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Uhrf2 is important for DNA damage response in vascular smooth muscle cells

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ABSTRACT

Emerging evidence shows that Uhrf1 plays an important role in DNA damage response for maintaining genomic stability. Interestingly, Uhrf1 has a paralog Uhrf2 in mammals. Uhrf1 and Uhrf2 share similar domain architectures. However, the role of Uhrf2 in DNA damage response has not been studied yet. During the analysis of the expression level of Uhrf2 in different tissues, we found that Uhrf2 is highly expressed in aorta and aortic vascular smooth muscle cells. Thus, we studied the role of Uhrf2 in DNA damage response in aortic vascular smooth muscle cells. Using laser microirradiation, we found that like Uhrf1, Uhrf2 was recruited to the sites of DNA damage. We dissected the functional domains of Uhrf2 and found that the TTD, PHD and SRA domains are important for the relocation of Uhrf2 to the sites of DNA damage. Moreover, depletion of Uhrf2 suppressed DNA damage-induced H2AX phosphorylation and DNA damage repair. Taken together, our results demonstrate the function of Uhrf2 in DNA damage response.

1. Introduction

The Uhrf family proteins including Uhrf1 and Uhrf2 are multi-functional domain containing proteins. Both Uhrf1 and Uhrf2 have an ubiquitin-like domain (UBL), a tandem Tudor domain (TTD), a plant homeodomain (PHD), a SET and Ring associated (SRA) domain and a really interesting new gene (Ring) domain [1]. The multiple functional domains indicate the complicated biological functions of Uhrf1 and Uhrf2. Among these two proteins, Uhrf1, a 793 amino acid nuclear polypeptide, has been well studied. It has been shown that Uhrf1 plays a role for the maintenance of genomic DNA methylation and the high order of the chromatin through its TTD domain and SRA domain [2–6]. The SRA domain of Uhrf1 binds to hemi-methylated DNA and plays a crucial role in copying pre-existing methylation patterns onto newly replicated DNA by recruiting DNMT1 to replication sites [7–9]. The TTD domain mediates the binding of Uhrf1 to H3K9me3 and plays a role in maintaining this histone modification in heterochromatin [4,5]. In contrast, the PHD domain of Uhrf1 specifically binds to unmodified histone H3 and links Uhrf1 to regulation of euchromatic gene expression [10]. Because of the intriguing functions of Uhrf1 in the chromatin regulation, Uhrf1 is also examined in the DNA damage response in which active chromatin remodeling process protects genomic stability by facilitating DNA damage repair. Accumulated evidence suggests that Uhrf1 is critical for the maintenance of chromosome integrity critical in response to DNA double strand breaks [11–13]. For example, murine ES cells lacking Uhrf1 are hypersensitive to various DNA damage agents such as X-ray, UV, alkaline agents that induce DNA base damage and hydroxyurea that induces DNA replication stress [14]. Moreover, depletion of human Uhrf1 also results similar phenotypes [15].

Unlike Uhrf1, the function of Uhrf2 is largely unknown. Sequence analyses reveal that Uhrf2 has similar domain architecture with Uhrf1 [16–18]. Both the TTD and SRA domains of Uhrf2 also recognize the same epigenetic marks that are recognized by Uhrf1 [17]. Thus, it is likely that Uhrf2 may have similar biological functions of Uhrf1 but in different biological context or different physiological process. In this study, we focused on the function of Uhrf2 in DNA damage response. We found that unlike Uhrf1, Uhrf2 is highly expressed in aortic vascular smooth muscle cells and regulates DNA damage repair in aortic vascular smooth muscle cells.

2. Materials and methods

2.1. Antibodies and plasmids

N terminal of mouse Uhrf2 (residues 1–400) was expressed as GST-Uhrf2 to immunize rabbit and generate polyclonal antibody. Monoclonal anti-beta-actin (AC-15) and anti-Flag (M2) antibodies were purchased from Sigma. Anti-phospho-H2A.X monoclonal

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antibody (JBW301) was purchased from Upstate. Mouse Uhrf2 and deletion mutants were cloned into the SBP vector.

