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Dub3 controls DNA damage signalling by direct deubiquitination of H2AX

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ABSTRACT

A crucial event in the DNA damage response is the phosphorylation and subsequent ubiquitination of H2AX, required for the recruitment of proteins involved in DNA repair. Here we identify a novel regulator of this process, the ubiquitin hydrolase Dub3. Overexpression of wild type, but not catalytic inactive, Dub3 decreases the DNA damage-induced monoubiquitination of H2A(X) whereas downregulation of Dub3 has the opposite effect. Dub3 overexpression abrogates focus formation of 53BP1 and BRCA1 in response to genotoxic stress. However, focus formation of MDC1 and γH2AX, earlier events in this response, are unaffected by Dub3 overexpression. We show that Dub3 counteracts H2AX E3 ligases RNF8 and RNF168. Moreover, Dub3 and H2AX interact and Dub3 deubiquitinates H2AX *in vitro*. Importantly, overexpression of Dub3 delays H2AX dephosphorylation and recovery of MDC1 focus formation at later time points after DNA damage, whereas H2AX dephosphorylation at later time points is faster after Dub3 depletion. Altogether these results show that Dub3 regulates a correct DNA damage response by controlling H2AX ubiquitination.

1. Introduction

The cellular response to DNA damage, which preserves genomic integrity, consists of a signalling pathway in which post-translational modifications of the histones surrounding the DNA lesion play a central role. Initially this pathway was merely thought to function as a kinase cascade, in which master kinases ATM and ATR function upstream and phosphorylate, via mediator proteins, numerous targets, among which the downstream Chk2 and Chk1 kinases. In the last decade however, other protein alterations, such as ubiquitination, emerged as key modifications in the control of DNA damage response (DDR) signalling.

One of the earliest events in this response is the rapid accumulation of numerous signalling and repair proteins around the DNA lesion. This protein recruitment can be visualised under the microscope, as so-called foci, and is thought to provide a platform for bringing ATM/ATR and their substrates together to trigger subsequent repair and other signalling events. Critical for the recruitment of these

Abbreviations: ETP, etoposide; CI, catalytic inactive; DDR, DNA damage response; Dub, deubiquitylating enzyme; HU, hydroxyurea; IR, ionizing radiation; WT, wild type.

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proteins is the phosphorylation of H2AX by ATM in the area surrounding the lesion. MDC1 then recognizes phosphorylated H2AX (γH2AX) with its BRCT domains and ATM phosphorylates the MDC1 S/T-Q cluster, before mediating the subsequent recruitment of ubiquitin ligase RNF8, which initiates the ubiquitination of H2A(X) (Huen et al., 2007; Kolas et al., 2007; Mailand et al., 2007; Stucki et al., 2005). A second ubiquitin ligase, RNF168, is recruited by recognition of RNF8 ubiquitination products and with the help of E2 ubiquitin-conjugating enzyme Ubc13, amplifies the ubiquitination response, which triggers the recruitment of 53BP1 and Rap80/BRCA1, among other proteins. These complexes then promote DNA repair by non-homologous end joining or homologous recombination (Doil et al., 2009; Stewart et al., 2009). The role of RNF8 in recruitment of BRCA1 is different than for 53BP1. The BRCA1 complex is recruited by binding of the tandem ubiquitin interaction motifs to the polyubiquitin chains generated (Kim et al., 2007; Sato et al., 2009; Sobhian et al., 2007; Wang et al., 2007; Wu et al., 2009), whereas 53BP1 requires binding of its Tudor domain to methylated histone H4 on lysine 20 (Botuyan et al., 2006; Sanders et al., 2004). In undamaged conditions, the Tudor domain of lysine demethylase JMJD2A binds this methylated histone. Following DNA damage, degradation of JMJD2A in an RNF8-dependent manner exposes histone H4K20, thereby enabling 53BP1 recruitment (Mallette et al., 2012). In addition, an ubiquitin-dependent recruitment (UDR) motif was recently identified in the C-terminus of 53BP1, which interacts with ubiquitinated H2A on Lys15, a product of RNF168 activity (Fradet-Turcotte et al., 2013).

Given the importance of H2AX ubiquitination in triggering the DDR, a tight regulation is expected. Indeed, several ubiquitin hydrolases were shown to counterbalance the ubiquitination cascade by RNF8 and RNF168 in a direct or more indirect manner. Exogenous USP3 deubiquitinates H2A and H2B (Nicassio et al., 2007) and overexpression of USP16 and USP44 reverses RNF8/RNF168-mediated ubiquitination (Mosbech et al., 2013; Shanbhag et al., 2010). Interestingly, the Rap80/BRCA1 complex contains the deubiquitinating enzyme BRCC36, which functions to counteract Ubc13-RNF8 activity to provide a balanced level of ubiquitin levels around the DNA lesions (Shao et al., 2009). Otub1 was shown to suppress RNF168-dependent ubiquitination in a non-catalytic manner, by interacting with and inhibiting Ubc13 (Nakada et al., 2010). Finally, 19S proteasome ubiquitin protease POH1 was shown to restrict 53BP1 recruitment by counteracting RNF8/RNF168-mediated polyubiquitination and retention of JMJD2A on the chromatin (Butler et al., 2012).

As we reasoned that additional regulators might exist to ensure a strict control of this response, we performed a screening using a library of expression vectors for the majority of human deubiquitylating enzymes (Dubs) and identified ubiquitin hydrolase Dub3/USP17L2 as a novel regulator of this cascade by directly deubiquitinating H2A(X). Overexpression of wild type, but not a catalytic inactive version of the enzyme, decreases the DNA damage-induced mono-ubiquitination of H2A(X) and abrogates focus formation of 53BP1 and BRCA1. Most importantly, our results indicate that regulating Dub3 (activity) might be a mechanism to guarantee a correct DDR and to allow checkpoint recovery.

