/kg/h) was enough to suppress NE in the serum and the administered Sivelestat was systemically effective during that period. Under the effect of Sivelestat, the serum concentrations of pro-inflammatory cytokines, such as IL-1β and IL-6, were significantly suppressed, even in patients without complications. In cases of esophageal cancer, marked elevation of cytokine levels is observed during and shortly after operation, and hypercytokinemia produces excessive stress and may trigger postoperative complications.[1] Several reports on the relationships between the severity of sepsis, trauma and burns and SIRS have recently been published.[1,32] SIRS is thought to be induced by hypercytokinemia, and the longer the duration of SIRS, the more likely it is to progress to multiple organ dysfunction syndrome.[1,33–35] Considering these results, the reason why Sivelestat improved postoperative clinical courses, even in patients without complications, was that Sivelestat decreased hypercytokinemia, leading to an improvement of the state of SIRS. Further investigations are warranted in order to clarify how Sivelestat decreased hypercytokinemia on the molecular basis.

HMGB1 is a nuclear DNA-binding protein acting as a proinflammatory cytokine when released in the extracellular space from necrotic cells, activated macrophages and dendritic cells.[36–38] In a previous study, we have demonstrated that surgical stress of transthoracic esophagectomy itself induced an increase in HMGB1 in serum, even in patients without complications, and higher levels of HMGB1 in the immediate postoperative period correlated with elevated preoperative serum HMGB1 concentrations and a complicated clinical course, indicating that elevations in HMGB1 might play a contributory role in the development of postoperative organ system dysfunction.[39] Furthermore, HMGB1 is one of the key mediators of ALI/ARDS.[40] In the present study, serum HMGB1 concentrations were significantly suppressed in the Sivelestat-treated group. Although the actual mechanism of the suppression of serum HMGB1 by Sivelestat treatment was unclear, the suppression of serum HMGB1 concentrations might contribute to the improvement of postoperative clinical courses and respiratory function in the Sivelestat-treated group.

Regarding cytokine modulation in esophagectomy, only preoperative administrations of corticosteroid, ulinastatin, and gabexate mesilate have been reported to be effective.[1,2,14] However, although Sivelestat could be more effective when administered as early as insults occur as described before, the postoperative administration of Sivelestat was still effective in this study. It is possible to speculate that Sivelestat modulates downstream of the inflammatory cascade, while corticosteroid does so upstream of it.[2,36,38] Development of cytokine modulators can modulate the downstream inflammatory cascade and may offer a clinically relevant prophylaxis and treatment to surgical stress-induced complications. From the preliminary data of the present study, we can speculate that an early phase administration of Sivelestat after major surgical insults may reduce the risk of serious complications in patients at risk. To investigate the preventive effect of the Sivelestat treatment for postoperative complication in patients with radical esophagectomy, a large-scale prospective randomized trial would be required.

In the present study, improved surgical techniques arising from greater experience and more practice, and not Sivelestat alone, may account for the more favorable clinical courses after transthoracic

esophagectomy in the treatment group, since the control group was earlier and the treatment group was later. A randomized double-blinded trial would be warranted to prove this efficacy in a future study.

Acknowledgments

We wish to thank Kazuhito Kawabata for measuring the serum NE activities and Tetsuji Yamashita for measuring the serum TNFα, IL-1β, and IL-6 concentrations. The authors are indebted to Professor J. Patrick Barron of the International Medical Communications Center of Tokyo Medical University, for his review of this manuscript. We also wish to thank the Ministry of Labour Health and Welfare, Japan for financial support in the form of Health Sciences Research Grants (Comprehensive Research on Aging and Health 14-015).

References

1 Ono S, Aosasa S, Mochizuki H. Effects of a protease inhibitor on reduction of surgical stress in esophagectomy. Am J Surg 1999; 177: 78–82.
2 Sato N, Koeda K, Ikeda K *et al.* Randomized study of the benefits of preoperative corticosteroid administration on the postoperative morbidity and cytokine response in patients undergoing surgery for esophageal cancer. Ann Surg 2002; 236: 184–90.
3 Avendano C E, Flume P A, Silvestri G A, King L B, Reed C E. Pulmonary complications after esophagectomy. Ann Thorac Surg 2002; 73: 922–6.
4 Stein H J, Siewert J R. Improved prognosis of resected esophageal cancer. World J Surg 2004; 28: 520–5.
5 Griffin S M, Shaw I H, Dresner S M. Early complications after Ivor Lewis subtotal esophagectomy with two-field lymphadenectomy: risk factors and management. J Am Coll Surg 2002; 194: 285–97.
6 Ando N, Ozawa S, Kitagawa Y, Shinozawa Y, Kitajima M. Improvement in the results of surgical treatment of advanced squamous esophageal carcinoma during 15 consecutive years. Ann Surg 2000; 232: 225–32.
7 Whooley B P, Law S, Murthy S C, Alexandrou A, Wong J. Analysis of reduced death and complication rates after esophageal resection. Ann Surg 2001; 233: 338–44.
8 Fang W, Kato H, Tachimori Y, Igaki H, Sato H, Daiko H. Analysis of pulmonary complications after three-field lymph node dissection for esophageal cancer. Ann Thorac Surg 2003; 76: 903–8.
9 Karl R C, Schreiber R, Boulware D, Baker S, Coppola D. Factors affecting morbidity, mortality, and survival in patients undergoing Ivor Lewis esophagogastrectomy. Ann Surg 2000; 231: 635–43.
10 Law S, Wong K H, Kwok K F, Chu K M, Wong J. Predictive factors for postoperative pulmonary complications and mortality after esophagectomy for cancer. Ann Surg 2004; 240: 791–800.
11 McCulloch P, Ward J, Tekkis P P. Mortality and morbidity in gastro-oesophageal cancer surgery: initial results of ASCOT multicentre prospective cohort study. BMJ 2003; 327: 1192–7.
12 Bailey S H, Bull D A, Harpole D H *et al.* Outcomes after esophagectomy: a ten-year prospective cohort. Ann Thorac Surg 2003; 75: 217–22.
13 Atkins B Z, Shah A S, Hutcheson K A *et al.* Reducing hospital morbidity and mortality following esophagectomy. Ann Thorac Surg 2004; 78: 1170–6.
14 Sato N, Endo S, Kimura Y *et al.* Influence of a human protease inhibitor on surgical stress induced immunosuppression. Dig Surg 2002; 19: 300–5.
15 Kitagawa Y, Van Eeden S F, Redenbach D M *et al.* Effect of mechanical deformation on structure and function of polymorphonuclear leukocytes. J Appl Physiol 1997; 82: 1397–405.
16 Lee W L, Downey G P. Neutrophil activation and acute lung injury. Curr Opin Crit Care 2001; 7: 1–7.
17 Razavi H M, Wang L F, Weicker S *et al.* Pulmonary neutrophil infiltration in murine sepsis: role of inducible nitric oxide synthase. Am J Respir Crit Care Med 2004; 170: 227–33.
18 Lin X, Yang H, Sakuragi T *et al.* Alpha-chemokine receptor blockade reduces high mobility group box 1 protein-induced lung inflammation and injury and improves survival in sepsis. Am J Physiol Lung Cell Mol Physiol 2005; 289: L583–L590.
19 Guo L, Yamaguchi Y, Ikei S, Sugita H, Ogawa M. Neutrophil elastase inhibitor (ONO-5046) prevents lung hemorrhage induced by lipopolysaccharide in rat model of cerulein pancreatitis. Dig Dis Sci 1995; 40: 2177–83.
20 Matsuzaki K, Hiramatsu Y, Homma S, Sato S, Shigeta O, Sakakibara Y. Sivelestat reduces inflammatory mediators and preserves neutrophil deforemability during simulated extracorporeal circulation. Ann Thorac Surg 2005; 80: 611–7.
21 Zeiher B G, Artigas A, Vincent J L *et al.* Neutrophil elastase inhibition in acute lung injury: results of the STRIVE study. Crit Care Med 2004; 32: 1695–702.
22 Kawabata K, Hagio T, Matsumoto S *et al.* Delayed neutrophil elastase inhibition prevents subsequent progression of acute lung injury induced by endotoxin inhalation in hamsters. Am J Respir Crit Care Med 2000; 161: 2013–8.
23 Jian M Y, Koizumi T, Tsushima K, Fujimoto K, Kubo K. Effects of granulocyte colony-stimulating factor (G-CSF) and neutrophil elastase inhibitor (ONO-5046) on acid-induced lung injury in rats. Inflammation 2004; 28: 327–36.
24 Hagio T, Matsumoto S, Nakao S, Matsuoka S, Kawabata K. Sivelestat, a specific neutrophil elastase inhibitor, prevented phorbol myristate acetate-induced acute lung injury in conscious rabbits. Pulm Pharmacol Ther 2005; 18: 285–90.
25 Tamakuma S, Ogawa M, Aikawa N *et al.* Relationship between neutrophil elastase and acute lung injury in humans. Pulm Pharmacol Ther 2004; 17: 271–9.
26 Bone R C, Balk R A, Cerra F B *et al.*: definitions for sepsis and organ failure and guidelines for the use of innovative therapies in sepsis. Chest 1992; 101: 1644–55.
27 Bernard G R, Artigas A, Brigham K L *et al.* The American-European Consensus Conference on ARDS. Definitions, mechanisms, relevant outcomes, and clinical trial coordination. Am J Respir Crit Care Med 1994; 149: 818–24.
28 Knaus W A, Draper E A, Wagner D P, Zimmerman J E. APACHE II: a severity of disease classification system. Crit Care Med 1985; 13: 818–29.
29 Vincent J L, Moreno R, Takala J *et al.* The SOFA (sepsis-related organ failure assessment) score to describe organ dysfunction/failure. Intensive Care Med 1996; 22: 707–10.
30 Yamada S, Inoue K, Yakabe K, Imaizumi H, Maruyama I. High mobility group protein 1 (HMGB1) quantified by ELISA with a monoclonal antibody that does not cross-react with HMGB2. Clin Chem 2003; 49: 1535–7.
31 Zeiher B G, Matsuoka S, Kawabata K, Repine J E. Neutrophil elastase and acute lung injury: prospects for Sivelestat and other neutrophil elastase inhibitors as therapeutics. Crit Care Med 2002; 30: S281–S287.
32 Endo S, Inada K, Yamada Y *et al.* Plasma levels of interleukin-1 receptor antagonist (IL-1ra) and severity of illness in patients with burns. J Med 1996; 27: 57–71.
33 Bone R. Toward a theory regarding the pathogenesis of the systemic inflammatory response syndrome: what we do and do not know about cytokine regulation. Crit Care Med 1996; 24: 163–72.
34 Haga Y, Beppu T, Doi K *et al.* Systemic inflammatory response syndrome and organ dysfunction following gastrointestinal surgery. Crit Care Med 1997; 25: 1994–2000.
35 Smail N, Messiah A, Edouard A *et al.* Role of systemic inflammatory response syndrome and infection in the occurrence of early multiple organ dysfunction syndrome following severe trauma. Intensive Care Med 1995; 21: 813–6.
36 Wang H, Bloom O, Zhang M *et al.* HMG-1 as a late mediator of endotoxin lethality in mice. Science 1999; 285: 248–51.

37 Hatada T, Wada H, Nobori T *et al.* Plasma concentrations and importance of high mobility group box protein in the prognosis of organ failure in patients with disseminated intravascular coagulation. Thromb Haemost 2005; 94: 975–9.
38 Wang H, Yang H, Tracey K J. Extracellular role of HMGB1 in inflammation and sepsis. J Intern Med 2004; 255: 320–31.
39 Suda K, Kitagawa Y, Ozawa S *et al.* Serum concentrations of high-mobility group box chromosomal protein 1 before and after exposure to the surgical stress of thoracic esophagectomy: a predictor of clinical course after surgery? Dis Esophagus 2006; 19: 5–9.
40 Ueno H, Matsuda T, Hashimoto S *et al.* Contributions of high mobility group box protein in experimental and clinical acute lung injury. Am J Respir Crit Care Med 2004; 170: 1310–6.

www.nature.com/clinicalpractice/rheum

Synoviolin, protein folding and the maintenance of joint homeostasis

Naoko Yagishita, Satoshi Yamasaki, Kusuki Nishioka and Toshihiro Nakajima*

SUMMARY

Rheumatoid arthritis is a disease associated with painful joints that affects approximately 1% of the population worldwide, and for which no specific cure is available. Among other functions, the endoplasmic reticulum (ER) has an important role in protein folding. When the level of unfolded proteins in the ER exceeds the folding capacity of this organelle, defective proteins are eliminated by ER-associated degradation (ERAD), an ATP-dependent ubiquitin–proteasome degradation process, to reduce the burden on the ER. Synoviolin is an E3 ubiquitin ligase that is involved in ERAD. This protein is a pathogenic factor for arthropathy; it is overexpressed in the synovial cells of patients with rheumatoid arthritis. This overexpression results in a 'hyper-ERAD' state, in which the cell deals with accumulated unfolded proteins excessively. Rheumatoid synovial cells produce large amounts of various proteins such as cytokines and proteases, which consequently might confer an autonomous proliferation property on the cells. At least 30% of all newly synthesized, ER-sorted proteins are unfolded. Although degradation of unfolded proteins consumes large amounts of ATP and would seem an unconventional process, it is essential for joint homeostasis.

KEYWORDS ER, hyper-ERAD, rheumatoid arthritis, synovial cells, synoviolin

REVIEW CRITERIA

Data for this review were identified by searching the PubMed database for papers published since 1997. Only peer-reviewed, full-text, English-language journals were included. The search terms used were "rheumatoid arthritis", "endoplasmic reticulum", "synoviolin", "unfolded protein", "ERAD", "apoptosis", and "p53". Additional papers were identified from the authors' databases.

N Yagishita is an Associate Director in the Department of Locomotor Science at the Institute of Medical Science at St Marianna University School of Medicine, Kawasaki, Kanagawa, Japan, where S Yamasaki is an Associate Director and T Nakajima is Head of the Department and Vice Director of the Institute. K Nishioka is a Director of the Institute.

