

UVA irradiation induces Nrf2 activation in dermal fibroblasts: Protective role in UVA-induced apoptosis

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Abbreviations: UV, ultraviolet; Nrf2, NF-E2-related Factor 2; Keap1, Kelch-like-ECH-associated protein 1; ROS, reactive oxygen species; GSH, glutathione; γ -GCS, γ -glutamylcysteine synthetase; GST, glutathione S-transferase; NQO1, NADPH quinone oxidoreductase-1; HO-1, heme oxygenase-1; TRX, thioredoxin; ICAM-1, intercellular adhesion molecule-1; MMP, matrix metalloproteinase; GPX, glutathione peroxidase

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Abstract

Ultraviolet (UV) radiation is one of the most important environmental factors involved in the pathogenesis of skin aging and cancer. Many harmful effects of UV radiation are associated with the generation of reactive oxygen species, and cellular antioxidants act to prevent the occurrence and reduce the severity of UV-induced photoaging and diseases of the skin. Transcription factor Nrf2 and its cytoplasmic anchor protein Keap1 are central regulators of the cellular antioxidant response. We investigated in this study effects of UV irradiation on the activation of Nrf2 in dermal fibroblasts. We found that UVA irradiation, but not UVB, causes nuclear translocation and accumulation of Nrf2. The nuclear accumulation of Nrf2 induced by UVA was enhanced by the photosensitizer hematoporphyrin, suggesting that Nrf2 activation is mediated by singlet oxygen. To evaluate the protective role of Nrf2 against UVA radiation, we examined UVA-induced apoptosis using dermal fibroblasts derived from *nrf2* and/or *keap1* gene knockout mice. While disruption of *nrf2* increased the number of apoptotic cells following UVA irradiation, disruption of *keap1* decreased the apoptotic cell number compared with wild-type controls. These findings thus demonstrate that the Nrf2-Keap1 pathway plays an important role in the protection of the skin against UVA irradiation.

(198 words)

Introduction

The skin acts as a physiological barrier protecting the organism against pathogens and chemical or physical damage. The major environmental cause of skin damage is ultraviolet (UV) radiation. UV irradiation of the skin leads to acute reactions, such as erythema, sunburn (Ley, 1985), and chronic reactions that include premature skin aging (Fisher *et al*, 1996) and skin tumors (Ananthaswamy and Pierceall, 1990). UV affects the skin in different ways depending on its wavelength; while UVB (290-320 nm) radiation may only reach the epidermis and the upper dermis, UVA (320-400 nm) penetrates deeper into the skin and elicits its effects in the deep dermis. UVB has the strongest cytotoxic and mutagenic effects (Ichihashi *et al*, 2003) as the bases in DNA directly absorb incident photons within this narrow wavelength range. This can result in DNA damage, particularly the formation of cyclobutane pyrimidine dimers (CPDs) and photoproducts that induce DNA mutation in skin cells, leading to the development of skin tumors. In contrast, the effects of UVA are primarily oxidative in nature. DNA is not a chromophore for UVA radiation (Rosenstein and Mitchell, 1987), but may be damaged indirectly by reactive oxygen species (ROS) generated by endogenous photosensitizers (Cadet *et al*, 1997). Exposure of mammalian skin to UVA increases the cellular levels of ROS, which damage lipids, proteins, and nucleic acids in both epidermal and dermal cells and probably contribute to both photocarcinogenesis and photoaging (Scharffetter-Kochanek *et al*, 1997). Photoexcited sensitizers are likely to generate ROS, including superoxide and singlet oxygen. In addition, photooxidation reactions, including the formation of 8-oxo-7,8-dihydroguanine, occur on UVA irradiation of cellular DNA (Wamer and Wei, 1997).

Cells can be protected against the adverse effects of UV irradiation by a number of both enzymatic and non-enzymatic antioxidants. Antioxidants, such as polyphenols (Vayalil *et al*,

2003) and vitamin E (Packer and Valacchi, 2002), applied topically or in the diet have been shown to protect against photooxidative damage of the skin. The endogenous antioxidant capacity of the skin is a major determinant in its response to oxidative stress-mediated damage. Low intracellular levels of glutathione (GSH) have been shown to result in elevated sensitivity to UV irradiation (Tyrrell and Pidoux, 1988). Thus, antioxidants constitute an important group of pharmacological agents capable of preventing the occurrence and reducing the severity of UV irradiation-induced skin diseases and skin aging. Furthermore, as the skin is the outermost organ exposed directly to the prooxidative environment, including solar UVA and UVB radiation, the skin is equipped with an elaborate system of antioxidant substances and enzymes, including a network of redox active antioxidants.

Nrf2 (NF-E2-related Factor 2) and Keap1 (Kelch-like-ECH-associated protein 1) are key proteins in the coordinate transcriptional induction of various antioxidant-metabolizing enzymes. Nrf2 is a member of the NF-E2 family of nuclear basic leucine zipper transcriptional activators, and Keap1 is a cytoplasmic protein homologous to the *Drosophila* actin-binding protein Kelch (Chui *et al*, 1995; Itoh *et al*, 1999b). Under normal physiological conditions, Nrf2 is largely bound to Keap1 and retained in the cytoplasm. On disruption of the Nrf2-Keap1 complex by inducers, Nrf2 undergoes rapid translocation to the nucleus where it activates its target genes in heterodimeric combinations with other transcription factors, such as small maf proteins (Itoh *et al*, 1999a). After translocation into the nucleus, Nrf2 recognizes and binds to anti-oxidant response elements (ARE) in the promoter regions of its target genes and induces phase II detoxification enzymes and antioxidant proteins, such as γ -glutamylcysteine synthetase (γ -GCS) (Wild *et al*, 1999), cystine/glutamate exchange transporter (Sasaki *et al*, 2002), glutathione S-transferase (GST) (Hayes *et al*, 2000), NADPH quinone oxidoreductase-1 (NQO1) (Venugopal and Jaiswal,

1996), heme oxygenase-1 (HO-1) (Alam *et al*, 1999), and thioredoxin (TRX) (Ishii *et al*, 1999). Furthermore, Nrf2 was recently shown to have a protective effect against apoptosis induced by Fas signaling (Morito *et al*, 2003) and mitochondrial toxins (Lee *et al*, 2003). Therefore, we focused on the Nrf2-Keap1 system as a putative major component of the protective machinery involved in protection of the skin against UV-induced oxidative damage.

In the present study, we examined whether UV irradiation of cells triggers activation of Nrf2, which can protect the cells against UV-induced apoptosis. Nrf2 nuclear translocation was investigated in dermal fibroblasts irradiated with UVA or UVB. Furthermore, we used dermal fibroblasts derived from *nrf2*^{+/+} and *nrf2*^{-/-} mice to examine the protective effect of Nrf2 against UV-induced apoptosis.

Materials and Methods

Mice — Newborn (1-2 d old) ICR, *nrf2* null mutant mice in an ICR/129SV background (Itoh *et al*, 1997), *keap1* null mutants in a B6/129SV background, and *nrf2::keap1* double null mice in a 129SV/C57BL6/ICR background (Wakabayashi *et al*, 2003) were used for the dermal skin fibroblasts culture experiments. All experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals of the University of Tsukuba, Japan.

Primary fibroblast culture — Newborn mouse pups were killed by decapitation, and washed with 75% ethanol. Their limbs and tails were amputated, and the trunk skin was removed. The skins were then stretched and flattened with the dermal side down and floated on 0.25% trypsin (Invitrogen, Carlsbad, CA) overnight at 4°C. The dermis was split from the epidermis, minced with sterile scissors, and stirred in collagenase solution in an incubator at 37°C for 30 min. The solution was filtered through sterile Nytex gauze and centrifuged at 300×g for 10 min. The pellet was resuspended in Dulbecco's modified Eagle's medium (Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum (Sigma), 100 U of penicillin (Sigma), and 100 µg of streptomycin (Sigma), and then distributed into culture dishes. Cells were maintained in a humidified 5% CO₂-95% air incubator at 37°C. All experiments were carried out with subcultures at passage 3-5.

UV irradiation — Before UV irradiation, the cells were washed and covered with phosphate-buffered saline (PBS). The cells were irradiated with UVA using FL20SBLB lamps (Toshiba, Tokyo) with a peak emission at 352 nm. Wavelengths below 320 nm were removed

using an ATG filter UV-35 (Asahi Technoglass, Tokyo). UVB irradiation was performed using FL20SE lamps with emission at 280-320 nm with a peak at 312.5 nm. After UV irradiation, the culture medium was replaced with fresh medium and the cells were incubated at 37°C under an atmosphere of 5% CO₂ for the indicated periods for each experiment. In some cases, sham-irradiation was also carried out.