2. Materials and methods

2.1. Cell culture and treatments

293T, U2OS, 639V, HCT116 and HeLa cells were grown using standard procedures. Unless stated otherwise, cells were treated with 2 mM hydroxyurea (HU, 16 h), 2Gy of ionizing radiation (IR, 1 h) or 20 μM of etoposide (ETP, 1 h).

2.2. Plasmids, siRNA oligos and transfection

Plasmid DNA was transfected into cells using the calcium phosphate transfection method or using jetPRIME (Polyplus). Simultaneous transfection of plasmids and siRNA oligonucleotides was performed using Metafectene Pro (Biontex).

pMEF Flag-Dub3 wild type (WT) and C89S (CI) were kindly provided by J.F. Burrows (Queen's University, Belfast, Northern Ireland). Dub3 WT and CI were cloned into peGFP-C1 to generate GFP-fusion expression vectors. pMEF Flag-Dub3 C89S/H334Q/D350N, another catalytic inactive version, was obtained using the QuikChange Site-Directed Mutagenesis Kit (Agilent Technologies).

pcDNA3.1 Flag-H2AX was kindly shared by L. Penengo (University of Piemonte Orientale A. Avogadro, Novara, Italy), RNF168-GFP by G.S. Stewart (University of Birmingham, United Kingdom), Flag-HA-USP3, Flag-HA-BRCC36 and Flag-HA-USP16 by R.A. Greenberg (University of Pennsylvania, USA), Flag-HA-USP44 and Flag-HA-Otub1 by J.W. Harper (Harvard Medical School, Boston, USA; Addgene plasmids #22604 and #22551 (Sowa et al., 2009) and HA-RNF8 by T.M. Thomson (IBMB, Barcelona, Spain). USP28 cDNA was a kind gift from G. Marfany (Barcelona University, Barcelona, Spain) and was cloned in pCMVTag2B (Agilent Technologies) to generate Flag-tagged protein.

siRNA oligonucleotides (Sigma) were transfected into cells using Lipofectamine RNAiMax (Invitrogen) according to the manufacturers instructions. Sequences of oligonucleotides were as follows:

Luc CGUACGCGGAAUACUUCGAdTdT
Dub3 CCUCCGUGAUGUUGCUUGAdTdT

2.3. Antibodies and other reagents

Antibodies obtained from commercial sources were as following: γH2AX (mouse), Ub-H2AX and FK2 from Millipore, γH2AX (rabbit) and β-actin from Genscript, H2AX from Bethyl, Ku86 (C-20) and FANCD2 (FI17) from Santa Cruz Biotechnology, Flag (M2) from Sigma, 53BP1 (Ab172580) from Abcam, HA (12CA5) from Roche.

Rabbit polyclonal anti-GFP (Warmerdam et al., 2009), anti-MDC1 (Kakarougkas et al., 2013a), anti-BRCA1 (Kakarougkas et al., 2013b) and anti-USP28 (Martín et al., 2014) have been described previously.

The antibody against Dub3 was generated by injecting rabbits with a His-tagged antigen (amino acid 1–250) that was obtained by expression in bacteria and purified with a Ni-NTA resin (Qiagen) following manufacturers recommendations.

2.4. Cell lysis and histone extraction

Whole-cell extracts were prepared by washing cultures in PBS before boiling cells in Laemmli buffer for 5 min.

To extract histones, cells were twice washed in cold PBS. Then cells were resuspended in Triton Extraction Buffer (TEB: PBS containing 0.5% Triton X-100, 2 mM phenylmethylsulfonyl fluoride and 0.02% NaN_3) at a cell density of 10^7 cells/ml and incubated on ice for 10 min. Nuclei were centrifuged at 2000 rpm for 10 min at 4 °C, after which the supernatant was removed. After another wash in TEB, the pellet was resuspended in 0.2 N HCl at a density of 4×10^7 nuclei/ml and incubated overnight at 4 °C. The samples were centrifuged at 2000 rpm for 10 min at 4 °C to pellet debris. The supernatant was kept at −20 °C.

Protein concentrations were determined using the BCA protein assay (Novagen).

2.5. Chromatin fractionation

Biochemical fractionation of cells was performed as previously described (Méndez and Stillman, 2000; Smits et al., 2006). Soluble cytoplasmic and soluble nuclear fractions were pooled to one soluble fraction.

2.6. Immunofluorescence

For immunostaining, cells were fixed in 2% paraformaldehyde containing 0.2% Triton X-100 for 20 min at room temperature and then permeabilized with 0.1% Triton X-100 for 5 min. Samples were blocked in 1% FCS and immunostained with antibodies as indicated.

In all instances, more than 100 cells were analysed for each point and error bars on graphs represent the standard error of the mean of three independent experiments. Cells with more than 5 foci were scored as positive.

Images were made using a Cell Observer fluorescent microscope equipped with Axiovision software (Zeiss).

