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NDR1 modulates the UV-induced DNA-damage checkpoint and nucleotide excision repair

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ABSTRACT

Nucleotide excision repair (NER) is the sole mechanism of UV-induced DNA lesion repair in mammals. A single round of NER requires multiple components including seven core NER factors, xeroderma pigmentosum A–G (XPA–XPG), and many auxiliary effector proteins including ATR serine/threonine kinase. The XPA protein helps to verify DNA damage and thus plays a rate-limiting role in NER. Hence, the regulation of XPA is important for the entire NER kinetic. We found that NDR1, a novel XPA-interacting protein, modulates NER by modulating the UV-induced DNA-damage checkpoint. In quiescent cells, NDR1 localized mainly in the cytoplasm. After UV irradiation, NDR1 accumulated in the nucleus. The siRNA knockdown of NDR1 delayed the repair of UV-induced cyclobutane pyrimidine dimers in both normal cells and cancer cells. It did not, however, alter the expression levels or the chromatin association levels of the core NER factors following UV irradiation. Instead, the NDR1-depleted cells displayed reduced activity of ATR for some set of its substrates including CHK1 and p53, suggesting that NDR1 modulates NER indirectly via the ATR pathway.

1. Introduction

Genome integrity is essential to organismal survival and is controlled by the DNA damage response (DDR), which detects DNA lesions and promotes their repair [1,2]. Defects in the DDR mechanisms generally increase sensitivity to DNA-damaging agents and cause diseases including cancer [3,4]. Nucleotide excision repair (NER) is the primary pathway that removes the helix-distorting DNA strand damage induced by ultraviolet (UV) irradiation or chemical carcinogens [5]. Genetic defects in NER lead to severe disorders such as xeroderma pigmentosum (XP), which is associated with a predisposition to skin cancer as well as to other somatic cancers [6]. The regulation of NER is an attractive avenue to prevent the detrimental consequences of impaired NER. The XP A–G (XPA–XPG) proteins are the core factors involved in the NER pathway. The NER system consists of a series of reactions requiring the XP proteins, including lesion recognition and verification and dual incision followed by DNA re-synthesis and ligation [7]. While the core NER proteins that carry out the damage recognition, excision, and repair reactions have been identified and extensively characterized, the regulatory pathways that govern the NER threshold levels have not been fully elucidated.

Damage verification, performed mainly by XPA, is the key regulatory step in the NER process [8]. The steady-state level of XPA is mainly controlled by the circadian clock [9], HERC2 ubiquitin ligase [10], and ATR kinase [11]. Clock-controlled, high-amplitude oscillations of XPA mRNA and protein levels result in daily oscillations of NER activity in various mouse organs [9,10]. As a complex system comprising a transcriptional activator (Clock/Bmal1) and repressor (Cry/Per), the circadian clock generates a circadian rhythm of XPA gene expression, whereas HERC2 functions as an E3 ubiquitin ligase, targeting XPA for degradation in a proteasome-dependent manner [10]. The half-life of the XPA protein is approximately 4 h in the absence of DNA damage, whereas it is much longer in the presence of DNA damage [10]. Upon UV-induced DNA damage, ATR kinase phosphorylates XPA [12], stabilizing XPA by preventing its association with HERC2 [11]. Hence, ATR activity in response to UV-induced DNA damage can be utilized to a certain extent as a surrogate marker for NER activity.

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Members of the nuclear-Dbf2-related (NDR) family of Ser/Thr kinases are highly conserved from yeasts to humans. Humans express four related NDR kinases: NDR1 (alternatively STK38), NDR2 (or STK38L), LATS1 (large tumor suppressor-1), and LATS2 [13]. The NDR kinases function in processes intimately linked to cell cycle regulation, including centrosome duplication, apoptosis, and the alignment of mitotic chromosomes, although most biological NDR substrates remain to be identified [13].

