USP7/HAUSP stimulates repair of oxidative DNA lesions

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ABSTRACT

USP7 is involved in the cellular stress response by regulating Mdm2 and p53 protein levels following severe DNA damage. In addition to this, USP7 may also play a role in chromatin remodelling by direct deubiquitylation of histones, as well as indirectly by regulating the cellular levels of E3 ubiquitin ligases involved in histone ubiquitylation. Here, we provide new evidence that USP7 modulated chromatin remodelling is important for base excision repair of oxidative lesions. We show that transient USP7 siRNA knockdown did not change the levels or activity of base excision repair enzymes, but significantly reduced chromatin DNA accessibility and consequently the rate of repair of oxidative lesions.

INTRODUCTION

Base excision repair (BER) is the major cellular pathway for repair of DNA base lesions and DNA single-strand breaks induced by endogenous and exogenous mutagens (1,2). The enzymology of BER has been studied in detail and the majority of BER proteins have been identified, cloned and characterized. However, the mechanism of BER at the chromatin level is less understood. Using DNA substrates containing reconstituted nucleosomes, it has been shown that the nucleosome structure is mainly inhibitory for BER and therefore chromatin remodelling is required to allow access of repair enzymes to the DNA damage (3). Major progress has recently been achieved in understanding the enzymology of chromatin remodelling in response to DNA double-strand breaks (4,5) and ultraviolet (UV) damage (6,7). However, it is not clear whether the same principles apply to all DNA lesions since very little is known about the mechanisms involved in BER-related chromatin remodelling.

Ubiquitylation and deubiquitylation of histones play an important role in chromatin remodelling and several E3 ubiquitin ligases and deubiquitylation enzymes have been reported as histone modifiers (8). In particular it has been demonstrated that USP7, also known as HAUSP, is able to deubiquitylate purified histone H2B in an *in vitro* reaction (9). USP7 is a ubiquitin specific protease, that recognizes and removes ubiquitin molecules from proteins. Although USP7 has several substrates, the most studied are the E3 ubiquitin ligase Mdm2 and p53. Mdm2 downregulates p53 by ubiquitylating it and thus labelling it for proteasomal degradation. However, Mdm2 can also self-ubiquitylate which promotes its own degradation therefore releasing p53 from its regulatory control, although this is inhibited by USP7, which persistently deubiquitylates Mdm2 (10–12). Since USP7 participates in the cellular DNA damage response and possibly in chromatin remodelling, it is important to analyse whether it plays any role in DNA repair. In this study, we address the role of USP7 in BER of oxidative DNA lesions.

MATERIALS AND METHODS

Cells, plasmids and antibodies

All experiments were performed in HeLa cells which were cultured as a monolayer in DMEM medium. The mammalian expression plasmid encoding the *usp7* gene containing an N-terminal Flag tag was purchased from Addgene (Cambridge, USA; Addgene database plasmid 16655). For overexpression studies, HeLa cells were grown on 10-cm dishes for 24 h to 70–80% confluency and then treated with 10 µl Lipofectamine transfection reagent (Invitrogen, Paisley, UK) in the presence of 2 µg of USP7 expression plasmid for a further 24 h. USP7 antibodies were purchased from Bethyl Laboratories (Montgomery, USA), Mdm2 antibodies were from AbD Serotec (Kidlington, UK), H2B and DNA PKcs antibodies were from Santa Cruz (California, USA), antibodies to ubiquitylated histone H2B were purchased from 2B Scientific (Upper Heyford, UK), RNF20/Bre1, ATM and ATR antibodies were from Abcam (Cambridge, UK) and anti-phospho-histone H2A.X (Ser

139) antibodies were purchased from Millipore (Watford, UK). Antibodies against human Pol β, XRCC1, APE1 and PARP-1 were raised in rabbit and purified by affinity chromatography. Tubulin and actin antibodies were purchased from Sigma-Aldrich (Gillingham, UK) and antibodies raised against poly(ADP-ribose) polymers were purchased from Trevigen (Gaithersburg, USA).