2.7. Protein purification and immunoprecipitations

For protein purification of ubiquitinated Flag-H2AX, Flag-Dub3 WT/CI and Flag-USP28, 293T cells were transfected with the corresponding expression vectors. Cells overexpressing Flag-H2AX were treated with UV (40 J/m^2, 1 h) to increase ubiquitylation of H2AX. Cells were lysed in EB150 lysis buffer (50 mM Hepes pH 7.5, 150 mM NaCl, 5 mM EDTA, 2 mM $MgCl_2$, 0.5% NP40, 10% glycerol) for 20 min on ice. In case of Flag-H2AX, the buffer was supplemented with protease inhibitors and 2 mM N-ethylmaleimide (NEM, Sigma) and extracts were sonicated 8 times 15 s. After centrifugation 13,000 rpm for 20 min, extracts were incubated with anti-Flag M2 agarose (Sigma) for 2 h at 4 °C, followed by 4 washes with lysis buffer and 1 wash in elution buffer (50 mM Tris–HCl pH 7.5). Proteins were eluted in elution buffer supplemented with 330 μg/ml Flag (DYKDDDDK) peptide (Genscript) for 1.5 h at 4 °C. Supernatant was collected, 10% glycerol added and aliquots were stored at 20 °C.

For immunoprecipitations, purified proteins were mixed in EB150 lysis buffer, supplemented with protease and phosphatase inhibitors and incubated with Dub3 antibody cross-linked to protein A-sepharose CL-4B (GE Healthcare) for 3 h at 4 °C. After 3 washes with EB150 buffer, the proteins were eluted with 150 mM Glycine pH 2.3, after which sample buffer was added.

2.8. Dub activity assay

Cells were lysed in lysis buffer (50 mM Tris–HCl pH 7.4, 5 mM $MgCl_2$, 250 mM sucrose, 1 mM DTT, 2 mM ATP and 0.1% NP40) for 20 min on ice. The extracts were centrifuged at 13,000 rpm for 20 min and incubated with 50 μM HA-Ubiquitin-Vinyl Sulfone (Boston Biochemicals) for 1 h at 37 °C, when indicated in the presence of 2 mM NEM. Subsequently the samples were incubated with anti-HA affinity matrix (Roche Diagnostics) for 2 h at 4 °C, followed by 4 washes with lysis buffer, after which sample buffer was added and analysis by western blotting for the Dub of interest.

2.9. In vitro *deubiquitin assay*

Purified ubiquitylated H2AX and Dub were mixed in buffer (50 mM Tris–HCl pH 7.5 and 4 mM DTT) and incubated for 2 h at 37 °C. Sample buffer was added to stop the reaction and samples were analysed by western blotting.

2.10. Colony survival assay

To determine cellular sensitivity to DNA damaging agents, HeLa cells were transfected with empty vector or GFP-Dub3 WT by jetPRIME. 24 h later, 1000 cells were seeded in 6 cm dishes. The next day, cells were treated with different concentrations of camptothecin (CPT) for 7 h. Following 10 days in culture, cells were fixed, stained and colonies were counted. Triplicate cultures were scored for each treatment and the error bars present the standard error of the mean of three independent experiments.

3. Results and discussion

3.1. Dub3 catalytic activity reduces monoubiquitination of H2A(X) and H2B

To identify novel regulators of the DNA damage response, we performed a screening using a library of expression vectors for the majority of human Dubs, using H2AX monoubiquitination by western blotting as readout. Cells were treated with hydroxyurea (HU) to trigger checkpoint activation. We identified several candidate proteins that reduce the level of H2AX monoubiquitination after genotoxic stress (data not shown), among which Dub3/USP17L2 (hereafter called Dub3) was the most obvious one. The overexpression of Dub3 leads to a decrease of H2AX ubiquitination, as demonstrated by western blotting using an antibody specific for ubiquitinated H2AX. This result was confirmed in westerns with antibodies against total H2AX or phosphorylated H2AX (γH2AX), in which the mono-ubiquitinated histone runs with lower mobility (Figure 1A). This effect is due to the catalytic activity of Dub3, as expression of a catalytic

