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Depletion of Wip1 Phosphatase Sensitizes Murine Skin Cells to UV-B Irradiation

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Abstract—UV irradiation is a major natural and artificial stress factor that may cause severe skin injury. UV irradiation induces DNA damage, which, eventually, may lead to cell death, senescence or oncogenic mutations and tumor growth. Wip1 is a phosphatase involved in the regulation of DNA damage response and oncogenic stress. Here, we studied response to UV-B irradiation in wild-type and Wip1-depleted murine cells of epidermal and mesenchymal lineages. We found that both cell types, skin keratinocytes and fibroblasts, responded to UV-B in a similar manner with increased cytotoxicity in Wip1–/– cells. The number of nuclear foci of histone γH2A-X, a DNA damage marker and aWip1 target protein, was higher in Wip1–/– cells before and after UV-B. We observed a twofold increase in cell number with active caspase-3 in Wip1-deficient keratinocytes. Thus, Wip1 deficiency sensitizes cells to UV-B irradiation by promoting cell death, possibly by caspase-3 dependent apoptosis.

Keywords: UV-irradiation, Wip1 knockout mice, keratinocytes, fibroblasts, DNA damage
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INTRODUCTION

Ultraviolet (UV) irradiation, which is widespread both in nature (e.g., sunlight) and the technogenic sphere (e.g., germicidal lamps), can do serious damage to cells and tissues. In human beings, as with other mammals, skin injuries that depend on racial differences cause benign or malignant skin tumors over time (Leiter and Garbe, 2008; Polefka et al., 2012). There are three subtypes of UV light: long- (UV-A, 315–400 nm), medium- (UV-B, 280–315 nm) and short-wave (UV-C, 100–280 nm). The most serious damages are evoked with UV-C. However, this UV type is absorbed by the terrestrial atmosphere and does not reach the Earth's surface. The least harmful is UV-A, a key component of the sunlight. UV-B, which is partially blocked by the atmosphere, is less present than UV-A in the sunlight reaching the Earth's surface. However, it is a strong carcinogen that induces DNA damage. DNA damage produced by UV-B may cause mutations resulting in malignant transformation in skin and other tissues (Budden and Bowden, 2013). DNA repair of UV-induced damage and prevention of malignant transformation by genes involved in DNA damage response and tumor suppressors, such as p53, is of great importance. Moreover, precise regulation of these proteins is also particularly important (Zhou and Elledge, 2000; Marechal and Zou, 2013; Menon and Povirk, 2014).

Wip1 is a serine–threonine phosphatase with oncogene properties. It is encoded by *Ppm1d* gene and is a transcriptional target of p53 (Lu et al., 2008). Stress conditions—e.g., DNA damage—trigger p53 activation coordinating various systems of stress response, including stimulation of the gene expression for Wip1 phosphatase. Wip1 dephosphorylates activation sites on participant molecules of the cascade stress response and inhibits p53 activity, which switches cellular molecular systems over from stress conditions into the rest state. Cell response to DNA damage is tightly associated with systems of tumor growth inhibition, and parts of the system are very important for effective use of anticancer chemotherapeutic drugs. Thereby, Wip1 may be a negative regulator of tumor suppressors and a modulator of tumor response on anticancer therapy (Lu et al., 2005, Fujimoto et al., 2006). Wip1 has displayed procancer properties in the development of mammary cancer, and cancer of the lymphoid system and intestine (Bulavin et al., 2004; Harrison et al., 2004). In intestinal tumors, Wip1 is active in stem cells. It accumulates in stem cells of intestine epithelium, probably due to the high level of activity of p53-cascade in these cells. Genetic manipulations directed to Wip1 gene ablation

prevent stem cell malignant transformation by inhibiting the triggering of an apoptotic death program with oncogenic stress produced by deregulation of β-catenin oncogene. Mice with Wip1 deletion are more resistant to tumorigenesis in animals with modeled intestinal polyposis produced by mutation in the tumor suppressor gene APC (Demidov et al., 2007). As a whole, Wip1 expression and its role in the maintenance of homeostasis in skin under stress conditions is poorly examined.

The goal of this study was to assay the impact of Wip1 gene knockout on skin cell sensitivity to UV-B irradiation in vitro and in vivo.

