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# News & Views

# Is SS-A/Ro52 a Hydrogen Peroxide-Sensitive Signaling Molecule?

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## ABSTRACT

**SS-A/Ro52 (Ro52) protein is one of the targets of autoantibodies in Sjogren's syndrome and systemic lupus erythematosus. Ro52 structurally belongs to the RING-B-box/coiled-coil family, which appears to carry out diverse functions, but the physiological function of Ro52 remains largely unknown. Here, the authors demonstrate that hydrogen peroxide but not other oxidative stressors induced translocation of Ro52 protein from the cytoplasm to the nucleus and this phenomenon was attenuated by inhibition of MAP kinases, ERK in particular. These findings raise the possibility that SS-A/Ro52 may function as a hydrogen peroxide-selective, oxidative stress-sensitive signaling molecule that is activated via the MAP kinase pathway.** *Antioxid. Redox Signal.* 9, 385–391.

## OXIDATIVE STRESS AND RO52

AUTOIMMUNE DISEASES develop within a context that includes both genetic and environmental factors. Infections, ultraviolet (UV) irradiation, coldness, and emotional stress are all well-known triggers and/or exacerbating factors in autoimmune disease. Of those, the environmental factors have the potential to induce some degree of oxidative stress, while infection and inflammation activate monocytes and neutrophils, which generate large amounts of reactive oxygen species (ROS) such as hydrogen peroxide ($H_2O_2$). All have the capacity to disrupt immune system homeostasis.

SS-A/Ro52, which is comprised of at least two types of ribonucleoproteins, the 52 kDa protein Ro52 and the 60 kDa protein Ro60, is one of the targets of autoantibodies in Sjogren's syndrome (SS) and systemic lupus erythematosus and is also closely associated with subacute cutaneous lupus erythematosus and neonatal lupus erythematosus. Although the mechanism by which anti-Ro52 autoantibodies are generated is not fully understood, some evidence suggests that Ro52 is expressed within apoptotic blebs during apoptosis evoked by various stimuli, including UV-B, viral infection, and TNF-α, among others (2). The Ro52 antigens within apoptotic blebs would be effectively phagocytosed by dendritic cells, resulting in the activation of Ro52-specific antibody-producing B cells. On the other hand, we recently reported an alternative mechanism by which oxidative stress caused by UV-B and diamide induces the appearance of Ro52 on the surface of keratinocytes without inducing apoptosis (23). In the same study, we also found that, in contrast to UV-B and diamide, $H_2O_2$ did not induce cell surface expression of Ro52, which suggests that the effects of $H_2O_2$ on Ro52 protein differ from those of UV-B and diamide.

## STRUCTURE OF RO52 AND ITS POSTULATED ROLES

Ro52 belongs to the rapidly growing RING-B-box/coiled-coil (RBCC) family, also denoted TRIM (tripartite motif), which appears to carry out a variety of highly diverse functions within cells (17, 19, 29). The Ro52 RBCC region consists of an N-terminal $Zn^{2+}$ binding region containing a RING

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finger and a B-box, followed by a coiled-coil region and a putative leucine zipper domain. RING-B-box structure is supposed to be involved in protein–protein interaction, and coiled-coil motif to promote oligomerization by forming supercoiled dimers, trimers, or tetramers, depending on sequence constraints and biological context. In addition, at the C-terminus of the Ro52 there is a well-conserved B30.2 domain, the function of which is not yet known (6). These functional regions of Ro52 also can serve as epitopes for autoantigens in autoimmune diseases. For instance, disease-related antibodies bind Ro52 in a conformation-dependent manner at both the coiled-coil region and the $Zn^{2+}$-binding RING-B-box region.