2.2. Cell cultures and transfection

Mouse vascular smooth cell line, MOVAS, was purchased from ATCC. Cells were grown in DMEM (Invitrogen) supplemented with 10% calf serum and 1% penicillin/streptomycin. Subconfluent smooth muscle cells were transfected with 4 μg plasmid DNA by using 30 μl Effectene and 24 μl Enhancer provided by the Effectene transfection kit (Qiagen). After 48 h, the cells were subjected to laser microirradiation or Western blotting.

2.3. Retroviral vectors construction, virus production, and infection

Retrovirus shRNA vectors targeting Uhrf2 (Uhrf2-shRNA) and Mock were constructed by inserting short hairpin RNA templates into pMSCV-neo-U6 [19]. The short hairpin RNA templates specifically targeting Uhrf2 were designed, synthesized and annealed as previously reported. The targeting sequence of Uhrf2-shRNA is 5′-CAAATATGCTCCAGAAGAA-3′, and the sequence of mock is 5′-AATAGTGTATACGGCATGC-3′. The Uhrf2-shRNA and mock were transfected into packaging 293T cell with two other helper packaging plasmids pMD-MLV-OGP (gag-pol) and pVSV-G (env). 48 h after transfection, cell culture medium was harvested, and the viral particles were concentrated by ultracentrifugation at 50,000*g* for 3 h and then resuspended in expansion medium. MOVAS cells were infected with retrovirus at a multiplicity of infection (MOI) of 10 for 4 h in the presence of 8 μg/ml polybrene. After infection, MOVAS cells were allowed to recover for 24 h and then selected in expansion medium with 800 μg/ml G418 (Sigma Aldrich) for 1 week and maintained in the medium with 400 μg/ml of G418.

2.4. Laser microirradiation, immunofluorescence staining and microscope image acquisition

For laser microirradiation, cells were grown on 35-mm glass bottom dishes (MatTek Corporation). Laser microirradiation was performed on OLYMPUS IX71 inverted fluorescence microscope with a Micropoint® Laser Illumination and Ablation System (Photonic Instruments). The laser output was set to 40%, which can reproducibly give a focused γH2AX stripe. The GPF strips were recorded at indicated time points and then analyzed with Image J software. For the time course analysis of laser microirradiation, samples were subjected to continuous microirradiation along certain paths within the indicated time interval. Then, the samples were subjected to immunofluorescence staining with indicated antibodies. For immunofluorescence staining, cells were fixed in 3% paraformaldehyde for 10 min and permeabilized with 0.5% Triton X-100 in phosphate-buffered saline (PBS) for 5 min at room temperature. Samples were blocked with 8% goat serum and then incubated with the primary antibody for 1 h. Samples were washed for three times and incubated with the secondary antibody for 30 min. The coverslips were mounted onto glass slides and visualized with OLYMPUS IX71 inverted fluorescence microscope. All the images were acquired with cellSens standard (Version 1.3) software under OLYMPUS IX71 inverted fluorescence microscope equipped with a UPlanSApo 60×/1.35 oil immersion objective at room temperature. Identical contrast and brightness adjustments were used on images for all given experiments.

2.5. Neutral comet assay

Single-cell gel electrophoretic comet assays were performed under neutral conditions [20]. Briefly, MOVAS cells transfected with Uhrf2-shRNA were irradiated with 20 Gy and incubated in culture medium at 37 °C for 4 h. For cellular lysis, the slides were immersed in neutral lysis solution (2% sarkosyl, 0.5 M EDTA, 0.5 mg/ml proteinase K, pH 8.0) overnight at 37 °C. On the second day, after electrophoresis at 15 V for 25 min (0.6 V/cm), the slides were stained for 20 min with 10 μg/ml propidium iodide and viewed in a fluorescence microscope. The comet tail moment was analyzed by CometScore software.

2.6. Protein extraction and Western blotting

Protein samples from the mouse tissues were extracted by using total protein extraction kit (Millipore, #2140). For the culture cells, the protein samples were extracted by NETN100 buffer (0.5% Nonidet P-40, 50 mM Tris–HCl pH 8.0, 2 mM EDTA, and 100 mM NaCl). Western blotting was performed following standard protocol as described anywhere else with indicated antibodies.