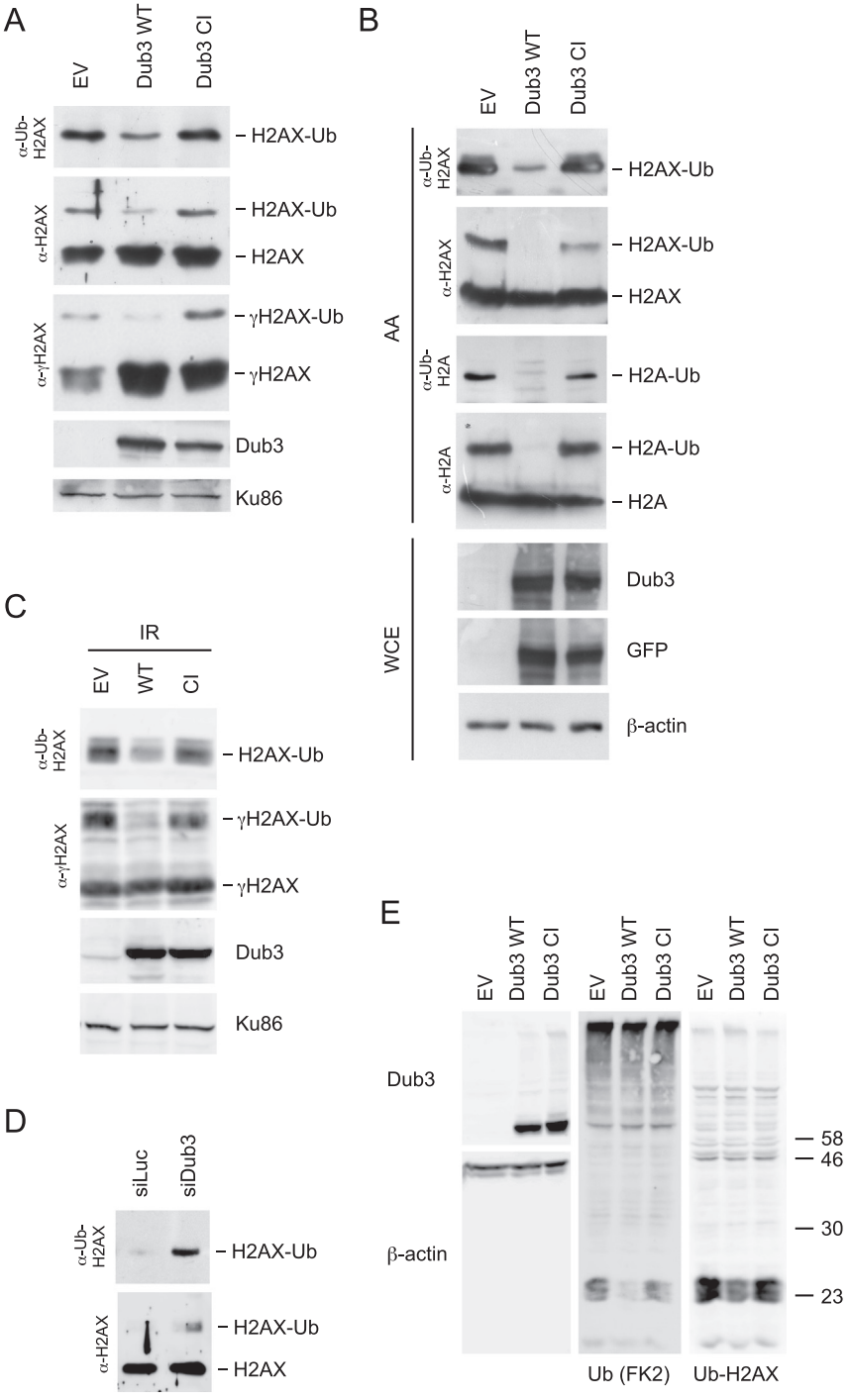

Figure 1 – *Dub3 affects H2A(X) monoubiquitination*. (A) 293T cells were transfected with empty vector (EV) or a Dub3 wild type (WT) or catalytic inactive (CI) expression vector. The next day, cells were treated with HU (2 mM, 16 h). Whole cell extracts were prepared and analysed by western blotting using the indicated antibodies. (B) 293T cells were transfected with EV, GFP-Dub3 WT or GFP-Dub3 CI, treated with HU after which acid extraction (AA) was performed and whole cell extracts (WCE) were prepared. Western blotting was performed with the indicated antibodies. (C) As in (A), but cells were treated with IR (10 Gy) and harvested 1 h later. (D) 639V cells were twice transfected with siRNA oligos for Luciferase (Luc) or Dub3. 48 h later, cells were lysed and extracts were analysed by western blotting using the indicated antibodies. (E) 293T cells, transfected with empty vector (EV), Dub3 WT or Dub3 CI. Western blot analysis with the indicated antibodies. Ubiquitinated H2AX (23kD) can be seen in the FK2 western.

inactive version of Dub3 (CI, C89S) did not reduce the monoubiquitination of H2AX (Figure 1A). In addition, this negative control demonstrated that the decrease of H2AX ubiquitination by Dub3 wild type (WT) is unlikely to be a nonspecific effect of overexpression. The activity of Dub3 WT and two different CI versions was verified using an assay with artificial substrate (Figure S1A). Analysing histones after acid extraction demonstrated that overexpression of Dub3 wild

type (WT) also diminished the monoubiquitination of H2A (Figure 1B). Expression of Dub3 WT, but not CI, also decreased ionizing radiation (IR)-induced H2AX monoubiquitination (Figure 1C). Importantly, depletion of Dub3 by siRNA resulted in increased ubiquitination of H2AX in the absence of exogenous DNA damage (Figure 1D, knock down efficiency shown in Figure S1B). Together these results demonstrate a major role for Dub3 in controlling the ubiquitination of H2A and H2AX. Other ubiquitin hydrolases have been reported controlling the ubiquitination status of H2AX: USP3, Otub1, BRCC36, USP16 and USP44 (Butler et al., 2012; Mosbech et al., 2013; Nakada et al., 2010; Nicassio et al., 2007; Shanbhag et al., 2010; Shao et al., 2009). When comparing the activity of Dub3 to that of the other Dubs by overexpression, we observed that Dub3 was among the most efficient ones affecting H2AX ubiquitination upon similar expression levels of the ubiquitin hydrolases (Figure S1C). Finally, to exclude the possibility that overexpression of Dub3 has a pan-cellular effect, we analysed the conjugated ubiquitin signal using the FK2 antibody. Indeed, the effect of Dub3 appeared to be specific to the histone, also recognised by the FK2 antibody (Figure 1E).

3.2. *Recruitment of 53BP1 and BRCA1 to sites of DNA lesions is regulated by Dub3*

DNA damage-induced ubiquitination of H2AX is a critical event for the recruitment of important mediator proteins 53BP1 and BRCA1 to sites of DNA lesions (Huen et al., 2007; Mailand et al., 2007). We therefore studied the consequences of Dub3 expression on DNA damage-induced focus formation of these and other DDR proteins by transfecting U2OS cells with GFP-tagged versions of Dub3, WT or CI, after which focus formation was analysed by immunofluorescence. Figure 2A demonstrates that the IR-induced focus formation by 53BP1 was completely abrogated by WT, but not CI Dub3. Flag-tagged Dub3 expression resulted in the same effect and also 53BP1 foci induced by treating cells with etoposide was completely inhibited by expression of Dub3 WT (Figure S1D and data not shown). Dub3 WT equally affected IR-induced focus formation of BRCA1 and local accumulation of conjugated ubiquitin (FK2 immunofluorescence) upon etoposide treatment was also inhibited by overexpression of WT Dub3 (Figures 2B, 2E and S1E). Conversely, depletion of Dub3 increased FK2 immunofluorescence in the absence of exogenous damage, as compared to control cells (Figure S1F). Since histone H2A is the most abundant ubiquitinated protein in cells (Jason et al., 2002), it is likely that changes in FK2 staining upon modulating Dub3 levels represent alterations in H2A(X) ubiquitination. If Dub3 directly controls H2AX ubiquitination, earlier events in the DDR should not to be affected by Dub3 overexpression. Indeed, phosphorylation of H2AX and recruitment of MDC1 into IR-induced foci, two events that occur before the ubiquitination of H2AX (Mailand et al., 2007), are not affected under these conditions (Figure 2C–E). Together these data strongly suggest that Dub3 directly impacts on the ubiquitination of H2AX. Interestingly, these immunofluorescence experiments demonstrated that Dub3 predominantly localizes in the nucleus (Figure 2A–D). Cellular fractionating showed that part of Dub3 associated with chromatin, which is consistent with its function of controlling histone ubiquitination (Figure S1G).