## $H_2O_2$ AS A SECOND MESSENGER

The cytotoxicity of $H_2O_2$ is well established; their generation leads to cellular stress responses, apoptosis, aging, and death. In recent years, however, the "bad reputation" of $H_2O_2$ has been changing, as these molecules are now being recognized as inevitable and essential for appropriate cell growth and development. It has been shown, for example, that at low concentrations $H_2O_2$ exerts mitogenic effects and can imitate the actions of growth factors (22, 24). Furthermore, these effects are not simply reactions to an artificial exposure to exogenous $H_2O_2$. Upon stimulation of specific cell surface receptors by their native ligands, cells themselves produce $H_2O_2$ and use it as a second messenger for signal transduction and amplification (1, 15). Initiation of such receptor signaling not only requires the activation of kinases but, more importantly, the inhibition of phosphatases (8), and $H_2O_2$ is a critical element within such regulatory circuits.

A variety of proteins are known to be oxidized and thus modified by $H_2O_2$. Among these are redox-regulated mediators and transcriptional factors (*e.g.*, p53, Jun, Fos, and NF-κB), some of which are reportedly translocated from the cytoplasm to the nucleus in response to oxidative stress. For example, when stimulated with $H_2O_2$, NF-κB moves from the cytoplasm to the nucleus following tyrosine phosphorylation of its p65 subunit and IκBα (28). Likewise, thioredoxin (Trx), a ubiquitous antioxidant protein and regulator of NF-κB activity, also moves from cytoplasm to the nucleus in response to various oxidative stresses (27).

## UV-B BUT NOT $H_2O_2$ INDUCES CELL SURFACE EXPRESSION OF RO52

We first investigated the behavior of Ro52 after $H_2O_2$ treatment, since we speculated that $H_2O_2$ may be the key to unveil the largely unknown roles of Ro52 protein. We previously showed that translocation of Ro52 antigen to the cell surface occurs upon UV-B irradiation but not $H_2O_2$ treatment in both primary human keratinocytes and HaCaT cells (23). We therefore primarily used the latter in the following experiments. Initially, we confirmed that purified IgGs from monospecific sera from SS patients specifically reacted to Ro52 (Fig. 1A) and then determined whether Ro52 antigen was expressed on the surface of HaCaT cells after UV-B irradiation or exposure to $H_2O_2$. In addition, flow cytometric analysis showed that following UV-B irradiation (200 mJ/cm$^2$), the surfaces of HaCaT cells stained with IgG specific for Ro52 antigen (anti-Ro52 Ab) (Fig. 1B). By contrast, Ro52 antigen was not expressed on the cell surface after exposure to $H_2O_2$ (200 μ*M*), though 10% of the cells were apoptotic (data not shown).

## $H_2O_2$ BUT NOT UV-B OR DIAMIDE INDUCES TRANSLOCATION OF RO52 FROM THE CYTOPLASM TO THE NUCLEUS

We next analyzed the subcellular distribution of Ro52 after exposure to various oxidative stresses using HaCaT cells transiently transfected with pEGFP-N1 or pRo52–EGFP, a plasmid vector that Ro52 gene was cloned N-terminally into pEGFP-N1. We found that before exposing cells to $H_2O_2$, the Ro52–EGFP fusion protein was mainly localized in cytoplasm, although there was a weak nuclear signal. Control EGFP was ubiquitously and homogenously expressed in the cytoplasm and nucleus. After $H_2O_2$ treatment, there was