We used the immunoprecipitation of XPA followed by mass spectrometry to show that NDR1 interacts with XPA. In the absence of DNA damage, NDR1 mainly localized in the cytoplasm, while XPA presented exclusively in the nucleus. Upon UV irradiation, NDR1 colocalized with XPA in the nucleus. The depletion of NDR1 caused delayed cyclobutane pyrimidine dimer (CPD) repair but did not affect 6-4 photoproduct (6–4 PP) repair. The activity of ATR, postulated from the phosphorylation levels of ATR substrate proteins including p53 and CHK1, was significantly reduced in the NDR1-depleted cells. We concluded that NDR1 is an effector protein in the NER pathway, modulating ATR activity in response to UV damage.

2. Materials and methods

2.1. Cell culture, reagents, and treatments

Human lung carcinoma (A549) and primary dermal fibroblast (PDF) cells (American Type Culture Collection, Manassas, VA, USA) were cultured according to the manufacturer's instructions. X-tremeGENE reagent (Roche, Grenzach-Wyhlen, Germany) was used to transfect Flag-tagged XPA plasmids or siRNAs according to the manufacturer's directions. ON-TARGET plus SMARTpool siRNA duplexes [XPA (L-005067-00-0050), NDR1 (L-004674-00-0050), and Cyclophilin-B (D-001820-01-20) as a control] were purchased from Dharmacon (Lafayette, CO, USA). For UV-C irradiation, confluent cells were exposed to the prescribed dose using a germicidal lamp emitting primarily UV-C light. A UV-C sensor (UV Products, Upland, CA, USA) was used to calibrate the fluence rate of the incident light. For immunofluorescence staining, cells were grown on a glass cover slip coated with poly-D-lysine and laminin (BD Biosciences, San Jose, CA, USA) and either left untreated or treated with UV irradiation before fixation. If necessary, the cells were treated with 15 ng/ml leptomycin B (Sigma, St. Louis, MO, USA), a nuclear export inhibitor, 1 h before UV irradiation. The CellTiter-Fluor Cell Viability Assay kit (Promega, Madison, WI, USA) was used to assess cell viability according to the manufacturer's protocol.

2.2. Subcellular fractionation

Whole-cell lysates and cytoplasmic and nucleoplasmic extracts were isolated as described previously [14]. A chromatin-enriched fraction was prepared using the pellet obtained following nucleoplasm extraction by treatment with 10 U DNase I (Promega) and 100 U micrococcal nuclease (Promega) for 30 min at 30 °C. The combined protein lysates obtained before the elution of the chromatin fraction were considered soluble fractions. All buffers were supplemented with phosphatase inhibitor cocktail (Sigma) and protease inhibitor mixture (Sigma) unless indicated.

2.3. Immunoslot blotting of UV-induced DNA lesions

Genomic DNA was obtained using a QIAamp DNA Mini Kit (Qiagen, Hilden, Germany), and 100 μg (for CPD) or 500 μg was vacuum-transferred to a nitrocellulose membrane using a BioDot SF Microfiltration apparatus (BioRad, Hercules, CA, USA). The DNA was crosslinked to the membrane by incubation at 80 °C for 2 h under vacuum. Monoclonal antibodies that recognize CPD (Kamiya, Seattle, WA, USA) and 6–4 PP (Cosmo Bio, Tokyo, Japan) were used to detect DNA lesions.

2.4. Immunoblotting and immunoprecipitation

Conventional immunoblotting was used to determine protein levels. The antibodies included those against XPA (Kamiya); XPB–XPD, CHK1, and ATR (Santa Cruz Biotechnology, Santa Cruz, CA, USA); XPE, p-p53 S15, p-CHK1 S345, and GAPDH (Cell Signaling Technology, Danvers, MA, USA); NDR1, XPF, and XPG (Abcam, Cambridge, UK); p-MCM2 S108, and p-RPA2 S33 (Bethyl Laboratory, Montgomery, TX, USA); and p-H2AX S139 (Millipore, Billerica, MA, USA). For XPA or NDR1 immunoprecipitation, 1 mg whole-cell lysate was incubated with 1 μg anti-XPA or anti-NDR1 conjugated to Protein A/G-agarose beads (Sigma) for 12 h at 4 °C with rotation. After washing with lysis buffer, the proteins were eluted from the beads by boiling in SDS sample buffer and resolved on 10% SDS-PAGE gels followed by immunoblotting with XPA or NDR1 antibody. Mass spectrometry was performed by the Korea Research Institute of Bioscience & Biotechnology Mass Spectrometry Core Facility.