2.7. RT-PCR

The mRNA expression level of Uhrf2 was measured by RT-PCR as described anywhere else. Quantitative-PCR was performed using Power SYBR green PCR master mix in 7300 real time PCR systems (Applied Biosystems). The mean value was calculated by three independent experiments. The primers used in this experiment are listed as follows: β-actin-forward: 5′-ACTGTGCCCATCTAC GAGGGGTATG-3′, β-actin-reverse: 5′-CGTAGCACAGCTTCTCCTTAA TGTC-3′, mouse Uhrf2-forward: 5′-CAGCTGCTAGTTCGTCCAGACTCC-3′, mouse Uhrf2-reverse: 5′-ACCAAGGCCGACATCTCTGGCATCC-3′.

2.8. Statistical analysis

All the experiments were performed at least three times. Results were analyzed using unpaired two-tailed Student's *t* test and data expressed as mean ± s.d. *p* values less than 0.05 were considered statistically significant.

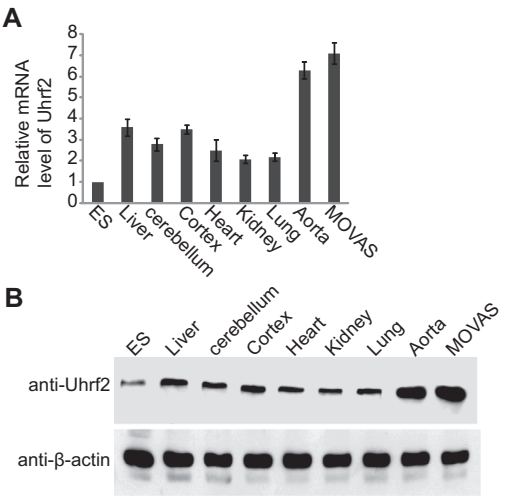

Fig. 1. The expression pattern of Uhrf2 in mouse tissues. The expression of Uhrf2 in somatic cells is higher than that in ES cells. Highest expression of Uhrf2 was observed in mouse aorta and mouse aorta-derived smooth muscle cell (MOVAS). The level of Uhrf2 mRNA was examined by RT-PCR and normalized to β-actin (A). Error bars indicate s.d. ($n = 3$). The protein level of Uhrf2 was examined by Western blotting (B). β-actin was used as the protein loading control.

3. Results and discussion

Previous studies demonstrated that unlike Uhrf1, Uhrf2 is mainly expressed in differentiated cells [17]. To study the biological function of Uhrf2, we further determined the expression of Uhrf2 in different mouse tissues. Consistent with previous reports, the expression level of Uhrf2 is higher in differentiated mouse tissues than that in ES cells. Interestingly, the highest expression of Uhrf2 was observed in aorta that mainly contains aortic vascular smooth muscle cells (Fig. 1). We further examined the level of Uhrf2 in the in vitro cultured mouse vascular smooth muscle cell (MOVAS) that was derived from mouse aorta. Again, we found the high expression level of Uhrf2 in MOVAS, similar to that in aorta (Fig. 1). Moreover, we could not detect both the RNA and protein level of Uhrf1 in aortic or MOVAS, which is consistent with previous report that Uhrf1 is mainly expressed in embryonic stem cells [17]. Thus, these results suggest that Uhfr2 is likely to replace Uhrf1 and play an important role in chromatin remodeling in MOVAS.