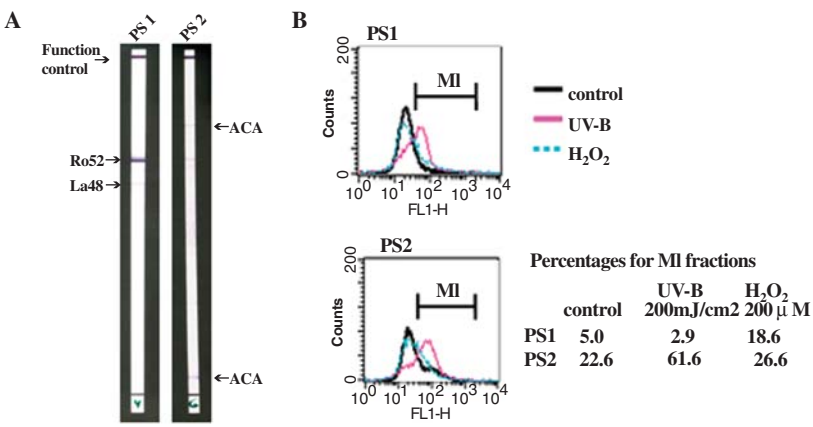


**FIG. 1. UV-B induces expression of Ro52 on the surface of keratinocytes.** (**A**) Western analysis of IgG fractions purified from patients' sera. Patient serum sample 1 (PS1) contained anti-Ro52 and anti-La48 Abs; Patient serum sample 2 (PS2) contained anti-Ro52 and anti-centromere Abs. ACA, anti-centromere antibody. (**B**) Flow cytometric analysis of the cell-surface expression of Ro52 (*left panel*). HaCaT cells were incubated with $H_2O_2$ (200 μ*M*) for 24 h or irradiated with UV-B (200 mJ/cm$^2$), then incubated for an additional 24 h. The cells were then labeled with IgG fractions purified from PS1 or PS2. The percentages of cells in the M1 fraction after UV-B or $H_2O_2$ exposure are shown in the *right panel*.

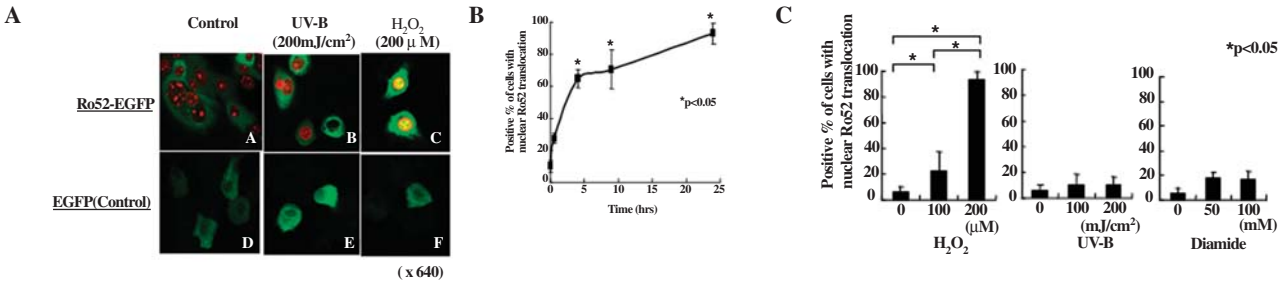


**FIG. 2. Confocal microscopic analysis showing that $H_2O_2$ but not UV-B irradiation induces nuclear translocation of Ro52.** (**A**) HaCaT cells transiently transfected with pEGFP-Ro52 or pEGFP-N1 were incubated with $H_2O_2$ (200 μ*M*) for 24 h or irradiated with UV-B (200 mJ/cm$^2$) and then incubated for an additional 24 h. EGFP-tagged Ro52 protein emits *green fluorescence*, while propidium iodide stains the nuclei *red*. In the merged images overlapping pixels appear as *yellow.* (**B**) Time-dependence of the nuclear translocation of Ro52 in HaCaT cells. Cells stably transfected with EGFP-Ro52 were incubated with $H_2O_2$ (50 μ*M*) for up to 24 h. Percentages of nuclear Ro52-positive cells were calculated. (**C**) Dose-dependence of nuclear translocation of Ro52 in HaCaT cells. Cells stably transfected with EGFP-Ro52 were incubated with either $H_2O_2$ or diamide for 24 h, or were irradiated with UV-B and cultured for 24 h thereafter. Percentages of nuclear Ro52-positive cells were then calculated. Results represent means ± SD from three independent experiments; *$p < 0.05$.