RNA interference

HeLa cells were grown on 10-cm dishes for 24 h to 30–50% confluency and then treated with 10 μl Lipofectamine reagent (Invitrogen, Paisley, UK) in the presence of 400 pmol of siRNA duplexes for a further 72 h. The following siRNA sequences were used: both 5′-ACCCUUG GACAAUAUUCCU-3′ and 5′-AGUCGUUCAGUCGU CGUAU-3′ for USP7 (13) or 5′-AAGCCAUUGCUUUU GAAGUUA-3′ for Mdm2 (14). For Mdm2 knockdown, cells were incubated with siRNA duplexes in transfection medium for 8 h, the medium was changed and transfection with Mdm2 siRNA was repeated after 36 h.

Whole-cell extracts

Whole-cell extracts (WCEs) were prepared by Tanaka's method (15) with some modifications as described previously (16).

Western blotting

Western blots were performed by standard procedure as recommended by the vendor (Novex, San Diego, USA). Blots were visualized using the Odyssey image analysis system (Li-cor Biosciences, Cambridge, UK).

Alkaline single-cell gel electrophoresis (Comet) assay

The comet assay was performed as recently described (17,18).

In vitro repair reactions

To analyse DNA ligase, DNA polymerase and AP endonuclease activities, a 5′-FAM labelled duplex oligonucleotide containing either a nick with 3′-OH and 5′-phosphate ends, a 1-nt gap with 3′-OH and 5′-phosphate ends, or an AP site, respectively were used as substrates. To prepare the nick substrate, a 20-mer FAM-labelled oligonucleotide (5′-FAM-CGATCAAGCTTATTGGGTAC-3′) was annealed in the presence of a 1.5-fold excess of a 20-mer 5′-phosphorylated oligonucleotide (5′-pAGAAGAAGAA GAAGAAGAGA-3′) and a 2-fold excess of a 40-mer oligonucleotide (5′-TCTCTTCTTCTTCTTCTTCTGTA CCCAATAAGCTTGATCG-3′). To prepare the gap substrate, a 16-mer FAM-labelled oligonucleotide (5′-FAM-C AATAGAGTAACACGG-3′) was annealed in the presence of a 1.5-fold excess of a 19-mer 5′-phosphorylated oligonucleotide (5′-pCGACCAGTCCCTGCCA ATC-3′) and a 2-fold excess of a 36-mer oligonucleotide (5′-GATTGGCAGGGACTGGTCGGCCGTGTTACTC TATTG-3′). The AP site containing substrate was generated from a 36-mer FAM-labelled oligonucleotide containing a uracil residue (5′-FAM-CAATAGAGTAA

CACGGUCGACCAGTCCCTGCCAATC-3′) that was annealed in the presence of a 2-fold excess of a 36-mer oligonucleotide (5′-GATTGGCAGGGACTGGTCGGC CGTGTTACTCTATTG-3′). All oligonucleotide duplexes were created by heating to 95°C for 3 min followed by slow-cooling to room temperature. The AP site was generated just before use by treating the uracil containing duplex oligonucleotide substrate with uracil DNA glycosylase and purifying the DNA over a Biospin P-30 column (Biorad, Hemel Hempstead, UK). Repair reactions contained 600 fmol duplex oligonucleotide substrate and were performed by incubation of WCEs in 10 μl buffer containing 50 mM HEPES-KOH (pH 7.8), 50 mM KCl, 10 mM $MgCl_2$, 0.5 mM EDTA, 1 mM DTT, 8.5% glycerol, 0.25 mM NAD^+, 1 μg of carrier DNA (single-stranded 30-mer oligonucleotide) and were supplemented with either 2 mM ATP, 25 mM phosphocreatine, 2.5 μg creatine phosphokinase (ligase and AP endonuclease reactions) or 20 μM dCTP, 20 μM dATP, 20 μM dGTP, 20 μM dTTP (polymerase reactions). Reactions were incubated for 20 min at 37°C prior to the addition of 10 μl of formamide loading dye. Reactions containing an AP site were treated with 200 mM $NaBH_4$ for 10 min on ice prior to the addition of loading dye. Samples were then heated to 95°C for 3 min prior to electrophoresis on a 20% denaturing polyacrylamide gel and gels were visualized using the Molecular Imager FX system (BioRad, Hemel Hempstead, UK).