Preparation of protein extracts — Whole-cell extracts were obtained using RIPA buffer. Nuclear extracts were prepared according to the method described by Schreiber et al. (Schreiber et al, 1989) with some modifications. Both extracts were supplemented with a solution containing complete protease inhibitor cocktail (Roche, Mannheim, Germany) according to the manufacturer's recommendations. The protein concentrations were determined by BCA protein assay (Pierce, Rockford, IL) or Bradford assay (Bio-Rad Laboratories, Hercules, CA).

Western blotting analysis — Whole-cell and nuclear extracts mixed with sample buffer were electrophoresed on 6% SDS-polyacrylamide gels and then blotted onto nitrocellulose membranes (Bio-Rad Laboratories). The blots were blocked in Tris-buffered saline (TBS) containing 3% non-fat dry milk at room temperature for 1 h. Blots were incubated with anti-Nrf2 antibody (Ishii et al, 2000) in TBS containing 3% non-fat dry milk at 4°C overnight. After washing twice in TBS containing 0.05% Tween 20 (TTBS), blots were incubated with goat anti-rabbit IgG HRP (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h at room temperature in TBS containing 3% non-fat dry milk. Immunoreactive proteins were detected using Supersignal West Pico Chemiluminescent Substrate (Pierce). As internal reference for relative protein amounts a second

western blot was done using the same protein lysates and an anti-lamin A/C antibody (Santa Cruz Biotechnology).

Immunostaining for Nrf2 — Fibroblasts were grown on slides for 24 h and irradiated with UVA. After 4 h of irradiation, cells were washed with PBS and fixed in 4% paraformaldehyde solution at room temperature for 10 min and then in 100% cold acetone for 10 min on ice. After washing with PBS, cells were incubated with 2% goat serum in PBS (pH7.4) for 1 h at 25°C. The cells were first treated with polyclonal rabbit anti-Nrf2 antibody (1:1000) in PBS containing 2% goat serum for 24 h at 4°C, and subsequently with goat anti-rabbit IgG-FITC (1:200, Santa Cruz Biotechnology) in PBS containing 2% goat serum for 1 h at room temperature. After washing with PBS, slides were mounted with fluorescent mounting medium (DakoCytomation, Glostrup, Denmark). The cells were then examined by fluorescence microscopy (Nikon, Tokyo).

Preparation of RNA and Northern blot hybridization — Total cellular RNA was prepared from primary murine dermal fibroblasts 5 h after irradiation with UVA (7 J/cm^2) using an Isogen RNA extraction kit (Nippon Gene, Toyama, Japan) according to the manufacturer's protocol. The RNA samples (10 μg) were denatured with formaldehyde and formamide, and electrophoresed on 1% agarose gels containing 2.2 M formaldehyde, then transferred onto Zeta-Probe GT membranes (Bio-Rad). Hybridization was performed at 68°C using ^{32}P -labeled cDNA corresponding to GCSH or 18S rRNA.

Flow cytometric analysis — Analysis of the early stages of apoptosis was performed using an

Annexin-V/Propidium iodide (PI) staining kit (Roche, Mannheim, Germany). Briefly, cells were harvested 5 h after UVA irradiation (15 J/cm^2 for nrf2 null cells and 20 J/cm^2 for keap1 null or Nrf2/Keap1 null cells), washed with PBS, and stained for 15 min with Annexin-V and PI in the buffer provided by the manufacturer. Flow cytometry was performed using a FACSCalibur (Becton-Dickinson, Franklin Lakes, NJ). Fluorescence associated with Annexin-V-FITC was recorded using a FL1-H ($530 \pm 15 \text{ nm}$) filter and that associated with PI was recorded using an FL3-H filter (over 650 nm). Data analyses were performed using Cell Quest software.

Results

UVA increased but UVB decreased nuclear accumulation of Nrf2 in mouse dermal fibroblasts — We first examined whether Nrf2 was accumulated in the nucleus after UVA irradiation of dermal fibroblasts. Fibroblasts were irradiated with UVA at a dose of 10 J/cm² and incubated for various periods before performing the nuclear extraction. As shown in Fig. 1A, UVA irradiation resulted in accumulation of Nrf2 in the nuclear fraction of fibroblasts after 2 and 4 h. We also examined the accumulation of Nrf2 at the whole-cell extract level. UVA irradiation increased the accumulation of Nrf2 not only in the nucleus but also at the level of the whole cell (Fig. 1B). This finding strongly suggested that UVA not only triggers translocation of Nrf2 into the nucleus, but also releases Nrf2 from the degradation pathway and stabilizes Nrf2 in the cytoplasm.

Next, we examined the effect of UVB on the Nrf2 activation. As shown in Fig. 2A, the level of Nrf2 in the nucleus was decreased in UVB-irradiated cells as compared with sham-irradiated controls. The levels of Nrf2 in the nuclear extracts were markedly reduced following UVB irradiation at doses in excess of 10 mJ/cm² (Fig. 2B). This is in a clear contrast against the situation for UVA irradiation (Fig. 1).

Subcellular distribution of Nrf2 following UVA irradiation — We next examined the subcellular localization of Nrf2 after UVA irradiation directly by immunostaining for Nrf2. In UVA-untreated control cells, Nrf2 was located mainly in the cytoplasm, especially in the perinuclear area (Fig. 3A), whereas intense nuclear staining was observed in the UVA-irradiated cells (10 J/cm²) 4 h after irradiation (Fig. 3B). This observation along with the results of the

immunoblot analysis (Fig. 1) thus demonstrates that the UVA irradiation promotes the accumulation of Nrf2 in the nucleus.

Hematoporphyrin treatment enhanced UVA-induced nuclear accumulation of Nrf2 in dermal fibroblasts — To confirm roles ROS play in the activation of Nrf2, we investigated the effects of the photosensitizer, hematoporphyrin, on the activation of Nrf2 following UVA irradiation. Fibroblasts were pretreated with hematoporphyrin, which generates ROS such as singlet oxygen ($^1\text{O}_2$), in cells in response to UVA absorption (Poh-Fitzpatrick, 1986). As shown in Fig. 4, pretreatment of cells with hematoporphyrin resulted in nuclear accumulation of Nrf2 following irradiation with UVA at a lower dose (2 J/cm^2) as compared to non-treated cells in a dose-dependent manner from 0.25 M to 1.0 M of hematoporphyrin. This dose-dependent effect of hematoporphyrin on the induction of Nrf2 activation in UVA-treated cells indicated the important role of ROS in UVA-induced Nrf2 activation.

Induction of γ -GCS mRNA by UVA irradiation — The heterodimeric protein γ -GCS is the rate-limiting enzyme in the *de novo* synthesis of glutathione, a scavenger of both electrophiles and ROS (Griffith and Mulcahy, 1999). The expression of γ -GCS has been shown to be controlled mainly by Nrf2 (Thimmulappa *et al*, 2002). To examine the effects of UVA irradiation on expression of Nrf2 target genes at the transcriptional level, RNA was extracted from cultured dermal fibroblasts following exposure to UVA at a dose of 7 J/cm^2 and analyzed by Northern blotting analysis for mRNA of the typical Nrf2 target genes, γ -GCS heavy and light chains. As shown in Fig. 5, UVA irradiation significantly increased the levels of both γ -GCS

heavy and light chain transcripts. These findings suggest that UVA-induced activation of Nrf2 is linked to induction of the target gene, γ -GCS.

Nrf2 deficiency enhanced sensitivity of cultured dermal fibroblasts to UVA-induced apoptosis — We examined the role of Nrf2 in UVA-induced apoptosis using dermal fibroblasts derived from *nrf2* null mutant mice. Each experiment was performed using fibroblasts at passage 3-5. Fibroblasts were exposed to UVA at a dose of 15 J/cm², incubated for 5 h, and labeled with annexin V-FITC and propidium iodide (PI). The numbers of annexin-positive/PI-negative, annexin-positive/PI-positive, and annexin-negative/PI-positive cells, as well as unlabeled cells, were determined by flow cytometry. Figure 6 shows the results of comparison between Nrf2-expressing wild-type cells and *nrf2* null mutant cells. After UVA exposure, 11.3% of *nrf2* null cells (annexin-positive/PI negative cells) were in the early stages of apoptosis as compared with only 5.9% of wild-type cells (Fig. 6c, d, R2). The numbers of double-labeled cells in the late stages of apoptosis were also increased in the UVA-treated *nrf2* null mutant cells (Fig.6c, d, R3). Unlabeled cells representing the live population (R1) showed only slight decreases in number from 90.1% to 86.7% in the wild-type cultures as compared to a decrease from 90.5% to 78.6% in cultures of *nrf2* null mutant cells.

Keap1 null phenotype protected dermal fibroblasts from UVA-induced apoptosis — Keap1 is a key regulator of Nrf2 function. Keap1 binds to Nrf2 under physiological unstressed condition, resulting in the retention of Nrf2 in the cytoplasm. When cells encounter a stimulator, Keap1 releases Nrf2, allowing its rapid translocation into the nucleus. A recent study demonstrated that *keap1*-null mutation in mice led to constitutive Nrf2 activation (Wakabayashi *et al*, 2003).