Correspondence
*Department of Locomotor Science, Institute of Medical Science, St Marianna University School of Medicine, 2-16-1 Sugao Miyamae-ku, Kawasaki Kanagawa 216-8512, Japan
nakashit@marianna-u.ac.jp

Received 6 August 2007 **Accepted** 6 November 2007
www.nature.com/clinicalpractice
doi:10.1038/ncprheum0699

INTRODUCTION

Rheumatoid arthritis (RA) affects nearly 1% of the adult population worldwide, and has a tremendous negative effect on quality of life.[1] It is a disease that is associated not only with painful joints but also with generalized symptoms related to the whole body, such as febricula, malaise and anorexia. Although RA is a serious condition, there is no specific cure as yet; this is mainly because the exact etiology of RA remains poorly understood. The burden of musculoskeletal diseases on society has been recognized throughout the world, and RA is defined as one of the most important diseases in the Bone and Joint Decade, which was launched in 2000 by the WHO to reduce the social and financial costs of musculoskeletal disorders to society.

The pathologic features of RA include chronic inflammation of systemic joints, which is associated with overgrowth of synovial cells. This inflammation, which eventually causes cartilage and bone destruction in the joint, is thought to result from activation of the cytokine system by inflammatory cells.[2,3] During the course of inflammation, synovial cells, macrophages, T cells and B cells contribute to the production of cytokines such as interleukin (IL)-1, IL-6, IL-10, tumor necrosis factor (TNF) and transforming growth factor β (TGF-β).[2,3] These cytokines, in turn, stimulate the overgrowth of synovial cells to form a mass of synovial tissue, called a pannus, which invades the bone and cartilage through osteoclast activation and protease production.[2–6] Because RA is considered to be an autoimmune disease, medical treatments that target inflammation have been applied; however, nearly 25% of patients with RA do not respond to anticytokine therapy or anti-inflammatory therapies.[7,8] On the basis of these observations, we have focused on synovial cells and have been attempting to clarify the mechanism of hyperplasia in rheumatoid synovial cells. In this Review, we outline what is known about the function in RA of synoviolin, an E3 ubiquitin ligase that is involved in endoplasmic reticulum

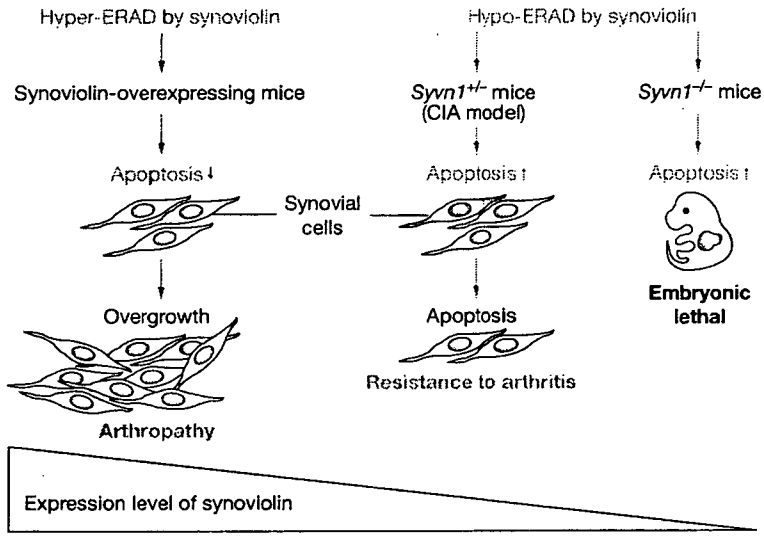

Figure 1 The functions of synoviolin. 'Gain-of-function' of synoviolin (in mice overexpressing synoviolin) and hyper-ERAD results in the development of spontaneous arthropathy through the anti-apoptotic effects of synoviolin in synovial cells. By contrast, *Syvn1*$^{+/-}$ (Syno$^{+/-}$) mice, in which there is a 'loss-of-function' of synoviolin and low levels of ERAD, show resistance to CIA, and *Syvn1*$^{-/-}$ (Syno$^{-/-}$) mice die *in utero* because of enhanced apoptosis. The expression level of synoviolin is, therefore, important for its biological roles under physiological and pathological conditions. Abbreviations: CIA, collagen-induced arthritis; ER, endoplasmic reticulum; ERAD, ER-associated degradation.

(ER)-associated degradation (ERAD), an ATP-dependent ubiquitin–proteasome process that functions to reduce the burden of excess unfolded proteins on the ER. Synoviolin is overexpressed in the synovial cells of patients with RA, where it is thought to have anti-apoptotic effects.

SYNOVIOLIN IS A NOVEL PATHOGENIC FACTOR FOR ARTHROPATHY

Synoviolin was cloned by immunoscreening using antibodies against rheumatoid synovial cells. This ER-resident E3 ubiquitin ligase[9–12] is the mammalian homolog of Hrd1p/Del3p, whose substrates include misfolded carboxypeptidase yscY[13] and 3-hydroxy-3-methylglutaryl-coenzyme A reductase.[14] Synoviolin is overexpressed in the rheumatoid synovium of human patients, and approximately 30% of mice that overexpress synoviolin develop spontaneous arthropathy as a result of reduced apoptosis of synoviocytes. Conversely, synoviolin-heterozygous (*Syvn1*$^{+/-}$ [Syno$^{+/-}$]) mice show resistance to the development of collagen-induced arthritis, owing to enhanced apoptosis of synovial cells. On the basis of the 'gain-of-function' and 'loss-of-function' results, synoviolin is considered a novel pathogenic factor in arthropathy that functions through its anti-apoptotic effects (Figure 1).

SYNOVIOLIN IN EMBRYOGENESIS

In another study, we reported that synoviolin is essential for embryogenesis (Figure 1).[15] Synoviolin-deficient homozygous mice (*Syvn1*$^{-/-}$ [Syno$^{-/-}$]) died *in utero* at around embryonic day 13.5, which indicates that synoviolin is indispensable for embryogenesis. The *Syvn1*$^{-/-}$ fetuses exhibited smaller livers and fewer hepatocytes than their wild-type counterparts. This hypocellularity was caused by enhanced apoptosis of hepatocytes during fetal life, and resulted in defective erythropoiesis in a non-cell-autonomous manner, causing severe anemia. The results were indicative of defective nursing activity of erythrocytes in the liver of *Syvn1*$^{-/-}$ fetuses. This phenomenon resembles that of RA—that is, RA bone marrow stromal cells, similar to hepatocytes, have nurse-cell-like activity; RA is a disease that accelerates this nursing activity.[16,17] Our data indicate a close relationship between embryogenesis and RA,[18] and indicate that a new approach to treating RA could potentially be developed by the analysis of the *Syvn1*$^{-/-}$ mouse, the phenotype of which is opposite to that involved in RA.

SYNOVIOLIN TARGETS P53

By analyzing the profile of protein expression in cells from *Syvn1*$^{-/-}$ mice, we identified that synoviolin targets the tumor suppressor p53 for ubiquitination.[19] Synoviolin sequesters and ubiquitinates p53 in the cytoplasm and negatively regulates its cellular levels and, hence, biological functions, including transcription, cell-cycle regulation and apoptosis (Figure 2). Importantly, these p53 regulatory functions are unique to synoviolin and were not carried out by other E3 ubiquitin ligases, such as MDM2, PIRH2 and COP1.[20–23] Although p53 is reported to be involved in RA, its precise function in this context is still controversial.[24–28] Mice that lack p53 do not develop spontaneous arthropathy, but have severe collagen-induced arthritis;[29,30] therefore, we consider that the severity of arthritis is probably determined by the synoviolin–p53 control pathway, whereas the onset of spontaneous arthropathy is caused by a p53-independent pathway in these models. Further research might uncover new pathogenic mechanisms in RA.

HYPER-ERAD BY SYNOVIOLIN

Newly synthesized proteins are transported to the ER of eukaryotic cells, where they are correctly folded. Under normal conditions, the folding and transport capacity of the ER matches the requirement of the secretory pathway. Increased levels of unfolded proteins result in ER stress. To maintain the efficacy of intracellular protein folding when faced with overwhelming environmental insults, cells are equipped with a self-protective mechanism that enables them to survive when there is a need for additional folding. ER stress triggers, therefore, a cellular response that is termed the 'unfolded protein response' (UPR) to overcome this problem.[31–33] During the UPR, the synthesis of new proteins is globally inhibited and, simultaneously, the expression of genes that encode ER chaperone proteins is upregulated to provide sufficient proteins to refold the misfolded proteins correctly.[31–33]

When even the UPR cannot deal with this problem, however, the excess of misfolded proteins is eliminated by the ubiquitin- and proteasome-dependent degradation process of ERAD. This approach spares the cells from death caused by ER dysregulation (Figure 3).[31–33] Synoviolin is involved in ERAD as a ubiquitin ligase,[9–12] an enzyme that couples the small protein ubiquitin to lysine residues on a target protein, marking that protein for destruction by the proteasome, and has a role in the evasion of cell death. As overexpression of synoviolin leads to an ERAD hyperactivation state in rheumatoid synovial cells, we consider RA to be a hyper-ERAD disease.[34,35]

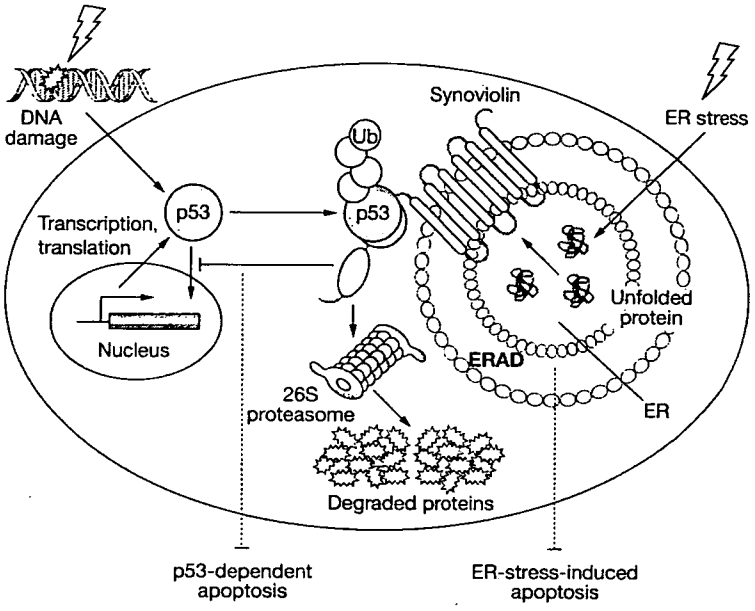

Figure 2 Synoviolin is involved in the inhibition of two apoptotic pathways. Synoviolin can sequester and ubiquitinate the tumor suppressor p53 in the cytoplasm, and thereby negatively regulate its biological functions in transcription, cell-cycle regulation and apoptosis (the figure shows the upregulation of p53 in response to DNA damage) by targeting it for proteasomal degradation. This regulation diverges from regulation by other E3 ubiquitin ligases for p53 (e.g. MDM2, PIRH2 and COP1). Synoviolin regulates, therefore, not only apoptosis in response to ER stress (through ERAD) but also a p53-dependent apoptotic pathway. Abbreviations: ER, endoplasmic reticulum; ERAD, ER-associated degradation; Ub, ubiquitin.

Hyper-ERAD is a characteristic of rheumatoid synovial cells

Rheumatoid synovial cells exhibit autonomous proliferation properties and aberrant production of proteins such as cytokines and proteases (Figure 4).[36] Here, we discuss these two characteristics from the perspective of hyper-ERAD by overexpression of synoviolin. Regarding autonomous proliferation, hyper-ERAD can prevent ER-stress-induced apoptosis, which consequently leads to synovial hyperplasia. Indeed, excess apoptosis of synovial cells is noted in *Synv1*$^{+/-}$ mice with collagen-induced arthritis—cells from these mice show impaired ERAD owing to the lack of synoviolin.[9] In other words, synovial cells from *Synv1*$^{+/-}$ mice with collagen-induced arthritis cannot avoid ER-stress-induced apoptosis because they lack synoviolin; this, in turn, prevents overgrowth of synovial cells. Put differently, we consider that, normally, rheumatoid synovial cells are programmed to survive on the basis of synoviolin expression, even though they should die when exposed to ER stress. In this regard, we have demonstrated that ER stress exists in the pannus; however, rheumatoid synovial cells seem more resistant to ER-stress-induced apoptosis than other cells.[37] Other investigators have implicated ERAD dysfunction in various disorders,[38,39] such as the occurrence of certain inherited neurodegenerative disorders through the production of proteins with expanded polyglutamine tracts.[40–42] Furthermore, mutation of the *parkin* gene, a well-known ubiquitin ligase protein that is involved in ERAD, is thought to result in neuronal death of the substantia nigra in patients with autosomal recessive juvenile Parkinsonism.[43] Here, we hypothesize that ERAD hyperactivation is the cause of diseases such as RA that are characterized by proliferation.[34,35]

Turning to the overproduction of proteins such as cytokines and proteases by rheumatoid

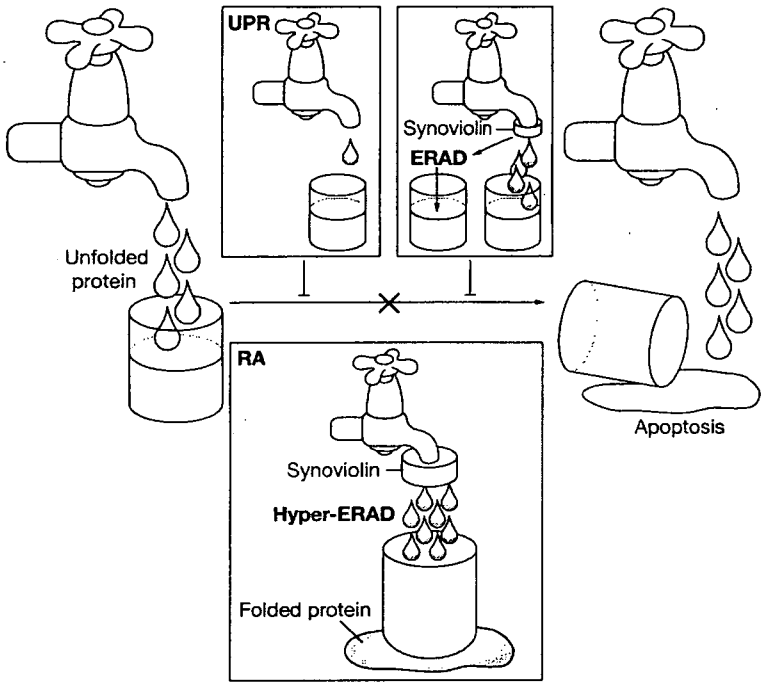

Figure 3 Defense mechanisms for ER stress. If the cellular level of unfolded protein induced by ER stress (likened to muddy water in a glass) exceeds the folding capacity, this results in apoptotic cell death (equivalent to the spillage of muddy water). There are two defense mechanisms in place—the so-called ER stress responses—to avoid this problem. One is the UPR, which inhibits the synthesis of new proteins globally and also upregulates the expression of genes that encode ER chaperone proteins to refold the misfolded proteins; this mechanism is equivalent to closing the tap. The other is ERAD, which eliminates the unfolded proteins by the ubiquitin–proteasome degradation system and recycles the amino acids; this control mechanism resembles a sewage disposal facility. In RA, overexpressed synoviolin acts like a filter that accelerates the purification of water, with consequent flooding of the glass with fresh water (i.e. folded proteins, including cytokines, proteases and so on). Abbreviations: ER, endoplasmic reticulum; ERAD, ER-associated degradation; RA, rheumatoid arthritis; UPR, unfolded protein response.

synovial cells, the production of properly folded proteins could be enhanced by hyper-ERAD, as this process promotes the breakdown of unfolded proteins and possibly accelerates protein synthesis through the abrogation of the UPR by clearance of unfolded proteins.[44] As increased protein synthesis leads to increased protein influx into the ER, hyper-ERAD further accelerates this process. This cycle results in excess production and secretion of proteins such as cytokines, chemokines, matrix proteins and proteases from rheumatoid synovial cells (Figure 4).[1–3,36]

Normally, synovial cells maintain joint homeostasis by forming synovial fluid, which provides nutrition for the joint structure; however, hyper-ERAD in rheumatoid synovial cells causes an oversupply of this nutritious fluid, which leads to autonomous outgrowth. Considered together, RA could also be viewed as a disease caused by a disorder of the ERAD system (Figure 3), the correction of which could result in a broad arrest of the various factors that constitute the complex pathology of RA.