Previous studies showed that Uhrf1 is recruited to DNA damage sites and participates in DNA damage response [11]. Since Uhrf2 has similar domain architecture with Uhrf1 and is highly expressed in MOVAS, we asked whether Uhrf2 is involved in DNA damage response in MOVAS. We treated MOVAS with laser microirradiaion to induce DNA damage. The DNA damage sites were examined by immunefluorescence staining of γH2AX, a surrogate marker of the DNA damage sites. Interestingly, we found that Uhrf2 was colocalized with γH2AX following laser microirradiation, suggesting that Uhrf2 clearly relocates to the DNA damage sites. Moreover, Uhrf2 was retained at DNA damage sites for more than 20 min following laser microirradiation, suggesting that it may regulates DNA damage repair (Fig. 2A). Since Uhrf2 has 5 major functional domains that are associated with different biochemical activities of Uhfr2, we asked which domain of Uhrf2 mediates the relocation to the sites of DNA damage. We generated a series of Uhrf2 mutants to internally delete each of these functional domains and expressed these mutants in MOVAS (Fig. 2B). Like full length Uhrf2, deletion of the UBL domain and the Ring domain did not affect the relocation of Uhrf2 to the sites of DNA damage. In contrast, deletion the TTD, PHD or SRA domain abrogated the recruitment of Uhrf2 to the sites of DNA damage, suggesting that these three domains may function together to mediate the relocation of Uhrf2 (Fig. 2B). Previous studies suggest that the TTD, PHD and SRA domains of Uhrf1 recognize different epigenetic marks and are involved in chromatin remodeling [1]. In particular, the SRA domain of Uhfr1 binds methylated DNA and regulates DNA methylation

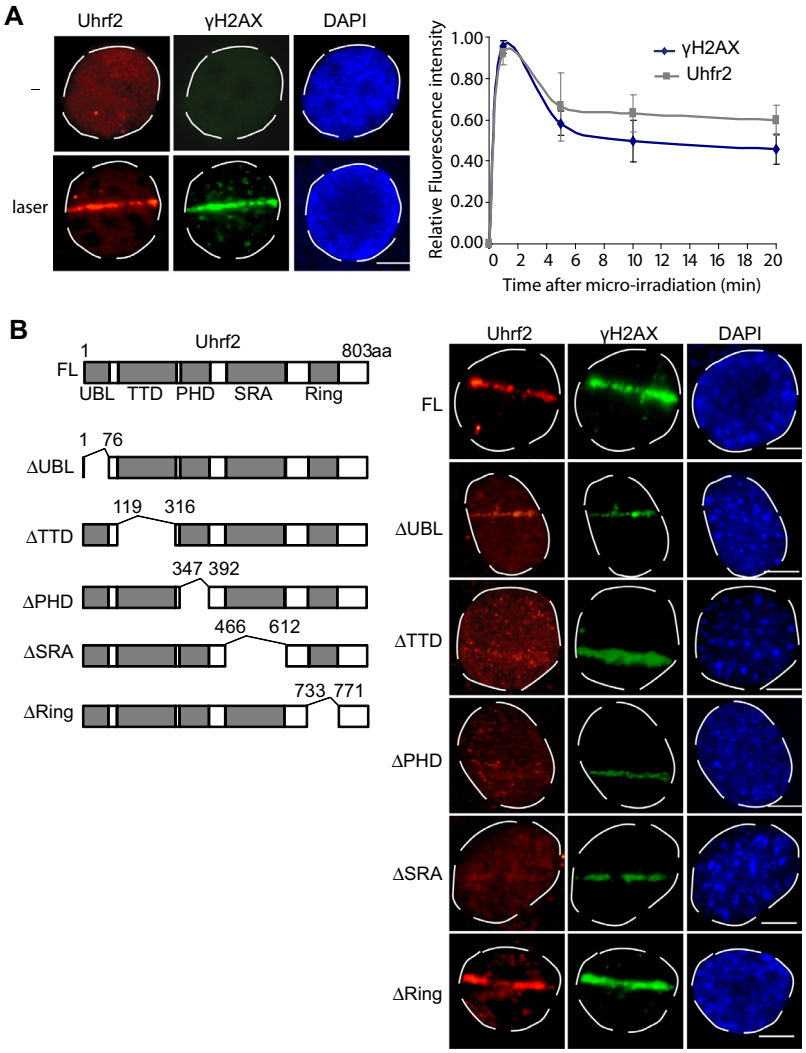

Fig. 2. Uhrf2 is recruited to the laser-induced DNA damage sites. (A) Immunofluorescence staining was performed in MOVAS with or without laser microirradiation. γH2AX was used as the marker of double-strand DNA breaks. Bar: 5 μm. (B) Schematic drawing shows the domain architecture of Uhrf2 and each internal deletion mutant. FL: full length; aa: amino acid. Deletion of the TTD, PHD or SRA domain abolished the recruitment of Uhrf2 to the sites of DNA damage. Bar: 5 μm.