To investigate the effects of Keap1-deficiency on UVA-induced apoptosis, we exposed cultured *keap1*-null mutant fibroblasts to UVA and analyzed the cells by flow cytometry. As shown in Fig. 7, *keap1*-null mutant cells showed significantly reduced UVA-induced annexin labeling. Wild-type cultures contained 15.6% annexin-positive/PI-negative apoptotic cells, which is very high incidence as compared with only 5.9% in the *keap1*-null mutant (Fig. 7c, d, R2). The number of cells double-labeled with annexin and PI was also decreased in UVA-treated *keap1*-null cells (Fig. 7c, d, R3). The number of unlabeled cells representing the live population (R1) was decreased markedly from 88.8% to 71.3% in the wild-type cultures but decreased only from 91.0% to 84.9% in the *keap1*-null mutant cell population. These findings indicate the protective effect of Keap1-deficiency, which results in constitutive Nrf2 activation in cells against UVA-induced apoptosis.

Keap1-deficiency fails to compensate for susceptibility of cells to UVA-induced apoptosis caused by Nrf2-deficiency — Next, we examined the sensitivity of *nrf2::keap1* double null mutant fibroblasts to UVA radiation. The number of UVA-induced annexin-positive/PI-negative apoptotic cells was much higher (19.3%) in *nrf2::keap1* double mutant cells as compared with wild-type controls (11.1%) (Fig. 8c, d). More than 84.0% of the wild-type cells were unlabeled as compared with 71.7% of the *nrf2::keap1* double null mutant cells. The high degree of susceptibility to UVA irradiation observed in *nrf2::keap1* double null mutant cells was similar to that of *nrf2* single mutant cells, indicating that Keap1-deficiency could not rescue the cells from the high susceptibility to UVA-induced apoptosis caused by Nrf2 deficiency. This observation suggested that Nrf2 is the major interacting molecule of Keap1 in the cytoplasm.

Discussion

The present study clearly indicates that Nrf2 undergoes translocation and accumulation in the nucleus in response to UVA irradiation. The nuclear accumulation of Nrf2 is enhanced by the addition of photosensitizer hematoporphyrin to the culture and this fact suggests that the Nrf2 activation is mediated by singlet oxygen ($^1\text{O}_2$). We also examined UVA-induced apoptosis using dermal fibroblasts derived from germ line *nrf2* and/or *keap1* gene mutant mice. The disruption of *nrf2* increased the number of apoptotic cells following the UVA irradiation, while the disruption of *keap1* decreased the apoptotic cell number compared with wild-type controls. These results thus unequivocally demonstrate that the Nrf2-Keap1 pathway plays an important role in the skin protection against UVA irradiation.

Importantly, the results of this study also show that, in contrast to the response to the UVA irradiation, nuclear accumulation of Nrf2 is not induced by the UVB irradiation. Consistent with this observation, it was previously reported that UVB radiation does not affect the GST expression (Seo *et al*, 1996), which is under the Nrf2 regulation. Whereas the precise reason for these differential effects of UVA and UVB irradiation on the Nrf2 activation is not clear at present, following observations may be pertinent to interpret these observations. It has been suggested that UVA mainly causes oxidative stresses rather than direct photodamage to DNA (Ichihashi *et al*, 2003). In fact, gene expression patterns induced by UVA radiation are different from those induced by UVB (Zhang, H. and Rosdahl, 2003). Indeed, UVA irradiation activates the expression of a wide variety of cytoprotective genes, including HO-1 (Keyse and Tyrrell, 1989), ICAM-1 (intercellular adhesion molecule-1) (Grether-Beck *et al*, 1996), MMP-1 (matrix metalloproteinase-1) (Scharffetter *et al*, 1991), MMP-2, MMP-3 (Herrmann *et al*, 1993), and NQO1 (Grether-Beck *et al*, 1997). In contrast, UVB irradiation has been reported to cause

direct DNA damage (Ichihashi *et al*, 2003) in addition to the oxidative cell damage.

Both UVA and UVB irradiations provoke apoptosis of the dermal cells. The mechanism of apoptosis induced by UVA has been suggested to be different from that induced by UVB; UVA induces apoptosis mainly through down-regulation of Bcl-2 expression, while UVB-induced apoptosis accompanies the accumulation of p53 (Wang *et al*, 1998). In this regard, Godar *et al*. classified the mechanisms of UV-induced apoptosis as either immediate or delayed (Godar, 1999a, b). Previous studies also demonstrated that UVB induces the release of cytochrome C from mitochondria, which triggers DNA damage (Kulms and Schwarz, 2002). The release starts 20 hours after the irradiation, suggesting that the UVB irradiation may cause the delayed apoptosis. One likely interpretation of these results is that if the extent of UVB-induced DNA damage proceeds beyond the limit that can be repaired, the cells undergo the apoptotic cell death pathway to prevent the aberrant cell generation. On the other hand, UVA irradiation provokes apoptosis of cultured dermal fibroblasts *in vitro* much sooner (Figs. 6- 8). While the UVA irradiation is much less mutagenic than UVB, it fully activates the Nrf2-Keap1 pathway within 4 hours after exposure to UVA. The precise mechanisms generating the differential effects of UVA and UVB on Nrf2 activation is an interesting issue that should be clarified.

Through the study exploiting *nrf2* and/or *keap1* knockout lines of mice, we showed that the Nrf2-mediated induction of a set of cytoprotective genes is an important process for the protection of the dermal cells from UVA-induced oxidative stress. The protection of cells from UVA-induced apoptosis was significantly diminished by the disruption of *nrf2*, whereas *keap1*-null mutant fibroblasts showed a higher level of protection as compared to that of wild-type fibroblasts. Furthermore, the compound disruption of *nrf2::keap1* resulted in the increase of apoptotic cells as compared to the wild-type control fibroblasts. We envisage that

the mechanisms by which Nrf2 protect the skin dermal cells from apoptosis may be complex. Recent studies indicated that *nrf2*-null mutant cells are prone to apoptosis induced by the H₂O₂ stimulation (Zhang, D.D. and Hannink, 2003). Similarly, neurons of *nrf2*-null mutant mice are sensitive to mitochondrial toxin-induced apoptosis (Itoh *et al*, 1997). Overexpression of Nrf2 was shown to protect the cells from Fas-induced apoptosis (Kotlo *et al*, 2003). Importantly, it has been found that Fas-induced apoptosis of Jurkat T cells accompanies rapid and specific export of glutathione from the apoptotic cells (van den Dobbelsteen *et al*, 1996), and conversely Nrf2 regulates the sensitivity of cells to Fas-inducible apoptosis by affecting intracellular glutathione levels (Morito *et al*, 2003). The results of oligonucleotide microarray analyses have shown that *nrf2*-null mutant mice have lower basal level expression of Nrf2 target genes (Thimmulappa *et al*, 2002). Catalase, superoxide dismutase, and glutathione peroxidase (GPX), all of which eliminate ROS, are also dependent on Nrf2 (Lee *et al*, 2003). Decreases in the expression level of antioxidant enzymes and proteins may result in the increased susceptibility to UVA irradiation of *nrf2*-null mutant fibroblasts.

The damaging effects of UVA appear to be due to absorption by endogenous sensitizers, such as flavins and porphyrins, leading to the production of ROS (Tyrrell and Keyse, 1990). In this study, we used the exogenous porphyrin derivative, hematoporphyrin, to evaluate the involvement of ROS in UVA-induced Nrf2 nuclear translocation. The results indicate that UVA-induced nuclear translocation of Nrf2 was enhanced by ¹O₂ hematoporphyrin. ¹O₂ is the main ROS produced by photosensitization of hematoporphyrin. We surmise that electrophilic ¹O₂ reacts directly with the cysteine residues of Keap1, which act as the sensor for reactive electrophiles and oxidants (Itoh *et al*, 1999b; Dinkova-Kostova *et al*, 2002; Zhang, D.D. and Hannink, 2003; Wakabayashi *et al*, 2004). Oxidation of these cysteine residues of Keap1 by ¹O₂

generated by UVA irradiation would lead to the nuclear translocation of Nrf2.

In summary, the present study provided the first evidence that UVA irradiation induces the nuclear accumulation of Nrf2 in dermal fibroblasts. Based on the observation that *nrf2* and *nrf2::keap1* mutant fibroblasts are sensitive to UVA-induced apoptosis, whereas *keap1*-null mutant fibroblasts are highly resistant to the apoptosis induced by UVA, we conclude that the Nrf2-Keap1 system plays a critical role in the protection of dermal cells from the deleterious effects provoked by the exposure to UVA.

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Figure Legends

Figure 1.

UVA increases both whole-cell and nuclear Nrf2 accumulation in murine dermal fibroblasts.