A PHYSIOLOGICAL ROLE FOR UNFOLDED PROTEINS?

Despite the central dogma of strict regulation, protein folding is not regulated exquisitely. Specifically, approximately 30% of newly synthesized, ER-sorted proteins fail to be folded.[45] When 20% of total proteins are processed in the ER, more than 6% of these proteins become unfolded. As mentioned above, some of them are refolded by ER chaperone proteins; however, the proteins that remain unfolded must be constantly eliminated from the ER by ERAD, the ubiquitin–proteasome system.[31–33] This system consists of the small polypeptide ubiquitin, a framework of enzymes that mediate the covalent attachment of ubiquitin to proteolytic substrates, and the 26S proteasome, which digests the modified proteins into peptides. The formation of ubiquitin conjugates requires the successive action of three classes of enzymes: an E1 (activating enzyme) first 'activates' ubiquitin in an ATP-dependent manner, forming a high-energy thioester bond between ubiquitin and E1; the activated ubiquitin is then transferred to an E2 (conjugating enzyme); E3 ubiquitin ligase then transfers ubiquitin to the target proteins. Through repeated reactions of this cycle, a polyubiquitin chain is formed on the target protein, which is recognized by the 26S proteasome and, ultimately, degraded.[46,47]

A large amount of ATP is, therefore, consumed during the course of this process to deal with a single, unfolded protein formed by the ambiguous protein folding system in the ER. Why was this 'wasteful' system never selected against in evolution? On the basis of our research in rheumatoid synovial cells, we believe that unfolded proteins might serve a physiological function. That is, the energy required to deal with unfolded proteins suppresses cell rebellion. Normal synovial cells produce normal levels of nutritious fluid for the joint, as these cells must also contend with a normal amount of unfolded proteins in the ER. These synovial cells lack the ability to overgrow, and ultimately die because of the accumulation of unfolded proteins, forming just a monolayer of

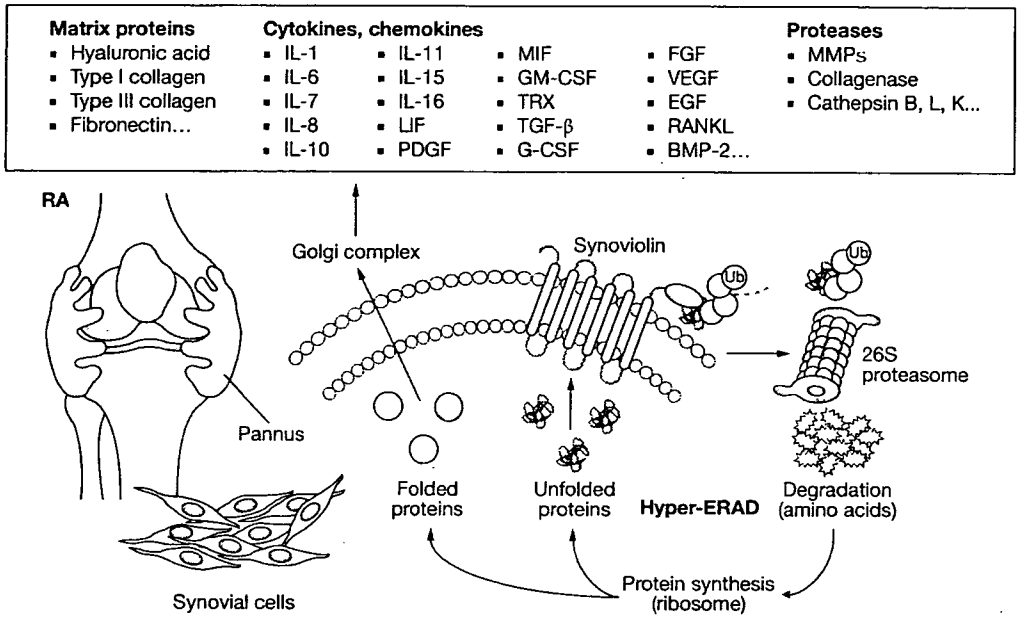

Figure 4 Aberrant protein production by hyper-ERAD. Synovial fibroblasts in patients with RA secrete large amounts of various proteins. This results in the excessive elimination of unfolded proteins—that is, hyper-ERAD—by overexpression of synoviolin, an E3 ubiquitin ligase that functions in the ubiquitin–proteasome pathway. As the breakdown of unfolded proteins leads to abrogation of the UPR, protein production and secretion might consequently be promoted. Abbreviations: BMP, bone morphogenetic protein; EGF, epidermal growth factor; ERAD, endoplasmic-reticulum-associated degradation; FGF, fibroblast growth factor; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; IL, interleukin; LIF, leukemia inhibitory factor; MIF, macrophage inhibitory factor; MMP, matrix metalloproteinase; PDGF, platelet-derived growth factor; RA, rheumatoid arthritis; RANKL, receptor activator for nuclear factor κB ligand; TGF-β, transforming growth factor β; TRX, thioredoxin; Ub, ubiquitin; UPR, unfolded protein response; VEGF, vascular endothelial growth factor.

synoviocytes. A normal joint therefore escapes the rebellion of synovial cells, autonomous proliferation and aberrant protein production, by dealing with wastefully produced unfolded proteins. Although it seems an outlandish system, as it wastes a large amount of ATP, it is indispensable for maintaining joint homeostasis. If the ERAD system becomes abnormal, however, and synoviolin is overexpressed, synovial cells undergo autonomous proliferation and produce excessive amounts of proteins such as cytokines and proteases, which results in RA.

The human genome encodes 22,000 proteins with their own physiological function;[48] and any of these proteins can be present in an unfolded form. Although the unfolded protein is a generic singular protein, all ER proteins have the potential to exhibit a noninherent function as an unfolded protein, perhaps identical to that discussed above—a function that is not determined by the genetic code.

CONCLUSIONS

On the basis of our research, we consider that synovial cells utilize unfolded proteins, which are produced by ER stresses, to maintain a monolayer form. In joints, where a complex environment is formed by the synovium, cartilage and bone, synovial cells exploit the 'function' of unfolded proteins in using cellular energy to perform their own duty while being continuously exposed to other joint elements. When this system becomes defective (that is, once hyperactivation of ERAD occurs, which excessively eliminates unfolded proteins), however, synovial cells acquire the properties of aberrant protein production and autonomous proliferation—the main pathology of RA. Ideally, therefore, there should be a 'futile' system in which ~30% of ER proteins should be unfolded in order to maintain the normal function of synovial cells and, hence, joint homeostasis.

Synoviolin, an E3 ubiquitin ligase that functions in ERAD, is considered a novel pathogenic factor

in arthropathy. It is overexpressed in synovial cells in patients with RA, which results in hyper-ERAD, in which the cell deals with accumulated unfolded proteins excessively. As well as its involvement as a ubiquitin ligase in ERAD, synoviolin is thought to function as an anti-apoptotic factor through sequestration of the tumor suppressor p53.

Regarding the relationship between synoviolin and RA, Toh *et al.*[49] reported that elevated peripheral blood levels of synoviolin were associated with a lack of response to infliximab treatment. Downregulation of synoviolin might, therefore, be a suitable therapy for patients with RA who do not respond to anti-TNF therapy. To reduce the level of synoviolin, it is important to elucidate its transcriptional regulation so that this process can be suppressed. We have identified a crucial site for synoviolin expression in the synoviolin proximal promoter, the Ets-binding site, and found that the GA-binding protein (GABP) α–β complex is essential for its transcriptional regulation.[50] The GABP α–β complex is a transcription factor that functions downstream of signaling by mitogen-activated protein kinases such as c-Jun N-terminal kinase (JNK) and extracellular signal-regulated kinase (ERK). Consistent with these data, Gao *et al.*[51] found that ERK signaling is important in the upregulation of synoviolin expression in response to TNF and IL-1β in mice. Thus, targeting synoviolin expression would, theoretically, be a potential therapeutic approach to RA, although the means by which this might be achieved remain a challenge.

KEY POINTS

- Synoviolin, an E3 ubiquitin ligase that is associated with endoplasmic reticulum-associated degradation (ERAD), is highly expressed in rheumatoid synovial cells and is involved in the onset of rheumatoid arthritis (RA)
- Synoviolin targets the tumor suppressor protein p53 for proteasomal degradation, and can regulate both ER-stress-induced and p53-dependent apoptotic pathways
- Rheumatoid synovial cells undergo autonomous proliferation and aberrant protein production caused by hyper-ERAD by overexpression of synoviolin
- Approximately 30% of newly synthesized proteins are unfolded; the cell utilizes ATP to degrade these proteins, which enables synovial cells to maintain a single-layer structure

References

1 Firestein GS (2003) Evolving concepts of rheumatoid arthritis. *Nature* **423:** 356–361
2 Arend WP (2001) Physiology of cytokine pathways in rheumatoid arthritis. *Arthritis Rheum* **45:** 101–106
3 McInnes IB and Schett G (2007) Cytokines in the pathogenesis of rheumatoid arthritis. *Nat Rev Immunol* **7:** 429–442
4 Stanczyk J *et al.* (2006) Synovial cell activation. *Curr Opin Rheumatol* **18:** 262–267
5 Huber LC *et al.* (2006) Synovial fibroblasts: key players in rheumatoid arthritis. *Rheumatology (Oxford)* **45:** 669–675
6 Knedla A *et al.* (2007) Developments in the synovial biology field 2006. *Arthritis Res Ther* **9:** 209
7 Spencer-Green G (2000) Etanercept (Enbrel): update on therapeutic use. *Ann Rheum Dis* **59 (Suppl 1):** i46–49
8 St Clair EW (2002) Infliximab treatment for rheumatic disease: clinical and radiological efficacy. *Ann Rheum Dis* **61 (Suppl 2):** ii67–69
9 Amano T *et al.* (2003) Synoviolin, an E3 ubiquitin ligase, as a novel pathogenic factor for arthropathy. *Genes Dev* **17:** 2436–2449
10 Kaneko M *et al.* (2002) Human HRD1 protects against ER stress-induced apoptosis through ER-associated degradation. *FEBS Lett* **532:** 147–152
11 Nadav E *et al.* (2003) A novel mammalian endoplasmic reticulum ubiquitin ligase homologous to the yeast Hrd1. *Biochem Biophys Res Commun* **303:** 91–97
12 Kikkert M *et al.* (2004) Human HRD1 is an E3 ubiquitin ligase involved in degradation of proteins from the endoplasmic reticulum. *J Biol Chem* **279:** 3525–3534
13 Bordallo J *et al.* (1998) Der3p/Hrd1p is required for endoplasmic reticulum-associated degradation of misfolded lumenal and integral membrane proteins. *Mol Biol Cell* **9:** 209–222
14 Shearer AG and Hampton RY (2004) Structural control of endoplasmic reticulum-associated degradation: effect of chemical chaperones on 3-hydroxy-3-methylglutaryl-CoA reductase. *J Biol Chem* **279:** 188–196
15 Yagishita N *et al.* (2005) Essential role of synoviolin in embryogenesis. *J Biol Chem* **280:** 7909–7916
16 Shimaoka Y *et al.* (1998) Nurse-like cells from bone marrow and synovium of patients with rheumatoid arthritis promote survival and enhance function of human B cells. *J Clin Invest* **102:** 606–618
17 Tomita T *et al.* (1999) Establishment of nurse-like stromal cells from bone marrow of patients with rheumatoid arthritis: indication of characteristic bone marrow microenvironment in patients with rheumatoid arthritis. *Rheumatology* **38:** 854–863
18 Yagishita N *et al.*: What do synoviolin deficient mice tell us? In *Bone Marrow in RA Patients* (Eds Ochi T and Lipsky PE), in press
19 Yamasaki S *et al.* (2007) Cytoplasmic destruction of p53 by the endoplasmic reticulum-resident ubiquitin ligase 'Synoviolin'. *EMBO J* **26:** 113–122
20 Haupt Y *et al.* (1997) Mdm2 promotes the rapid degradation of p53. *Nature* **387:** 296–299
21 Kubbutat MH *et al.* (1997) Regulation of p53 stability by Mdm2. *Nature* **387:** 299–303
22 Leng RP *et al.* (2003) Pirh2, a p53-induced ubiquitin-protein ligase, promotes p53 degradation. *Cell* **112:** 779–791
23 Dornan D *et al.* (2004) The ubiquitin ligase COP1 is a critical negative regulator of p53. *Nature* **429:** 86–92