RESULTS

Changes in cellular USP7 levels modulate the repair of oxidative lesions without affecting the activities or amounts of BER enzymes

To examine the effect of USP7 on the repair of oxidative DNA lesions, we treated HeLa cells with USP7 siRNA for 72 h (Figure 1A; approximate 70% efficient knockdown), and then analysed by the alkaline comet assay the rate of repair of DNA strand breaks and alkali labile sites induced by hydrogen peroxide. Treatment of HeLa cells with USP7 siRNA alone appeared to slightly increase the basal level of DNA strand breaks, although this effect was not statistically significant (Figure 1B; see 'Control'). Following treatment of the cells with hydrogen peroxide, a similar level of DNA damage is induced in both lipofectamine only (Lipo) and USP7 siRNA transfected cells (see time 0). Cells treated with lipofectamine only then efficiently repair the majority of hydrogen peroxide-induced DNA lesions within the first 2 h following treatment. However, we detected a notable delay in the rate of repair of hydrogen peroxide-induced DNA lesions (about 2-fold at all time points within the first hour) after USP7 knockdown (Figure 1B). Conversely, when we overexpressed USP7 protein (about 2-fold over cellular level, Figure 1C) we observed an increased rate of repair of hydrogen peroxide-induced DNA damage (Figure 1D). This result was quite unexpected since the current opinion is that ubiquitylation of histones should stimulate the process of chromatin opening and therefore histone deubiquitylation should negatively regulate this process.
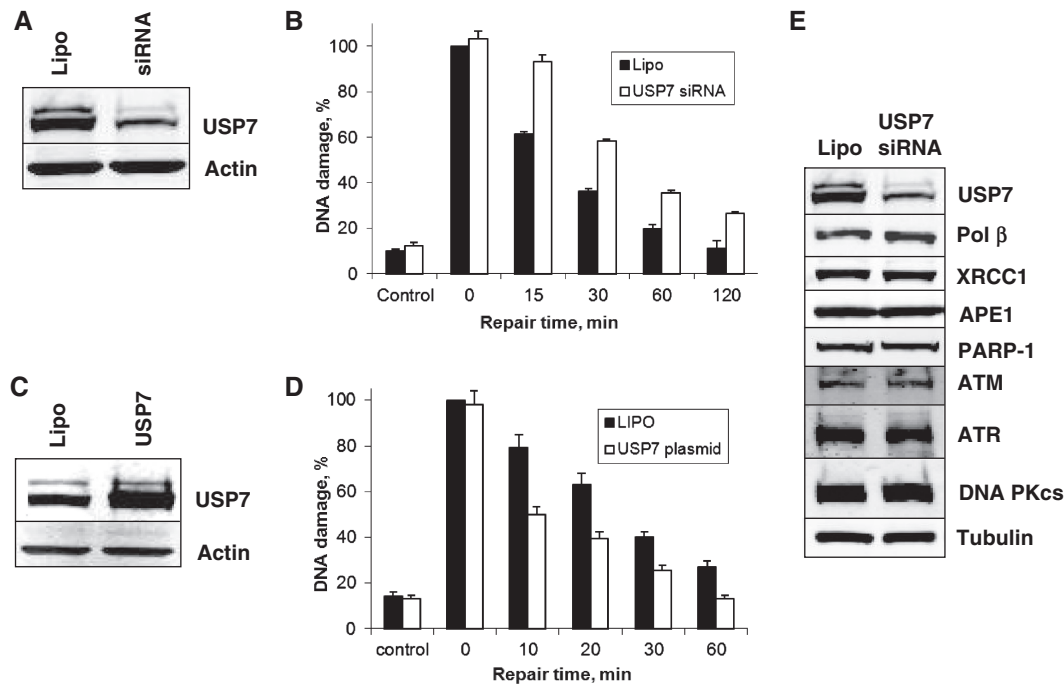
Figure 1. USP7 knockdown causes a delay in the repair of hydrogen peroxide induced DNA lesions but does not affect steady-state levels of BER enzymes. (**A, B** and **E**) HeLa cells were grown on 10-cm dishes for 24 h to 30–50% confluency and then treated with Lipofectamine transfection reagent (10 μl) in the absence (Lipo) or presence of USP7 siRNA (400 pmol) for a further 72 h. (A and E) Whole-cell extracts were prepared and analysed by 10% SDS–PAGE and Western blotting with the antibodies indicated. (B) Alternatively, cells were treated in suspension with 25 μM hydrogen peroxide for 5 min, allowed to repair for 0–120 min and the levels of DNA strand breaks and alkali labile sites were then analysed by the alkaline single cell gel electrophoresis (Comet) assay. (**C** and **D**) HeLa cells were grown on 10-cm dishes for 24 h to 70–80 % confluency and then treated with Lipofectamine transfection reagent (10 μl) in the absence (Lipo) or presence of a mammalian expression plasmid encoding *usp7* (2 μg) for a further 24 h. (C) Whole-cell extracts were prepared and analysed by 10% SDS–PAGE and Western blotting with the antibodies indicated. (D) Alternatively, the cells were treated in suspension with 25 μM hydrogen peroxide for 5 min, allowed to repair for 0–120 min and the levels of DNA strand breaks and alkali labile sites were then analysed by the Comet assay. Comet assay values are expressed as percentage DNA damage, whereby the percentage tail DNA values were normalized against control cells treated with Lipofectamine transfection reagent and with hydrogen peroxide at time 0 min, which was set to 100%. Standard deviations from at least three independent experiments are shown.