[7,9]. Interestingly, activation of DNA methylation has been observed at the sites of DNA damage, suggesting that DNA methylation may play a key role in chromatin remodeling during DNA damage repair [21].

To study the biological function of Uhrf2-medicated chromatin remodeling in response to DNA damage, our study is focused on the phosphorylation of H2AX. H2AX is a variant of canonical histone H2A and evenly incorporated into the genome. Following DNA double strand breaks, H2AX close to the DNA damage sites is phosphorylated by a group of PI3-like kinases such as ATM, ATR and DNAPKcs at Ser 139 [22]. The phosphorylated H2AX, also named γH2AX, is not only a surrogate maker of DNA double strand breaks, but also stabilizes a groups of DNA damage repair factors at DNA damage sites and facilitates DNA damage repair [23]. Loss of H2AX impairs DNA damage repair and induces genomic instability [24,25]. Previous study shows that high order of chromatin regulates the phosphorylation of H2AX and DNA damage repair [26]. Thus, we wonder whether Uhrf2 also regulates the phosphorylation of H2AX and DNA damage repair. We first used shRNA to knock down the Uhrf2 expression in MOVAS cells (Fig. 3A). Then DNA double strand breaks (DSBs) were induced by 10 Gy of γ-irridiation in the Uhrf2 knock-down MOVAS or control cells. The immunofluorescence staining was used to detect the ionizing radiation-induced foci formation (IRIF) of γH2AX in the Uhrf2 knock-down MOVAS and control MOVAS. The IRIF of γH2AX was significantly decreased in the Uhrf2 knock-down MOVAS compared to that in the control MOVAS (Fig. 3B). Spontaneous foci of γH2AX also exist in the control cells because the spontaneous DNA damage may occur in every living cell. Endogenous genotoxic stress such as replication errors will induce spontaneous foci formation of γH2AX in control cells. Thus, to exclude the spontaneous foci of γH2AX and quantitatively measure the IR-induced foci formation of γH2AX, we considered cells with more than 10 γH2AX foci as γH2AX positive cells. Our results show that lacking Uhrf2 significantly reduced the IRIF of γH2AX (Fig. 3B). Since γH2AX plays a key role in DNA damage repair, we examined whether the role of Uhrf2 in DNA damage repair using the comet assay, a standard assay for measuring DNA breaks. With 20 Gy of IR treatment, unrepaired DNA fragments scored as the "comet tail" were observed in the Uhrf2 knock-down MOVAS. However, in the control MOVAS, DNA breaks induced by IR treatment were significantly repaired. Thus, the "comet tail" was significantly reduced (Fig. 3C).

To further confirm the function of Uhrf2 in DNA damage repair and exclude the off-target effect of shRNA treatment, we constructed the RNAi-resistant Uhrf2 expressing vector. Following knock-down of endogenous Uhrf2 in MOVAS, we expressed the RNAi-resistant full length Uhrf2 or each domain deletion mutant. After 10 Gy of IR treatment, the IRIF of γH2AX was examined by fluorescence staining. Only the full-length Uhrf2 and the ΔUBL mutant could rescue the IRIF of γH2AX (Fig. 4A). Moreover, the "comet tail" was significantly reduced by expressing the full-length Uhrf2 or the ΔUBL mutant but not other mutant, suggesting

