The EMBO Journal (2009) 28, 3207–3215 | © 2009 European Molecular Biology Organization | All Rights Reserved 0261-4189/09
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THE EMBO JOURNAL

Ubiquitin ligase ARF-BP1/Mule modulates base excision repair

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Base excision repair (BER) is the major cellular pathway involved in removal of endogenous/spontaneous DNA lesions. Here, we study the mechanism that controls the steady-state levels of BER enzymes in human cells. By fractionating human cell extract, we purified the E3 ubiquitin ligase Mule (ARF-BP1/HectH9) as an enzyme that can ubiquitylate DNA polymerase β (Pol β), the major BER DNA polymerase. We identified lysines 41, 61 and 81 as the major sites of modification and show that replacement of these lysines to arginines leads to increased protein stability. We further show that the cellular levels of Pol β and its ubiquitylated derivative are modulated by Mule and ARF and siRNA knockdown of Mule leads to accumulation of Pol β and increased DNA repair. Our findings provide a novel mechanism regulating steady-state levels of BER proteins.

The EMBO Journal (2009) **28,** 3207–3215. doi:10.1038/emboj.2009.243; Published online 27 August 2009
Subject Categories: proteins; genome stability & dynamics
Keywords: ARF; base excision repair; DNA polymerase β; Mule; ubiquitylation

Introduction

Base excision repair (BER) is the major cellular system involved in removal of DNA lesions induced by endogenous and exogenous DNA damaging agents, as well as those arising because of the chemical instability of the DNA molecule (Lindahl, 1993; Friedberg *et al*, 2006). BER is accomplished by a subset of enzymes that recognize and process DNA damage of different complexity, including DNA base lesions and single strand DNA breaks (reviewed in Dianov and Parsons, 2007). Among the BER proteins, DNA polymerase β (Pol β) has a central role by filling the one nucleotide gap arising during the repair process (Sobol *et al*, 1996; Dianov *et al*, 1999; Allinson *et al*, 2001). The regulation of cellular Pol β levels is vital, as both under or overproduction of Pol β leads to deficient repair or increased rate of mutagenesis, respectively, and both have been linked to increased cancer susceptibility (Canitrot *et al*, 1999; Cabelof *et al*, 2003; Chan *et al*, 2007). In normal cells, Pol β is mainly located in the nucleus, however if the mechanism controlling the steady-state level of Pol β is broken or misbalanced, this may lead to abnormally high levels and accumulation of Pol β in the nucleus, cytoplasm or both, which has been shown to frequently occur in cancer cells (Albertella *et al*, 2005).

We have recently shown that steady-state levels of BER enzymes are controlled by two processes: DNA damage-dependent stabilization of BER enzymes involved in DNA repair and ubiquitylation-dependent degradation of BER enzymes that are not. We also purified the major cellular ubiquitin ligase involved in polyubiquitylation of Pol β and identified it as carboxyl terminus of Hsc70 interacting protein (CHIP; Parsons *et al*, 2008). These data provided a mechanism responsible for Pol β degradation. However, it was not clear how CHIP discriminates between repair proteins that are excessive and should be labelled for proteasomal degradation, and those that are required for DNA repair but are not yet relocated to the sites of DNA damage. We speculated that DNA damage-associated monoubiquitylation of Pol β that precedes polyubiquitylation by CHIP, may be the missing link that serves as a tuning mechanism providing adjustment of the levels of BER enzymes to the amount of DNA lesions. We hypothesize that such a mechanism should be able to ascertain the steady-state level of BER enzymes that would be enough to maintain efficient repair of endogenous DNA lesions, but will also prevent excessive accumulation of BER proteins. We therefore sought to identify the ubiquitin ligase that is able to monoubiquitylate Pol β and whose activity is regulated by DNA damage. Here, we report that Mule (also known as ARF-binding protein 1/ARF-BP1 or Hect H9), whose activity is regulated by ARF protein in response to DNA damage (Chen *et al*, 2005), is an E3 ubiquitin ligase that ubiquitylates Pol β and modulates its steady-state level.

Results

Mule ubiquitylates Pol β at lysines 41, 61 and 81

We recently purified CHIP as an E3 ubiquitin ligase that is involved in polyubiquitylation of BER enzymes (Parsons *et al*, 2008). Further analysis of the dependence of the ubiquitin conjugating (E2) enzymes involved in ubiquitylation of Pol β by active fractions purified from HeLa cells showed that in addition to CHIP, which was identified in these fractions and requires H5/H6 E2s for activity (Figure 1A), active fractions at the final steps of purification (Mono Q column; Parsons *et al*, 2008) also contained E3 ligase activity dependent on H7 E2 (Figure 1B). This H7-dependent ubiquitylation generates three major protein bands that crossreact with Pol β antibodies (Figure 1C, lane 4), suggesting three potential sites for monoubiquitylation

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Received: 17 March 2009; accepted: 23 July 2009; published online: 27 August 2009

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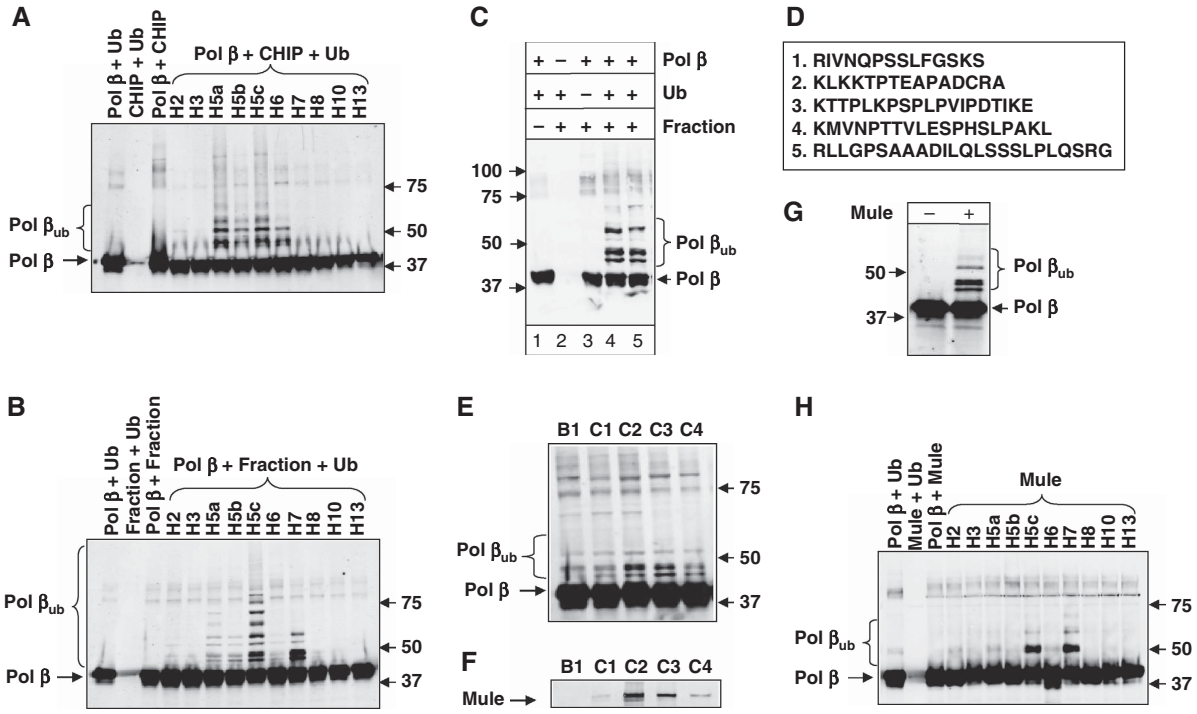