Consequently, a knockdown of USP7 should result in more efficient chromatin remodelling and activation of DNA repair rather than the reverse effect found in our experiments.

In many cases, the steady-state levels of proteins is an equilibrium between ubiquitylation that promotes protein degradation and deubiquitylation that support protein stability. We therefore hypothesized that USP7 may have an indirect effect on DNA repair. We have recently reported that ubiquitylation and proteasomal degradation of BER enzymes controls their steady-state levels (16,18). We thus assumed that USP7 may deubiquitylate one of the major BER proteins and thus would be required for its stability. Accordingly, an siRNA knockdown of USP7 should lead to a reduced amount of BER enzymes and impair DNA repair. However, we did not find any changes in the steady-state levels of neither key BER proteins including Pol β, XRCC1, APE1 and PARP-1 nor in DNA damage signalling or double-strand break repair proteins including ATM, ATR and DNA PK$_{CS}$ (Figure 1E). To exclude the possibility that USP7 deubiquitylation may affect BER protein activity, we prepared WCEs from control and USP7 siRNA knockdown cells and examined AP endonuclease, DNA polymerase and DNA ligase activities using duplex oligonucleotide substrates containing an AP site, a 1-nt gap and a single-strand break, respectively. We were able to show that neither APE1, Pol β nor DNA ligase activity were dramatically altered following USP7 knockdown (Figure 2A–C). We thus conclude that, most probably, USP7 affects BER by controlling the protein(s) responsible for chromatin remodelling.

USP7 silencing by siRNA reduces chromatin accessibility and repair by modulating Mdm2 levels