2.5. Immunofluorescence

Immunofluorescence was performed as described previously [15]. Briefly, cells grown on cover slips were fixed in 4% (w/v) paraformaldehyde in phosphate buffered saline (PBS) for 20 min at room temperature. The cells were then washed three times with PBS containing 0.1% Triton X-100 (PBST), permeabilized in 0.5% (v/v) NP-40 in PBS for 10 min, washed once in PBST, and blocked with 3% (w/v) bovine serum albumin in PBS for 30 min. The primary antibodies were incubated for 1 h at room temperature and then washed three times with PBST. After incubation with the appropriate secondary antibody, the cover slip was washed three times and embedded in Vectashield (Vector Labs, Burlingame, CA). Images were captured using a fluorescence microscope (Nikon, Japan).

2.6. λ-Phosphatase assay

10 μg of chromatin lysates prepared without phosphatase inhibitor were incubated with either 100 units of λ-phosphatase (New England Biolabs, Ipswich, MA) alone or together with phosphatase inhibitor cocktail (Sigma) in a 25 μl phosphatase reaction buffer provided by the manufacturer for 10 min at 37 °C. SDS-PAGE was then preformed on all samples, followed by immunoblot analysis using anti-XPA and anti-pRPA2 S33 antibodies.

3. Results

In a previous study, we found that HERC2 binds specifically to XPA and ubiquitinates XPA for proteolysis [10]. We also identified many XPA binding proteins including replication protein A (RPA), which is a well-known XPA-interacting partner [16], and NDR1, which has not been reported in the process of DNA repair (Fig. 1A). In order to confirm the physical interaction between XPA and NDR1, we performed an immunoprecipitation analysis. The XPA coprecipitated with NDR1 and *vice versa* (Fig. 1B). Next, we analyzed the subcellular localization of the two proteins using immunofluorescence. In the absence of DNA damage, NDR1 and XPA localized mostly in the cytoplasm and nucleus, respectively (Fig. 1C). Upon UV irradiation, the two proteins colocalized in the nucleus. Because NDR1 is known as a cytoplasmic-nuclear