MATERIALS AND METHODS

Mice. C57Bl/6 wild-type (Wip1+/+) mice and with *Ppm1d* gene (Wip1 phosphatase) knockout (Wip1−/−) were used in experiments. Mice were kept under standard vivarium conditions (22°C, 12-h light/dark cycle). They were given unlimited access to water and food.

Keratinocytes were isolated from newborn mice of wild type and with *Ppm1d* gene knockout as described in (Rheinwald, 1980; Spichkina et al., 2006) with modifications. Skin removed from newborn mice was placed into a mixture of 0.5% dispase II (Sigma, United States) and 0.2% collagenase I (Gibco, Life Technologies, United States) with dermal side facing down and incubated at 4°C overnight. On the next day, the epidermis was carefully separated, minced, and placed into 0.125% trypsin for 5 min at 37°C. Trypsin activity was blocked with bovine serum (HyClone, United States). The cells were filtered through a 70-μm filter, centrifuged (600 *g*, 5 min), and suspended in the keratinocyte culture medium. Cell suspension was seeded in 35-mm plates (6×10^4 cells/plate) coated with collagen type I (100 μg/mL in acetic acid) and incubated in KBM Gold standard keratinocyte medium (Lonza, Switzerland) containing KGM-Gold SingleQuots (Lonza, Switzerland) and 60 μM $CaCl_2$.

Embryonic fibroblasts were established from 12-day mouse embryos. Embryos removed from the body were purified, washed with sterile PBS, minced, and placed into 0.25% trypsin for 5 min at 37°C. Trypsin cell suspension was collected and sedimented pieces were again covered with trypsin. The procedure was repeated. Trypsin activity was blocked with bovine serum. Cell suspension was centrifuged (600 *g*, 5 min). The cells were suspended in DMEM medium and seeded into 35-mm plates. The cells were maintained in DMEM medium with 10% fetal bovine serum.

Irradiation. Three-month-old mice in were irradiated in the back with hair removed by UV-B (5000 J/m^2) using Vilber single/double 15W UV-B VL 115M and C equipment (France). Irradiation was measured with a Vilber VLX-3W CX312 sensor (France). Irradiated animals were examined for hair recovery every 2 days. Cultured cells were irradiated the same technique (dose 500 J/m^2).

Cell viability was assessed with MTT-test or staining with vital dye trypan blue. For MTT-test the cells were seeded in 24-well plates (2×10^4 cells/well), irradiated and cultivated for a certain period of time. Then the medium was replaced with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (thiazolyl blue) in sterile PBS (0.5 mg/mL) (Sigma, United States). Cells were incubated for 1 h at 37°C. The dye conversion into violet formasan was controlled with light microscopy. After 1-h incubation, the solution was replaced with DMSO and incubated for about 30 min until formasan completely dissolved in DMSO. Formasan was quantified spectrophotometrically at 572 nm.

For trypan blue staining, the cell suspension was obtained with trypsin treatment and washed with PBS. Ninety microliters of cell suspension in PBS was added with 10 μL trypan blue for 10 min at room temperature. The number of stained (dead) and unstained (live) cells were counted with Gorajev's camera under a light microscope.

Immunofluorescence. Cells cultivated on coverslips were fixed with 4% formaldehyde for 20 min at room temperature. Coverslips were washed with sterile PBS and permeabilized with 0.2% Triton X-100. Unspecific binding was blocked with 3% BSA/TBS-T for 1.5 h. The cells were then incubated with primary antibodies in 3% BSA/TBS-T to cleaved (active) caspase 3 (R&D Systems, United States) or γH2A-X (Cell Signaling, United States) overnight at 4°C. The cells were then washed, treated with secondary goat antirabbit antibodies conjugated with AlexaFluor 488 (Invitrogen, United States), and mounted into ProLong Gold reagent (Invitrogen, United States) with DAPI to visualize nuclei. Images were captured with a Leica TCS SL confocal microscope. Fluorescence intensity was measured with ImageJ software. The cell number with γ-H2A-X foci and foci number per cell were counted with a Leica TCS SL confocal microscope (100 cells were counted).