To evaluate chromatin accessibility for BER enzymes, we analysed synthesis of poly(ADP-ribose) polymers (PAR) following treatment of cells with hydrogen peroxide. PARP-1 is one of the first molecules to bind DNA single-strand breaks that arise directly, and generated as an intermediate through DNA repair, after treatment of cells with DNA-damaging agents. After binding, PARP-1 is activated and catalyses synthesis of PAR (19). Cells transfected with lipofectamine (Lipo) only and cells transfected with lipofectamine and USP7 siRNA duplexes for 72 h were treated with hydrogen peroxide and collected at different time points after treatment. WCEs prepared from these cells were subjected to electrophoresis and
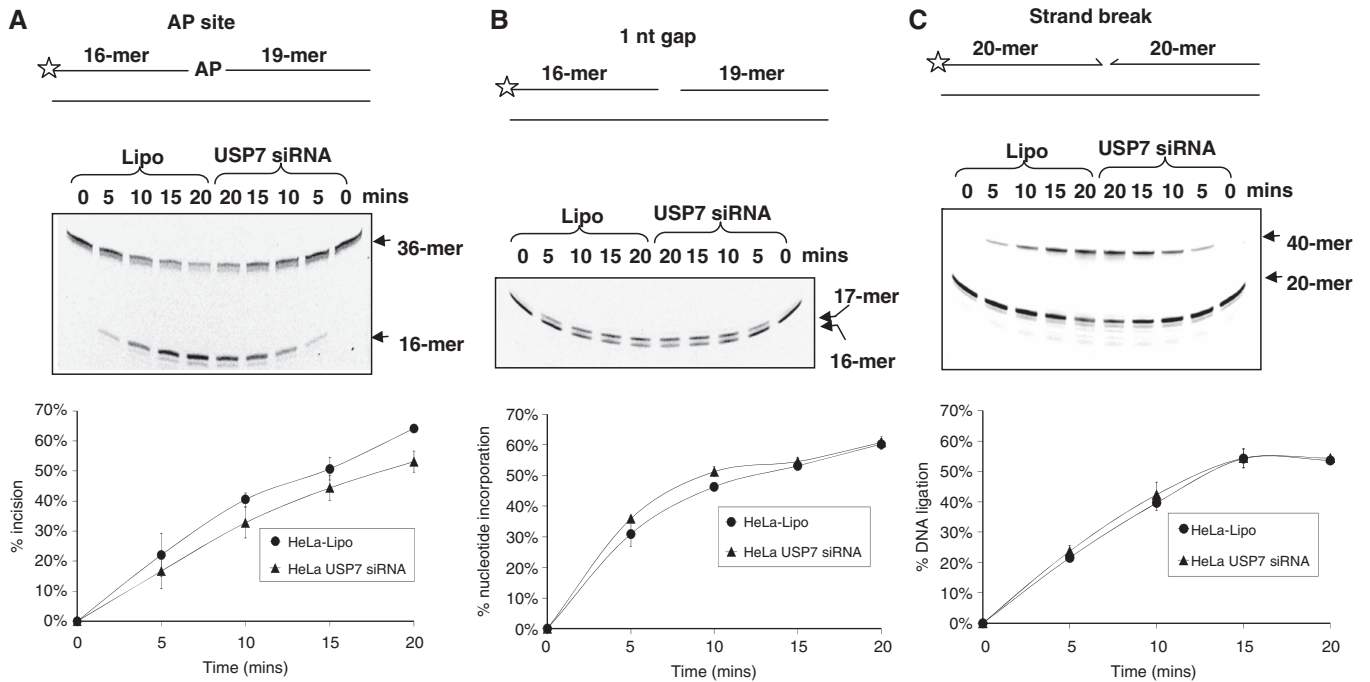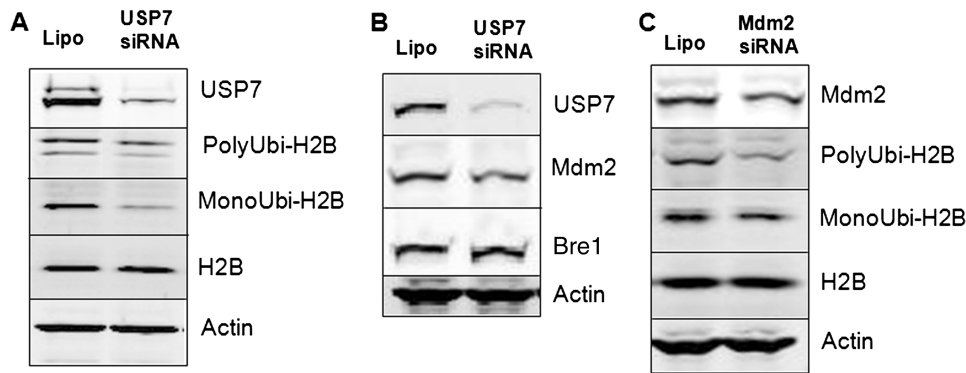
Figure 2. USP7 knockdown did not dramatically change the activity of BER enzymes. (**A–C**) HeLa cells were grown on 10-cm dishes for 24 h to 30–50% confluency and then treated with Lipofectamine transfection reagent (10 µl) in the absence (Lipo) or presence of USP7 siRNA (400 pmol) for a further 72 h and whole-cell extracts were prepared. The extracts (5 ng, 2 µg or 4 µg) were then incubated with 600 fmol FAM-labelled duplex oligonucleotide substrates containing either (A) an AP site, (B) a 1-nt gap or (C) a nick, respectively for 0–20 min at 30°C prior to separation by 20% denaturing PAGE and analysis by fluorescent imaging. Shown are a representative gel from three independent experiments and the percentage incision, one nucleotide incorporation and DNA ligation at the various time points were quantified, plotted and are shown in the graphs below, including standard deviations.