marked accumulation of Ro52–EGFP but not EGFP within the nucleus (Fig. 2A). This apparent $H_2O_2$-evoked translocation of Ro52 was both time- and dose-dependent, occurring within as little as 4 h, with about 90% of cells responding to 200 μ*M* $H_2O_2$ within 24 h (Fig. 2B) in HaCaT cell line stably transfected with pRo52EGFP (HaCaT–Ro52EGFP). On the other hand, neither UV-B irradiation nor diamide treatment elicited translocation of Ro52–EGFP to the nucleus. These findings were confirmed by Western analysis, which showed that in wild-type HaCaT cells, nuclear levels of Ro52 protein increased after $H_2O_2$ treatment but not after UV-B irradiation, and that nuclear levels of Ro52 were similarly increased in normal keratinocytes from neonatal foreskin after $H_2O_2$ treatment (Fig. 3). In both UV-B and $H_2O_2$ treatment, Ro52 expression in the cytoplasm of HaCaT cells increased, suggesting that these two sources of oxidative stress commonly increased the protein synthesis of Ro52 while the intracellular trafficking of Ro52 protein by these two stimuli were different. Notably, this accumulation of nuclear Ro52 elicited by $H_2O_2$ was attenuated by the antioxidant NAC in a concentration-dependent manner (Fig. 4).

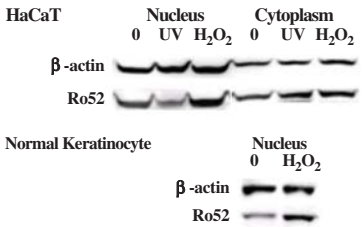


**FIG. 3. Detection of nuclear Ro52 after $H_2O_2$ treatment.** HaCaT cells and normal keratinocytes were incubated with $H_2O_2$ (200 μ*M*) for 24 h or irradiated with UV-B (200 mJ/cm$^2$) and then incubated for an additional 24 h. The cells were then lysed, separated into cytoplasmic and nuclear extracts, and subjected to SDS-PAGE and Western blot analysis. To detect Ro52 protein, the membranes were probed with IgG purified from sera positive for anti-Ro52 Ab (SP1 in Fig. 1). The membranes were then stripped and reprobed with mouse anti-human mAb against β-actin.

## MAP KINASE INHIBITORS ATTENUATE $H_2O_2$-INDUCED NUCLEAR ACCUMULATION OF RO52

Because the MAP kinase cascade is reportedly activated by $H_2O_2$ (25), we also evaluated the extent to which MAP kinase activity contributes to $H_2O_2$ -induced nuclear translocation of Ro52. HaCaT–Ro52EGFP cells were pretreated with PD98059, SB203580, or SP600125, specific inhibitors of ERK, p38, and JNK, respectively, after which $H_2O_2$-induced translocation of Ro52 was examined. We found that all three inhibitors dose-dependently reduced the percentage of cells showing $H_2O_2$-induced nuclear Ro52 translocation, but the reduction caused by ERK inhibition was particularly noteworthy. This suggests that the translocation of Ro52 is mediated via a MAP kinase pathway, one most likely regulated by ERK (Fig 5A).

Finally, to test whether protein synthesis is required for the nuclear translocation of Ro52, we pretreated HaCaT–Ro52EGFP cells with cycloheximide (CHX) before exposing them to $H_2O_2$ (Fig. 5B). We found that CHX partially inhibited Ro52 accumulation within the nucleus, suggesting that new synthesis of Ro52 and/or Ro52-related proteins facilitates translocation of Ro52 into the nucleus.

## CONCLUSIONS AND OPEN QUESTIONS

### *Is Ro52 a stress-responding molecule situated downstream of MAP kinase pathway?*

$H_2O_2$ reportedly stimulates the activity of ERK1/2 more than JNK or p38 (5, 14, 20), and this $H_2O_2$–ERK1/2 pathway has been implicated in the activation of the redox-responsive transcription factors NF-κB (12) and AP1 (13). We observed