Fibroblasts were irradiated with UVA in PBS and incubated for the indicated times. Nuclear extracts and whole-cell extracts were prepared as described in Materials and Methods. Equal amounts of protein were analyzed by SDS-PAGE and Western blotting with anti-Nrf2 polyclonal antibody. A. Nuclear extracts prepared 1, 2, and 4 h after UVA irradiation (0 or 10 J/cm²) were analyzed. B. Nuclear extracts and whole-cell extracts prepared 4 h after UVA irradiation (0 or 10 J/cm²) were analyzed. rNrf2, recombinant Nrf2 protein loaded as a molecular weight control. Antibody against lamin A/C was used as a loading control.

Figure 2.

UVB decreases Nuclear Nrf2 accumulation in murine dermal fibroblasts. Fibroblasts were irradiated with UVB in PBS and incubated for 4 h. Nuclear extracts were prepared as described in Materials and Methods. Equal amounts of protein were analyzed by SDS-PAGE and Western blotting with anti-Nrf2 polyclonal antibody. A. Nuclear extracts prepared 4 h after UVB irradiation at various doses (0, 15, 30, 60, 100, and 200 mJ/cm²) were analyzed. Antibody against lamin A/C was used as a loading control. B. Nuclear extracts prepared 4 h after UVB irradiation at various doses (0, 2, 5, 10, and 15 mJ/cm²) were analyzed.

Figure 3.

Nuclear accumulation of Nrf2 in response to UVA irradiation.

Cultured dermal fibroblasts were treated with or without UVA irradiation at a dose of 10 J/cm^2 . After 4 h of irradiation, cells were fixed and immunostained with anti-Nrf2 primary antibody followed by FITC-labeled secondary antibody. A, Non-irradiated fibroblasts; B, UVA-irradiated fibroblasts.

Figure 4.

Nuclear accumulation of Nrf2 in the presence of hematoporphyrin in murine dermal

fibroblasts. Fibroblasts were irradiated with UVA (2 J/cm^2) in PBS with hematoporphyrin and incubated in conventional medium for the indicated times. Nuclear extracts were prepared as described in Experimental Procedures. Equal amounts of protein were analyzed by SDS-PAGE and Western blotting with anti-Nrf2 polyclonal antibody. Antibody against lamin A/C was used as a loading control.

Figure 5.

Induction of γ -GCS mRNA by UVA irradiation. Total RNAs were prepared from cultured dermal fibroblasts 5 h after exposure to UVA at a dose of 7 J/cm^2 . Levels of mRNA for the typical Nrf2 target genes, i.e., γ -GCS heavy chain (GCSH; upper panel) and γ -GCS light chain (GCSL; middle panel) were determined by Northern blotting analysis. The membranes were subsequently hybridized with a probe for the loading control (18S; lower panel).

Figure 6.

Detection of UVA radiation-induced cell death in Nrf2-deficient and wild-type murine

dermal fibroblasts. Fibroblasts were irradiated with UVA at a dose of 15 J/cm^2 , incubated for 5 h in conventional medium, and labeled with annexin and propidium iodide (PI). The distributions of live and dead cells were determined by FACScalibur analysis of annexin and PI-labeled cells. Live cells were unlabeled with annexin and PI (R1), while annexin labeling (R2) represents the population undergoing apoptosis. Cells showing annexin and PI double labeling (R3) represent those that have already died by apoptosis. Ten thousand cells were analyzed in each sample. a, Non-irradiated wild-type cells. b, Non-irradiated nrf2 null mutant cells. c, UVA-irradiated wild-type cells. d, UVA-irradiated nrf2 null mutant cells.

Figure 7. **Detection of UVA radiation-induced cell death in Keap1-deficient and wild-type murine mouse dermal fibroblasts.**

Wild-type keap1 or keap1-deficient cells were treated and analyzed as described in Figure 6 except that the dose of UVA irradiation was 20 J/cm^2 . a, Non-irradiated wild-type cells. b, Non-irradiated keap1 null mutant cells. c, UVA-irradiated wild-type cells. d, UVA-irradiated keap1 null mutant cells.

Figure 8. **Detection of UVA radiation-induced cell death in wild-type or double Nrf2- and Keap1-deficient murine dermal fibroblasts.**

Wild-type or double mutant nrf2- and keap1-deficient cells were treated and analyzed as described in Figure 6 except that the dose was 15 J/cm^2 . a, Non-irradiated wild-type cells. b, Non-irradiated nrf2/keap1 double null mutant cells. c, UVA-irradiated wild-type cells. d, UVA-irradiated nrf2/keap1 double null mutant cells.

References

- Alam J, Stewart D, Touchard C, Boinapally S, Choi AM, Cook JL: Nrf2, a Cap'n'Collar transcription factor, regulates induction of the heme oxygenase-1 gene. *J Biol Chem* 274: 26071-26078, 1999
- Ananthaswamy HN, Pierceall WE: Molecular mechanisms of ultraviolet radiation carcinogenesis. *Photochem Photobiol* 52: 1119-1136, 1990
- Cadet J, Berger M, Douki T, Morin B, Raoul S, Ravanat JL, Spinelli S: Effects of UV and visible radiation on DNA-final base damage. *Biol Chem* 378: 1275-1286, 1997
- Chui DH, Tang W, Orkin SH: cDNA cloning of murine Nrf 2 gene, coding for a p45 NF-E2 related transcription factor. *Biochem Biophys Res Commun* 209: 40-46, 1995
- Dinkova-Kostova AT, Holtzclaw WD, Cole RN, *et al*: Direct evidence that sulfhydryl groups of Keap1 are the sensors regulating induction of phase 2 enzymes that protect against carcinogens and oxidants. *Proc Natl Acad Sci U S A* 99: 11908-11913, 2002
- Fisher GJ, Datta SC, Talwar HS, Wang ZQ, Varani J, Kang S, Voorhees JJ: Molecular basis of sun-induced premature skin ageing and retinoid antagonism. *Nature* 379: 335-339, 1996
- Godar DE: Light and death: photons and apoptosis. *J Investig Dermatol Symp Proc* 4: 17-23, 1999a
- Godar DE: UVA1 radiation triggers two different final apoptotic pathways. *J Invest Dermatol* 112: 3-12, 1999b
- Grether-Beck S, Buettner R, Krutmann J: Ultraviolet A radiation-induced expression of human genes: molecular and photobiological mechanisms. *Biol Chem* 378: 1231-1236, 1997
- Grether-Beck S, Olaizola-Horn S, Schmitt H, *et al*: Activation of transcription factor AP-2 mediates UVA radiation- and singlet oxygen-induced expression of the human

intercellular adhesion molecule 1 gene. Proc Natl Acad Sci U S A 93: 14586-14591, 1996

Griffith OW, Mulcahy RT: The enzymes of glutathione synthesis: gamma-glutamylcysteine synthetase. Adv Enzymol Relat Areas Mol Biol 73: 209-267, 1999

Hayes JD, Chanas SA, Henderson CJ, *et al*: The Nrf2 transcription factor contributes both to the basal expression of glutathione S-transferases in mouse liver and to their induction by the chemopreventive synthetic antioxidants, butylated hydroxyanisole and ethoxyquin. Biochem Soc Trans 28: 33-41, 2000

Herrmann G, Wlaschek M, Lange TS, Prenzel K, Goerz G, Scharffetter-Kochanek K: UVA irradiation stimulates the synthesis of various matrix-metalloproteinases (MMPs) in cultured human fibroblasts. Exp Dermatol 2: 92-97, 1993

Ichihashi M, Ueda M, Budiyanoto A, *et al*: UV-induced skin damage. Toxicology 189: 21-39, 2003

Ishii T, Itoh K, Sato H, Bannai S: Oxidative stress-inducible proteins in macrophages. Free Radic Res 31: 351-355, 1999

Ishii T, Itoh K, Takahashi S, *et al*: Transcription factor Nrf2 coordinately regulates a group of oxidative stress-inducible genes in macrophages. J Biol Chem 275: 16023-16029, 2000

Itoh K, Chiba T, Takahashi S, *et al*: An Nrf2/small Maf heterodimer mediates the induction of phase II detoxifying enzyme genes through antioxidant response elements. Biochem Biophys Res Commun 236: 313-322, 1997

Itoh K, Ishii T, Wakabayashi N, Yamamoto M: Regulatory mechanisms of cellular response to oxidative stress. Free Radic Res 31: 319-324, 1999a

Itoh K, Wakabayashi N, Katoh Y, Ishii T, Igarashi K, Engel JD, Yamamoto M: Keap1 represses nuclear activation of antioxidant responsive elements by Nrf2 through binding to the amino-terminal Neh2 domain. Genes Dev 13: 76-86, 1999b