western blot analysis with PAR antibodies. Although the amount of PARP-1 protein did not change after USP7 knockdown (Figure 1E), we reproducibly observed decreased PAR synthesis in USP7 knockdown cells immediately after, and following repair of, hydrogen peroxide induced DNA damage, although the amount of PARP-1 did not change following USP7 knockdown (Figure 3A). This indicates a reduced accessibility of PARP-1 to the DNA strand break and therefore reduced binding and activation of PARP-1. Similarly, we found no or very little phosphorylation of histone H2AX after hydrogen peroxide treatment, confirming the reduced accessibility of DNA repair proteins to the DNA damage within chromatin after USP7 knockdown (Figure 3B).

Although it has been previously demonstrated that USP7 can deubiquitylate histone H2B *in vitro*, there is no evidence that this occurs in living cells (9). To address this, we monitored the ubiquitylation status of H2B in the presence and absence of USP7. Unexpectedly, we found a reduced level of H2B ubiquitylation after USP7 knockdown, suggesting that most probably USP7 regulates the cellular levels of one of the ubiquitin ligases involved in H2B ubiquitylation, rather than directly deubiquitylates chromatin associated histone H2B, since following USP7 knockdown an increase in H2B ubiquitylation would be observed in this case (Figure 4A). Two major E3 ligases have been shown to ubiquitylate histone H2B, namely Mdm2 (20) and Bre1 (21,22). Although the role of USP7 in Mdm2 stability is
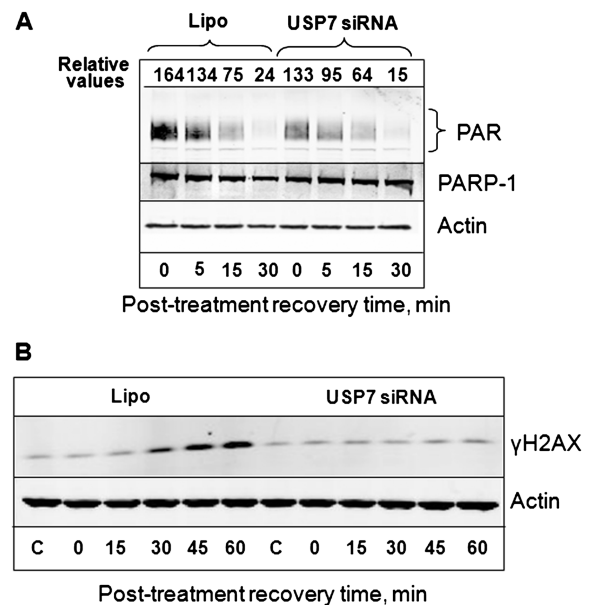
Figure 3. USP7 knockdown reduced chromatin accessibility. HeLa cells were grown on 10-cm dishes for 24 h to 30–50% confluency and then treated with Lipofectamine transfection reagent (10 µl) in the absence or presence of USP7 siRNA (400 pmol) for a further 72 h. Cells were then treated with 150 µM hydrogen peroxide for 15 min and allowed to repair for 0–120 min. Whole-cell extracts were prepared and analysed by 10% SDS–PAGE and western blotting with (**A**) PAR, PARP-1 or (**B**) γH2AX antibodies. Actin antibodies were used to demonstrate equal loading.

Figure 4. USP7 modulates histone H2B ubiquitylation by controlling the steady state levels of Mdm2. HeLa cells were grown on 10-cm dishes for 24 h to 30–50% confluency and then treated with Lipofectamine transfection reagent (10 μl) in the absence or presence of (**A** and **B**) USP7 siRNA or (**C**) Mdm2 siRNA and incubated for a further 72 h (an additional transfection with Lipofectamine and Mdm2 siRNA was performed 36 h after the first treatment). Cells were harvested, pelleted by centrifugation, whole-cell extracts were prepared and analysed by 10% SDS-PAGE and western blotting with the antibodies indicated.

well known, its role in Bre1 stability has not yet been examined. We therefore knocked down USP7 and, as expected, found reduced amounts of Mdm2, as previously reported (23,24). However, we did not observe any changes in the cellular levels of Bre1 following USP7 knockdown suggesting that, most probably, Mdm2 is ubiquitylating histones and that the observed defects in DNA repair and chromatin accessibility following USP7 knockdown observed in our experiments is due to the reduction in the levels of Mdm2 through self-ubiquitylation (Figure 4B). In support of this, when we knocked down Mdm2 using siRNA, which was only about 50% efficient, we observed reduced ubiquitylation of histone H2B and a slight increase in the amount of total histone H2B (Figure 4C).