Figure 1 Purification of an E3 ubiquitin ligase for Pol β and identification as Mule. *In vitro* ubiquitylation of Pol β (5 pmol) by (**A**) CHIP (15 pmol) or (**B**) active fraction purified from HeLa whole cell extracts in the presence of E1 (0.7 pmol), ubiquitin (0.6 nmol) and various E2 enzymes (9.5 pmol) analysed by 10% SDS–PAGE and immunoblotting using Pol β antibodies. (**C**, **E**) *In vitro* ubiquitylation of Pol β (5 pmol) by active fractions purified from HeLa whole cell extracts in the presence of E1 (0.7 pmol), H7 (9.5 pmol) and either ubiquitin (lanes 1–4, 0.6 nmol) or mutant ubiquitin (lane 5, 0.6 nmol) unable to form polyubiquitin chains. Samples were analysed by 10% SDS–PAGE and immunoblotting using Pol β antibodies. (**D**) Peptide sequences detected by nanoLC-MS/MS from the final chromatography fractions (C2 and C3) that correspond to the 482 kDa Mule protein (SwissProt Nr. Q7Z6Z7, Mascot Score: 192). (**F**) Analysis of final chromatography fractions purified from HeLa whole cell extracts by 10% SDS–PAGE and immunoblotting using Mule antibodies showing correlation with *in vitro* ubiquitylation activity. *In vitro* ubiquitylation of Pol β (5 pmol) by truncated Mule (3.5 pmol) in the presence of E1 (0.7 pmol), ubiquitin (0.6 nmol) and (**G**) H7 (9.5 pmol) or (**H**) various E2 enzymes (9.5 pmol) analysed by 10% SDS–PAGE and immunoblotting using Pol β antibodies. Molecular weight markers are indicated on the side of appropriate figures and the positions of ubiquitylated Pol β (Pol β_{ub}) are shown.

because all three ubiquitylation-specific bands were observed when mutant ubiquitin, unable to form polyubiquitin chains, was used (Figure 1C, lane 5). Proteins from active fractions from the final purification step (Mono Q column) were subjected to nanoLC-MS/MS tandem mass spectrometry, which showed Mule as a potential E3 ubiquitin ligase involved in H7-dependent Pol β ubiquitylation (Figure 1D). Indeed, when active fractions were tested for the presence of Mule, we found a strong correlation between H7-dependent ubiquitylation activity in these active fractions and the amount of Mule protein detected by immunoblotting (Figure 1E and F). Although Mule is a 4374 amino acid long protein with a molecular mass of 482 kDa, E3 ubiquitin ligase activity has been mapped within the C-terminal 370 amino acid HECT domain (Chen *et al*, 2005; Zhong *et al*, 2005). We therefore purified recombinant truncated human Mule protein containing the HECT domain (Adhikary *et al*, 2005) and found that it was able to ubiquitylate Pol β in an *in vitro* ubiquitylation system reconstituted with purified enzymes (Figure 1G) and that this reaction requires H5c or H7 E2 enzymes (Figure 1H). We also showed that FLAG-Pol β expressed in HeLa cells can co-precipitate with Mule showing an interaction between the two proteins in human cells (Supplementary Figure S1).

We have earlier mapped the ubiquitylation region for CHIP within the 8 kDa domain of Pol β (Parsons *et al*, 2008) and, using a similar approach, we also localized the ubiquitylation site for Mule within the 8 kDa domain (Figure 2A–C). We next identified the ubiquitylated 8 kDa domain band by Coomassie staining (Figure 2D), excised the band for analysis by tandem mass spectrometry and found that *in vitro* ubiquitylation occurs at lysines 41, 61 and 81 (Supplementary Figure S2). Site-directed mutagenesis of individual lysines to arginines or double mutation combinations showed that a knockout of one or two ubiquitylation sites (with the exception of lysine 41 that resulted in a reduction of one of the major ubiquitylation bands) did not block ubiquitylation and only replacement of all three lysines to arginines completely abolished *in vitro* ubiquitylation of mutated Pol β by both an active fraction purified from HeLa cells (Figure 2E) and recombinant truncated Mule (Figure 2F). We next generated mammalian vectors expressing wild type and mutant Pol β protein in which lysines 41, 61 and 81 were replaced with arginines. After transfection of equal amounts of the expressing plasmids in HeLa cells, we found that the mutant protein lacking the major ubiquitylation sites showed an average of 1.8-fold increased stability compared with the wild-type protein (Figure 2G). Taken together, these experiments demonstrate that Mule is able to ubiquitylate Pol β at lysines 41, 61 and 81 and that this ubiquitylation results in reduced protein stability.