3.3. *Dub3 antagonizes RNF8 and RNF168 function*

The ubiquitination of H2A(X) upon genotoxic stress is initiated by RNF8, where after RNF168 is recruited to amplify histone ubiquitination (Doil et al., 2009; Huen et al., 2007; Mailand et al., 2007; Stewart et al., 2009). To study if Dub3 counteracts RNF8 and RNF168 function, these E3 ligases were overexpressed in the presence and absence of Dub3 and H2AX ubiquitination was analysed. As shown in Figure 3A, overexpression of RNF8 only, or RNF8 and RNF168 together, led to elevated H2AX ubiquitination as compared to the control. However, co-expression of Dub3 reverted this increase in ubiquitination of H2AX, indicating that the ubiquitin hydrolase counteracts both RNF8 and RNF168 ligase activities. We next investigated if Dub3 could affect the recruitment of these E3 ligases to the sites of DNA lesions. Interestingly, whereas the percentage of cells with HA-RNF8 etoposide-induced foci did not significantly change after expression of Dub3, the percentage of cells with GFP-RNF168 foci was inhibited by around 50% upon co-expression of Dub3 WT. Co-expression of Dub3 CI did not affect RNF168 focus formation (Figure 3B). As DNA damage-induced localisation of RNF168 was reported to be dependent on RNF8 and ubiquitinated H2AX (Doil et al., 2009; Stewart et al., 2009), we believe that the inefficient DNA damage-induced focus formation of RNF168 in Dub3-overexpressing cells is a consequence of lower levels of H2AX ubiquitination instead of Dub3 directly affecting recruitment of RNF168 to sites of DNA lesions.

3.4. *Dub3 deubiquitinates H2AX in vitro*

To confirm that the effects of overexpression of Dub3 are due to direct de-ubiquitination of H2AX, we first determined a possible interaction between these two proteins by performing an immunoprecipitation protocol using purified proteins. Indeed, as shown in Figure 4A, H2AX co-immunoprecipitated in a Dub3 immunoprecipitation. Most importantly, using these purified proteins in an *in vitro* deubiquitination assay demonstrated that WT Dub3, but not CI, was able to deubiquitinate H2AX. Inhibition of Dub activity by N-ethylmaleimide (NEM) prevented deubiquitination of H2AX by Dub3 and to support the specificity of the assay, we demonstrate that USP28, an aspecific Dub, previously reported in the DDR (Zhang et al., 2006), was not able to deubiquitinate H2AX in these conditions (Figure 4B). These data demonstrate that Dub3 directly acts on H2AX by deubiquitination.

3.5. *Dub3 regulates correct DNA damage response functioning*

The identification of a novel, additional regulator of H2AX ubiquitination underscores the complexity and importance of this response to the maintenance of genome stability. It is expected that deregulation of this pathway has consequences for the DDR, for example an impaired DDR or repair upon overexpression of Dub3. This hypothesis was tested by studying H2AX phosphorylation (γH2AX) and MDC1 focus