RESULTS

Wild-type and Wip1−/− mice were UV-B irradiated three times with 24-h intervals to study Wip1 response. Before irradiation, hair on the back was

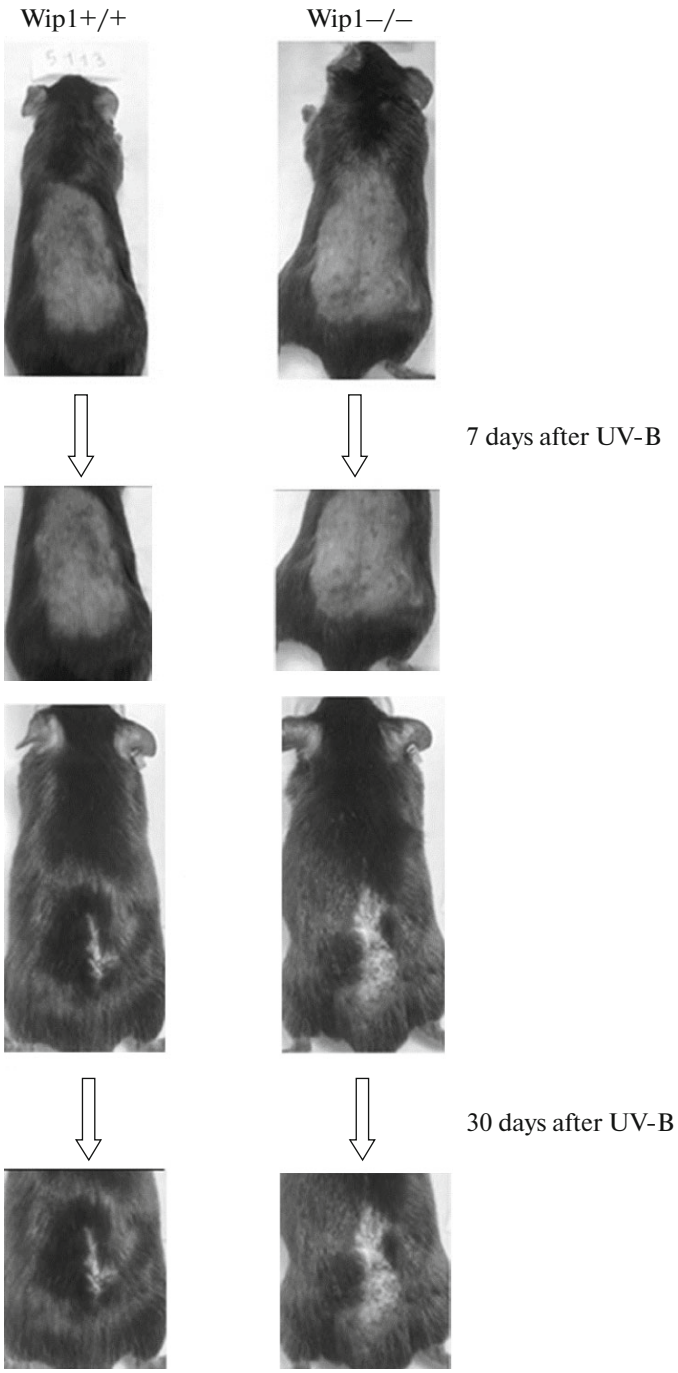

Fig. 1. Skin hair recovery in 3-month-old wild-type (Wip1+/+) and phosphatase gene Wip1 knockout (Wip1–/–) mice 7 and 30 days after UV-B exposure (5000 J/m^2).

removed. The UV dose was 5000 J/m^2. Skin state and hair recovery were evaluated every 2 days. We found that primary UV response was typical for the dose that we used and did not differ in wild-type and Wip1–/– mice. Irradiation caused hyperkeratosis, hyperplasia of the epidermis basal layer, and signs of inflammation. On the 30th day after irradiation, skin hair was recovered in wild-type mice, whereas this process was delayed in *Ppm1d* knockout animals (Fig. 1). Rapid recovery of hair growth is an indication of normal functioning of repair processes in cells.

Wip1 role in cell response on UV-B irradiation of wild type and *Ppm1d* knockout mice was studied in keratinocytes irradiated with UV-B dose (500 J/m^2). Cell viability was assayed with the MTT-test. It was