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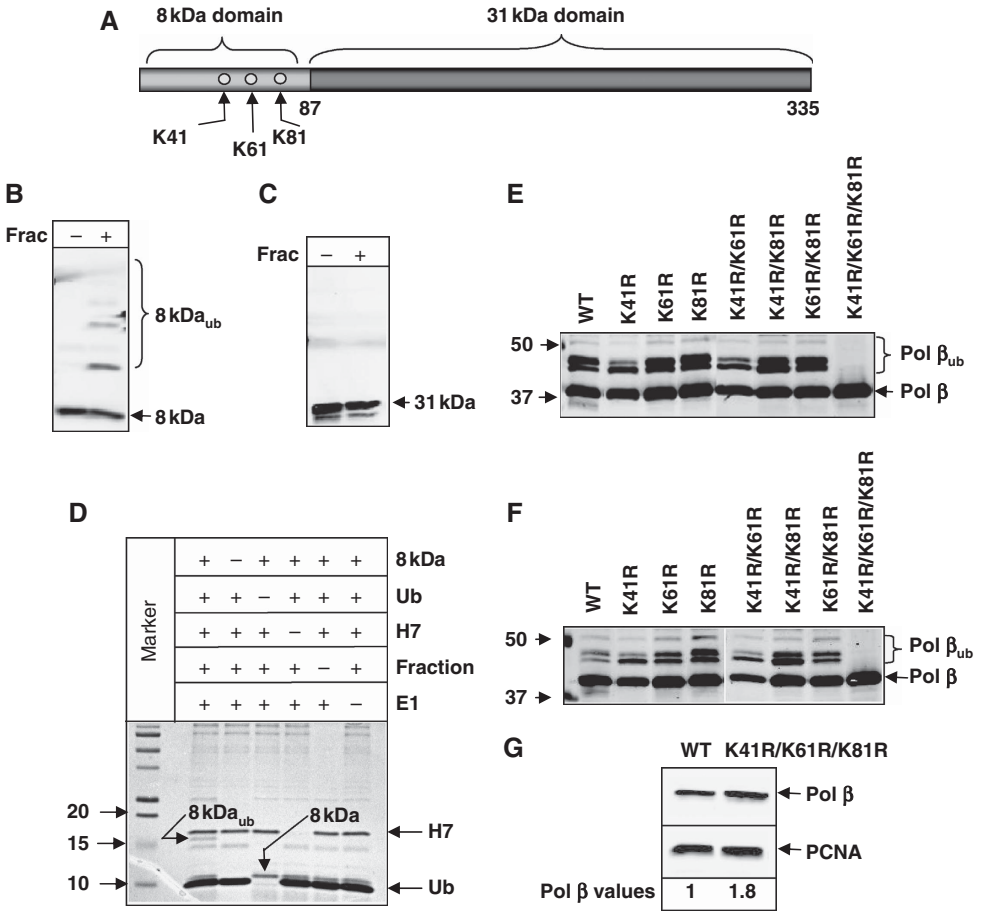

Figure 2 Identification of Mule ubiquitylation sites within Pol β. (**A**) Schematic diagram of the protein structure of Pol β showing the major sites (K41/K61/K81) of ubiquitylation by Mule that are present within the 8 kDa domain. *In vitro* ubiquitylation of (**B**) 8 kDa and (**C**) 31 kDa Pol β domains by active fraction purified from HeLa whole cell extracts in the presence of E1 (0.7 pmol), H7 (9.5 pmol) and ubiquitin (0.6 nmol) analysed by 10% SDS–PAGE and immunoblotting using Pol β antibodies. (**D**) *In vitro* ubiquitylation of 8 kDa Pol β domain by active fraction purified from HeLa whole cell extracts in the presence of E1 (0.7 pmol), H7 (9.5 pmol) and ubiquitin (0.6 nmol) analysed by Coomassie staining. The monoubiquitylated 8 kDa Pol β band was identified (8 kDa$_{ub}$; see arrow), excised and analysed by nanoLC-MS/MS to identify the sites of ubiquitylation (Supplementary Figure S2A–C). *In vitro* ubiquitylation of wild-type Pol β (WT) and various Pol β mutants using (**E**) active fraction purified from HeLa whole cell extracts or (**F**) purified truncated Mule (3.5 pmol) in the presence of E1 (0.7 pmol), H7 (9.5 pmol) and ubiquitin (0.6 nmol) analysed by 10% SDS–PAGE and immunoblotting using Pol β antibodies. Molecular weight markers are indicated on the left hand side of appropriate figures and the positions of ubiquitylated Pol β (Pol β$_{ub}$) and ubiquitylated 8 kDa domain Pol β (8 kDa$_{ub}$) are shown. (**G**) HeLa cells were transfected with mammalian vectors expressing wild type or K41/K61/K81 mutant FLAG-tagged-Pol β for 24 h, whole cell extracts were prepared and analysed by 10% SDS–PAGE and immunoblotting with FLAG or PCNA antibodies. The relative Pol β value is normalized to the amount of PCNA (average of three experiments).

Pol β ubiquitylation in living cells

To show that Pol β ubiquitylation occurs in living cells, we prepared HeLa whole cell extracts in the presence of *N*-ethylmaleimide (NEM), which is an effective inhibitor of deubiquitylating enzymes. We found that in HeLa extracts prepared with NEM, Pol β antibodies, in addition to Pol β itself, detected two other major protein bands just below 50 kDa (Figure 3A). As the molecular weight of monoubiquitylated Pol β is expected to be about 47 kDa, both proteins were considered as potential candidates for ubiquitylated Pol β (Pol β$_{ub}$). To identify which of the two bands was Pol β$_{ub}$ and to uncover its subcellular localization, we fractionated cellular components into cytoplasmic (C) and nuclear (N) protein fractions. The integrity of the extract preparations was tested for known cytoplasmic and nuclear protein markers to ensure the validity of this assay (Supplementary Figure S3A). Using this approach, we found that in HeLa cells one of the candidate proteins for Pol β$_{ub}$ was associated with the cytoplasmic fraction and the other with the nuclear protein fraction (Figure 3B, lanes 1 and 2). However, when siRNA targeted against Pol β was used, which causes an efficient knockdown of the Pol β protein (Supplementary Figure S3B), only one of these bands disappeared (Figure 3B, lanes 3 and 4), suggesting that the ~47 kDa protein isolated in the cytoplasmic fraction is modified Pol β. To provide further evidence that this protein was Pol β$_{ub}$, we partially purified this protein from cytoplasmic extracts by phosphocellulose and Mono Q chromatography, monitoring protein containing fractions with Pol β antibodies, and then probed the partially purified protein with antibodies directed against ubiquitin. We found that fractions containing the ~47 kDa protein crossreacting with Pol β antibodies also crossreacted with ubiquitin-specific antibodies (Figure 3C). We next transfected HeLa cells with a mammalian vector expressing FLAG-tagged-Pol β in the