24 Firestein GS *et al.* (1997) Somatic mutations in the p53 tumor suppressor gene in rheumatoid arthritis synovium. *Proc Natl Acad Sci USA* **94:** 10895–10900
25 Reme T *et al.* (1998) Mutations of the p53 tumour suppressor gene in erosive rheumatoid synovial tissue. *Clin Exp Immunol* **111:** 353–358
26 Inazuka M *et al.* (2000) Analysis of p53 tumour suppressor gene somatic mutations in rheumatoid arthritis synovium. *Rheumatology* **39:** 262–266
27 Muller-Ladner U and Nishioka K (2000) p53 in rheumatoid arthritis: friend or foe? *Arthritis Res* **2:** 175–178
28 Sun Y and Cheung HS (2002) p53, proto-oncogene and rheumatoid arthritis. *Semin Arthritis Rheum* **31:** 299–310
29 Yamanishi Y *et al.* (2002) Regulation of joint destruction and inflammation by p53 in collagen-induced arthritis. *Am J Pathol* **160:** 123–130
30 Simelyte E *et al.* (2005) Regulation of arthritis by p53: critical role of adaptive immunity. *Arthritis Rheum* **52:** 1876–1884
31 Ellgaard L *et al.* (1999) Setting the standards: quality control in the secretory pathway. *Science* **286:** 1882–1888
32 Schroder M and Kaufman RJ (2005) The mammalian unfolded protein response. *Annu Rev Biochem* **74:** 739–789
33 Rutkowski DT and Kaufman RJ (2004) A trip to the ER: coping with stress. *Trends Cell Biol* **14:** 20–28
34 Yamasaki S *et al.* (2005) Rheumatoid arthritis as a hyper-endoplasmic-reticulum-associated degradation disease. *Arthritis Res Ther* **7:** 181–186
35 Yagishita N *et al.* (2006) Role of Synoviolin in rheumatoid arthritis: possible clinical relevance. *Future Rheumatol* **1:** 31–36
36 Ritchlin C (2000) Fibroblast biology. Effector signals released by the synovial fibroblast in arthritis. *Arthritis Res* **2:** 356–360
37 Yamasaki S *et al.* (2006) Resistance to endoplasmic reticulum stress is an acquired cellular characteristic of rheumatoid synovial cells. *Int J Mol Med* **18:** 113–117
38 Zhao L and Ackerman SL (2006) Endoplasmic reticulum stress in health and disease. *Curr Opin Cell Biol* **18:** 444–452
39 Yoshida H (2007) ER stress and diseases. *FEBS J* **274:** 630–658
40 Bence NF *et al.* (2001) Impairment of the ubiquitin-proteasome system by protein aggregation. *Science* **292:** 1552–1555
41 Hirabayashi M *et al.* (2001) VCP/p97 in abnormal protein aggregates, cytoplasmic vacuoles, and cell death, phenotypes relevant to neurodegeneration. *Cell Death Differ* **8:** 977–984
42 Jana NR *et al.* (2001) Altered proteasomal function due to the expression of polyglutamine-expanded truncated N-terminal huntingtin induces apoptosis by caspase activation through mitochondrial cytochrome c release. *Hum Mol Genet* **10:** 1049–1059
43 Imai Y *et al.* (2000) Parkin suppresses unfolded protein stress-induced cell death through its E3 ubiquitin-protein ligase activity. *J Biol Chem* **275:** 35661–35664
44 Vabulas RM *et al.* (2005) Protein synthesis upon acute nutrient restriction relies on proteasome function. *Science* **23:** 1960–1963
45 Yewdell JW (2001) Not such a dismal science: the economics of protein synthesis, folding, degradation and antigen processing. *Trends Cell Biol* **11:** 294–297
46 Hershko A and Ciechanover A (1998) The ubiquitin system. *Annu Rev Biochem* **67:** 425–479
47 Pickart CM (2001) Mechanisms underlying ubiquitination. *Annu Rev Biochem* **70:** 503–533
48 International Human Genome Sequencing Consortium (2004) Finishing the euchromatic sequence of the human genome. *Nature* **431:** 931–945
49 Toh ML *et al.* (2006) Overexpression of synoviolin in peripheral blood and synoviocytes from rheumatoid arthritis patients and continued elevation in nonresponders to infliximab treatment. *Arthritis Rheum* **54:** 2109–2118
50 Tsuchimochi K *et al.* (2005) Identification of a crucial site for synoviolin expression. *Mol Cell Biol* **25:** 7344–7356
51 Gao B *et al.* (2006) The proinflammatory cytokines IL-1beta and TNF-alpha induce the expression of Synoviolin, an E3 ubiquitin ligase, in mouse synovial fibroblasts via the Erk1/2-ETS1 pathway. *Arthritis Res Ther* **8:** R172

Acknowledgments
We thank N Okamoto, Funsite, ITAKURA OFFICE and all members of Professor Nakajima's laboratory. This work was partially supported financially by the Japan Society for the Promotion of Science Grants-in-Aid for Scientific Research (Creative research conducted by university researchers individually or in groups Category A, Category C), Ministry of Health, Labour and Welfare Grants-in-Aid for Scientific Research (Research on Allergic disease and Immunology), the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation (04-3), the Kanagawa Nanbyo Foundation, Heiwa Nakajima Foundation, The Uehara Memorial Foundation, Takeda Science Foundation, Mitsui Life Insurance Co. Ltd. and the Sagawa Foundation for Promotion of Cancer Research.

Competing interests
The authors declared no competing interests.

[Cell Cycle 6:11, 1319-1323, 1 June 2007]; ©2007 Landes Bioscience

Perspective

The Roles of Synoviolin in Crosstalk Between Endoplasmic Reticulum Stress-Induced Apoptosis and p53 Pathway

Satoshi Yamasaki[1]
Naoko Yagishita[1]
Kusuki Nishioka[2]
Toshihiro Nakajima[1,*]

[1]Department of Genome Science; [2]Rheumatology, Immunology and Genetics Program; Institute of Medical Science; St. Marianna University School of Medicine; Kawasaki, Japan

*Correspondence to: Toshihiro Nakajima; Department of Genome Science; Institute of Medical Science; St. Marianna University School of Medicine; 2-16-1 Sugao Miyamae-ku; Kawasaki, Kanagawa 216-8512 Japan; Tel.: +81.44.977.8111 ext. 4113; Fax: +81.44.977.9772; Email: nakashit@marianna-u.ac.jp

Original manuscript submitted: 04/08/07
Manuscript accepted: 04/11/07

Previously published online as a *Cell Cycle* E-publication:
http://www.landesbioscience.com/journals/cc/abstract.php?id=4277

KEY WORDS

apoptosis, E3 ubiquitin ligase, rheumatoid arthritis, cancer, UPR, ERAD, hrd1

ABBREVIATIONS

GRP	glucose-regulated protein
eIF2α	eukaryotic translation initiation factor 2α
PERK	PKR-like ER kinase
CHOP	C/EBP homologues protein
XBP1	X box binding protein 1
ATF6	activating transcription factor 6
TRAF2	TNF receptor-associated factor 2
PUMA	p53 upregulated modulator of apoptosis
Cop1	constitutive photomorphogenesis protein 1
Parc	Parkin-like cytoplasmic protein

ACKNOWLEDGEMENTS

See page 1322.

ABSTRACT

Endopalsmic reticulum (ER) is specialized organelle to maintain the integrity of secreted and membranous proteins. ER also senses so-called "ER stress", which is a result of various internal and external stresses, and triggers apoptosis when the diverse attempts to accommodate with the stress are in fail. The impairment these ER functions has been implicated in several human diseases, in which aberrant ER stress induced apoptosis is observed. We discuss about another disease model related with ER mediated apoptosis based on the recent studies about Synoviolin, an E3 ubiquitin ligase inherently utilized for ER associated degradation (ERAD). In addition to its canonical role in ERAD, Synoviolin targets tumor suppressor gene p53 for proteasomal degradation, suggesting the crosstalk between ERAD and p53 mediated apoptotic pathway under ER stress. Together with the anti-apoptotic property of Synoviolin previously elucidated by both in vitro and in vivo analyses, its new function in p53 regulation may provide a new insight into the pathomechanism of proliferative diseases such as cancer or rheumatoid arthritis.

SYNOVIOLIN IN ER STRESS

Secreted and membranous proteins are modified in endoplasmic reticulum (ER) and each one of them is highly quality controlled by folding of newly synthesized proteins with ER resident chaperones (e.g., calnexin, calreticulin, GRP78, GRP94, protein disulfide isomerase).[1] If the influx of nascent protein is more than the folding capacity of ER, nascent peptide chains (unfolded proteins) accumulate in ER.[2-3] Similarly, glucose deprivation, DNA damage, inhibition of N-linked glycosylation or increase in protein synthesis can also perturb the proper post-translational modifications of nascent proteins.[3] The unfolded proteins produced under these conditions in the ER cause inverse effect on physiological homeostasis or protein integrity, a plight called the ER stress that is implicated with variety kinds of human diseases (reviewed in refs. 2 and 4–7.)

It is demonstrated that ER can sense the stress by several ER resident molecules (e.g., IRE1, PERK, ATF6).[2-3] They also initiate the unfolded protein response (UPR) to adapt various internal and external stresses for maintenance of protein integrity.[1-4] The ER stress induce phosphorylation of eIF2 α through the activation of the PERK, which results in attenuation of global translation to reduce the influx of proteins into ER and unfolded proteins.[8] On the other hand, PERK-eIF2α kinase axis upregulates expression of pro-apoptotic protein, CHOP, via activation of transcriptional factor ATF4.[9] ATF6, an ER resident transcriptional factor, is cleaved and translocates into nucleus to promote a group of stress inducible proteins including chaperones (e.g., Grp78, Grp97, protein disulphide isomerase) and transcription factors (XBP1).[10-12] ATF6 was also proved to induce the transcription of CHOP.[13] An ER resident enzyme, IRE1, possesses dual catalytic domains as serine-threonine kinase and endoribonuclease, activates XBP1 by generating its splicing variant (active form), which also contributes to transcription of chaperons and other molecule important for UPR and ERAD.[2,12] IRE1 is also involved in polyglutamine-induced cell death by activating ASK 1 through formation of an IRE1-TRAF2-ASK1 complex.[14] It is worthy to note that these molecules involved in UPR pathway have potential to carry out both prevention and promotion of apoptosis.

The UPR is closely related with another homeostatic mechanisms in ER known as ER associated degradation (ERAD), a system for disposal of the unfolded proteins.[15-18] The ERAD requires three steps, substrate transportation from the ER to the cytoplasm (dislocation), ubiquitination by specific ubiquitin ligases and proteolysis by proteasome in cytoplasm.[18] There are several ubiquitine ligase are reported involved in ERAD, and

Synoviolin is one of these ER-resident E3 ubiquitin ligases.[19-22] Synoviolin was introduced as a mammalian homolog of Hrd1p/Der3p that is inherent ubiquitin ligase for ERAD system. Misfolded carboxypeptide yscY (CPY*) and 3-hydroxy-3-methylglutaryl-coenzymeA reductase (HMGR), a key enzyme of the mevalonate pathway in yeast are known substrate for Synoviolin.[23-24] In mammal, Synoviolin seems to play more complicated roles, because it is essential for embryogenesis and suggested to be a pathogenic factor for arthritis.[19,25] The *synoviolin* homozygous knock out mouse is embryonic lethal due to severe anemia brought about by enhanced apoptosis in the liver where the embryonic erythrogenesis takes place. Since UPR, such as the induction of chaperon proteins, is not impaired in the knock out mice, the sole disruption of ERAD system in the mice can cause ER stress-induced apoptosis when the protein synthesis rapidly increases to build up fetal erythrogenesis in the liver.[25] The heterozygous knock out mouse can survive and show no phenotype in appearance, however, the mouse shows remarkable resistance to experimental arthritis model, which is induced by immunization of type II collagen (collagen induced arthritis: CIA).[19,25] Histological analyses demonstrate that the hyperplasia of the synovial tissue is suppressed in the mouse, which is usually observed in the CIA-induced wild type mice. Interestingly, enhanced apoptosis is detected in the synovial tissue of the CIA-induced heterozygous knock out mouse, indicating an important role of Synoviolin in antagonizing the apoptotic pathway. Contrary to this, Synoviolin overexpressing mouse exhibits overgrowth of synovial tissue that resembles rheumatoid arthritis (RA), a common chronic inflammatory joint disease in human.[19] In addition, the evidences that Synoviolin prevents ER stress induced apoptosis in cultured cells support the idea that Synoviolin is an anti-apoptotic factor in mammals probably due to protecting the cells from ER stress.[19,21] Based on these data, we proposed one pathogenic status with Synoviolin overexpression as "Hyper-ERAD", which might allow excess elimination of unfolded proteins and provide the cell with ER stress free condition.[19,26] It is estimated that 30 to 40% of the newly synthesized proteins failed to be folded properly in ER, which means most of the cells are facing at risk of ER stress unless enough ERAD constitutively clears these constantly produced unfolded proteins.[27] Since the positive correlation is known between ERAD and the production of secretory proteins, hyper-ERAD may allow the cell to over-produce secretary proteins.[28] A cell equipped with hyper-ERAD could be very refractory to ER stress, because unfolded proteins can be promptly eliminated by the equipped system even if the protein synthesis is upregulated or the environment becomes stressful for ER. When the cells become free from unfolded proteins, it is possible that UPR triggered translational attenuation or apoptosis signal are shut down. These phenotypes that might be resulted from hyper-ERAD (e.g., increased protein production, anti-apoptotic) are similar to the hallmarks of the proliferating diseases including neoplasm and arthritis.

p53 REGULATION BY SYNOVIOLIN

The mechanisms for the ER stress-induced apoptosis remain enigmatic, however, recent reports have succeeded to illustrate some pathways in the ER stress induced apoptosis (reviewed in refs. 29–32). ER resident caspase (caspase-12 in mouse and probably caspase-4 in human) is suggested to be activated in ER stress-induced apoptosis, even though their roles in human are still under debate.[33,34] One of the most characterized pathways in ER