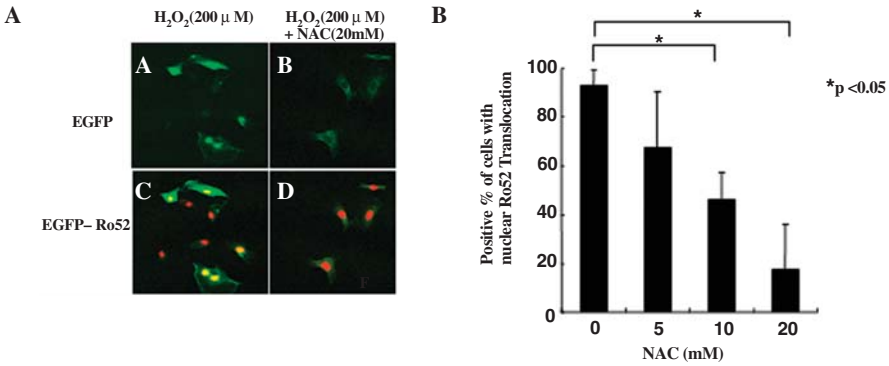


**FIG. 4. NAC inhibits $H_2O_2$-induced nuclear translocation of Ro52.** (**A**) HaCaT cells stably transfected with pEGFP-Ro52 were preincubated with NAC (20 m*M*) for 2 h and then incubated with $H_2O_2$ (200 μ*M*) for 24 h, after which confocal microscopic analysis was carried out. Each pair of panels (**A** and **C**, **B** and **D**) represents the same visual field. EGFP-tagged Ro52 emits *green fluorescence*, while propidium iodide stains the nuclei *red*. In the merged images overlapping pixels appear as *yellow* (*c, d*). (**B**) Dose-dependent inhibition of $H_2O_2$-induced Ro52 translocation by NAC. Results represent mean percentages ± SD of cells in which Ro52 localized in the nuclei in three independent experiments.

that nuclear translocation of Ro52 was strongly inhibited in cells treated with MAP kinase inhibitors, especially an ERK inhibitor, which suggests that the translocation of Ro52 is situated downstream of $H_2O_2$-evoked ERK1/2 activation. We hypothesize that Ro52 may convey an oxidative stress signal to the nucleus and/or that it functions as a transcription factor itself, although we have only limited information about the physiological function of Ro52.

It is interesting to compare $H_2O_2$ with UV-B in their selectivity to MAP kinase pathways. UV radiation is an important initiating factor underlying the generation of ROS, including $H_2O_2$, ·OH, ·O2⁻, and singlet oxygens ($^1O_2$), but the cytotoxicity of UV involves both oxygen-dependent and -independent damage to biomolecules. Iordanov *et al*. (7) reported that JNKs are important transcriptional mediators of stress responses to both UV and oxidative stressors in mammalian cells, and proposed that UV irradiation generates at least two autonomous signaling cascades: an oxygen-independent, ribosome-dependent cascade that leads to activation of JNKs, and an oxygen-dependent, ribosome-independent cascade that appears to involve UV-induced oxidative damage and leads to the activation of ERKs (7). Notably, among all of the MAP kinase superfamily members, ERKs show the weakest responses to UV irradiation. These data implicate that the difference in the effects of $H_2O_2$ and UV-B on the cellular trafficking of Ro52 might reflect their differences in the selectivity of MAP kinase pathways.

## *Ro52 needs help from unknown protein to enter into the nucleus*

It has been suggested that Ro52 is involved in transcriptional regulation of certain genes (4, 30). Ro52 may require an adaptor protein to enter the nucleus, since it does not contain nuclear localizing signal residues. For instance, Grp78/BiP, a stress-responsive protein located at the endoplasmic reticulum, may serve as a molecular chaperone for Ro52 (9). However, we did not find that Grp78 colocalizes with Ro52 before or after $H_2O_2$ treatment (data not shown). As CHX treatment dose-dependently inhibited the nuclear translocation of Ro52, an adaptor protein that is newly synthesized under the control of $H_2O_2$-mediated pathway is likely to exist but remains to be identified. On the other hand, Pourmand *et al*. reported that the hydrophilic domain of Ro52, including the leucine zipper motif, is crucial for retaining Ro52 within the cytoplasm, and that its retention there may result from its dimerization or from binding to other cytoplasmic molecules (18). It is also plausible that $H_2O_2$-medi-