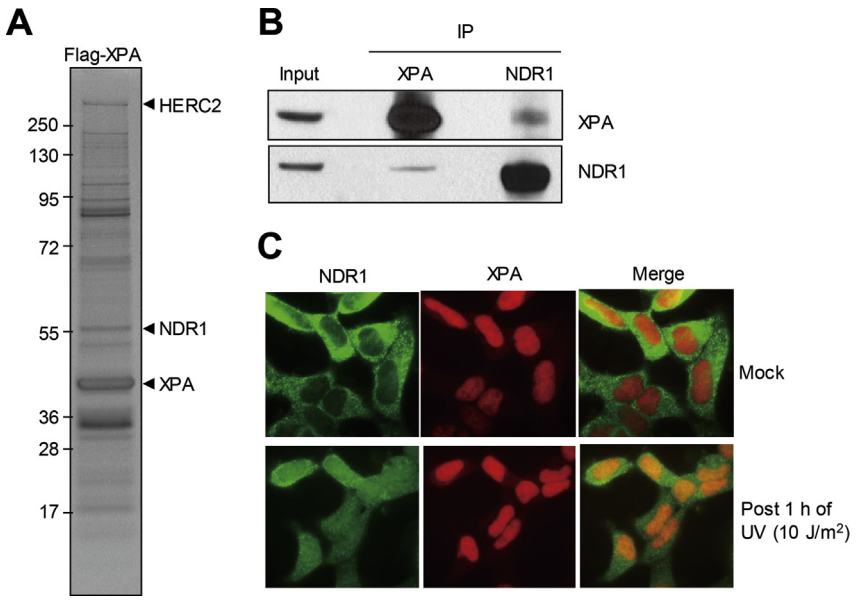

Fig. 1. Interaction of XPA and NDR1 in the nucleus. (A) Lysates from HEK293 cells stably expressing Flag-tagged XPA were mixed with anti-Flag agarose beads, and the bound proteins were precipitated. After vigorous washing, the bound proteins were visualized by SDS-PAGE and coomassie blue staining. The XPA-interacting proteins were identified by mass spectrometry. (B) Endogenous XPA or NDR1 was immunoprecipitated from A549 cell lysates followed by immunoblotting with the indicated antibodies. The input lanes contain 5% of the input protein. (C) Untreated or UV-treated A549 cells were immunostained with XPA and NDR1 antibodies and imaged using a fluorescent microscope.

shuttle protein, we hypothesized that DNA damage prevented the cytoplasmic export of NDR1, resulting in the accumulation of NDR1 in the nucleus.

Given that XPA is a core NER factor, and NDR1 interacts with XPA, we asked whether or not NDR1 has a role in the NER process. We employed siNDR1, an NDR1-specific siRNA, to decrease the NDR1 protein levels in A549 cells and human PDF cells. The siNDR1 treatment significantly reduced the NDR1 protein levels but did not change the nonspecific protein (designated NS) and GAPDH loading control levels (Fig. 2A). CPD and 6–4 PP are the two major lesions caused by UV radiation. Immunoslot blotting revealed that the NDR1 depletion reduced CPD repair (Fig. 2C, D) slightly in the A549 cells and significantly in the PDF cells, but it did not affect 6–4 PP repair (Fig. 2B). Because the NDR1 knockdown was more robust in