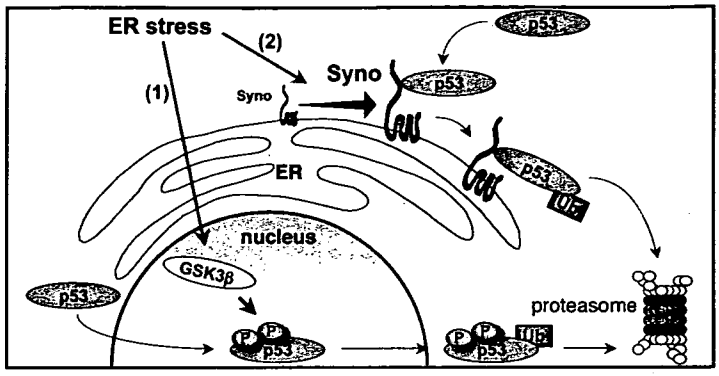

Figure 1. p53 regulatory pathway under ER stress. (1) Nuclear GSK3β, which is probably activated by ER resident kinase(s) after ER stress, phosphorylates p53 at serine 315 and 376. The phosphorylated p53 is ubiquitinated, exported to cytoplasm and degraded by proteasome. (2) ER stress induces Synoviolin expression by IRE and/or ATF6. Cytoplasmic p53 is trapped and ubiquitinated for proteasomal degradation by ER stress-induced Synoviolin (Syno) at ER before translocating into nucleus.

stress-induced apoptosis is carried out through BCL2 family proteins. The first report related with this pathway demonstrated the protective effect of BCL2 overexpression or Bax and Bak deficiency against ER stress-induced apoptosis.[35-37] Furthermore, BCL2 is inhibited by CHOP, which is transcriptionally induced by PERK-eIF2α-ATF4 pathway and post-translationaly activated by kinase pathway originated from IRE1-TRAF2 complex on ER after ER stress.[9,13,38] The other members of the BH3-only BCL-2 family proteins, PUMA and Noxa, have been identified as the pro-apoptotic molecules induced by ER stress.[39,40] Consistent with these facts, the activation of p53 is also observed in the ER stress, because both PUMA and Noxa are the evident transcriptional targets of p53.[40-42] However, the ER stress induced p53 activation is not straight forward, namely a mild or a short time ER stress tends to inhibit p53 dependent apoptosis and a severe or a long time stress promote it.[40,43,44]

The findings from Koromilas and our lab provide the evidence for striking mechanism in p53 regulation at ER (Fig. 1).[43-45] Qu et al. proved that ER stress inhibits p53 dependent apoptosis. The critical step for p53 suppression is accomplished by phosphorylation of p53 at serine 315 and 376 by glycogen synthase kinase-3β (GSK3 β).[43,44] The mechanism how the kinase is activated after ER stress remains unclear, but the C-terminus phosphorylation of p53 by the kinase exhibits remarkable inhibitory effect on nuclear localization and stabilization of p53 in cooperation with Hdm2, one of the most important negative regulators for p53.[46] On the other hand, we introduced Synoviolin as a potent negative regulator of p53.[45] Synoviolin can cytoplasmically capture p53 on ER and degrade it by ubiquitin-proteasome system. Synoviolin is totally independent of known p53 regulating ubiquitin ligases such as Hmd2, Pirh2, Cop1 or Parc in this process.[45,46] The importance of Synoviolin is underlined by the fact that the steady state level of p53 protein is strictly regulated by Synoviolin, because the half life of p53 protein is markedly prolonged in human cell line by siRNA for *synoviolin* as well as in the embryonic fibroblasts from *synoviolin* knock out mouse.[45] Since Synoviolin is known to be upregulated by IRE or ATF6, it can be said that the ER stress also indirectly dampen p53 through an ER resident ubiquitin ligase, Synoviolin.[21] Both GSK3 β and Synoviolin pathways invite the same consequences of p53, cytoplasmic localization and degradation in there, which might

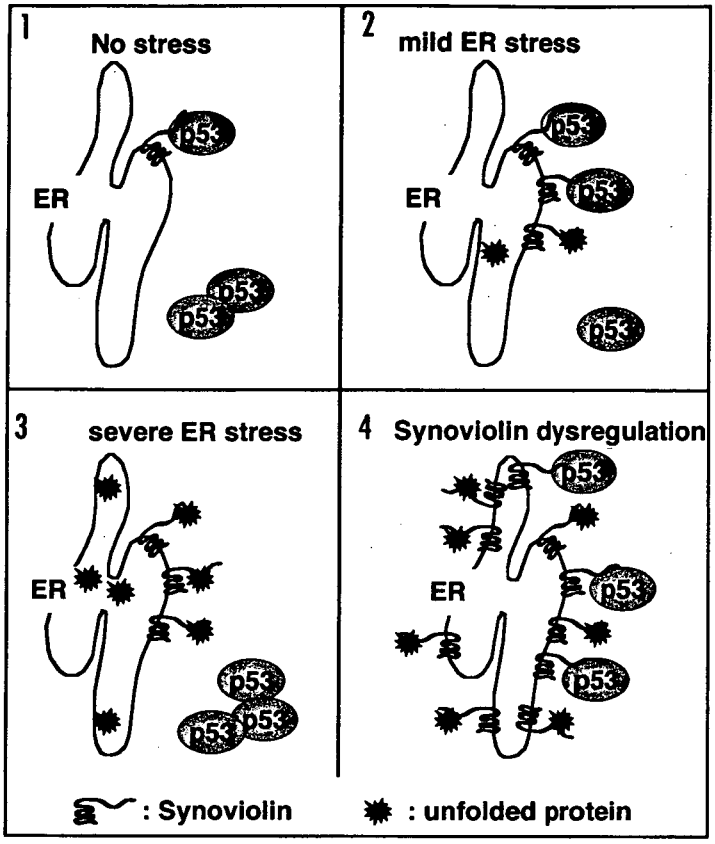

Figure 2. p53 regulatory status according to ER stress. (1) p53 is constitutively downregulated by Synoviolin (Syno) on ER. (2) ER stress induces Synoviolin, which is utilized for trapping p53 for degradation as well as its canonical role in ERAD. (3) Severe or prolonged ER stress produces the excess amount of unfodled proteins that may occupy most of Synoviolin. The trapped p53 are liberated from Synoviolin and translocate into nucleus to function as a transcriptional factor. (4) Neither ER stress nor p53 activation in Synoviolin dys-regulated cells. The aberrantly high expression of Synoviolin due to activation of particular signal pathway (e.g., JNK and ERK) simultaneously degrades p53 and unfolded proteins, which may block both p53 dependent and ER stress-induced apoptosis.

help to gain times for chaperones to refold the unfolded proteins to restore their ER functions before apoptosis is induced in these cells. The aberrant cytoplasmic localization of p53 are described in human cancers, thus the ER stress initiated p53 inhibition by Synoviolin or GSK3 β could be the mechanism how p53 is sequestered in cytoplasm of these cancers.[47,48] The milieu surrounding neoplasm cells also support this idea, namely the stressful external environment may give mild ER stress to tumor cells, and help to inhibit p53 by activating Synoviolin or GSK3 β. Actually, ER stress is demonstrated by the activation of ER stress inducible molecules in human tumor and arthritis.[19,49] On this point of view, blockade of Synoviolin or GSK3 β can be applied for adjuvant therapy for neoplasm therapy or arthritis through p53 activation.

IMPACT OF SYNOVIOLIN DYSREGULATION

There seems to be a transition in function of UPR proteins. IRE1 is one of the most characterized proteins in this aspect, because it renders anti-apoptotic effect by induction of ER chaperons through XBA1 activation, however, IRE1 can be pro-apoptotic by triggering ASK1-JNK pathway.[12,50] The severity or the duration of ER stress seems to determine the down stream pathway of IRE1. It is expected that severely damaged cells by ER stress could produce unfolded proteins that may be harmful to the systemic cells, therefore it is reasonable to change its roles from anti-apoptotic to pro-apoptotic as the cell damage becomes more severe. As mentioned before, the activity of p53 under ER stress also fluctuates according to the level of the stress. It seems that a mild ER stress de-stabilizes p53, but a severe one activates it.[40,43,44] The mechanism that determine the activity of p53 has not been fully figured out experimentally, however, previous data may unravel it by describing in detail the regulation and the function of Synoviolin under ER stress (Fig. 2). In unstressed cell, Synoviolin is thought to regulates steady state levels of p53 by sequestering and ubiquitinating it for proteasomal degradation in cytoplasm, because Synoviolin knocking down by siRNA clearly satbilizes the p53.[45] In the mild ER stress condition, Synoviolin is transcriptionally upregulated by IRE or ATF6 activation and the increased Synoviolin plays its canonical role in ERAD.[19-22] If the expression of Synoviolin is induced more than that is required to eliminate unfolded proteins, the free Synoviolin may capture more p53 than they do in unstressed cell. As a result, a mild ER stress may achieve p53 suppression (Fig. 2B). When a severe or a prolonged ER stress takes place, the unfolded proteins can exceed the capacity of upregulated Synoviolin. The overwhelming unfolded proteins may squelch Synoviolin and let it to liberate p53 for activation (Fig. 2C). This scenario can explain, at least in part, why p53 change its behavior according to the severity of ER stress.

ER stress has been implicated in the pathogenesis in human diseases.[2,4-7] Disruption of physiological UPR and/or ERAD or abnormal conformation of proteins have been demonstrated in a variety of human diseases including Alzheimer disease, Parkinson disease, neuronal damage by ischemia, prion disease, cystic fibrosis and diabetes mellitus. The cause of these degenerative changes in the diseases is explained by aberrant ER stress-induced apoptosis in the affected organs. Contrary to this, we would like to propose a novel model for proliferative diseases such as tumor or RA, which is also a hypothesis reply to an interesting question, "what will happen to cells if they are completely free from ER stress?" As previously described, Synoviolin has two functions, one is for ERAD and the other is for p53 blockade. Given that Synvoiolin is aberrantly upregulated in cells as shown in Figure 2D, they could provide the cells an ability to over-secrete and overgrow, because overexpressed Synoviolin will unburden the cells from ER stress by eliminating the unfolded protein even in the hostile milieu surrounding the cells such as hypoxia and/or starvation. In addition, ER stress free cells may be allowed to produce the harmful amount of secretary or membranous protein. Those acquired property will help the cells to proliferate, destruct and invade into surrounding tissues. The overexpressed Synoviolin is also expected to sequester p53 in cytoplasm and degrade it, which may rescues cells that are supposed to commit apoptosis. In this regard, our analyses about *synoviolin* promoter make the hypothesis plausible. The Ets binding site (EBS) is determined to be the crucial site for *synoviolin* transcription in vivo and in vitro, and GABP, a transcription factor known to be down stream of MAP kinases such as JNK and ERK, is proved to be bind to it.[51] The MAP kinase signals are activated in both neoplasm and RA, thus these signal may induce constitutively high expression of Synoviolin in these diseases.[52,53] Similar pathogenic mechanism for neoplasm progression has been actually implicated in a human disease. There is a clinical evidence that XBP-1 is highly upregulated in multiple

myeloma cell, a hematological neoplasm characterized by overproduction of monoclonal immunoglobin or Bence Jones proteins (reviewed in ref. 4). It is important that proteasomal inhibitors have been proved to be effective to this disease, indicating accumulation of unfolded proteins by proteasome blockade affect the proliferation of neoplasmic cells. Our speculation is that the increased unfolded proteins act as an inhibitor for Synoviolin and p53 is released and activated in proteasome inhibitor treated cells. Likewise, the therapy targeting Synoviolin could be a good strategy for the proliferative diseases refractory to the conventional treatment.

CONCLUSION

ER integrates many kinds of stresses and initiates a variety of signalings to adapt to the stresses. Among them, apoptosis pathway has become an important topic for its definite pathogenic potential in human diseases. P53, one of the most important molecules in tumor, has become a novel mediator of ER stress-induced apoptosis. It also can be said that ER regulates p53 pathway, because the ER specific stresses or molecules seem to play some roles in p53 regulation. We discuss about the dual role of Synoviolin on ER, one is the ERAD and the other is p53 inhibition in cytoplasm. In this regard, Synoviolin may upregulate two aspects of proliferative diseases, overproduction of secretory proteins and resistance to apoptosis. Both expression level and its enzyme activity of Synoviolin are required for ERAD and p53 regulation, the blockade of Synoviolin expression or activity may ameliorate the proliferative diseases such as neoplasm and RA. Further research is necessary to answer the important questions about the roles of Synoviolin in ER stress induced apoptosis. Does hyper-ERAD status really exist in human diseases? Can a specific inhibitor against Synoviolin cause p53 activation, and induce apoptosis of target cells? We hope that the continuous attempt to unveil the ER mediated apoptotic pathway will help to develop the new therapeutic approach to the refractory diseases.

Acknowledgements

We are grateful to Montminy MR, Verdine G, Nagata R, Shimizu H, Hishinuma I, Yokohama H, Kato H, Kitamura S, Yoshimatsu K, Yuichiro ITAKURA OFFICE and Takagi ES, for advice and encouragement, and to Takahashi H, Sato M, Otani S, Sugamiya A, Takagi N, Shinkawa S, Nakagawa Y, Sato Y, Yamanashi M and members of Toshi's Laboratory for the excellent technical assistance. This study was supported in part by LocomoGene Inc., Eisai Co., Ltd., National Institute of Biomedical Innovation, the Japanese Ministry of Education, Culture, Sports, Science and Technology, the Japanese Ministry of Health, Labour and Welfare, the Kato Memorial Trust for Nanbyo Research, the Japan Medical Association, Nagao Memorial Fund, Kanae Foundation for Life & Socio-medical Science, Japan Research Foundation for Clinical Pharmacology, Kanagawa Nanbyo Foundation, Kanagawa Academy of Science and Technology Research Grants, Japan College of Rheumatology, the Nakajima Foundation, Japan Society for Promotion of Science, New Energy and Industrial Technology Development Organization, Mochida Pharmaceutical Co. Ltd, Kanto Bureau of Economy, Trade and Industry, and the Uehara Memorial Foundation. H.F. is supported by Japan Society for the Promotion of Science.