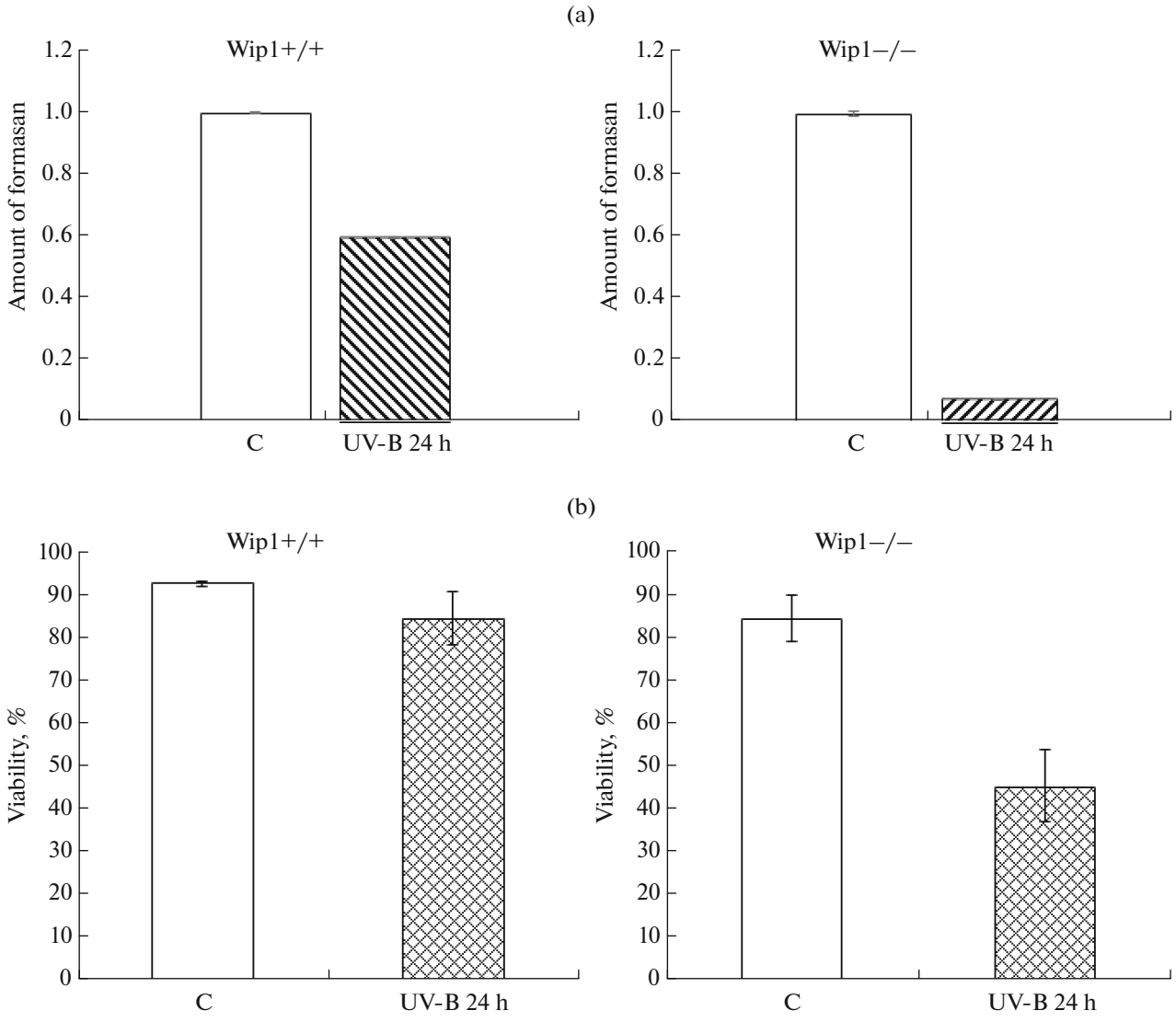

Fig. 2. Viability of (a) keratinocytes and (b) embryonic fibroblasts of wild-type (Wip1+/+) and phosphatase gene Wip1 knockout (Wip1−/−) 24 h after UV-B exposure (500 J/m^2). C—control. (a) Formasan amount (MTT-test); (b) viable cell number (not stained with trypan blue).

found that keratinocytes with knockout for Wip1 gene responded to UV irradiation by a drastically reduced viability relative to wild-type cells (Fig. 2a). The MTT-test showed that the substrate conversion into formasan after irradiation halved in wild-type cells and declined tenfold in knockout cells. It should be noted that Wip1−/− fibroblasts (mesenchymal cells) were also more sensitive to irradiation than wild-type cells (Fig. 2b).