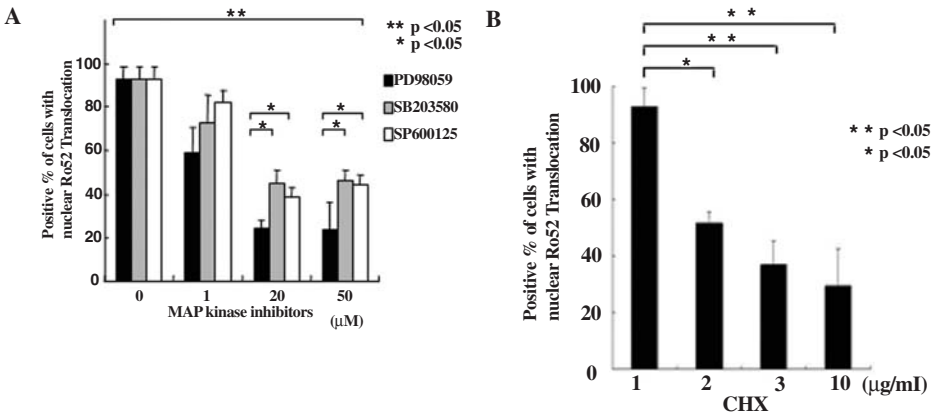


**FIG. 5. MAP kinases and CHX inhibited $H_2O_2$-induced nuclear translocation of Ro52.** (**A**) HaCaT cells stably transfected with pEGFP-Ro52 were pretreated for 1 h with PD98059, SB203580, or SP600125 before incubation with $H_2O_2$ (200 μ*M*) for 24 h, after which Ro52 was localized using confocal microscopy. (**B**) CHX treatment partially inhibited $H_2O_2$-induced nuclear translocation of Ro52. HaCaT cells were treated with $H_2O_2$ in the presence of CHX (1–10 μg/ml), after which Ro52 was localized using confocal microscopy. Results represent mean percentages ± SD of cells in which Ro52 accumulated in the nuclei. Data were obtained from three independent experiments.

ated signaling directly breaks the protein-protein interaction retaining Ro52 within the cytoplasm.

### *Are cysteine residues important in Ro52 function?*

$H_2O_2$ primarily targets cysteine residues in some proteins in contrast to more aggressive ROS molecules, which react with virtually all of the molecules they encounter. $H_2O_2$ appears to play a role in various intracellular signaling pathways and to fulfill the prerequisites for a second messenger, that is, it is a diffusible and ubiquitous, and it is both synthesized and destroyed in response to external stimuli (20, 26). Only certain intracellular proteins contain an oxidizable cysteine at a critical position where $H_2O_2$ can act on it. Interestingly, we noted that the N-terminal region of Ro52 contains four Cys–X–X–Cys motifs, which are all located at RING-B-box region (residues 1–128); Cys–Pro–Ile–Cys (residues16–19), Cys–Gln–Glu–Cys (residues 36–39), Cys–Pro–Val–Cys (residues 51–54), and Cys–Trp–Val–Cys (residues 111–114). Cys–X–X–Cys motif is famous for its susceptibility to oxidants, suggesting $H_2O_2$ can act on these Cys–X–X–Cys motifs to modulate the redox status of Ro52 protein. In other words, $H_2O_2$ may serve as a second messenger directly modulating the largely unknown function of Ro52. Further investigation for the roles of these Cys–X–X–Cys motifs may provide us a clue to address the questions: What is the physiological function of Ro52? Is Ro52 a stress-responding molecule? Furthermore, does $H_2O_2$ -induced nuclear accumulation of Ro52 exacerbate or control the development of SS?