References

1. Ellgaard L, Molinari M, Helenius A. Setting the standards: Quality control in the secretory pathway. Science 1999; 286:1882-8.
2. Schroder M, Kaufman RJ. The mammalian unfolded protein response. Annu Rev Biochem 2005; 74:739-89.
3. Rutkowski DT, Kaufman RJ. A trip to the ER: Coping with stress. Trends Cell Biol 2004; 14:20-8.
4. Marciniak SJ, Ron D. Endoplasmic reticulum stress signaling in disease. Physiol Rev 2006; 86:1133-49.
5. Lindholm D, Wootz H, Korhonen L. ER stress and neurodegenerative diseases. Cell Death Differ 2006; 13:385-92.
6. Zhao L, Ackerman SL. Endoplasmic reticulum stress in health and disease. Curr Opin Cell Biol 2006; 18:444-52.
7. Wu J, Kaufman RJ. From acute ER stress to physiological roles of the Unfolded Protein Response. Cell Death Differ 2006; 13:374-84.
8. Harding HP, Zhang Y, Ron D. Protein translation and folding are coupled by an endoplasmic-reticulum-resident kinase. Nature 1999; 397:271-4.
9. Harding HP, Novoa I, Zhang Y, Zeng H, Wek R, Schapira M, Ron D. Regulated translation initiation controls stress-induced gene expression in mammalian cells. Mol Cell 2000; 6:1099-108.
10. Haze K, Yoshida H, Yanagi H, Yura T, Mori K. Mammalian transcription factor ATF6 is synthesized as a transmembrane protein and activated by proteolysis in response to endoplasmic reticulum stress. Mol Biol Cell 1999; 10:3787-99.
11. Yoshida H, Haze K, Yanagi H, Yura T, Mori K. Identification of the cis-acting endoplasmic reticulum stress response element responsible for transcriptional induction of mammalian glucose-regulated proteins. Involvement of basic leucine zipper transcription factors. J Biol Chem 1998; 273:33741-9.
12. Yoshida H, Matsui T, Yamamoto A, Okada T, Mori K. XBP1 mRNA is induced by ATF6 and spliced by IRE1 in response to ER stress to produce a highly active transcription factor. Cell 2001; 107:881-91.
13. Yoshida H, Okada T, Haze K, Yanagi H, Yura T, Negishi M, Mori K. ATF6 activated by proteolysis binds in the presence of NF-Y (CBF) directly to the cis-acting element responsible for the mammalian unfolded protein response. Mol Cell Biol 2000; 20:6755-67.
14. Nishitoh H, Matsuzawa A, Tobiume K, Saegusa K, Takeda K, Inoue K, Hori S, Kakizuka A, Ichijo H. ASK1 is essential for endoplasmic reticulum stress-induced neuronal cell death triggered by expanded polyglutamine repeats. Genes Dev 2002; 16:1345-55.
15. Hampton RY. ER-associated degradation in protein quality control and cellular regulation. Curr Opin Cell Biol 2002; 14:476-82.
16. Friedlander R, Jarosch E, Urban J, Volkwein C, Sommer T. A regulatory link between ER-associated protein degradation and the unfolded-protein response. Nat Cell Biol 2000; 2:379-84.
17. Travers KJ, Patil CK, Wodicka L, Lockhart DJ, Weissman JS, Walter P. Functional and genomic analyses reveal an essential coordination between the unfolded protein response and ER-associated degradation. Cell 2000; 101:249-58.
18. Meusser B, Hirsch C, Jarosch E, Sommer T. ERAD: The long road to destruction. Nat Cell Biol 2005; 7:766-72.
19. Amano T, Yamasaki S, Yagishita N, Tsuchimochi K, Shin H, Kawahara K, Aratani S, Fujita H, Zhang L, Ikeda R, Fujii R, Miura N, Komiya S, Nishioka K, Maruyama I, Fukamizu A, Nakajima T. Synoviolin/Hrd1, an E3 ubiquitin ligase, as a novel pathogenic factor for arthropathy. Genes Dev 2003; 17:2436-49.
20. Bordallo J, Plemper R, Finger A, Wolf DH. Der3p/Hrd1p is required for endoplasmic reticulum-associated degradation of misfolded lumenal and integral membrane proteins. Mol Biol Cell 1998; 9:209-22.
21. Kaneko M, Ishiguro M, Niinuma Y, Uesugi M, Nomura Y. Human HRD1 protects against ER stress-induced apoptosis through ER-associated degradation. FEBS Lett 2002; 532:147-52.
22. Kikkert M, Doolman R, Dai M, Avner R, Hassink G, vanVoorden S, Thanedar S, Roitelman J, Chau V, Wiertz E. Human HRD1 is an E3 ubiquitin ligase involved in degradation of proteins from the endoplasmic reticulum. J Biol Chem 2004; 279:3525-34.
23. Shearer AG, Hampton RY. Structural control of endoplasmic reticulum-associated degradation: Effect of chemical chaperones on 3-hydroxy-3-methylglutaryl-CoA reductase. J Biol Chem 2004; 279:188-96.
24. Shearer AG, Hampton RY. Lipid-mediated, reversible misfolding of a sterol-sensing domain protein. EMBO J 2005; 24:149-59.
25. Yagishita N, Ohneda K, Amano T, Yamasaki S, Sugiura A, Tsuchimochi K, Shin H, Kawahara K, Ohneda O, Ohta T, Tanaka S, Yamamoto M, Maruyama I, Nishioka K, Fukamizu A, Nakajima T. Essential role of synoviolin in embryogenesis. J Biol Chem 2005; 280:7909-16.
26. Yamasaki S, Yagishita N, Tsuchimochi K, Nishioka K, Nakajima T. Rheumatoid arthritis as a hyper-endoplasmic-reticulum-associated degradation disease. Arthritis Res Ther 2005; 7:181-6.
27. Yewdell JW. Not such a dismal science: The economics of protein synthesis, folding, degradation and antigen processing. Trends Cell Biol 2001; 11:294-7.
28. Molinari M, Sitia R. The secretory capacity of a cell depends on the efficiency of endoplasmic reticulum-associated degradation. Curr Top Microbiol Immunol 2005; 300:1-15.
29. Boyce M, Yuan J. Cellular response to endoplasmic reticulum stress: A matter of life or death. Cell Death Differ 2006; 13:363-73.
30. Ferri KF, Kroemer G. Organelle-specific initiation of cell death pathways. Nat Cell Biol 2001; 3:E255-63.
31. Szegezdi E, Logue SE, Gorman AM, Samali A. Mediators of endoplasmic reticulum stress-induced apoptosis. EMBO Rep 2006; 7:880-5.
32. Chunyan X, Bailly-Maitre B, Reed JC. Endoplasmic reticulum stress: Cell life and death decisions. J Clin Invest 2005; 115:2656-64.

33. Hitomi J, Katayama T, Eguchi Y, Kudo T, Taniguchi M, Koyama Y, Manabe T, Yamagishi S, Bando Y, Imaizumi K, Tsujimoto Y, Tohyama M. Involvement of caspase-4 in endoplasmic reticulum stress-induced apoptosis and Abeta-induced cell death. J Cell Biol 2004; 165:347-56.
34. Nakagawa T, Zhu H, Morishima N, Li E, Xu J, Yankner BA, Yuan J. Caspase-12 mediates endoplasmic-reticulum-specific apoptosis and cytotoxicity by amyloid-beta. Nature 2000; 403:98-103.
35. Scorrano L, Oakes SA, Opferman JT, Cheng EH, Sorcinelli MD, Pozzan T, Korsmeyer SJ. BAX and BAK regulation of endoplasmic reticulum Ca2+: A control point for apoptosis. Science 2003; 300:135-9.
36. Zong WX, Lindsten T, Ross AJ, MacGregor GR, Thompson CB. BH3-only proteins that bind pro-survival Bcl-2 family members fail to induce apoptosis in the absence of Bax and Bak. Genes Dev 2001; 15:1481-6.
37. Zong WX, Li C, Hatzivassiliou G, Lindsten T, Yu QC, Yuan J, Thompson CB. Bax and Bak can localize to the endoplasmic reticulum to initiate apoptosis. J Cell Biol 2003; 162:59-69.
38. McCullough KD, Martindale JL, Klotz LO, Aw TY, Holbrook NJ. Gadd153 sensitizes cells to endoplasmic reticulum stress by downregulating Bcl2 and perturbing the cellular redox state. Mol Cell Biol 2001; 21:1249-59.
39. Reimertz C, Kogel D, Rami A, Chittenden T, Prehn JH. Gene expression during ER stress-induced apoptosis in neurons: Induction of the BH3-only protein Bbc3/PUMA and activation of the mitochondrial apoptosis pathway. J Cell Biol 2003; 162:587-97.
40. Li J, Lee B, Lee1 AS. Endoplasmic reticulum stress-induced apoptosis: Multiple pathways and activation of p53-upregulated modulator of apoptosis (PUMA) and NOXA by p53. J Biol Chem 2006; 281:7260-70.
41. Huang DC, Strasser A. BH3-Only proteins-essential initiators of apoptotic cell death. Cell 2000; 103:839-42.
42. Oda E, Ohki R, Murasawa H, Nemoto J, Shibue T, Yamashita T, Tokino T, Taniguchi T, Tanaka N. Noxa, a BH3-only member of the Bcl-2 family and candidate mediator of p53-induced apoptosis. Science 2000; 288:1053-8.
43. Qu L, Huang S, Baltzis D, Rivas-Estilla AM, Pluquet O, Hatzoglou M, Koumenis C, Taya Y, Yoshimura A, Koromilas AE. Endoplasmic reticulum stress induces p53 cytoplasmic localization and prevents p53-dependent apoptosis by a pathway involving glycogen synthase kinase-3β. Genes Dev 2004; 18:261-77.
44. Pluquet O, Qu L, Baltzis D, Koromilas AE. Endoplasmic reticulum stress accelerates p53 degradation by the cooperative actions of Hdm2 and Glycogen synthase kinase 3β. Mol Cell Biol 2005; 25:9392-405.
45. Yamasaki S, Yagishita N, Sasaki T, Nakazawa M, Kato Y, Yamadera T, Bae E, Toriyama S, Ikeda R, Zhang L, Fujitani K, Yoo E, Tsuchimochi K, Ohta T, Araya N, Fujita H, Aratani S, Eguchi K, Komiya S, Maruyama I, Higashi N, Sato M, Senoo H, Ochi T, Yokoyama S, Amano T, Kim J, Gay S, Fukamizu A, Nishioka K, Tanaka K, Nakajima T. Cytoplasmic destruction of p53 by the endoplasmic reticulum-resident ubiquitin ligase 'Synoviolin'. EMBO J 2007; 26:113-22.
46. Brooks CL, Gu W. p53 ubiquitination: Mdm2 and beyond. Mol Cell 2006; 21:307-315.
47. Moll UM, Ostermeyer AG, Haladay R, Winkfield B, Frazier M, Zambetti G. Cytoplasmic sequestration of wild-type p53 protein impairs the G_1 checkpoint after DNA damage. Mol Cell Biol 1996; 16:1126-37.
48. Schlamp CL, Poulsen GL, Nork TM, Nickells RW. Nuclear exclusion of wild-type p53 in immortalized human retinoblastoma cells. J Natl Cancer Inst 1997; 89:1530-6.
49. Shuda M, Kondoh N, Imazeki N, Tanaka K, Okada T, Mori K, Hada A, Arai M, Wakatsuki T, Matsubara O, Yamamoto N, Yamamoto M. Activation of the *ATF6*, *XBP1* and *grp78* genes in human hepatocellular carcinoma: A possible involvement of the ER stress pathway in hepatocarcinogenesis. J Hepatol 2003; 38:605-14.
50. Urano F, Wang X, Bertolotti A, Zhang Y, Chung P, Harding HP, Ron D. Coupling of stress in the ER to activation of JNK protein kinases by transmembrane protein kinase IRE1. Science 2000; 287:664-6.
51. Tsuchimochi K, Yagishita N, Yamasaki S, Amano T, Kato Y, Kawahara K, Aratani S, Fujita H, Ji F, Sugiura A, Izumi T, Sugamiya A, Maruyama I, Fukamizu A, Komiya S, Nishioka K, Nakajima T. Identification of a crucial site for synoviolin expression. Mol Cell Biol 2005; 25:7344-56.
52. Fang JY, Richardson BC. The MAPK signalling pathways and colorectal cancer. Lancet Oncol 2005; 6:322-27.
53. Meyer LH, Pap T. MAPK signalling in rheumatoid joint destruction: Can we unravel the puzzle? Arthritis Res Ther 2005; 7:177-8.

BRCA1 Ubiquitinates RPB8 in Response to DNA Damage

Wenwen Wu,[1] Hiroyuki Nishikawa,[1] Ryosuke Hayami,[1] Ko Sato,[1] Akeri Honda,[1] Satoko Aratani,[2] Toshihiro Nakajima,[2] Mamoru Fukuda,[1] and Tomohiko Ohta[1]

[1]Division of Breast and Endocrine Surgery and [2]Department of Genome Science, Institute of Medical Science, St. Marianna University School of Medicine, Kawasaki, Japan

Abstract

The breast and ovarian tumor suppressor BRCA1 catalyzes untraditional polyubiquitin chains that could be a signal for processes other than proteolysis. However, despite intense investigations, the mechanisms regulated by the enzyme activity remain only partially understood. Here, we report that BRCA1-BARD1 mediates polyubiquitination of RPB8, a common subunit of RNA polymerases, in response to DNA damage. A proteomics screen identified RPB8 as a protein modified after epirubicin treatment in BRCA1-dependent manner. RPB8 interacted with BRCA1-BARD1 and was polyubiquitinated by BRCA1-BARD1 *in vivo* and *in vitro*. BRCA1-BARD1 did not destabilize RPB8 *in vivo* but rather caused an increase in the amount of soluble RPB8. Importantly, RPB8 was polyubiquitinated immediately after UV irradiation in a manner sensitive to BRCA1 knockdown by RNA interference. Substitution of five lysine residues of RPB8 with arginine residues abolished its ability to be ubiquitinated while preserving its polymerase activity. HeLa cell lines stably expressing this ubiquitin-resistant form of RPB8 exhibited UV hypersensitivity accompanied by up-regulated caspase activity. Our findings suggest that ubiquitination of a common subunit of RNA polymerases is a mechanism underlying BRCA1-dependent cell survival after DNA damage. [Cancer Res 2007;67(3):951–8]

Introduction

Germ line mutation of the cancer susceptibility gene *BRCA1* causes familial breast and ovarian cancer. BRCA1 acts as a hub protein that coordinates many cellular pathways to prevent tumor progression. Accordingly, down-regulation of this key protein by mechanisms other than *BRCA1* gene mutation causes sporadic breast cancer (1). All cells defective in BRCA1 show genomic instability as evidenced by hypersensitivity to DNA damage, the presence of chromosomal abnormalities, and the loss of heterozygosity at multiple loci. These results are likely to stem from the failure of BRCA1 to function in DNA damage repair, transcriptional regulation, apoptosis induction, intra-S or G_2-M checkpoint function, and regulation of centrosome duplication (2–4).