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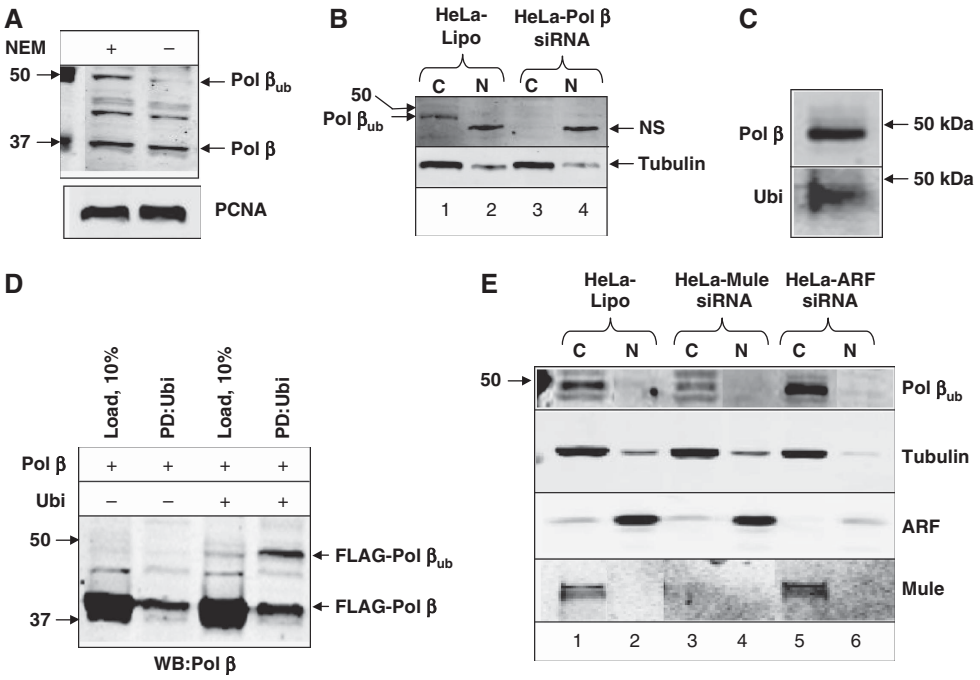

Figure 3 Identification of monoubiquitylated Pol β in human cell extracts and dependence on Mule and ARF. (**A**) Whole cell extracts were prepared from HeLa cells in the presence and absence of 1 mM NEM and analysed by 10% SDS–PAGE and immunoblotting with Pol β and PCNA antibodies. (**B**, **E**) HeLa cells were grown in 6 cm^2 dishes for 24 h to 30–50% confluency and then treated with Lipofectamine transfection reagent (10 μl) in the absence and presence of (B) Pol β or (E) Mule and ARF siRNA (200 pmol) for a further 72 h. Cells were pelleted by centrifugation, cytoplasmic (C) and nuclear (N) fractions were prepared and 40 μg protein in the C fraction and an equal volume of the N fraction were analysed by 10 % SDS–PAGE and immunoblotting with the antibodies indicated. (**C**) The ~47 kDa protein suspected to be ubiquitylated Pol β was partially purified from calf thymus by phosphocellulose and Mono Q chromatography and fractions crossreacting with Pol β antibodies were also probed with ubiquitin antibodies. (**D**) HeLa cells were transfected with a mammalian vector (1 μg) expressing FLAG-tagged-Pol β in the presence or absence of a mammalian vector expressing His-tagged-ubiquitin (1 μg) for 24 h, whole cell extracts were prepared, ubiquitylated proteins were precipitated with Ni-agarose beads and analysed by western blotting using FLAG antibodies. Molecular weight markers are indicated on the side of appropriate figures and the positions of ubiquitylated Pol β (Pol $β_{ub}$) are shown. NS corresponds to a non-specific protein recognized by the Pol β antibodies.

presence and absence of a vector expressing His-tagged-ubiquitin and precipitated ubiquitylated proteins with Ni-agarose beads. Western blot analysis of precipitated proteins using anti-FLAG antibodies identify a protein band specific for ubiquitylated Pol β only in cells expressing FLAG-tagged-Pol β and His-tagged-ubiquitin (Figure 3D, last lane). We thus conclude that this ~47 kDa protein is monoubiquitylated Pol β.

In vivo ubiquitylation of Pol β is dependent on Mule and ARF

HeLa cells are known to overexpress Mule (Adhikary *et al*, 2005) and we found that this correlates well with the increased levels of Pol $β_{ub}$, compared with several other cell lines tested (Supplementary Figure S3C; data not shown). To show that cellular ubiquitylation of Pol β is dependent on Mule, we knocked down Mule in HeLa cells by siRNA (>80% decrease; Figure 3E) and fractionated cell extracts into cytoplasmic and nuclear protein fractions. We found a reduced (two-fold, average of three experiments) amount of Pol $β_{ub}$ in the cytoplasmic fraction of Mule knockdown cells (Figure 3E; Supplementary Figure S3C, compare lanes 1 and 3). ARF is known to inhibit the ubiquitylation activity of Mule (Chen *et al*, 2005) and consistent with this inhibition, knockdown of ARF resulted in an increased (two-fold, average of three experiments) amount of Pol $β_{ub}$ (Figure 3E; Supplementary Figure S3C, compare lanes 1 and 5). Thus, we conclude that a significant proportion of the ubiquitylation of Pol β in living cells is mediated by Mule and that this ubiquitylation is controlled by ARF. We also conclude that the majority of ubiquitylated Pol β is localized in the cytoplasm. This is further supported by the observation that Mule is predominantly found in the cytoplasmic fraction (Figure 3E) and has been localized to the cytoplasm (Liu *et al*, 2007; Supplementary Figure S4A).

ARF and Mule modulate BER by controlling steady-state levels of Pol β

To show that Mule can modulate BER capacity, we knocked down Mule using a Mule-specific siRNA. After 72 h of siRNA treatment we observed a reduced level of Pol $β_{ub}$ but also a consistent ~1.8-fold increase in the level of Pol β extracted from both the cytoplasm and the nucleus (Figure 4A). This elevated level of Pol β in the nucleus after Mule siRNA is predicted to cause an elevation in the rate of DNA repair of cells after DNA damage treatment. Therefore, Mule knockdown of cells was followed by treatment with hydrogen peroxide and DNA repair ability of these cells was evaluated using the alkaline Comet assay. About an 80% knockdown of Mule (Figure 4B) did not change the overall steady-state level of DNA damage compared to Lipofectamine-only-treated cells (Figure 4D control bars). However, Mule knockdown