Diamide is a thiol-specific compound that has been shown to induce expression of a larger number of proteins, including heat shock proteins, than are induced by $H_2O_2$, which suggests that oxidation of intracellular thiols may trigger induction of certain stress-related proteins (21). Recent studies of Yap1p, a positive regulator of gene expression in *Saccharomyces cerevisiae*, may provide some insight into the differences in the oxidant-specific responses of Ro52 to $H_2O_2$ and diamide. In the absence of an oxidative challenge, Yap1p resides in the cytoplasm. Upon induction of stress by addition of oxidants like diamide or $H_2O_2$, nuclear export of Yap1p is inhibited, so that it rapidly accumulates within the nucleus (3, 11). Yap1p contains two clusters of cysteine residues, termed cysteine rich domains (CRDs), located at its N-and C-termini (n-CRD and c-CRD, respectively). Whereas diamide challenge produces disulfide bonds that form within either the n- or c-CRD, $H_2O_2$ induces formation of a disulfide bond between the n- and c-CRD (10). As mentioned above, Ro52 has a cysteine rich domain at its N-terminus. It is interesting to analyze if there is any difference in the disulfide bonds formation by $H_2O_2$ or diamide, which may result in modulating the intracellular localization of Ro52 in response to each oxidative stressor.

Taken together, our results suggest that $H_2O_2$ may be a useful tool with which to investigate the physiological function of Ro52, perhaps as a stress-responsive protein and nuclear signaling molecule. Thus, a better understanding of the structural characteristics that enable nuclear translocation of Ro52 protein is of particular interest.

## ACKNOWLEDGMENTS

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## ABBREVIATIONS

c-CRD, cysteine rich domains located at its C-terminus; ERK kinase, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; MAP kinase, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; NAC, *N*-acetyl-L-cysteine; n-CRD, cysteine rich domains located at its N terminus; RBCC, RING/B-box/coiled-coil; ROS, reactive oxygen species; SS, Sjogren's syndrome; TRIM, tripartite motif; Trx, thioredoxin.

## APPENDIX

## Notes

### 1. Reagents and antibodies

FITC-conjugated goat anti-human IgG, *N*-acetyl-l-cysteine (NAC), diamide, and cycloheximide (CHX) were purchased from Sigma (St. Louis, MO). PD98059, SP600125, and SB203580, inhibitors of mitogen-activated protein (MAP) kinase/ERK kinase (MEK), c-Jun N-terminal kinase (JNK), and stress-activated protein kinase-2/p38 alpha and beta, respectively, were purchased from Calbiochem-Novabiochem Corp (San Diego, CA). Monospecific sera positive for anti-Ro52 antibody (Ab) were collected from patients with SS. Control serum was obtained from a healthy donor. An ImmunoPure IgG Purification Kit (Pierce Chemical, Rockford, IL) was used to purify IgG from the serum samples. Mouse anti-human β-actin mAb was from Santa Cruz Biotechnology (Santa Cruz, CA).

### 2. UV-B irradiation

A bank of five fluorescent sunlamps (Toshiba FL20SE, Tokyo, Japan) emitting 280–370 nm light (mainly UV-B energy with a peak at 305 nm) was used for UV-B irradiation. Flux intensity was measured with a UVR-305/365D digital radiometer (Tokyo Optical, Tokyo, Japan).

### 3. Cell culture

Normal human epidermal keratinocytes derived from neonatal foreskin were obtained from Toyobo (Tokyo, Japan) and were maintained in Keratinocyte Growth Medium (Toyobo) supplemented with 10% heat-inactivated fetal calf serum (FCS). The HaCaT human keratinocyte cell line was kindly provided by Dr. N. E. Fusenig (German Cancer Research Center, Heidelberg, Germany) and maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL, Grand Island, NY) supplemented with 10% FCS. All cells were cultured at 37°C under a humidified atmosphere containing 5% $CO_2$. For all experiments, cells were plated in the wells of Lab-Tek chamber slides (Nalge Nunc, Taastrupgaardsvej, Denmark) and cultured in media devoid of 2-