- Keyse SM, Tyrrell RM: Heme oxygenase is the major 32-kDa stress protein induced in human skin fibroblasts by UVA radiation, hydrogen peroxide, and sodium arsenite. *Proc Natl Acad Sci U S A* 86: 99-103, 1989
- Kotlo KU, Yehiely F, Efimova E, *et al*: Nrf2 is an inhibitor of the Fas pathway as identified by Achilles' Heel Method, a new function-based approach to gene identification in human cells. *Oncogene* 22: 797-806, 2003
- Kulms D, Schwarz T: Independent contribution of three different pathways to ultraviolet-B-induced apoptosis. *Biochem Pharmacol* 64: 837-841, 2002
- Lee JM, Shih AY, Murphy TH, Johnson JA: NF-E2-related factor-2 mediates neuroprotection against mitochondrial complex I inhibitors and increased concentrations of intracellular calcium in primary cortical neurons. *J Biol Chem* 278: 37948-37956, 2003
- Ley RD: Photoreactivation of UV-induced pyrimidine dimers and erythema in the marsupial *Monodelphis domestica*. *Proc Natl Acad Sci U S A* 82: 2409-2411, 1985
- Morito N, Yoh K, Itoh K, Hirayama A, Koyama A, Yamamoto M, Takahashi S: Nrf2 regulates the sensitivity of death receptor signals by affecting intracellular glutathione levels. *Oncogene* 22: 9275-9281, 2003
- Packer L, Valacchi G: Antioxidants and the response of skin to oxidative stress: vitamin E as a key indicator. *Skin Pharmacol Appl Skin Physiol* 15: 282-290, 2002
- Poh-Fitzpatrick MB: Molecular and cellular mechanisms of porphyrin photosensitization. *Photodermatol* 3: 148-157, 1986
- Rosenstein BS, Mitchell DL: Action spectra for the induction of pyrimidine(6-4)pyrimidone photoproducts and cyclobutane pyrimidine dimers in normal human skin fibroblasts. *Photochem Photobiol* 45: 775-780, 1987

- Sasaki H, Sato H, Kuriyama-Matsumura K, *et al*: Electrophile response element-mediated induction of the cystine/glutamate exchange transporter gene expression. *J Biol Chem* 277: 44765-44771, 2002
- Scharffetter K, Wlaschek M, Hogg A, *et al*: UVA irradiation induces collagenase in human dermal fibroblasts in vitro and in vivo. *Arch Dermatol Res* 283: 506-511, 1991
- Scharffetter-Kochanek K, Wlaschek M, Brenneisen P, Schauen M, Blaudschun R, Wenk J: UV-induced reactive oxygen species in photocarcinogenesis and photoaging. *Biol Chem* 378: 1247-1257, 1997
- Schreiber E, Matthias P, Muller MM, Schaffner W: Rapid detection of octamer binding proteins with 'mini-extracts', prepared from a small number of cells. *Nucleic Acids Res* 17: 6419, 1989
- Seo KI, Cho KH, Park KC, Youn JI, Eun HC, Kim KT, Park SC: Change of glutathione S-transferases in the skin by ultraviolet B irradiation. *J Dermatol Sci* 13: 153-160, 1996
- Thimmulappa RK, Mai KH, Srisuma S, Kensler TW, Yamamoto M, Biswal S: Identification of Nrf2-regulated genes induced by the chemopreventive agent sulforaphane by oligonucleotide microarray. *Cancer Res* 62: 5196-5203, 2002
- Tyrrell RM, Keyse SM: New trends in photobiology. The interaction of UVA radiation with cultured cells. *J Photochem Photobiol B* 4: 349-361, 1990
- Tyrrell RM, Pidoux M: Correlation between endogenous glutathione content and sensitivity of cultured human skin cells to radiation at defined wavelengths in the solar ultraviolet range. *Photochem Photobiol* 47: 405-412, 1988
- van den Dobbelen DJ, Nobel CS, Schlegel J, Cotgreave IA, Orrenius S, Slater AF: Rapid and specific efflux of reduced glutathione during apoptosis induced by anti-Fas/APO-1

- antibody. *J Biol Chem* 271: 15420-15427, 1996
- Vayalil PK, Elmets CA, Katiyar SK: Treatment of green tea polyphenols in hydrophilic cream prevents UVB-induced oxidation of lipids and proteins, depletion of antioxidant enzymes and phosphorylation of MAPK proteins in SKH-1 hairless mouse skin. *Carcinogenesis* 24: 927-936, 2003
- Venugopal R, Jaiswal AK: Nrf1 and Nrf2 positively and c-Fos and Fra1 negatively regulate the human antioxidant response element-mediated expression of NAD(P)H:quinone oxidoreductase1 gene. *Proc Natl Acad Sci U S A* 93: 14960-14965, 1996
- Wakabayashi N, Dinkova-Kostova AT, Holtzclaw WD, *et al*: Protection against electrophile and oxidant stress by induction of the phase 2 response: Fate of cysteines of the Keap1 sensor modified by inducers. *Proc Natl Acad Sci U S A* 101: 2040-2045, 2004
- Wakabayashi N, Itoh K, Wakabayashi J, *et al*: Keap1-null mutation leads to postnatal lethality due to constitutive Nrf2 activation. *Nat Genet* 35: 238-245, 2003
- Wamer WG, Wei RR: In vitro photooxidation of nucleic acids by ultraviolet A radiation. *Photochem Photobiol* 65: 560-563, 1997
- Wang Y, Rosenstein B, Goldwyn S, Zhang X, Lebwohl M, Wei H: Differential regulation of P53 and Bcl-2 expression by ultraviolet A and B. *J Invest Dermatol* 111: 380-384, 1998
- Wild AC, Moinova HR, Mulcahy RT: Regulation of gamma-glutamylcysteine synthetase subunit gene expression by the transcription factor Nrf2. *J Biol Chem* 274: 33627-33636, 1999
- Zhang DD, Hannink M: Distinct cysteine residues in Keap1 are required for Keap1-dependent ubiquitination of Nrf2 and for stabilization of Nrf2 by chemopreventive agents and oxidative stress. *Mol Cell Biol* 23: 8137-8151, 2003
- Zhang H, Rosdahl I: Ultraviolet A and B differently induce intracellular protein expression in

human skin melanocytes--a speculation of separate pathways in initiation of melanoma.

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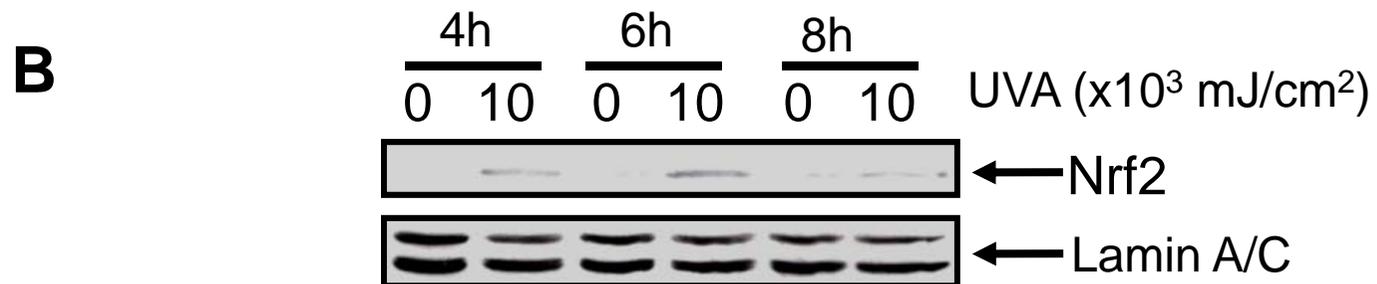
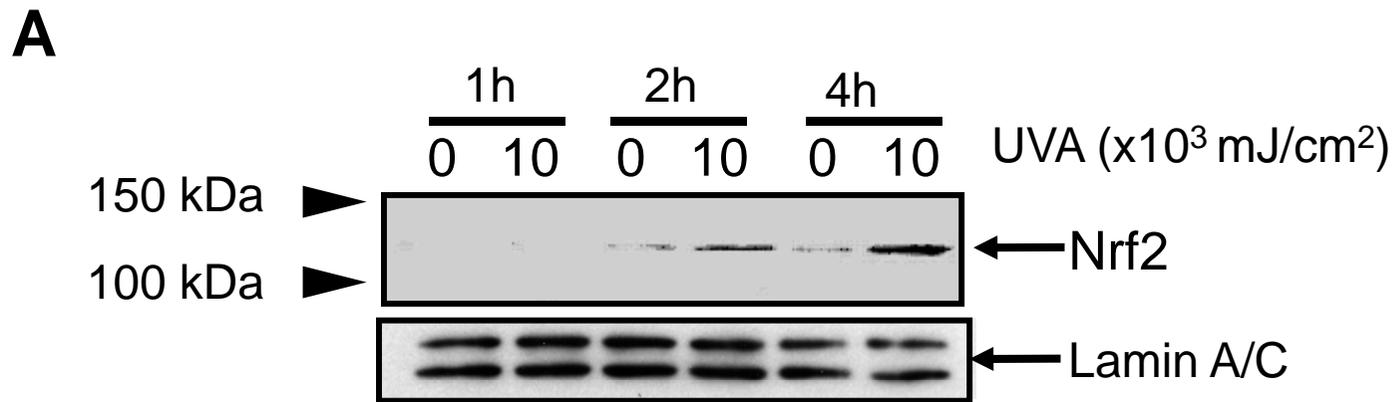