Phosphorylation of histone H2A-X, which is a target for Wip1 phosphatase, marks the sites of DNA breaks and is required for initiation of the cell response to UV irradiation, such as cell cycle arrest, repair and cell fate decision: survive or trigger a mechanism of programmed cell death (Cha et al., 2010; Moon et al., 2010). We assessed accumulation of H2A-X foci (γH2A-X) in cells after irradiation. A large number of nonirradiated Wip1−/− cells have γH2A-X foci. The average number of these foci in nuclei of these cells is four times higher than in wild-type cells (Fig. 3). The cell number with foci increased up to 90% after irradiation in both wild-type and knockout cells. The foci number in keratinocytes was about 18 per cell, which was three times more than in wild-type cells. Wip1 is a regulator of molecules involved in the response on DNA damage (H2A-X, as well as ATM, p53, Chk1 and 2), and its absence deregulates the response on UV-irradiation. Increased signals from damaged

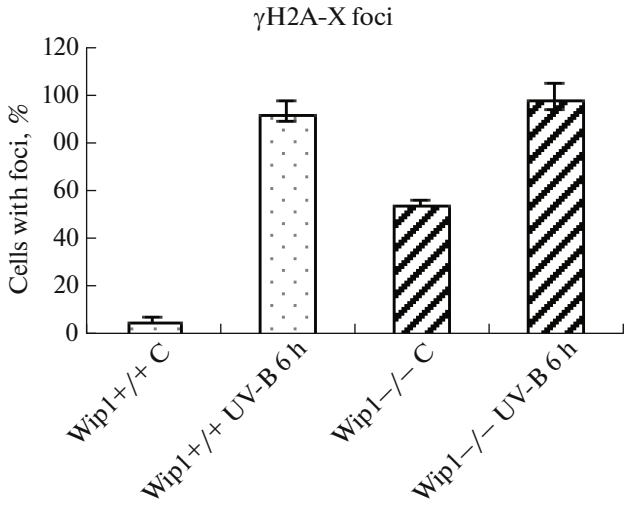

Fig. 3. Phosphorylated histone H2A-X foci (γH2A-X) in wild-type (Wip1+/+) and Wip1 knockout (Wip1−/−) keratinocytes 6 h after UV-B exposure (500 J/m^2). C—control, nonirradiated cells. γH2A-X foci were detected with immunofluorescence.

DNA in keratinocytes mediated with phosphorylated H2A-X may result in cell death rather than in repair. The mechanism of the cell death is probably apoptosis. Accumulation of active caspase 3, which is the final executioner of apoptosis, is twice as great in knockout cells than in control (Fig. 4). Nevertheless, the mechanism of Wip1−/− death after UV-B irradiation requires further investigation.

Thus, keratinocyte irradiation causes DNA damage. The Viability of cells with Wip1 knockout after irradiation is less than in wild-type ones. It is probably that Wip1 activity promotes resistance of skin cells to UV-B irradiation.

DISCUSSION

In this work, we demonstrated that the lack of serine/threonine phosphatase Wip1 in mouse tissues delayed repair processes in the mouse skin subjected to UV irradiation. One of the factors that explain this response in Wip1−/− mice is increased skin sensitivity to UV exposure. We found that a remarkably decreased viability was observed as a UV response in both keratinocytes and fibroblasts isolated from Wip1−/− mice. The level of active caspase 3 in these cells was increased. It should be noted that many knockout cells without irradiation exhibit γH2A-X foci in nuclei. The cell number with these foci increased after irradiation of both knockout and wild-type cells, but the number of γH2A-X foci was higher in nuclei of Wip1−/− keratinocytes. In increase in the histone phosphorylated form γH2A-X in Wip1−/− keratinocytes after UV exposure has been documented with Western-blotting (Lee et al., 2014). Wip1 implication in later stages of UV response requires further experiments.

In wild-type cells, the repair system provides survival of cells after their exposure to stress. Wip1 gene deficiency apparently enhances the signal from damaged DNA, as well as disturbing the regulation of repair systems that decreases the viability of these cells after irradiation more than in wild-type cells.

It is suggested that modified UV sensitivity of keratinocytes (MTT-test) stems not only from increased cell death, but also from inhibited proliferation, for example. Blockage of Wip1 activity (as a regulator of phosphorylation of H2A-X histone) is important both for repair system and cell cycle regulation. Regulation of the cell cycle is essential for cells with UV-damaged DNA to prevent cell proliferation with DNA damage. The cell may respond to irradiation with senescence, a program inhibiting cell proliferation. It has been demonstrated recently that Wip1 is involved in the negative regulation of cell senescence in mouse embryonic fibroblasts with Wip1 knockout (Sakai et al., 2014). Wip1 may prevent the development of senescence phenotype by regulation of DNA damage response (Sakai et al., 2014), as well as dephosphorylation of p38 kinase (Zubova et al., 2007). Supposedly, Wip1 is a certain link between DNA damage response and regulation of the cell cycle, as well as cellular senescence.