mercaptoethanol. For experiments with $H_2O_2$ or diamide, cells were incubated for up to 24 h in culture media containing various concentrations (0–200 μ*M*) of the indicated compound. Before UV-B irradiation, cells were washed and the medium was replaced with phosphate-buffered saline (PBS) free of any photoactive compounds. After irradiation, the cells were immediately reconstituted with fresh medium and incubated for 24 h. For experiments with the antioxidant NAC, cells were preincubated for 2 h with various concentrations of NAC, after which $H_2O_2$ was added to a concentration of 200 μ*M*, and the cells were incubated for an additional 24 h. To examine the effects of MAP kinases (ERK, p38, and JNK) on $H_2O_2$-induced phenomena, cells were pretreated for 1 h with the MAP kinase inhibitor PD98059 (20 μ*M*) for ERK, SB203580 (1 μ*M*) for p38 or SP600125 (20 μ*M*) for JNK before treatment with $H_2O_2$. Thereafter the cells were subjected to flow cytometric, Western or confocal microscopic analysis, as described below.

#### 4. Flow cytometric analysis

One million cells were incubated first with PBS containing human AB serum (20%) for 5 min and then with anti-Ro52 Ab or normal human IgG (control) for 30 min on ice. After washing with PBS containing 2% FCS, the cells were incubated with FITC-conjugated goat anti-human IgG for 30 min on ice. The labeled cell fraction was then determined using a FACS Calibur equipped with CellQuest analysis software (Becton Dickinson, San Jose, CA).

#### 5. Western blot analysis

The monospecificity of IgG purified from monospecific sera positive for anti-Ro52 Ab was determined by Western analysis using nuclear and mitochondrial antigens from HEp-2 cells as antigen sources (ANA/AMA Immunoblot Kit, DPC Biermann, Nauheim, Germany). The cells were lysed and separated into cytoplasmic and nuclear extracts using NE-PER®Nuclear and Cytoplasmic Extraction reagents (Pierce Chemical), after which the extracts and controls were subjected to SDS-PAGE using 5–20% Tris-glycine gels (PAGEL NPG-520L, ATTO, Tokyo, Japan). The separated proteins were transferred onto nitrocellulose membranes, which were then blocked with 20 mmol/L tris, 140 mmol/L NaCl, 0.5% Tween 20, and 5% milk (TBS-T/milk). The probes used were IgG purified from the monospecific sera positive for anti-Ro52 Ab and mouse anti-human β-actin mAb, which were incubated with the target for 1 h at room temperature. The blots were then washed three times with TBS-T and incubated with horseradish peroxidase-conjugated anti-protein G (PROzyme, San Leandro, CA) or FITC-conjugated anti-mouse IgG (Southern Biotechnology Associates, Inc., Birmingham, AL) for 1 h at room temperature. Finally, the blots were washed three times with TBS-T and developed with West Dura (Pierce Chemical).

#### 6. Plasmids and establishment of stable HaCaT-Ro52 transfectants

Ro52 gene cloned N-terminally into pEGFP-N1 vector (pRo52-EGFP) (Clontech, Palo Alto, CA) was kindly provided by Dr. Karl Albert Brokstad (Broegelmann Research Laboratory, University of Bergen) (16). pRo52-EGFP or empty pEGFP-N1 vector was then transfected into HaCaT cells using GenePORTER 2 Transfection Reagent (Gene Therapy Systems, San Diego, CA) according to the manufacturer's instructions. Two days after transfection, geneticin (1 mg/ml) was added to the culture medium, and single clones expressing EGFP or Ro52-EGFP were selected using the standard limiting dilution method to establish stable transfectant cultures.

#### 7. Microscopic analysis

Microscopy was carried out using a Carl Zeiss Pascal confocal laser scanning microscope (LSM5 Pascal Ver.3.0, Carl Zeiss, Göttingen, Germany) equipped with lasers emitting at 496 and 568 nm. At least 500 cells were examined; those with green fluorescence were identified as EGFP transfectants. Propidium iodide was used to visualize nuclei with red fluorescence. Each sample was run in duplicate, and experiments were repeated at least three times.

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