Involvement of BRCA1 in multiple cellular processes is logical given its enzymatic function as a ubiquitin ligase (E3). In this capacity, it has the potential to interact with numerous protein substrates and subsequently influence the biological response of a cell at many points. BRCA1 contains an NH_2-terminal RING finger domain, a common motif found in ubiquitin ligases. It acquires significant ubiquitin ligase activity when bound to another conformationally similar RING finger protein, BARD1, as a RING heterodimer (5–8). The most common polyubiquitin chain is linked through Lys^{48} of ubiquitin and serves as a signal for rapid degradation of substrates by the proteasome-dependent proteolysis pathway (9). However, BRCA1-BARD1 has the unique capacity to catalyze Lys^6-linked polyubiquitin chains, and the ubiquitination mediated by BRCA1-BARD1 could signal a process other than degradation (10–13). Deleterious missense mutations in the RING finger domain of BRCA1 found in familial breast cancer abolish the E3 ligase activity of BRCA1-BARD1 (6, 7, 14), indicating that the E3 ligase activity is important for role of BRCA1 as a tumor suppressor.

One of the most significant functional features of BRCA1 is that it is a component of the RNA polymerase II holoenzyme (15, 16). BRCA1 specifically interacts with a large fraction of hyperphosphorylated, processive polymerase II (IIO), in preference to the hypophosphorylated polymerase II (IIA) found at promoters (17). It has been proposed that BRCA1 binds polymerase IIO complexes as part of a genome scanning function for DNA damage (18). However, how BRCA1 affects the polymerase II complexes after DNA damage remains partially understood. In this study, we identify RPB8 (also called hRPB17 or *POLR2H*), a common subunit of all three types of RNA polymerases, as a substrate of BRCA1 E3 ligase and show that BRCA1 ubiquitinates RPB8 immediately after DNA damage. HeLa cell lines stably expressing a ubiquitin-resistant form of RPB8 exhibited UV hypersensitivity, a known phenotype of BRCA1 deficiency. These results indicate a significant role of ubiquitin ligase activity of BRCA1 for cell survival after DNA damage and provide a new aspect of a common subunit of RNA polymerases in DNA damage responses.

Materials and Methods

Two-dimensional difference gel electrophoresis. Methods for fluorescence two-dimensional difference gel electrophoresis (DIGE) and mass spectrometric analysis are reported in the Supplementary Data.

Plasmids. cDNA for full-length human RPB8 was amplified by PCR from a MCF10A cell cDNA library using Pfx polymerase (Stratagene, La Jolla, CA). Mammalian expression plasmids for BRCA1, BARD1, ubiquitin, and their mutants were previously described (7, 11). The point mutations to substitute the Lys residue(s) of RPB8 with Arg were produced by site-directed mutagenesis (Stratagene). All plasmids used were verified by DNA sequencing.

Cell cultures and transfections. T47D, HCC1937 breast carcinoma cells, HeLa cervical carcinoma cells, and 293T transformed human kidney cells were cultured in DMEM supplemented with 10% FCS and 1% antibiotic-antimycotic agent (Life Technologies, Inc., Grand Island, NY) in 5% CO_2 at 37°C. MCF10A normal human breast epithelial cells were grown in DMEM/Ham's F12 (1:1) medium supplemented with 2.5% FCS, 100 ng/mL cholera toxin, 20 ng/mL epidermal growth factor, 500 ng/mL hydrocortisone,

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

Requests for reprints: Tomohiko Ohta, Division of Breast and Endocrine Surgery, Department of Surgery, St. Marianna University School of Medicine, Kawasaki 216-8511, Japan. Phone: 81-44-977-8111; Fax: 81-44-976-5964; E-mail: to@marianna-u.ac.jp.

doi:10.1158/0008-5472.CAN-06-3187

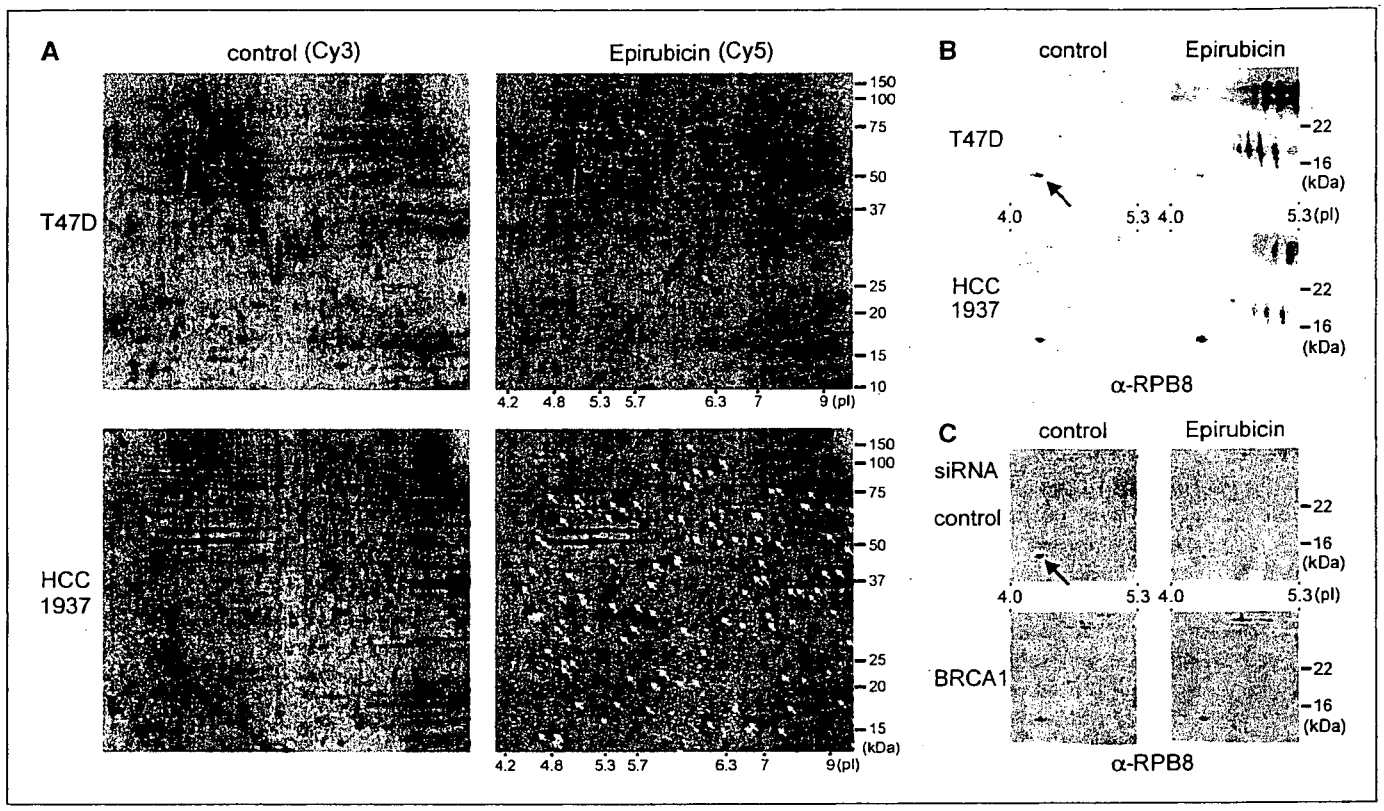

Figure 1. Proteomic screen for proteins affected by epirubicin treatment. *A*, T47D cells (*top*) and HCC1937 cells (*bottom*) were either untreated or treated with 0.2 μg/mL of epirubicin for 3 h and lysed with 7 mol/L urea/2 mol/L thiourea–containing buffer. Protein (50 μg) from untreated and epirubicin-treated cells was labeled with Cy3 (*left*) and Cy5 (*right*), respectively. The differently labeled samples were mixed together, resolved on a two-dimensional gel (pH range 3–10, *left* to *right*), and scanned with a fluorescence image analyzer. *Yellow arrows*, protein spots whose levels were significantly altered after epirubicin treatment. *Red arrows*, proteins that significantly decreased only in T47D cells after epirubicin treatment. The slower-migrating protein was identified as RPB8 and the faster one was myosin light chain. *B*, T47D cells or HCC1937 cells were treated as in *A* and lysed with 7 mol/L urea/2 mol/L thiourea–containing buffer. Lysates (500 μg) were resolved on a two-dimensional gel (pH range 3–10). A part of the gel was subjected to immunoblot with anti-RPB antibody. *Arrow*, RPB8. *C*, T47D cells were transfected either with control siRNA (*top*) or with siRNA for BRCA1 (*bottom*), treated with or without epirubicin, and subjected to anti-RPB8 immunoblotting as in *B*.

10 μg/mL insulin, and 1% antibiotic-antimycotic agent. For epirubicin treatment, cells were incubated in medium containing 0.2 μg/mL epirubicin (Pfizer, New York, NY). To examine the half-life of proteins *in vivo*, cells were incubated with 10 μg/mL cycloheximide (Wako, Osaka, Japan), a protein synthesis inhibitor, for the indicated time periods. 293T cells were transfected using the standard calcium phosphate precipitation method. To generate cell lines that stably expressed either wild-type (WT) or mutant FLAG-RPB8, HeLa cells were transfected using FuGENE6 (Roche, Indianapolis, IN) with pcDNA3 plasmids encoding each protein and selected with G418. For UV irradiation studies, cells were washed with PBS, irradiated with UV light (254 nm; UVP, Inc., Upland, CA) at the indicated doses, and grown in fresh medium for various times.

Antibodies. Mouse monoclonal antibodies to hemagglutinin (HA; Boehringer-Mannheim, Mannheim, Germany), Myc (BabCo, Richmond, CA), FLAG (Sigma, St. Louis, MO), polyubiquitin (Affiniti, Exeter, United Kingdom), conjugated ubiquitin (Affiniti; ref. 10), α- and β-tubulin (Neomarkers, Fremont, CA), and actin (Santa Cruz Biotechnology, Santa Cruz, CA) as well as rabbit polyclonal antibodies to BRCA1 (Santa Cruz Biotechnology), RPB1 (Covance), and cleaved caspase-3 (Cell Signaling Technology, Danvers, MA) were purchased commercially. Anti-FLAG cross-linked agarose beads (Sigma) were used for immunoprecipitation to detect *in vivo* ubiquitinated substrates. Rabbit polyclonal antibodies to BARD1 and RPC155 were generous gifts from Dr. Richard Baer (Columbia University, New York, NY) and Dr. Nouria Hernandez (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY), respectively. Rabbit polyclonal antibody to RPB8 was generated against full-length human glutathione *S*-transferase (GST)-RPB8 and purified by protein G agarose chromatography.

RNA interference. SMART pool BRCA1 small interfering RNA (siRNA) mix and control siRNA mix were purchased from Dharmacon Research, Inc. (Lafayette, CO). RNA duplexes (final concentration 50 nmol/L) were transfected into the cells with Oligofectamine (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Retrovirus expressing short hairpin RNA (shRNA) that targets BRCA1 mRNA sequence 5′-CUAGAAAUCUGUUGCUAUG-3′ was created by cotransfecting 293T cells with pGP vector, pE-ampho vector, and pSINsi-hU6 retroviral vector that has previously been subcloned with the oligonucleotide 5′-GATCCGCTAGAAATCTGTTGCTATGTTCAAGAGACATAGCAACAGATTTCTAGCTTTTTTAT-3′ according to the manufacturer's protocol (TaKaRa, Otsu, Japan). Oligonucleotide 5′-GATCCGTAAGGCTATGAAGAGATACTTCAAGAGAGTATCTCTTCATAGCCTTACTTTTTTAT-3′ was used for the retrovirus expressing control shRNA. For infection, HeLa cells were incubated with virus supernatants and fresh culture medium containing 8 μg/mL Polybrene (Sigma). Cells were analyzed 48 h after transfection or infection.

Immunoprecipitation and immunoblotting. Immunoprecipitation and immunoblotting methods were previously described (11). For the immunoblotting analysis after two-dimensional gel electrophoresis, cells were lysed with 7 mol/L urea/2 mol/L thiourea–containing buffer as described above. Soluble fractions were prepared with 0.5% NP40-based buffer as previously described (11). Denatured whole-cell lysates were prepared by boiling in Laemmli SDS-loading buffer with 0.1 mol/L DTT.

***In vitro* ubiquitin ligation assay.** Full-length His-FLAG-RPB8 was obtained from BL21/DE3 bacteria cells with isopropyl-L-thio-β-D-galactopyranoside induction by two-step purification using nickel agarose beads followed by anti-FLAG cross-linked agarose beads (Supplementary Fig. S3). Complexes of WT or I26A mutant of FLAG-BRCA1$^{1-772}$ with BARD1 were

purified from transfected 293T cells by anti-FLAG affinity chromatography and FLAG peptide elution. Both WT and I26A mutant complexes contained an ~1:1 ratio of BRCA1 and BARD1 proteins (Supplementary Fig. S3). Rabbit E1 (BIOMOL, Plymouth Meeting, PA) and mammalian ubiquitin (Boston Biochem, Cambridge, MA) were purchased commercially. The *in vitro* reaction was done as previously described (11) with a reaction mixture (30 μL) that contained 0.5 μg His-FLAG-RPB8, 40 ng E1, 0.3 μg UbcH5c, and 0.3 μg each of FLAG-BRCA1$^{1-772}$ and BARD1.

Runoff transcription assay. The runoff transcription assay used was described elsewhere (17). Briefly, the runoff template was created by annealing 50 pmol each of a 65-mer oligonucleotide 5′-ATTGGGTAAAGGAGAGTATTTGAGCGGAGGACAGTACTCCGGGTCCCCCCCCCCCCCCCCCCCCC-3′and a complementary 45-mer oligonucleotide 5′-GACCCGGAGTACTGTCCTCCGCTCTTTTACTCTCCTTTACCCAAT-3′ in a 200 μL annealing mixture containing 20 mmol/L Tris (pH 7.4), 1 mmol/L EDTA, and 0.2 mol/L NaCl. Runoff transcription reactions (20 μL) contained 8.25 mmol/L $MgCl_2$, 5 μg of bovine serum albumin, 250 nmol/L nucleotide triphosphates, 5 units of RNase inhibitor, 50 ng of poly(deoxyinosinic-deoxycytidylic acid), 0.05% NP40, 1 pmol of annealed oligonucleotides, and 0.5 μCi of [α-^{32}P]CTP. Equilibrated FLAG-RPB8 immunocomplexes bound to M2 beads (10 μL) were added to the reactions (20 μL) and incubated for 40 min at 30°C and stopped with 50 μL of PK buffer (300 mmol/L sodium acetate, 0.2% SDS, 10 mmol/L EDTA, 100 ng tRNA, and 10 μg proteinase K). Reactions were then incubated at 55°C for 20 min, extracted with phenol/chloroform, and precipitated with ethanol. Single-stranded RNA transcripts were resolved under denaturing conditions on 12% polyacrylamide/urea gels and scanned with the Typhoon 9400 image analyzer (Amersham, Piscataway, NJ).