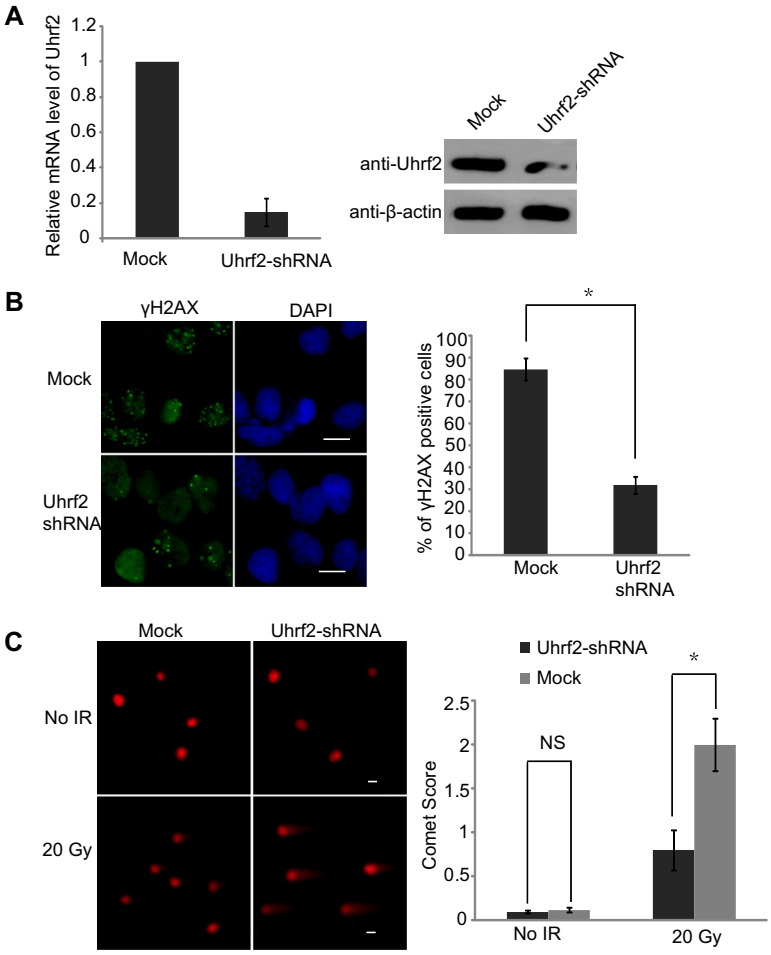

Fig. 3. Depletion of Uhrf2 impairs DNA damage repair in MOVAS. (A) RT-PCR and Western blotting results show the significant decrease of Uhrf2 in MOVAS by Uhrf2-shRNA. Error bars indicate s.d. ($n = 3$). (B) The IRIF of γH2AX is suppressed by knock-down of Uhrf2 in MOVAS. Uhrf2 knock-down cells and control cells were treated with 10 Gy of IR. Cells were fixed and examined by anti-γH2AX antibody. Bar: 10 μm. γH2AX foci positive cells were calculated. Results are averaged (±s.d.) from three independent experiments. *p Values <0.05. (C) Comet assays show that depletion of Uhrf2 impairs DNA damage repair in MOVAS. Representative images of neutral comet assays are shown. Bar: 10 μm. The moment of comet tail were quantitatively measured. *p Values <0.05; NS means no statistical significance.