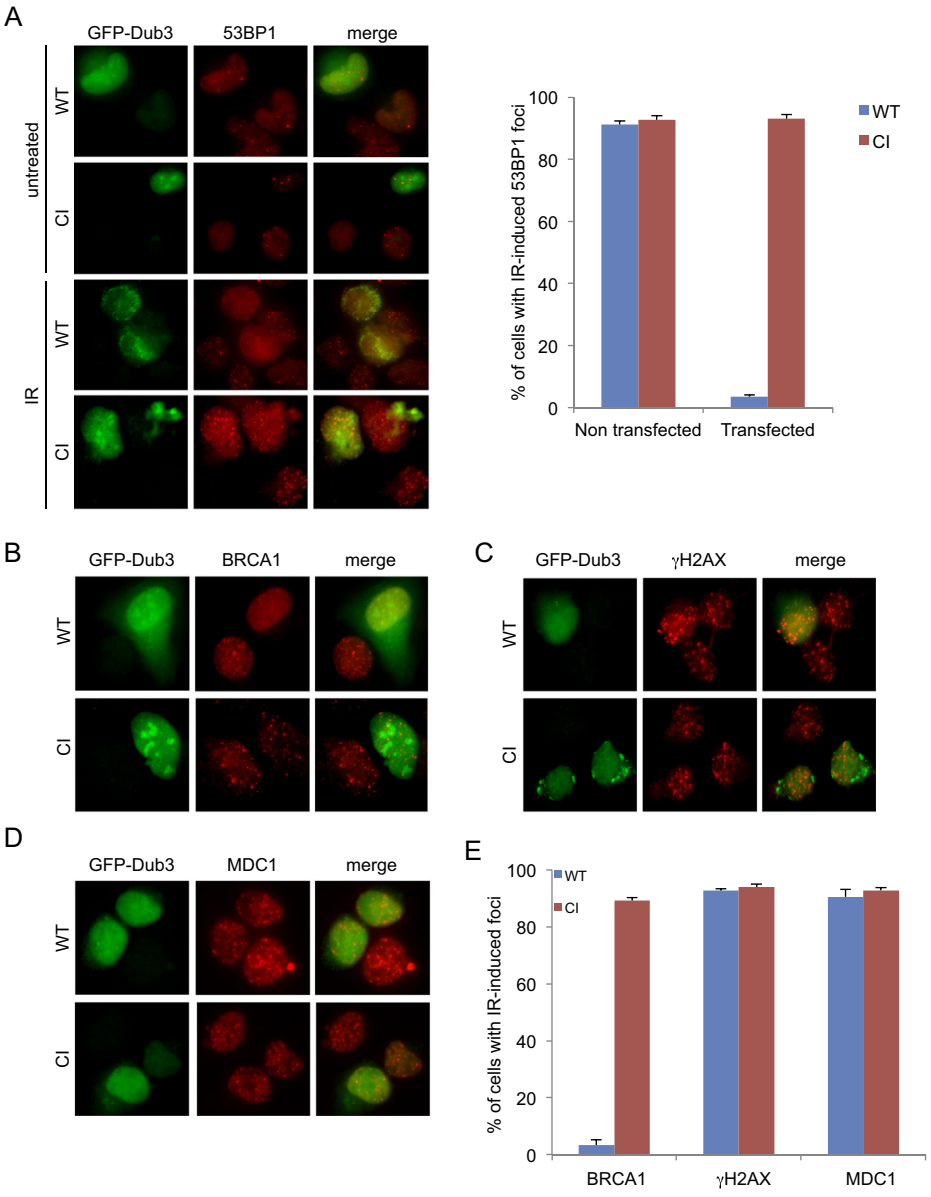

Figure 2 – *Overexpression of Dub3 abrogates DNA damage-induced focus formation of 53BP1 and BRCA1, but not γH2AX and MDC1.* (A) U2OS cells were transfected with GFP-Dub3 WT or CI. Cells were left untreated or treated with IR (2Gy). 1 h later cells were fixed and analysed by immunofluorescence using 53BP1 antibody. GFP-positive (transfected) and GFP-negative (non transfected) cells were scored for 53BP1 foci (right panel). (B) Cells were transfected as in A), irradiated and 1 h later analysed by immunofluorescence for BRCA1. (C) As in B), but now for γH2AX. (D) As in B), but now for MDC1. (E) Quantification of IR-induced focus formation of BRCA1, γH2AX and MDC1 in cells expressing wild type (WT) or catalytic inactive (CI) GFP-Dub3 (panels B–D).

formation, typical markers of an activated DNA damage response, after overexpression of Dub3. Western blot analysis demonstrated that H2AX phosphorylation was maintained high at late time points after overexpression of Dub3 WT, whereas in cells overexpressing Dub3 CI H2AX phosphorylation returned to basal levels at these times (Figure 4C), suggesting that the deregulation of H2AX ubiquitination results in a problematic DDR. Analysis of γH2AX and MDC1 focus formation at late time points showed the same outcome. Significantly more γH2AX-positive cells were identified at 6 h after etoposide treatment in Dub3 WT as compared to CI expressing cells (Figure 4D and F). Also MDC1 foci sustained at later time points after damage in cells expressing Dub3 WT but not in Dub3 CI (Figure 4E and F). Depletion of Dub3 had the opposite effect. Etoposide-induced phosphorylation of H2AX recovered quicker at later time points in Dub3-depleted cells as compared to control cells (Figure S2A). At these time points also γH2AX foci disappear quicker after Dub3 knockdown (Figure S2B). Finally, overexpression of Dub3 resulted in a slight but reproducible increased sensitivity for camptothecin in HeLa cells (Figure S2C).