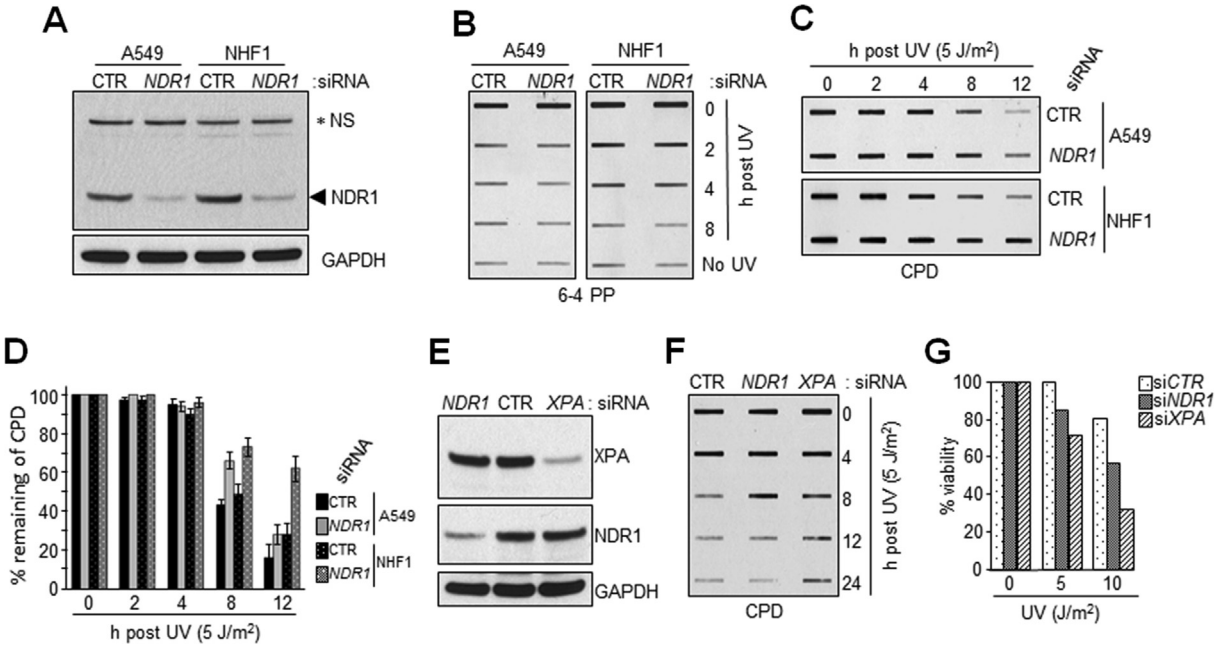

Fig. 2. Modulation of NER activity by NDR1. (A) Lysates from A549 and PDF cells transfected with the indicated siRNAs were immunoblotted with NDR1 antibody. GAPDH was used as a loading control. NS indicates nonspecific bands appearing in the NDR1 blot. (B, C) Cells treated with the siRNAs before UV irradiation (5 J/m^2) were allowed to carry out repair for the indicated times. Genomic DNA was then isolated and subjected to immunoslot blotting to detect residual 6–4 PP (B) or CPD (C). (D) Quantitative analysis of the data from C. (E) Lysates from PDF cells transfected with the siRNAs were immunoblotted with the indicated antibodies. GAPDH was used as a loading control. (F) Cells treated with the siRNAs before UV irradiation (5 J/m^2) were allowed to carry out repair for the indicated times. Genomic DNA was then isolated and subjected to immunoslot blotting analysis to detect residual CPD. (G) Cell viability after 24 h post-UV irradiation was assessed by fluorescence-based cell viability assay.

the PDF cells than in the A549 cells, all subsequent experiments were performed using PDF cells.

We hypothesized that if NDR1 is linked directly with XPA in the NER process, the NDR1 knockdown effect would be similar to an XPA knockdown effect. When the CPD repair kinetics of cells treated with siNDR1 were compared to those of cells treated with siXPA, the repair rate was faster (Fig. 2F) and the difference in cellular viability was marginal (Fig. 2G). We therefore concluded that NDR1 might be an auxiliary factor that affects NER kinetics via XPA.

In response to DNA damage, the stability of the XPA protein can be enhanced by ATR-mediated phosphorylation, which prohibits interaction between XPA and HERC2. In agreement with a previous report, XPA levels were not changed during the recovery period after UV irradiation, whereas the phospho-p53 levels were increased (Fig. 3A). In addition, NDR1 levels were not altered during the recovery period (Fig. 3A). The core NER factors (XPA–XPG) are supposed to be recruited immediately to DNA lesions. To determine if NDR1 affects the chromatin recruitment of NER factors, we biochemically fractionated cell lysates into soluble and chromatin portions. Thirty minutes after UV irradiation, all of the NER factors were recruited to the chromatin, and 5 h later, they started to disappear from the chromatin of cells treated with control siRNA (Fig. 3B). NDR1 did not move to the chromatin upon UV damage (Fig. 3B). Compared with the control cells, the NDR1-depleted cells showed more chromatin retention of XPA and less chromatin retention of XPG 5 h after UV irradiation. In this study the immunoblotting of XPA with total lysate showed a clear single band whereas the lysate obtained by means of subceulluar fractionation showed another XPA band (~35 kDa) below the authentic XPA band (~40 kDa), possibly due to the phosphorylation of XPA upon DNA damage condition as reportedly [12]. However, this newly appeared XPA band in chromatin fraction at 30 min post UV irradiation did not alter by λ phosphatase treatment while the phosphorylated RPA2 was completely disappeared by the phosphatase treatment (Fig. 3C), suggesting that at least the two XPA bands are unrelated to the phosphorylation status of XPA. It is also conceivable that the occurrence of multiband of XPA during immunblotting is probably due to the intrinsic property of XPA as an intrinsically unstructured protein as characterized previously [17] for binding of various types of DNA damage. After damage verification, the XPA was removed from the lesion to recruit the XPF and XPG endonucleases. Based on those results, NDR1 appears to regulate the removal of XPA from the chromatin and the subsequent chromatin loading of XPG without directly associating with the chromatin.