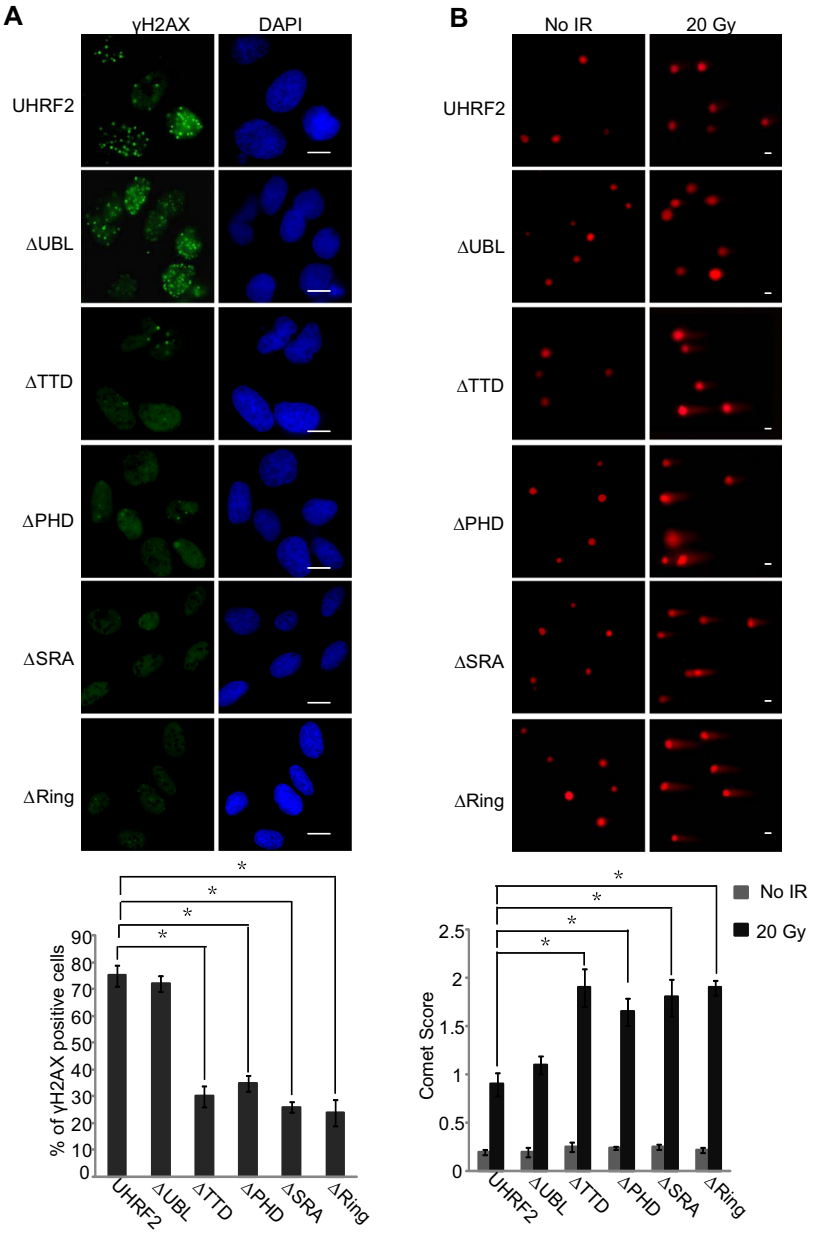

Fig. 4. Full length Uhrf2 rescues DNA damage repair in Uhrf2-depeleted MOVAS. (A) The IRIF of γH2AX is rescued by the full length Uhrf2 or the ΔUBL mutant but not by the other mutants. Bar: 10 μm. γH2AX foci positive cells were calculated. Results are averaged (±s.d.) from three independent experiments. *p Values <0.05. (B) Comet assays show that the full length of Uhrf2 or the ΔUBL mutant but not the other domain deletion mutants restores DNA repair in Uhrf2-depleted MOVAS. Representative images of neutral comet assays are shown. Bar: 10 μm. The moment of comet tail was quantitatively measured. *p Values <0.05.

that the full length Uhfr2 or the ΔUBL mutant but not other Uhrf2 mutants restores DNA damage repair in MOVAS (Fig. 4B). Thus, these results further support that Uhrf2 participates in DNA damage repair. It also indicates that the UBL domain may not be important for the Uhfr2-dependent DNA damage response, whereas other domains of Uhfr2 play important role. Since the TTD, PHD and SRA domains are required for the recruitment of Uhfr2 to DNA damage sites, loss of these domains affect the function of Uhfr2 in DNA damage repair. Besides the TTD, PHD and SRA domains, the Ring domain of Uhfr2 also plays an important role in DNA damage repair. Since the Ring domain of Uhfr2 is an E3 ubiquitin ligase, it is likely that the Ring domain may participate in DNA damage-induced protein ubiquitination events.

Taken together, our study demonstrates that Uhrf2 plays an important role in DNA damage response in MOVAS. Like Uhfr1, Uhrf2 can also be recruited to DNA damage sites, which is mediated by the TTD, PHD and SRA domains. The relocation of Uhfr2 to DNA damage sites facilitates the DNA damage-induced phosphorylation of H2AX and DNA damage repair. Since Uhrf2 is involved in chromatin remodeling, it is like that Uhrf2 regulates DNA damage-induced chromatin remodeling during DNA damage repair. Due to the functional similarity, Uhrf1 and Uhrf2 may be interchangeable and have redundant molecular cellular function. However, due to the different expression pattern, Uhrf2 is likely to act as a dominant role in MOVAS to maintain genomic stability in response to DNA damage.

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