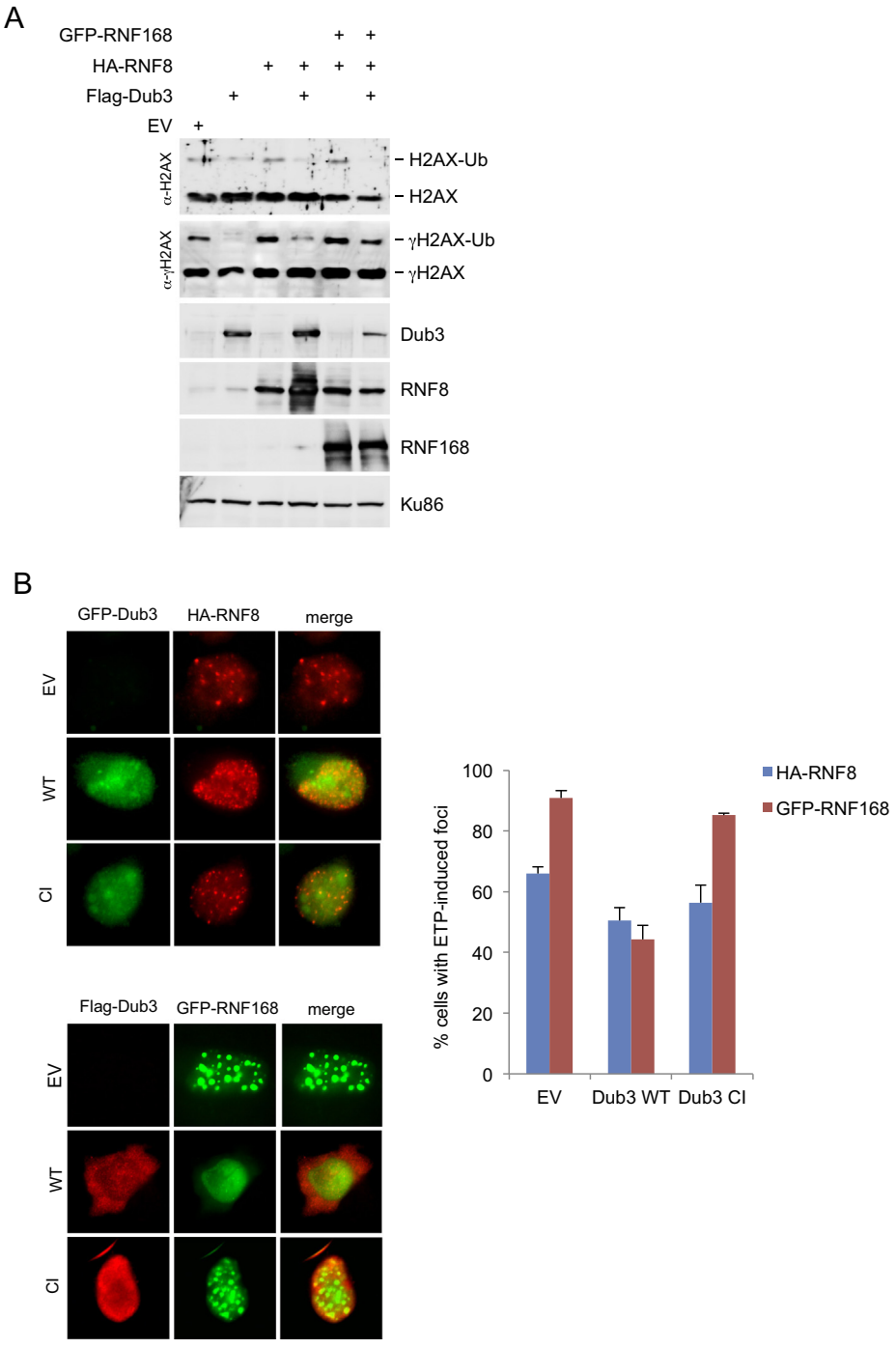

Figure 3 – *Dub3 inhibits RNF8 and RNF168 function and restrains RNF168 recruitment to sites of DNA lesions*. (A) 293T cells were transfected with the indicated plasmids. The next day, cells were treated with HU, lysed and analysed by western blotting using the indicated antibodies. (B) U2OS cells were transfected with empty vector (EV)/GFP-Dub3 (WT or CI) in combination with HA-RNF8 (upper left panel) or GFP-RNF168 (lower left panel). Cells were treated with etoposide (ETP, 20 μM) and 1 h later fixed. RNF8 foci were analysed in GFP-positive cells after immunofluorescence with an HA antibody. GFP-RNF168 foci were scored in Flag-positive cells. Right panel shows the quantification.

Together these data indicate that a balanced level of H2AX ubiquitination is required for a correct DNA damage response: activation upon the detection of DNA lesions and switch of when the lesions are repaired, to stimulate recovery.

The data presented in this paper characterize a novel role for Dub3 in the DNA damage response by direct deubiquitination of H2AX. Previously, other ubiquitin hydrolases have been reported to regulate the ATM-H2AX-RNF8-53BP1/BRCA1 pathway, either by direct deubiquitination of H2AX or indirectly: USP3, Otub1, BRCC36, USP16, POH1 and USP44 (Butler et al., 2012; Mosbech et al., 2013; Nakada et al., 2010; Nicassio et al., 2007; Shanbhag et al., 2010; Shao et al., 2009). The latter was very recently identified using a very similar approach as ours, a library of expression plasmids of human