The implication of Wip1 in the late stages of UV response needs further investigation. We have shown that, in mouse skin and other tissues, Wip1 is an

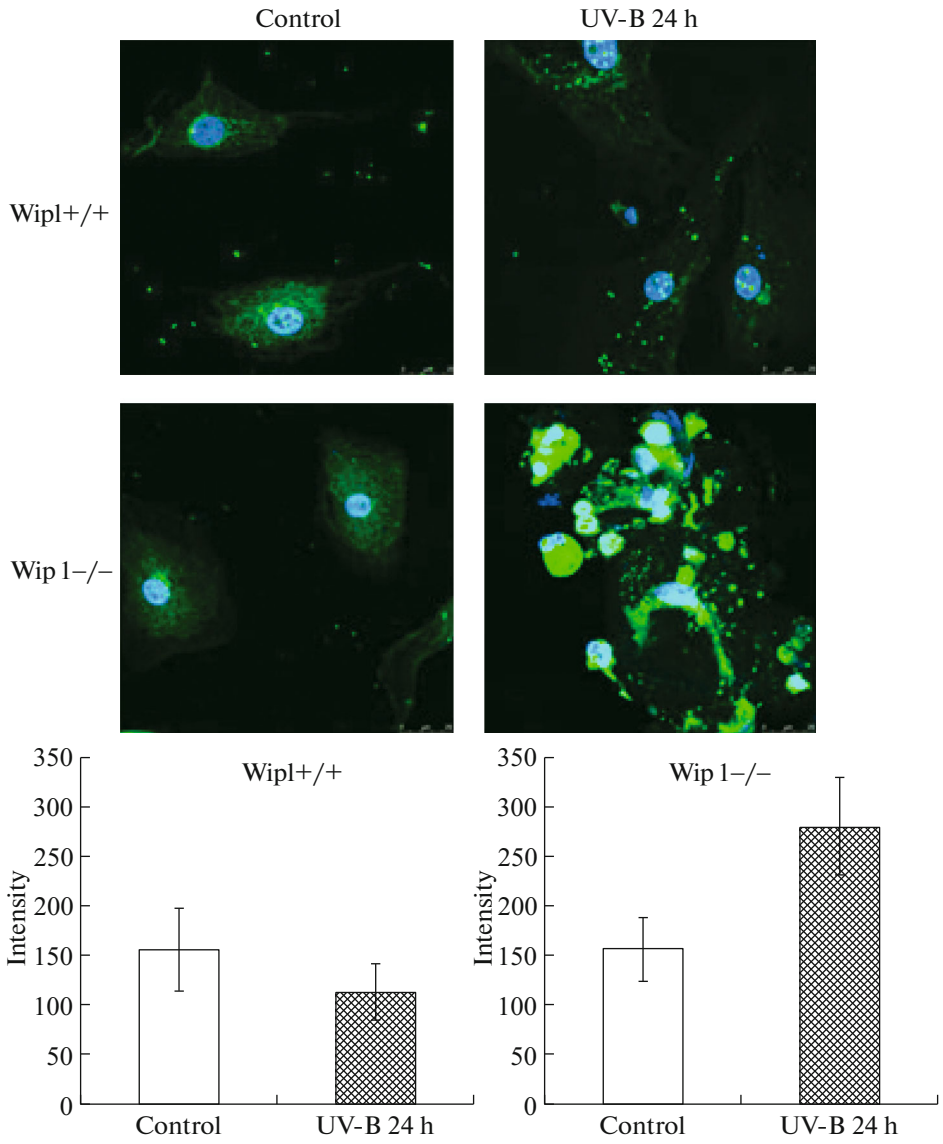

Fig. 4. Caspase 3 activity in wild-type (Wip1+/+) and Wip1 knockout (Wip1–/–) keratinocytes 6 h after UV-B exposure (500 J/m^2). C—control. (a) Immunofluorescence with antibodies to active caspase 3; ob. 100×. (b) Quantification of fluorescence intensity with ImageJ software.

important regulator of cell response to DNA damage required for normal DNA repair.

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