DISCUSSION

Chromatin remodelling is important for many DNA transactions, including transcription, replication and DNA repair. Although there is an increase in the number of studies addressing the role of different post-translational modifications in the response to DNA damage (25), the orchestration of chromatin remodelling and the precise role of individual post-translational modifications within proteins are still obscure. In this study, we demonstrated that USP7 is indirectly involved in the regulation of oxidative DNA damage repair by modulating Mdm2 cellular levels and consequently chromatin accessibility to DNA repair enzymes. The importance of this finding is highlighted by the fact that oxidative DNA lesions are generated endogenously within the cell and therefore require continuous repair. This means that USP7/Mdm2-dependent chromatin remodelling is a dynamic process that is continuously involved in chromatin opening and closing to support the repair of DNA lesions and thus retain DNA integrity and genome stability. We propose that, when DNA damage is detected, ubiquitylation of H2B by Mdm2 opens chromatin for DNA repair (Figure 5, pathway 1). Since USP7 forms a complex with Mdm2 and is required for Mdm2 stability
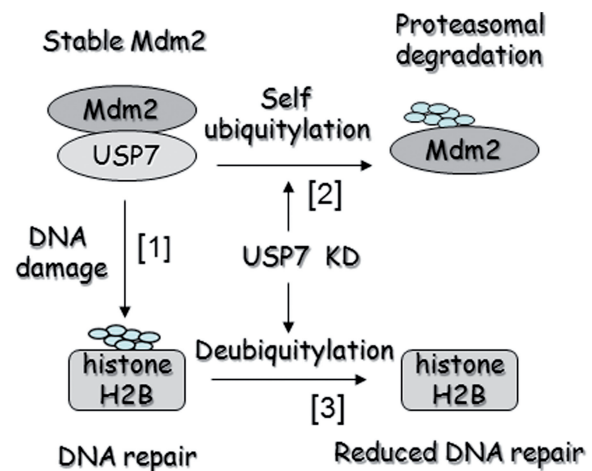
Figure 5. Proposed model for the effects of USP7 knockdown on histone H2B ubiquitylation status and DNA repair. Ubiquitylation of histone H2B by Mdm2 opens chromatin for DNA repair (1). Knockdown of USP7 promotes Mdm2 self-ubiquitylation and proteasomal degradation (2) and consequently the balance between ubiquitylation and deubiquitylation changes in favour of histone H2B deubiquitylation and results in reduced DNA repair (3).

(26,27), a knockdown of USP7 employed in our experiments, leads to Mdm2 self-ubiquitylation and proteasomal degradation (Figure 5, pathway 2). Consequently, the balance between ubiquitylation and deubiquitylation changes that results in H2B deubiquitylation and reduced DNA repair (Figure 5, pathway 3). Interestingly, the effect of USP7 and Mdm2 knockdown on histone H2B ubiquitylation is slightly different. While Mdm2 knockdown mainly reduced polyubiquitylation and to lesser degree monoubiquitylation, USP7 knockdown affected both poly- and monoubiquitylation (Figure 4). These data suggest that, in addition to Mdm2, USP7 may be regulating another ubiquitin ligase mainly responsible for H2B monoubiquitylation in response to DNA damage.

It is not clear whether USP7/Mdm2-dependent chromatin remodelling is also required for the processing of different DNA lesions that are repaired by other DNA

repair pathways. Since we detected the effect of USP7 knockdown on histone H2AX phosphorylation, it is quite possible that Mdm2 induced histone H2B ubiquitylation is important not only for BER of oxidative lesions but may also play a role in DNA repair in general. However, further studies are required to address this issue. Nevertheless, it is clear that in addition to Mdm2 and USP7, other ubiquitin ligases and deubiquitylation enzymes are involved in chromatin remodelling that support efficient repair of DNA lesions induced by endogenous and exogenous mutagens and thus play a role in maintaining genome stability.

FUNDING

Conflict of interest statement. None declared.

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