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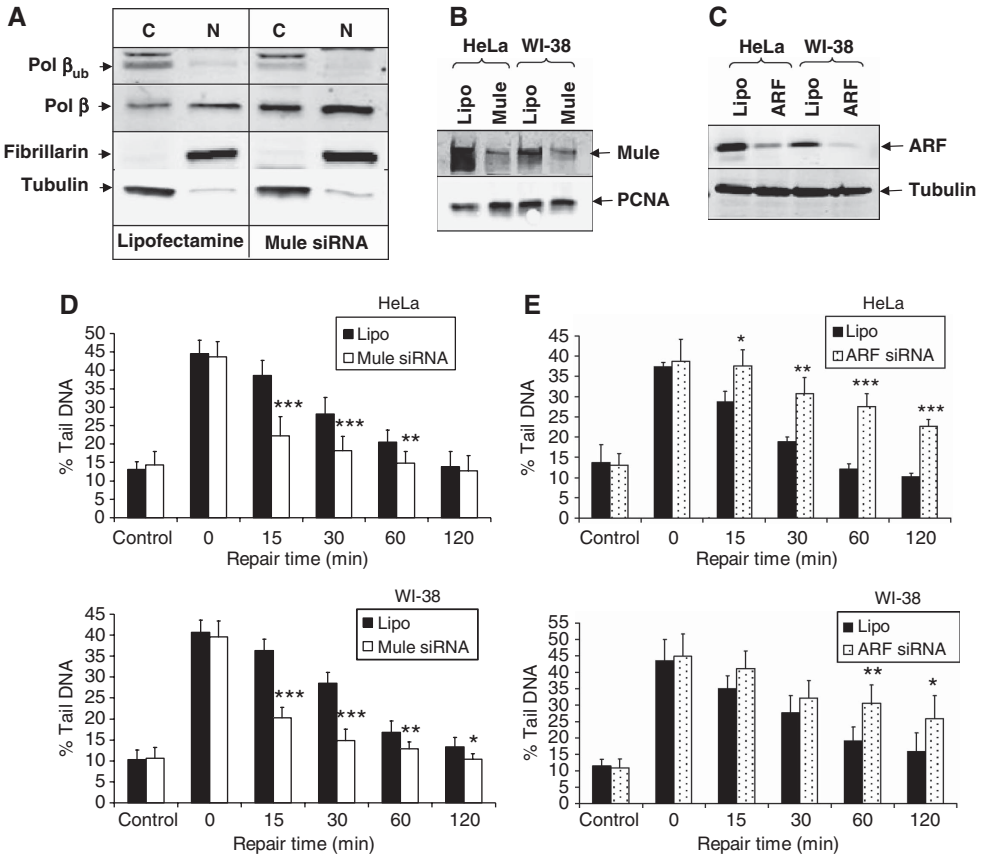

Figure 4 Modulation of BER by Mule and ARF. (**A**) HeLa cells were grown in 6 cm^2 dishes for 24 h to 30–50% confluency and then treated with Lipofectamine transfection reagent (10 μl) in the absence and presence of Mule siRNA (200 pmol) for a further 72 h. Cells were pelleted by centrifugation, cytoplasmic (C) and nuclear (N) fractions were prepared and 40 μg protein in the C fraction and an equal volume of the N fraction were analysed by 10% SDS–PAGE and immunoblotting with the antibodies indicated. HeLa and WI-38 cells were grown in 6 cm^2 dishes for 24 h to 30–50% confluency and then treated with Lipofectamine (10 μl) in the absence and presence of Mule (**B**) or ARF (**C**) siRNA (200 pmol) for a further 72 h. Whole cell extracts were prepared and analysed by 10% SDS–PAGE and immunoblotting with the antibodies indicated. Alternatively, after 72 h with Lipofectamine or (**D**) Mule or (**E**) ARF siRNA the cells were treated with 20 μM hydrogen peroxide for 5 min, allowed to repair for 0–120 min and the levels of single strand breaks and alkali labile sites then analysed by the alkaline single cell gel electrophoresis (Comet) assay. Shown are the mean % tail DNA values with standard deviations from at least three independent experiments. Statistically significant results comparing Lipofectamine and siRNA-treated cells are represented by $*P<0.02$, $**P<0.005$ and $***P<0.001$, as analysed by Student's *t*-test.

increased the rate of repair of hydrogen peroxide-induced DNA lesions (Figure 4D) confirming that the increased level of Pol β in these cells modulated by Mule knockdown correlates with increased DNA repair capacity. As expected, ARF knockdown (Figure 4C) had the opposite effect with slower repair rates of hydrogen peroxide-induced DNA damage observed in both cell lines compared to Lipofectamine-only-treated cells (Figure 4E) or cells treated with scrambled siRNA (data not shown).

As Mule controls many other cellular functions, it was important to show that the effect on DNA repair was dependent on Pol β. We therefore used isogenic Pol β-proficient (Pol $\beta^{+/+}$) and Pol β-deficient (Pol $\beta^{-/-}$) mouse embryonic fibroblasts. Although Pol β-deficient cells are able to quite efficiently repair hydrogen peroxide-induced DNA damage, they accomplish the repair process using a backup pathway involving DNA polymerase δ (Fortini *et al*, 1998) and correspondingly this repair pathway should not respond to Mule knockdown. As predicted, we found that in response to Mule knockdown (Figure 5A), similar to the effect of Mule on HeLa and WI-38 cells, Pol $\beta^{+/+}$ cells showed an increase in the rate of repair of hydrogen peroxide-induced DNA damage compared to Lipofectamine-only-treated cells (Figure 5B). In contrast, the repair rate of hydrogen peroxide-induced DNA damage in Pol $\beta^{-/-}$ cells did not change in response to Mule knockdown (Figure 5B). These data suggest that the increased DNA repair response to Mule downregulation is Pol β-dependent.

CHIP-dependent degradation of monoubiquitylated Pol β

In vitro experiments suggested that monoubiquitylation of Pol β by Mule stimulates polyubiquitylation catalysed by CHIP, as active fractions purified from HeLa cells (containing both CHIP and Mule) in the presence of H5c E2 conjugating enzyme, which can be used by both ubiquitin ligases, polyubiquitylates Pol β much more efficiently than the individual proteins (compare Figure 1B with Figure 1A and H). Similarly, in an *in vitro* ubiquitylation reaction reconstituted with purified recombinant proteins, monoubiquitylation of Pol β by Mule stimulates polyubiquitylation by CHIP (Figure 6A). We therefore proposed that monoubiquitylated Pol β is further polyubiquitylated by CHIP and thus labelled

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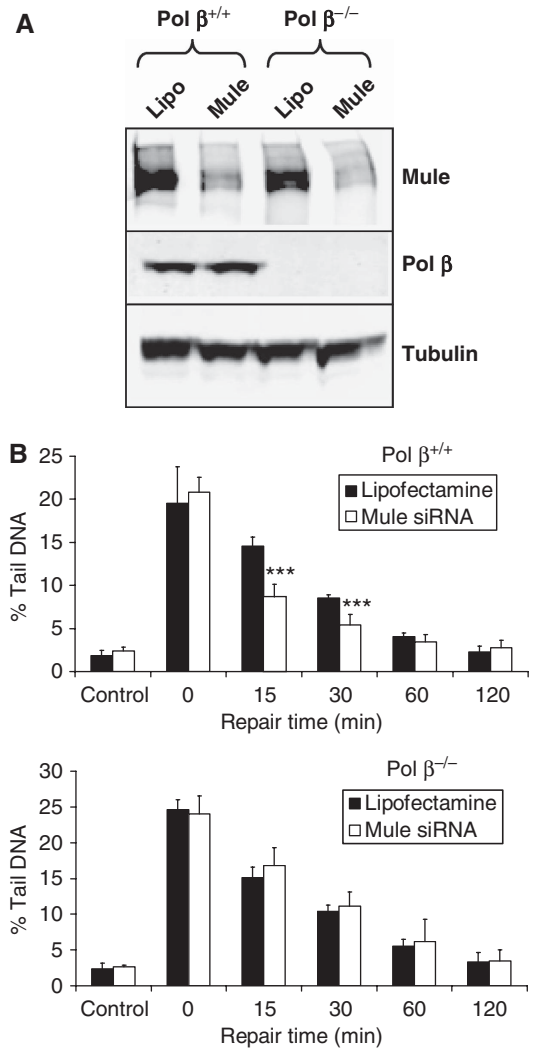