For insight into the NDR1-mediated NER modulation, we analyzed the phosphorylation status of target proteins of ATR, the master cell cycle-checkpoint kinase, which is required to arrest the cell cycle to secure time for the DNA repair. The phosphorylation levels of most of the ATR substrate proteins, including CHK1, p53, and H2AX, were reduced in the NDR1-depleted cells (Fig. 4A), a result which was further confirmed by immunofluorescence staining with phospho-H2AX and phospho-CHK1 (Fig. 4B). However, the other ATR substrate, MCM2, merely altered by NDR1 expression. Since MCM2 is a critical regulator for origin firing during S phase, the NDR1-mediated ATR regulation may operate during other cell cycles such as G1 or G2. Alternatively, NDR1 is not involved in the ATR-mediated regulation of origin firing during DNA damage response. It was reported previously that NDR1 can be retained in the nucleus following treatment with the nuclear export inhibitor leptomycin B. Leptomycin B did not intensify the UV-induced phospho-CHK1 levels (Fig. 4C), however, suggesting that the levels of NDR1 retained in the nucleus were already sufficient to activate ATR kinase.

4. Discussion

The myriad genome protection mechanisms comprised by the DDR counteract the deleterious effects of DNA damage and preserve genome stability. The primary role of the DDR is the activation of cell cycle checkpoints to arrest cell cycle progression and thereby boost distinct repair pathways, depending on the type of DNA damage [18]. Upon UV-induced DNA damage, the ATR pathway coordinates a series of DDR mechanisms such as the activation of CHK1 and the modulation of NER activity [19]. ATR kinase was recently proposed to directly promote NER activity by phosphorylating XPA at serine 196 (S196). The phosphorylation of XPA appears to have an important role in mediating NER processes. A phospho-deficient XPA mutant (S196A) exhibited greater UV sensitivity compared with the wildtype, while the phospho-mimic form (S196D) showed tolerance to UV damage [12]. In addition, the down regulation of ATR in the presence of UV damage resulted in the destabilization of XPA, and S196A expressed in XPA cells compromised NER activity [11]. The molecular mechanisms by which ATR activity is modulated during the UV response require more clarity, however.

We found that NDR1 modulates NER activity by regulating the ATR-mediated DDR pathway. NDR1 is normally localized in the cytoplasm, but it was retained in the nucleus after UV irradiation.

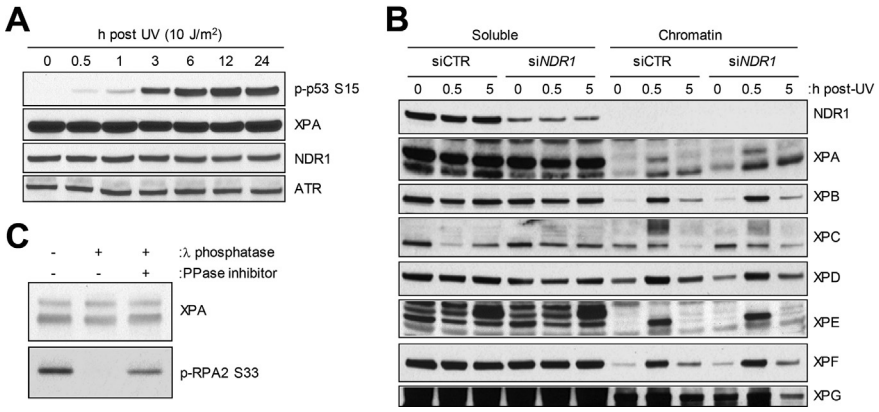

Fig. 3. NDR1 partly modulates the removal of XPA from chromatin. (A) Levels of the indicated proteins after UV damage were assessed by immunoblotting. (B) Chromatin association and dissociation of core NER factors were analyzed from lysates fractionated into soluble and chromatin fractions by immunoblotting. (C) The chromatin lysates obtained from cells transfected with the control siRNA and allowed 30 min for recovery after UV-irradiated were treated with either λ phosphatase alone or together with phosphatase (PPase) inhibitor to analyze the property of XPA bands.