Fig.1

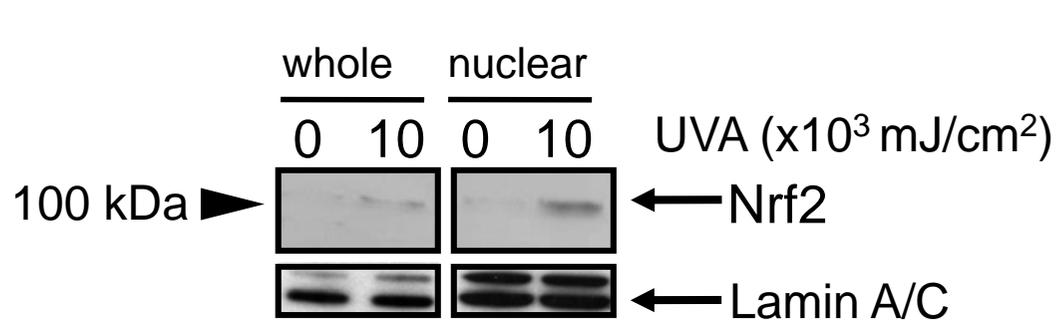
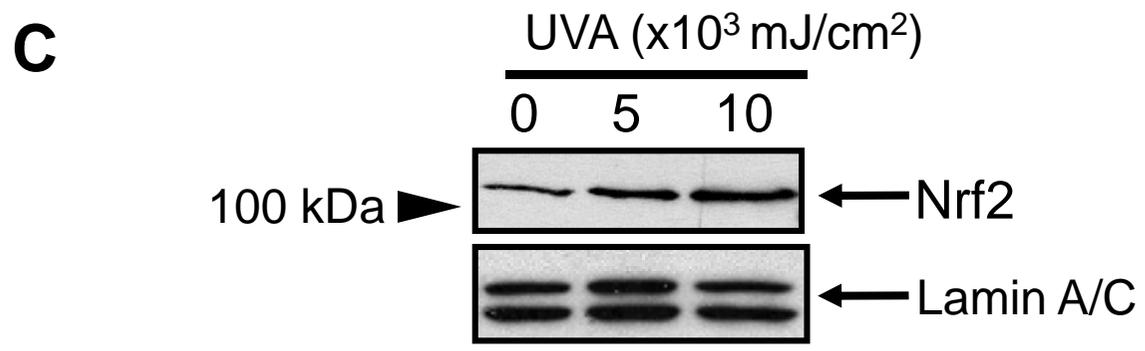


Fig.1

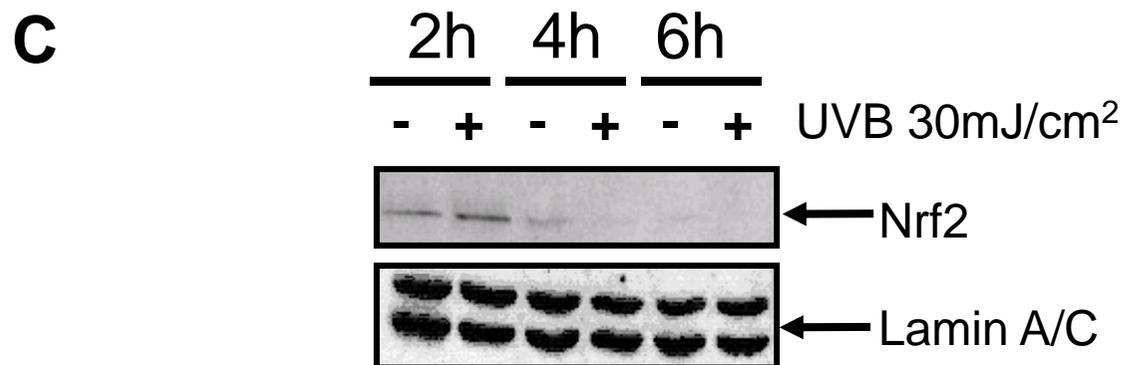
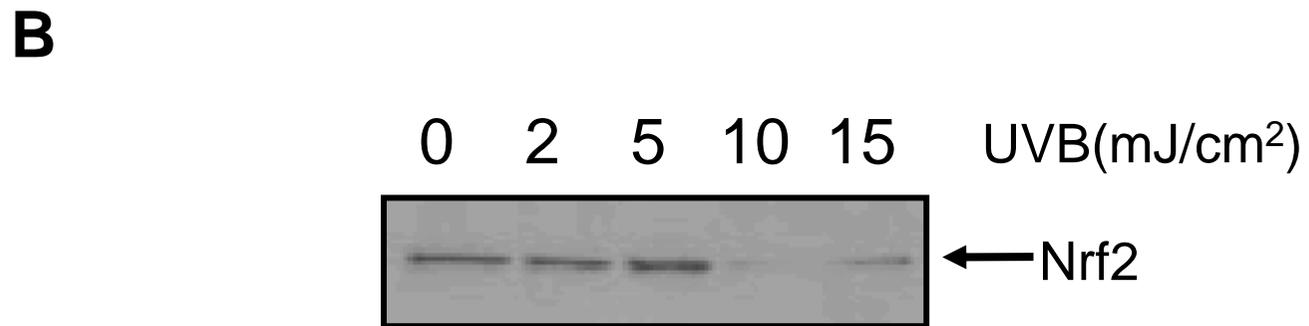
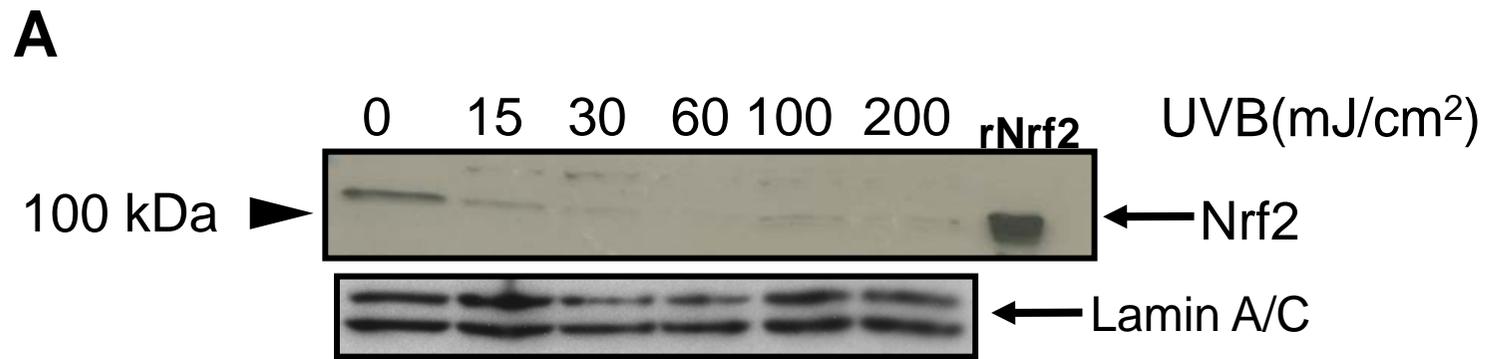
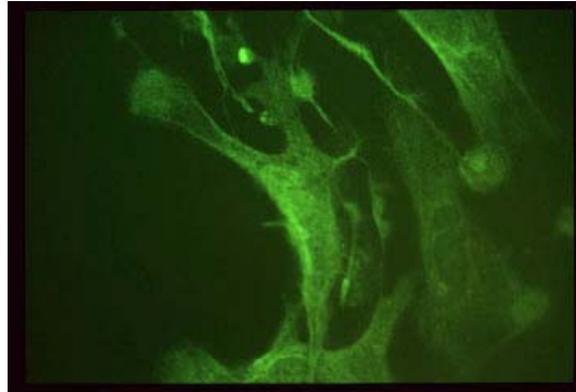