Figure 5 Modulation of BER by Mule depends on Pol β. Isogenic Pol β-proficient (Pol $\beta^{+/+}$) and Pol β-deficient (Pol $\beta^{-/-}$) cells were grown in 6 cm^2 dishes for 24 h to 30–50% confluency and then treated with Lipofectamine transfection reagent (10 μl) in the absence and presence of Mule siRNA (200 pmol) for a further 48 h. (**A**) Whole cell extracts were prepared and analysed by 10% SDS–PAGE and immunoblotting with the antibodies indicated. (**B**) Alternatively, cells were treated with 30 μM hydrogen peroxide for 5 min, allowed to repair for 0–120 min and the levels of single strand breaks and alkali labile sites then analysed by the alkaline single cell gel electrophoresis (Comet) assay. Shown are the mean % tail DNA values with standard deviations from at least three independent experiments. Statistically significant results comparing Lipofectamine and siRNA-treated cells are represented by $***P<0.001$, as analysed by Student's *t*-test.

for proteasomal degradation. If this is the case, then a reduction in the levels of CHIP should result in an increased level of Pol β_{ub}. Indeed, when we knocked down CHIP by siRNA and monitored accumulation of Pol β_{ub}, we found increasing amounts of Pol β_{ub} at 48 and 72 h after transfection with CHIP siRNA (Figure 6B). In agreement with these results, knockdown of CHIP or Mule resulted in an increased stability of Pol β; however, Pol β stability in CHIP–Mule double knockdown cells was only slightly higher (Figure 6C), suggesting that both proteins operate on the same pathway, although CHIP has some Mule-independent ubiquitylation activity on Pol β, as we observed earlier in *in vitro* experiments (Figure 1A). We also found that overexpression of CHIP in HeLa cells accelerates the degradation of Pol β_{ub} (Figure 6D). These data support the idea that CHIP is further ubiquitylating Pol β_{ub} after Mule and thus promoting its degradation by the proteasome.

Discussion

The results presented herein show a previously unknown link between ARF, Mule and DNA repair and provide a new mechanism regulating the steady-state levels of BER proteins. Theoretically, the cellular pool of Pol β should consist of several forms: newly synthesized Pol β located in the cytoplasm (ubiquitylated or not), Pol β relocated to the nucleus but not yet associated with chromatin and chromatin-associated Pol β involved in DNA repair. We propose that the dynamics of this pool is controlled by Mule and ARF, which determines the fate of the newly synthesized cytoplasmic Pol β. If Pol β is ubiquitylated by Mule and later polyubiquitylated by CHIP, then it will be degraded by proteasome. However, if it escapes ubiquitylation it would be translocated to the nucleus and allowed to participate in DNA repair. Thus, in this scenario ARF and Mule control the stream of Pol β and direct it either to degradation or to repair. As this mechanism operates with only a small amount of cytoplasmic Pol β (about 15–20%), it is quite clear that the ARF–Mule-dependent accumulation of BER proteins can only be accomplished within a certain time frame and cannot be considered as an emergency cellular response to cope with dramatic changes in DNA damage levels induced by exogenous mutagens. Therefore, most likely other mechanisms are involved in the BER response to such severe DNA damage (Woodhouse *et al*, 2008). Most probably Mule–ARF-dependent regulation of BER has evolved as a tuning mechanism to bring steady-state levels of BER enzymes in accord with an individual's genomic DNA damage background and can only effectively respond to minor fluctuations in endogenous DNA lesions.

We have shown earlier that steady-state levels of BER enzymes are controlled by two processes: DNA damage-dependent stabilization of BER enzymes involved in DNA repair and CHIP-dependent degradation of BER enzymes that are not (Parsons *et al*, 2008). We now show that Mule and ARF are important components of this DNA damage-dependent regulation. Mule is predominantly a cytoplasmic protein (Liu *et al*, 2007; Figure 3E; Supplementary Figure S4A) whereas ARF is located in the nucleoli (Weber *et al*, 1999; Supplementary Figure S4B). It is also known that in response to DNA damage ARF is released from the nucleoli (Lee *et al*, 2005; Supplementary Figure S4C), interacts with Mule and inhibits its ubiquitin ligase activity (Chen *et al*, 2005; Gallagher *et al*, 2006), providing a molecular link between DNA damage and Mule activity.

We propose that as endogenous DNA damage is always occurring, the amount of Pol β required for DNA repair is regulated by Mule ubiquitylation, which is controlled by DNA damage through the release of ARF (Figure 7). At low levels of endogenous DNA damage, the majority of newly synthesized Pol β is ubiquitylated by Mule and CHIP in the cytoplasm and degraded by the proteasome before being relocated to the nucleus (Figure 7, left side). As long as

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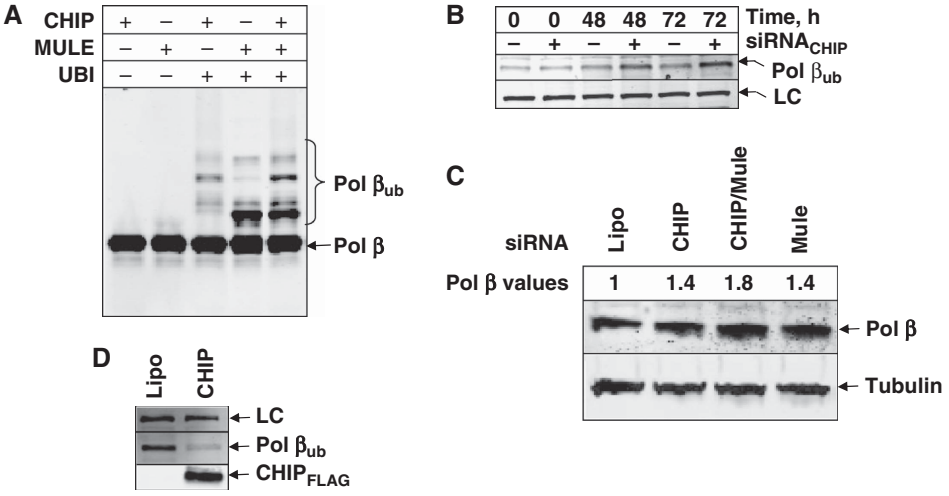