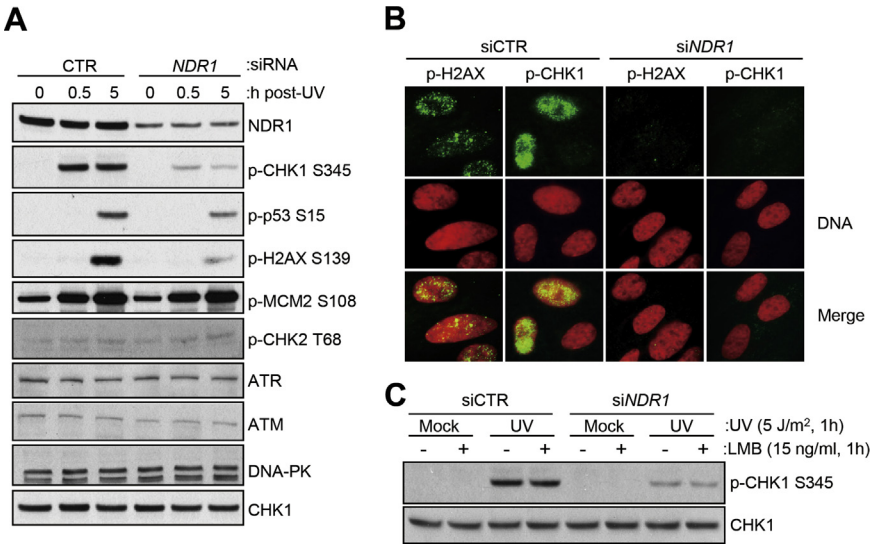

Fig. 4. Modulation of ATR activity by NDR1. (A) Cells transfected with the indicated siRNAs were exposed to UV and allowed to recover for the indicated times. Protein levels in the cell lysates were analyzed by immunoblotting using the indicated antibodies. (B) At 5 h post-UV, the cells transfected with the siRNAs were fixed and immunostained for phospho-H2AX and phospho-CHK1. The DNA was counterstained with Hoechst dye. (C) PDF cells transfected with the siRNAs were treated with leptomycin B for 1 h before UV irradiation and subsequently incubated for 5 h before harvest. The cell lysates were analyzed by immunoblotting with phosphor-CHK1 and CHK1 antibodies.

NDR1 was previously characterized as a cell cycle regulator, and we hypothesized that NDR1 functions as a transducer of DNA damage signals to ATR. UV irradiation mainly produces two types of DNA lesions, CPD and 6–4 PP. The structural distortion of the DNA helix caused by 6–4 PP lesions is more drastic than that caused by CPD lesions. Hence, 6–4 PP lesions are a more favorable substrate for NER than CPD lesions [20]. We found that NDR1 affects the repair of CPD lesions but not that of 6–4 PP lesions. Follow-up studies to understand how NDR1 discriminates between the different types of DNA lesions will shed light on the sophisticated modulation of ATR activity during DDR and the crosstalk between cell cycle checkpoints and DNA repair pathways.

In the last 20 years, most of the core NER machinery has been elaborately described, shifting attention to the mechanisms that facilitate NER in a spatiotemporal context and to cooperative interactions between NER and other DDR pathways. While increased NER activity protects the genome against the accumulation of DNA lesions, thereby maintaining genome integrity, it might be beneficial to reduce the NER capacity in patients with cancer who are undergoing chemotherapy, because doing so might help ensure the efficient action of DNA damage-inducing drugs such as cisplatin. In that regard, we expect that the transient suppression of NER through the pharmacological manipulation of NER core factors or regulatory pathways will synergize with DNA-damaging agents to optimize chemotherapeutic outcomes.

Conflict of interest

The authors declare no conflicts of interest.

Acknowledgments

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Transparency document

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