Results

Identification of RPB8 as a protein modified in BRCA1-positive cells after epirubicin treatment. To search for candidate substrates for the BRCA1-BARD1 E3 ligase in response to DNA damage, we used two-dimensional DIGE technology. Breast cancer–derived, BRCA1–positive T47D cells and BRCA1-defective HCC1937 cells were incubated for 3 h with epirubicin, a topoisomerase II inhibitor that induces DNA double strand breaks. Cells were lysed with 7 mol/L urea/2 mol/L thiourea-containing buffer, and the proteomes were compared with untreated cells using two-dimensional DIGE. Interestingly, whereas the expression levels of only a few proteins were affected by the epirubicin treatment in T47D cells, that of ~100 proteins were altered in HCC1937 cells (Fig. 1*A*). Conversely and even more interesting, two proteins whose expression levels were dramatically reduced in T47D cells were not changed in HCC1937 cells (Fig. 1*A*, *red arrows*). Therefore, we speculated that the reduction could depend on the presence of BRCA1. The protein spots were in-gel digested and subjected to nanoscale capillary liquid chromatography-tandem mass spectrometry (LC/MS/MS) analysis. LC/MS/MS analysis revealed that the samples were RPB8, a common subunit of three types of RNA polymerases, and myosin light chain. RPB8 is a very acidic, small protein with a calculated molecular mass of 17.1 kDa and an isoelectric point of 4.34 (19). One of the most significant functional features of BRCA1 is that it is a component of the RNA polymerase II holoenzyme (15, 16). Therefore, we focused on RPB8 for further analyses.

To confirm our mass spectrometry data, we generated a rabbit polyclonal antibody to GST-RPB8 for immunoblot analysis. Cells were treated as in Fig. 1*A*, and immunoblot analysis of the proteins resolved by two-dimensional gels verified that the protein spot was indeed RPB8. It was again severely reduced by epirubicin treatment only in T47D cells (Fig. 1*B*). The difference in RPB8 expression in response to epirubicin treatment could be due to the different genetic backgrounds of these two cell lines, not just the absence or presence of BRCA1. Therefore, we next compared RPB8 expression between isogenic cells with and without knockdown of BRCA1

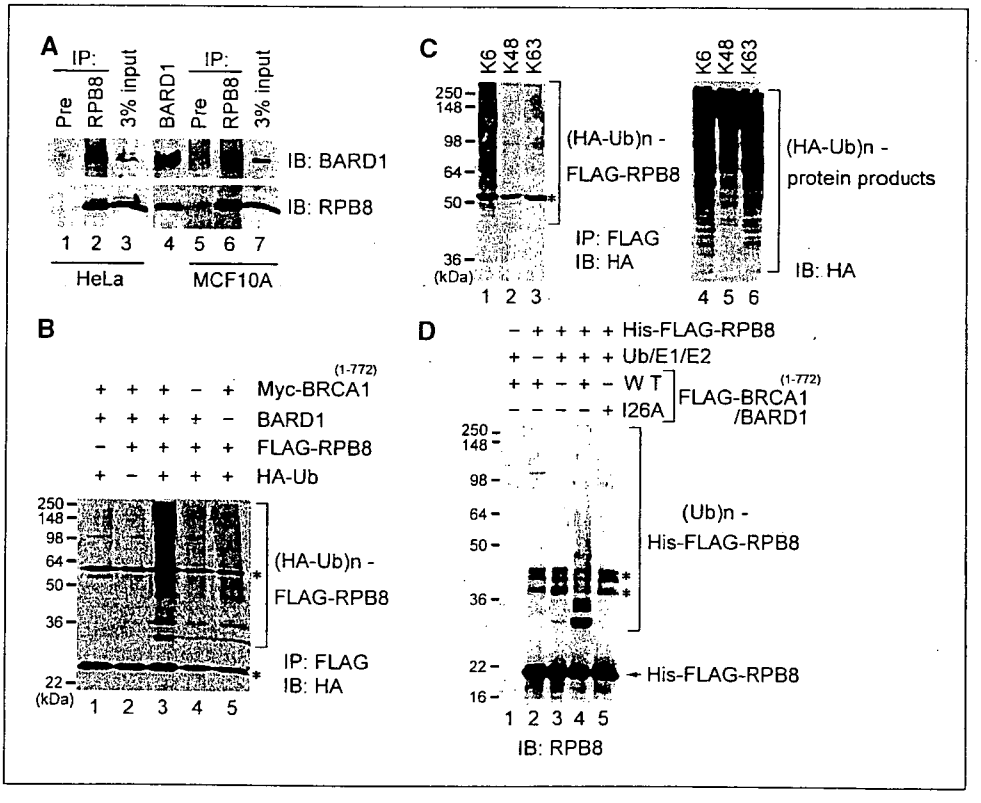

Figure 2. RPB8 and BARD1 interaction, and RPB8 ubiquitination by BRCA1-BARD1. *A*, endogenous RPB8 interacts with BARD1. Lysates prepared from HeLa (*lanes 1–3*) or MCF10A (*lanes 5–7*) cells were immunoprecipitated (*IP*) with anti-RPB8 or preimmune serum (*Pre*) and analyzed by immunoblotting (*IB*) using the indicated antibodies. A portion of the cell lysates corresponding to 3% of the input for immunoprecipitation as well as lysate from 293T cells transfected with BARD1 (*lane 4*) were also loaded. *B*, 293T cells transfected with the indicated plasmids were boiled in 1% SDS lysis buffer, diluted to 0.1% SDS, and immunoprecipitated with anti-FLAG antibody–cross-linked beads. Precipitated FLAG-RPB8 was resolved by 12.5% SDS-PAGE followed by immunoblotting with anti-HA antibody. *, IgG. *C*, polyubiquitination of RPB8 was detected as in *A*, except that HA-ubiquitin (*HA-Ub*) with a single lysine residue was transfected as indicated (*lanes 1–3*). A portion of the cell lysate was subjected to immunoblotting with anti-HA antibody to detect total HA-ubiquitin–conjugated proteins in cells as a control for protein expression (*lanes 4–6*). *, IgG. *D*, bacterially purified His-FLAG-RPB8 was incubated in the presence of ATP with ubiquitin, E1, E2/UbcH5c, and either WT or I26A mutant of FLAG-BRCA1$^{1-772}$/BARD1 complex as indicated and immunoblotted with anti-RPB8 antibody. *, nonspecific products copurified with His-FLAG-RPB8.

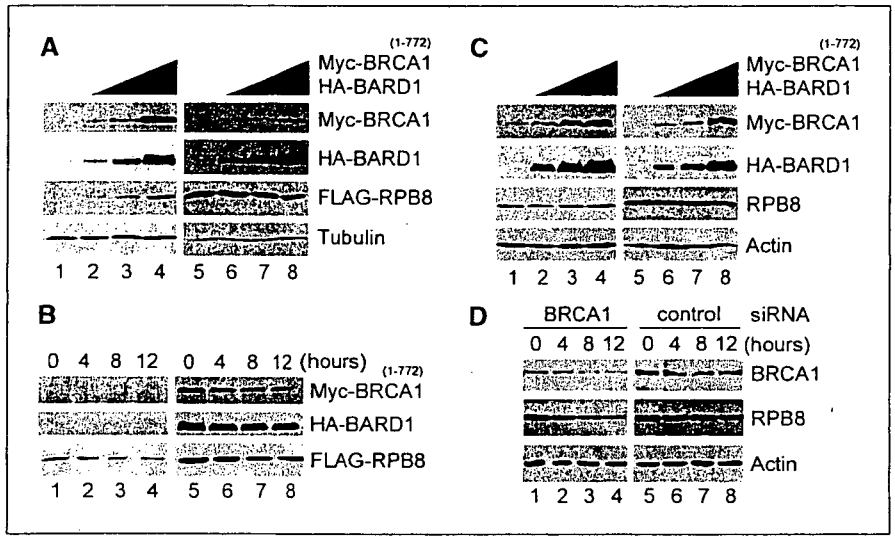

Figure 3. BRCA1-BARD1 did not destabilize RPB8 *in vivo* but rather caused an increase of RPB8 in the soluble fraction. *A*, 293T cells were transfected with plasmids encoding FLAG-RPB8 (*lanes 1–8*, 0.3 μg) and increasing amounts of Myc-BRCA1$^{1-772}$ and HA-BARD1 (*lanes 2* and *6*, 2 μg; *lanes 3* and *7*, 4 μg; *lanes 4* and *8*, 7.35 μg each). Total plasmid DNA was adjusted to 15 μg per plate by adding the parental pcDNA3 vector. The steady-state level of each protein in the soluble fraction (*lanes 1–4*) and whole-cell lysates (*lanes 5–8*) was analyzed by immunoblot using anti-Myc, anti-HA, anti-FLAG, or anti-tubulin antibodies. *B*, 293T cells were transfected with plasmids encoding FLAG-RPB8 (0.2 μg) and either parental pcDNA3 vector (2 μg, *lanes 1–4*) or Myc-BRCA1$^{1-772}$ and HA-BARD1 (1 μg each, *lanes 5–8*). Thirty-six hours after transfection, cells were incubated with cycloheximide (10 μmol/L) and chased for the indicated lengths of time. Soluble fractions of the cell lysates were then immunoblotted with Myc, HA, or FLAG antibody. *C*, steady-state levels of RPB8 were analyzed as in *A*, except that FLAG-RPB8 was not transfected and anti-RPB8 antibody was used to detect endogenous RPB8. *D*, T47D cells were transfected either with siRNA for BRCA1 (*lanes 1–4*) or control siRNA (*lanes 5–8*). Cells were incubated with cycloheximide (10 μmol/L) and chased for the indicated lengths of time. The soluble fraction of the cell lysates was then immunoblotted with the indicated antibodies.

expression. T47D cells were transfected with either control siRNA or BRCA1 siRNA and then treated as in Fig. 1*A*. The siRNA-transfected cells were successfully silenced for BRCA1 expression (Supplementary Fig. S1). Immunoblot analysis of the proteins resolved by two-dimensional gels showed that RPB8 was reduced by epirubicin treatment only in control cells, not in cells with BRCA1 knockdown, supporting the idea that this modification depends on BRCA1 expression (Fig. 1*C*). The reduction of RPB8 at its normal migrating position could be due to protein degradation or to covalent modification.

BRCA1-BARD1 interacts with and ubiquitinates RPB8. The polymerase II holoenzyme interacts with BRCA1 and BARD1 (15, 16). Consistent with the previous reports, a significant amount of endogenous BARD1 coimmunoprecipitated with RPB8 isolated from HeLa cells or MCF10A cells compared with controls (Fig. 2*A*). The same results were observed with MCF7, T47D, and 293T cells (data not shown). Exogenously expressed RPB8 also interacted with BRCA1 and BARD1 (Supplementary Fig. S2). Then, we tested whether RPB8 is ubiquitinated by BRCA1-BARD1 *in vivo*. FLAG-RPB8 was coexpressed in 293T cells with HA-ubiquitin, Myc-BRCA1$^{1-772}$, and BARD1. Cells were collected 36 h after transfection and boiled in 1% SDS–containing buffer, and FLAG-RPB8 was immunoprecipitated. Immunoblotting of the RPB8 precipitates resolved by SDS-PAGE using anti-HA antibody showed a ladder characteristic of polyubiquitinated RPB8 (Fig. 2*B*). Omission of FLAG-RPB8, HA-ubiquitin, Myc-BRCA1$^{1-772}$, or BARD1 all abolished the RPB8 ladders, supporting the idea of BRCA1-BARD1–dependent RPB8 ubiquitination.

BRCA1-BARD1 is the only known E3 ligase to catalyze Lys^6-linked polyubiquitin chains (10, 11, 13). To show that the *in vivo* RPB8 ubiquitin ladders were directly due to BRCA1-BARD1 ligase activity, we verified that RPB8 was modified by ubiquitin through Lys^6 linkages. HA-tagged ubiquitins that have a single lysine residue available for conjugation were used for *in vivo* ubiquitination assays. As expected, BRCA1-BARD1–dependent RPB8 polyubiquitination was predominantly detected when HA-ubiquitin with only Lys^6 available, but not Lys^{48} or Lys^{63}, was coexpressed (Fig. 2*C*). However, it has been suggested that ubiquitin mutants could fold incorrectly and may cause artifacts (20). Recent quantitative analysis of *in vitro* ubiquitination revealed that even for cyclin B1 ubiquitination catalyzed by the anaphase-promoting complex, heterogeneous ubiquitin chains, including Lys^{63}, Lys^{11}, and Lys^{48}, or monoubiquitin attached to multiple lysine residues on the substrate. Further, some types of linkages are dependent on the combination of E2 and E3 enzymes (21). Thus, it is possible that ubiquitination mediated by BRCA1-BARD1 also resulted in multiple polyubiquitin chains, including Lys^6. The preference for Lys^6 ubiquitination observed in the *in vivo* experiment was not enough evidence to support the direct role of BRCA1-BARD1 for RPB8 ubiquitination. Therefore, we further tested whether BRCA1-BARD1 directly catalyzes RPB8 polyubiquitination by *in vitro* ubiquitination using recombinant RPB8 protein (Supplementary Fig. S3). His-FLAG-RPB8 incubated with ubiquitin, E1, E2/His-UbcH5c, and FLAG-BRCA1$^{1-772}$/BARD1 complex (Supplementary Fig. S3) resulted in a ladder and smear detected by anti-RPB8 immunoblot (Fig. 2*D*). Omission of substrate RPB8, ubiquitin/E1/E2, or FLAG-BRCA1$^{1-772}$/BARD1 complex, as well as substitution of BRCA1$^{1-772}$ with the E2-nonbinding mutant I26A, all abolished RPB8 ubiquitination. Hence, the results suggest that the RPB8 polyubiquitination is directly catalyzed by BRCA1-BARD1.

BRCA1-BARD1 does not destabilize RPB8 *in vivo*. Our previous results suggested that BRCA1-BARD1 catalyzed untraditional polyubiquitin chains that served as a signal for a process other than degradation (7, 11, 12). However, the reduced expression of RPB8 after epirubicin treatment detected by two-dimensional DIGE or two-dimensional immunoblot (Fig. 1) suggested the