Figure 6 Ubiquitylation of Pol β by Mule stimulates CHIP-dependent ubiquitylation and degradation. (**A**) *In vitro* ubiquitylation of Pol β (5 pmol) by truncated Mule (3.5 pmol) and/or CHIP (15 pmol) in the presence of E1 (0.7 pmol), ubiquitin (0.6 nmol) and H5c (9.5 pmol) analysed by 10% SDS–PAGE and immunoblotting using Pol β antibodies. (**B**) HeLa cells were grown in 6 cm^2 dishes for 24 h to 30–50% confluency, treated with Lipofectamine transfection reagent (10 μl) in the absence and presence of CHIP siRNA (200 pmol) for a further 48 and 72 h and then whole cell extracts prepared and analysed by immunoblotting with Pol β antibodies to identify Pol $β_{ub}$. (**C**) HeLa cells were grown in 6 cm^2 dishes for 24 h to 30–50% confluency and then treated with Lipofectamine transfection reagent (10 μl) in the presence and absence of Mule siRNA (200 pmol), CHIP siRNA (200 pmol) or both for 72 h, whole cell extracts were prepared and analysed by western blotting using Pol β or tubulin antibodies. Relative Pol β values are normalized to the amount of tubulin. (**D**) HeLa cells were grown in 6 cm^2 dishes for 24 h to 90–95% confluence, treated with Lipofectamine transfection reagent (10 μl) in the absence and presence of CHIP expressing plasmid (1.2 pmol) for a further 24 h. Whole cell extracts were then prepared and analysed by immunoblotting with Pol β or FLAG antibodies. LC corresponds to a loading control.

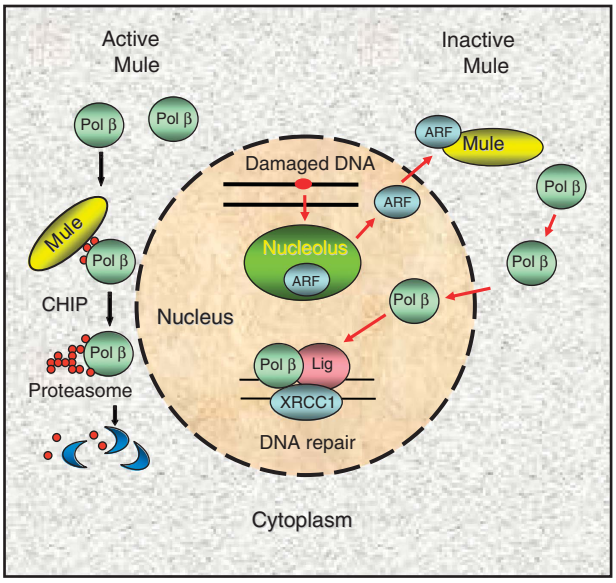

Figure 7 Proposed model for the regulation of Pol β steady-state levels by Mule, ARF and CHIP. If not required for DNA repair, Pol β is ubiquitylated by Mule that is then a target for CHIP mediated polyubiquitylation and subsequent degradation by the proteasome (left side of scheme). However, after detection of DNA damage ARF is released from the nucleoli into the cytoplasm in which it inhibits the activity of Mule, thus reducing Pol β degradation and upregulating DNA repair (right side of scheme). The repair of DNA damage will result in a decreased release of ARF, with a concomitant increased activity of Mule that will downregulate Pol β levels. A new adjustment cycle will therefore begin on the detection of increased levels of DNA damage.

DNA damage is detected, ARF that is normally located in the nucleoli (Korgaonkar *et al*, 2005) is released (Lee *et al*, 2005). This inhibits Mule activity, which will in turn lead to a reduced rate of Pol β monoubiquitylation by Mule and consequently a reduced CHIP-promoted degradation of Pol β (Figure 7, right side). The concomitant accumulation of Pol β, firstly in the cytoplasm and consequently in the nucleus, will lead to increased ability of the cell to perform DNA damage repair. This will lead to a reduced level of DNA lesions, reduced release of ARF, activation of Mule and ubiquitylation of Pol β, accomplishing a tuning cycle (Figure 7). This cycle can be repeated again and again to increase or decrease the steady-state level of DNA repair enzymes and keep it at the level required for efficient repair of existing DNA lesions. This model is supported by our results showing that prolonged downregulation of Mule by siRNA increases the amount of Pol β. However, it should be stressed again that substantial upregulation of the steady-state levels of Pol β will require many tuning cycles that limit the capacity of the system to respond to significant increases in DNA damage induced by exogenous mutagens.

It is clear that the cellular consequences of losing ARF may be dramatic because, in addition to controlling steady-state levels of BER enzymes, this protein is also involved in controlling apoptosis and p53-dependent cell-cycle arrest (reviewed in Gallagher *et al*, 2006; Sherr, 2006). The reduced repair capacity of ARF-deficient cells demonstrated in this study, in combination with the inability to undergo apoptosis or to block damaged cells from proliferation, significantly increases the probability of developing tumours. This is highly relevant because a deficiency in ARF has been found in many cancers (Sharpless and Chin, 2003; Kim and Sharpless, 2006). Our results provide a molecular mechanism to explain how the tumour suppressor ARF regulates DNA repair of endogenous DNA lesions by controlling the levels of Pol β.

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Materials and methods

Antibodies

Pol β antibodies were purified as described (Dianova *et al*, 2001). Mule and ARF antibodies were purchased from Bethyl Laboratories (Montgomery, USA), FLAG antibodies were purchased from Agilent Technologies (Stockport, UK), tubulin antibodies were purchased from Sigma-Aldrich (Gillingham, UK), PCNA antibodies were purchased from Santa Cruz (California, USA) and histone H4, fibrillarin and ubiquitin were from Abcam (Cambridge, UK).

Proteins

Ubiquitin, E1 and E2 enzymes were purchased from Boston Biochemicals (Cambridge, USA). The bacterial and mammalian expression vectors for truncated Mule and CHIP were kindly provided by Dr M Eilers and Prof J Hohfeld, respectively. The mammalian expression vectors for FLAG-Pol β was kindly provided by Dr R Sobol. Pol β expression mutants were constructed using the 'QuikChange' protocol (Stratagene, Amsterdam, Holland) and confirmed by sequencing. Histidine-tagged Pol β and CHIP were purified on Ni-NTA agarose (Qiagen, Crawley, UK) and GST-tagged truncated Mule was purified on a GSTrap FF column (GE Healthcare, Little Chalfont, UK). CHIP and Mule were further loaded onto a 1 ml Mono Q HR 5/5 column (GE Healthcare) in Buffer A (50 mM Tris–HCl (pH 8.0), 1 mM EDTA, 5% glycerol, 1 mM DTT, 1 mM PMSF) containing 50 mM KCl, proteins were eluted using a linear gradient of 50–1000 mM KCl, fractions were analysed using the Experion Electrophoresis System (Bio-Rad, Hemel Hempstead, UK) and those containing CHIP or truncated Mule were pooled, aliquoted and frozen at −80°C.