Fig.2

A

Control



B

UVA irradiation

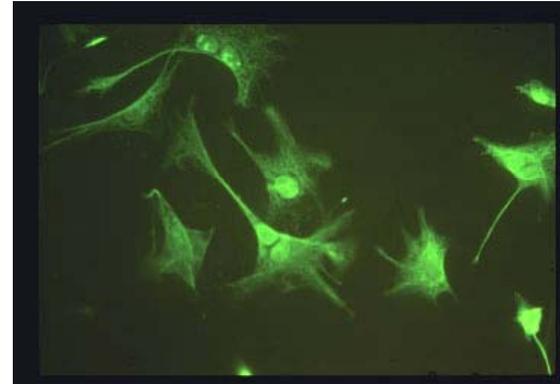


Fig.3

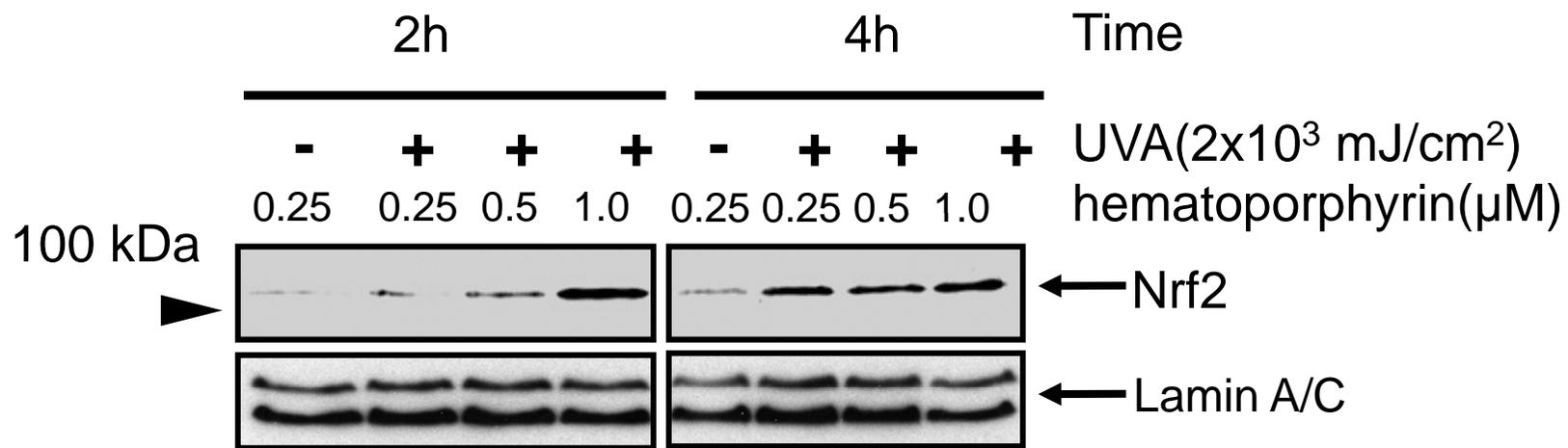


Fig.4

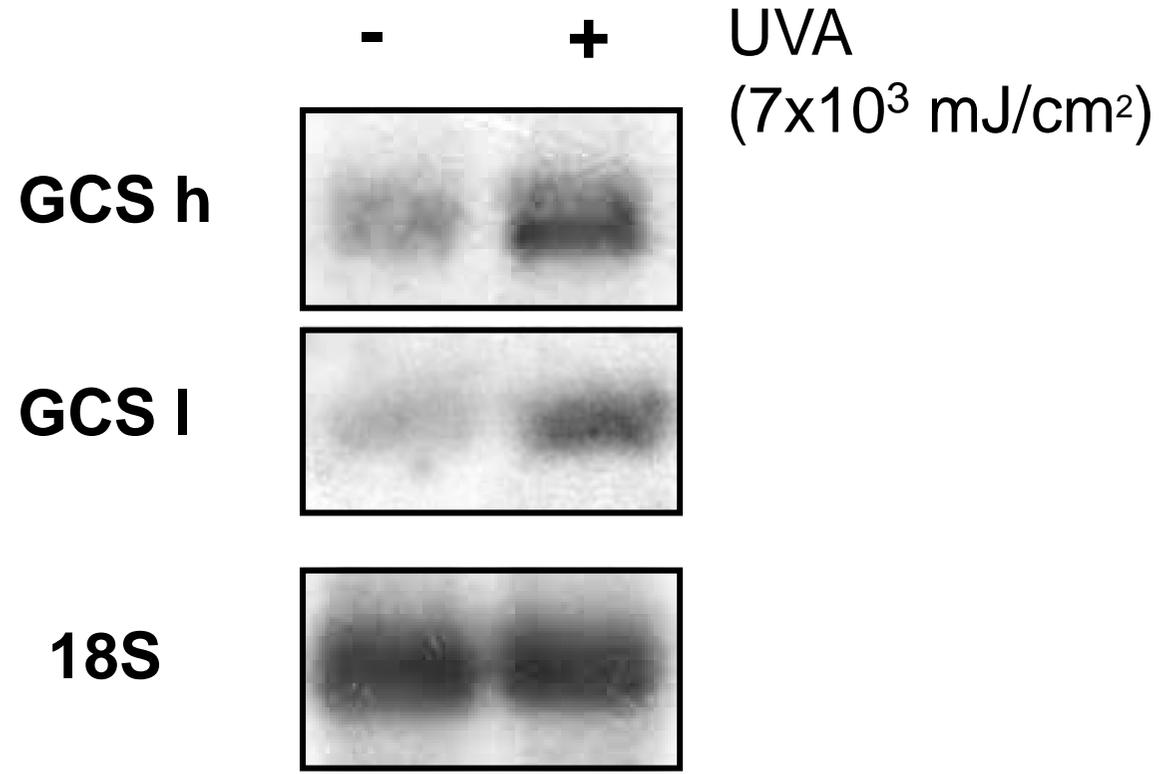


Fig.5

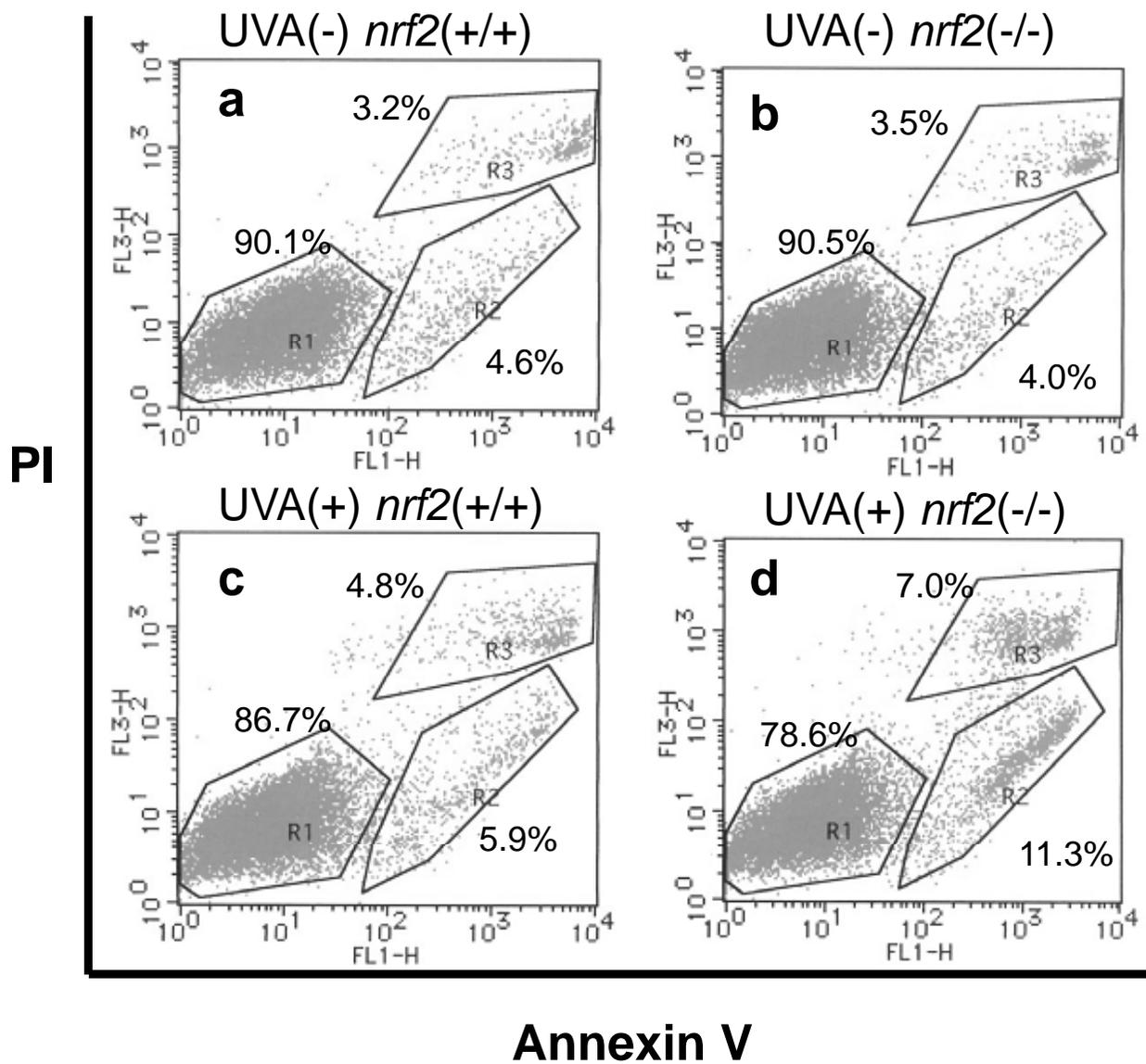


Fig. 6

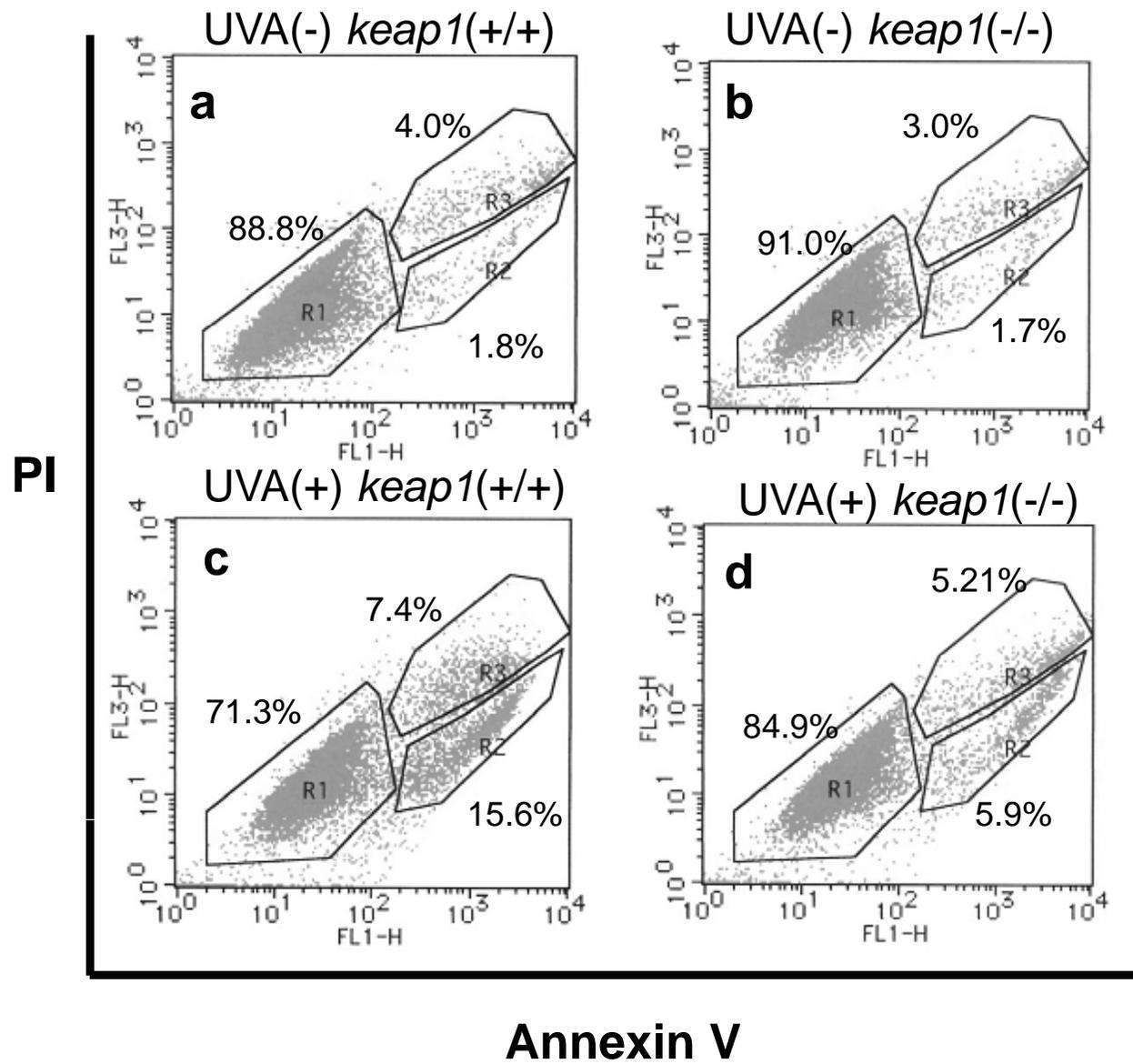


Fig.
7

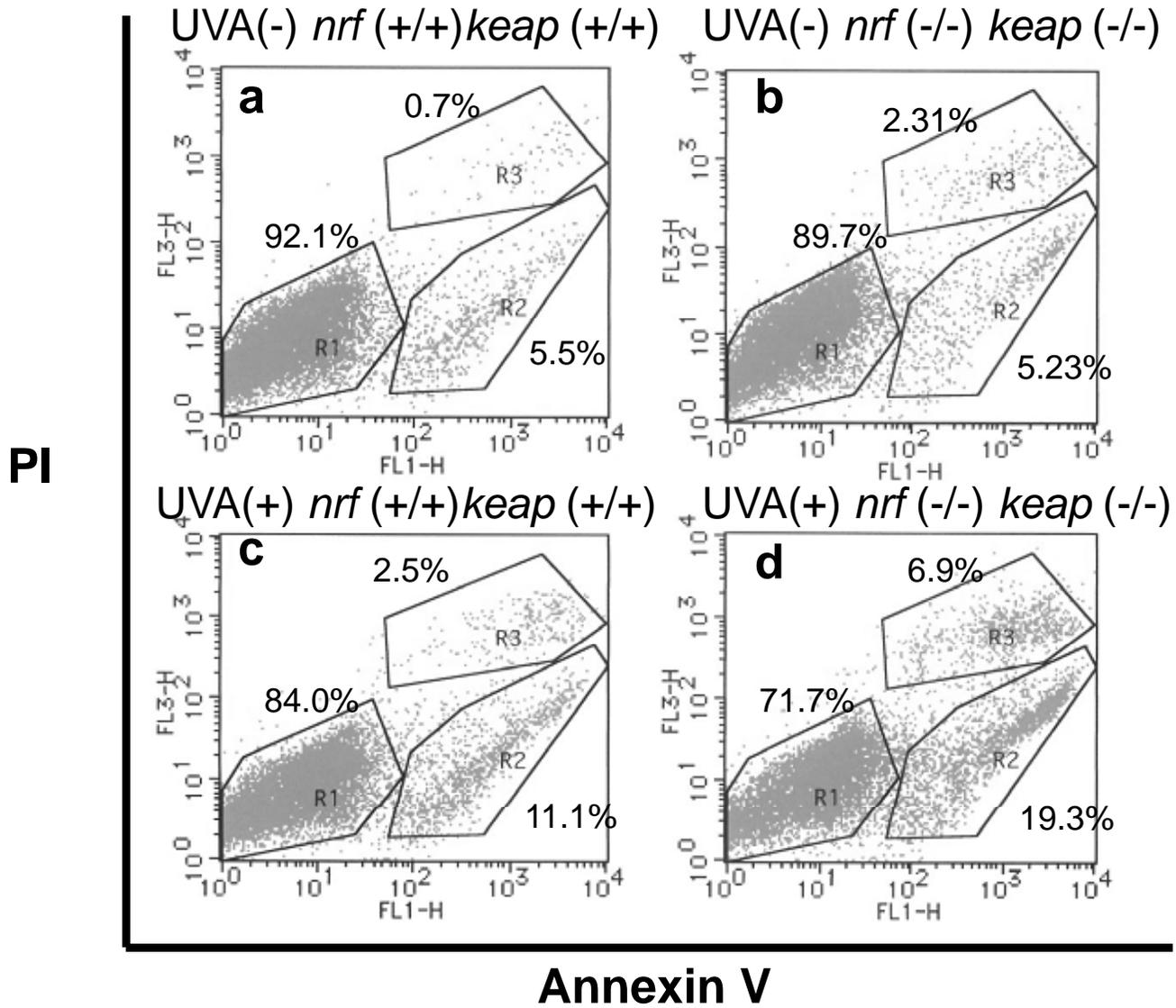


Fig. 8

Table. *Nfr2* deficiency enhanced sensitivity to UVA-induced apoptosis while *keap1* null phenotype reduced sensitivity to UVA-induced apoptosis.

| genotype | UVA (mJ/cm ²) | % apoptosis (R ₂ +R ₃) |
|------------------|---------------------------|---|
| <i>nrf2</i> +/+ | 0 | 5.78 ± 1.74 ^a |
| <i>nrf2</i> -/- | 0 | 5.72 ± 1.50 ^a |
| <i>nrf2</i> +/+ | 15,000 | 13.83 ± 3.28 ^a |
| <i>nrf2</i> -/- | 15,000 | 23.93 ± 6.49 ^a |
| <i>keap1</i> +/+ | 0 | 4.85 ± 2.25 ^a |
| <i>keap1</i> -/- | 0 | 6.27 ± 2.44 ^a |
| <i>keap1</i> +/+ | 20,000 | 19.51 ± 4.43 ^a |
| <i>keap1</i> -/- | 20,000 | 10.70 ± 5.15 ^a |

^a Mean ± SD

^b % apoptosis of irradiated *nrf2*^{-/-} cells is significantly more (p<0.05) than that of wild-type cells.

^c % apoptosis of irradiated *keap1*^{-/-} cells is significantly less (p<0.05) than that of wild-type cells.