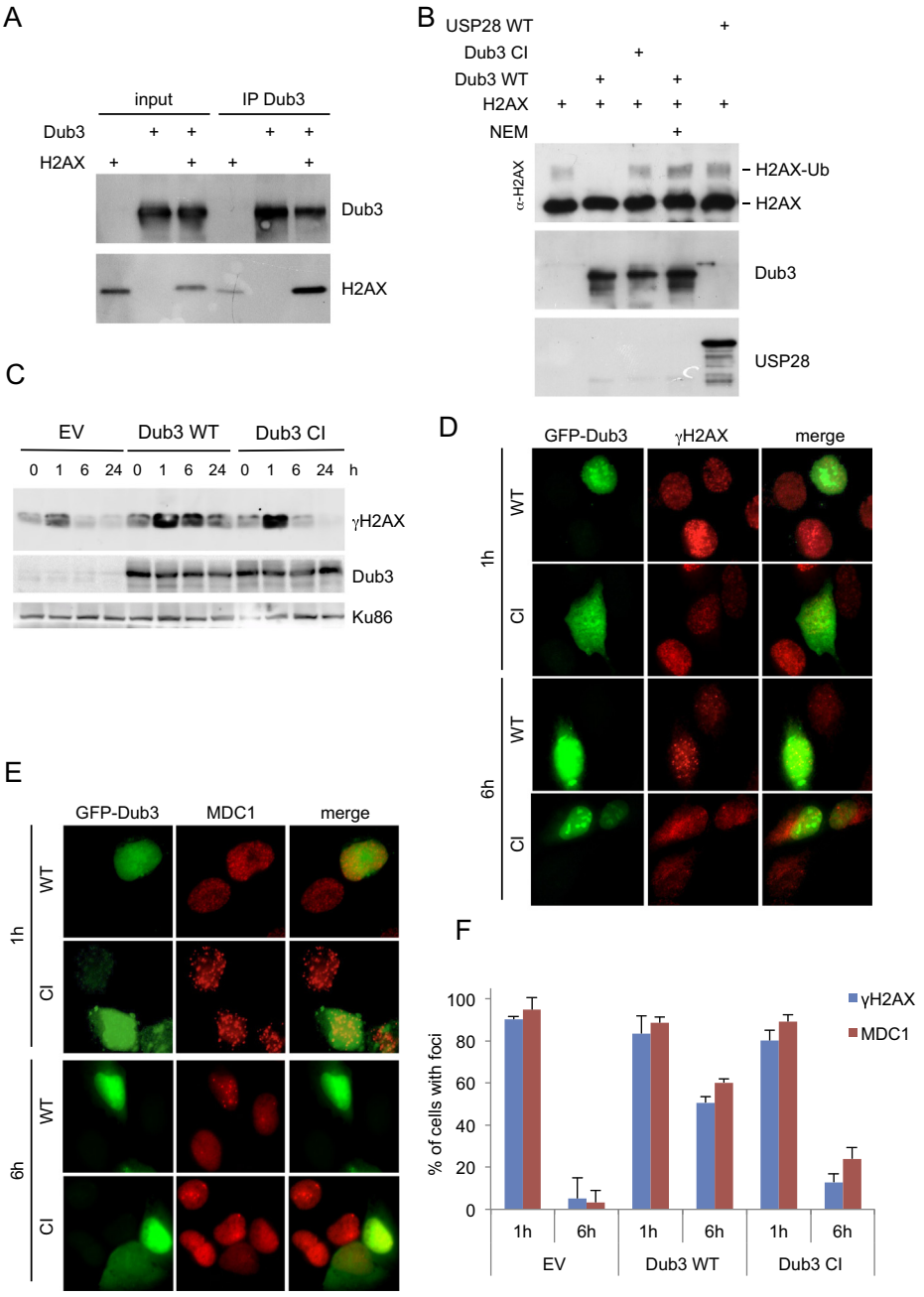

Figure 4 – *Excessive H2AX deubiquitylation by Dub3 binders a correct DNA damage response*. (A) Immunoprecipitation of purified H2AX and Dub3 with an anti-Dub3 antibody, followed by western blotting using the indicated antibodies. (B) *In vitro* deubiquitination assay of purified ubiquitylated H2AX and Dub3 WT, Dub3 CI or USP28 WT, analysed by western blotting using the indicated antibodies. (C) 293T cells were transfected with empty vector (EV), Dub3 WT or CI. After 36 h, cells were incubated with 1 μM ETP for 1 h, then washed and incubated with fresh medium for the indicated time periods. Then cells were lysed and western blotting using the indicated antibodies was performed. (D) U2OS cells were transfected with GFP-Dub3 WT or CI. Cells were treated with 1 μM ETP for 1 h. Cells were fixed or washed and left to recover for another 5 h. Cells were analysed by immunofluorescence. GFP-positive cells were scored for γH2AX foci. GFP-negative cells served as untransfected controls. (E) As in D) but now for MDC1. (F) Quantification of D) and E).

Dubs, in which Dub3 was absent (Mosbech et al., 2013). Although we cannot rule out cell type or tissue specificity for each of the different enzymes, the existence of more ubiquitin hydrolases regulating this pathway critical for maintaining genomic stability suggests functional redundancy. Our experiments show that the Dub3 H2AX deubiquitination effect was comparable or even stronger than most other of the described Dubs. Interesting, knockdown of only two of these known Dubs leads to an increase in ubiquitylated H2A(X), as we observed for Dub3 (Nicassio et al., 2007; Shao et al., 2009). Among them, depletion of BRCC36 increases the DNA damage-induced H2AX ubiquitination. Different to this,

depletion of Dub3 and USP3 already leads to elevated H2AX ubiquitination levels in undamaged conditions. This observation implies an active turnover of histone ubiquitination under such circumstances and points to Dub3 and USP3 as significant participants in this process.

Little is known about Dub3, although this ubiquitin Dub3 has been implicated in the DDR before by regulating the stability of Cdc25A, a phosphatase that activates Cyclin/Cdk complexes and is a downstream target of the DNA damage checkpoint (Mailand et al., 2000; Pereg et al., 2010). Given the effects of Dub3 overexpression on 53BP1 and BRCA1 focus formation, two events upstream the regulation of the DNA damage-induced cell cycle arrest, the direct interaction between Dub3 and H2AX and finally, the *in vitro* deubiquitination of H2AX by Dub3, the effect of Dub3 on H2AX appears independent of its role in controlling proteasome-dependent degradation of Cdc25A. By controlling the DDR at different levels, Dub3 emerges as a putative important regulator in maintaining genomic integrity, which is underscored by the oncogenic potential in xenograft tumour models (Pereg et al., 2010). However, this ubiquitin hydrolase does not seem to control all DNA damage-induced ubiquitination events, as for example overexpression of Dub3 does not affect FANCD2 monoubiquitination (Figure S2D). Future studies will focus on regulation of Dub3, especially in response to DNA damage, as this might be a mechanism to counterbalance DNA damage checkpoint activation, and/or to contribute in recovery of cell cycle progression.

3.6. Conclusion

This study describes the identification and characterization of Dub3 as a novel regulator of H2A(X). By directly controlling the levels of H2AX deubiquitination, Dub3 regulates the recruitment of DNA repair factors 53BP1 and BRCA1 to sites of DNA lesions and thereby ensures a correct DDR. These data demonstrate that a tight regulation of DNA damage checkpoint activation at multiple levels is crucial for the maintenance of genomic integrity.

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Appendix A. Supplementary data

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