Whole cell extracts

Whole cell extracts were prepared by Tanaka's method (Tanaka *et al*, 1992). Briefly, cells were resuspended in one packed cell volume of buffer containing 10 mM Tris–HCl (pH 7.8), 200 mM KCl, 1 mg/ml of each protease inhibitor (pepstatin, aprotinin, chymostatin and leupeptin), 1 mM DTT and 1 mM PMSF. Two packed cell volumes of buffer containing 10 mM Tris–HCl (pH 7.8), 600 mM KCl, 40 % glycerol, 0.1 mM EDTA and 0.2% Nonidet P-40 was then added and mixed thoroughly before rocking the cell suspension for 2 h at 4°C. The cell lysate was then centrifuged at 40 000 r.p.m. at 4°C for 20 min and the supernatant was collected, aliquoted and stored at −80°C.

Cell fractionation

Cell fractionation was performed as recently described (Woodhouse *et al*, 2008). Briefly, cell pellets were resuspended in two packed cell volumes of buffer containing 20 mM Tris–HCl pH 7.4, 2.5 mM $MgCl_2$, 0.5 % (v/v) Nonidet P-40, 1 mM PMSF, 1 mM DTT and 1 μg/ml each of aprotinin, pepstatin, chymostatin and leupeptin, and incubated on ice for 10 min. After centrifugation at 10 000 r.p.m. for 2 min, the supernatant containing cytoplasmic proteins (C) was collected. The nuclear pellet was similarly extracted with two packed cell volumes of buffer containing 100 mM KPO_4 pH 8.0, 0.5 M KCl, 5 mM $MgCl_2$, 1 mM EDTA, 0.75 % (v/v) Triton X-100, 10% (v/v) glycerol, 1 mM PMSF, 1 mM DTT and 1 μg/ml each of aprotinin, pepstatin, chymostatin and leupeptin and the supernatant containing nuclear proteins (N) was collected.

Western blots

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).

RNA interference

Cells were transfected using Lipofectamine 2000 transfection reagent (Invitrogen, Paisley, UK) according to the manufacturer's protocol. The siRNA sequences used to target the Pol β, Mule, ARF and CHIP transcripts were as follows: 5′-GAUCAGUAUUACUGUGG UG-3′ (Albertella *et al*, 2005), 5′-AAUUGCUAUGUCUCUGGGACA-3′ (Chen *et al*, 2005), 5′-GAACAUGGUGCGCAGGUUCTT-3′ (Eymin *et al*, 2006) and 5-GGAGCAGGGCAAUCGUCUG-3′ (Qian *et al*, 2006), respectively.

In vitro ubiquitylation assay

In vitro ubiquitylation was performed as recently described (Parsons *et al*, 2008). Briefly, assays were performed in a 15 μl reaction volume in the presence of E1 activating enzyme (0.7 pmol), the indicated E2 conjugating enzyme (9.5 pmol) and ubiquitin (0.6 nmol) or mutant ubiquitin unable to form polyubiquitin chains (Boston Biochemicals) in buffer containing 25 mM Tris–HCl (pH 8.0), 4 mM ATP, 5 mM $MgCl_2$, 200 μM $CaCl_2$, 1 mM DTT, 10 μM MG-132 for 1 h at 30°C. 1 × SDS–PAGE sample buffer was added, the samples were heated for 5 min at 95°C before separation of the proteins on a 10% SDS–polyacrylamide gel, followed by transfer to a PVDF membrane and immunoblot analysis with the indicated antibodies.

Alkaline single cell gel electrophoresis (Comet) assay

The Comet assay was performed as recently described (Woodhouse *et al*, 2008). Briefly, cells were trypsinized, treated or mock treated in suspension with hydrogen peroxide for 5 min on ice and embedded on a microscope slide in agarose (Bio-Rad, Hemel Hempstead, UK). The slides were incubated for various times at 37°C in a humidified chamber to allow for DNA repair, before lysis in buffer containing 2.5 M NaCl, 100 mM EDTA, 10 mM Tris–HCl pH 10.5, 1% (v/v) DMSO and 1% (v/v) Triton X-100 for 1 h at 4°C. The slides were then incubated in the dark for 30 min in cold electrophoresis buffer (300 mM NaOH, 1 mM EDTA, 1% (v/v) DMSO, pH 13) to allow the DNA to unwind before electrophoresis at 25 V for 25 min. After neutralization with 0.5 M Tris–HCl (pH 8.0), the slides were stained with SYBR Gold (Invitrogen, Paisley, UK) and analysed using the Komet 5.5 image analysis software (Andor Technology, Belfast, Northern Ireland).

Partial purification of monoubiquitylated Pol β

Calf thymus (10 g) was homogenized in 100 ml buffer containing 10 mM HEPES (pH 8.0), 10 mM KCl, 1.5 mM $MgCl_2$, 10% glycerol, 0.34 M sucrose, 0.5% Triton-X100, 1 mg/ml each of pepstatin, chemostatin and leupeptin, 1 mM DTT, 1 mM PMSF, 1 mM NEM and 10 μM MG-132 and centrifuged at 1300 *g* for 4 min at 4°C. The supernatant (soluble fraction) was clarified by centrifugation at 25 000 r.p.m. for 20 min at 4°C, filtered through 0.45 μm filters and fractionated by phosphocellulose chromatography using buffer containing 50 mM Tris (pH 8.0), 700 mM KCl, 1 mM EDTA, 5% glycerol, 1 mM DTT, 1 mM PMSF, 1 mM NEM. Proteins were eluted using buffer containing 1 M KCl, concentrated and diluted to 50 mM KCl and fractionated by Mono Q chromatography using a linear gradient of 50–1000 mM KCl. Fractions were analysed by 10% SDS–PAGE and immunoblotting with Pol β or ubiquitin antibodies.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

Acknowledgements

We thank Drs J Hohfeld, M Eilers and R Sobol for providing cells and expression constructs. This work was supported by the Medical Research Council, the Cancer Research UK and NIHR Biomedical Research Centre, Oxford.

Conflict of interest

The authors declare that they have no conflict of interest.

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